Anti-herbivore factors of grass endophytes: new prospects from metabolomics


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Abstract
A defensive role for several of the major alkaloids of Neotyphodium spp. endophytes in forage grasses has been confirmed in genetic experiments. However, many aspects of the activity of endophyte-infected grasses towards herbivores remain unaccounted for, suggesting there are further anti-herbivore factors yet to be uncovered. The development of metabolomics has provided new tools for the discovery of novel metabolites without preconceptions of the likely chemical structural class. Prospects for the application of new methods from metabolomics to further elucidate the defensive chemistry of associations of Neotyphodium spp. endophytes with their grass hosts are reviewed.

Keywords: Neotyphodium, Epichloë, defensive chemistry, invertebrate, vertebrate, gene deletion

Introduction
The development and commercial release of associations of selected endophytes with improved cultivars of forage grasses (Bouton & Easton 2004; Easton et al. 2001) provides an object lesson in how discoveries of natural mechanisms of chemical defence against herbivores can be manipulated to advance agricultural technology. However, while evidence of the defensive chemistry of symbioses of fungal endophytes of Neotyphodium spp. with host grasses of the Poaceae and their anti-herbivore effects has accumulated over some 25 years (below), many effects remain unaccounted for.

For example, some endophyte strains have been found to suppress root-feeding aphids (Aploneura lentisci) on perennial ryegrass (Popay et al. 2004), while the major known alkaloids are largely confined to the above-ground parts of the plant (Lane et al. 2000), and the relationship between the known alkaloid chemotype of endophyte strains and their in planta activity against insects is in many cases unclear. Thus the N. lolii strain AR1 in perennial ryegrass affords some resistance against black beetle (Heteronychus arator) (Popay & Baltus 2001) despite the absence of ergovaline to which this insect is known to be sensitive (Ball et al. 1997), and the effects on feeding and development of Spodoptera frugiperda larvae of different endophyte strains in three different host grasses could not be accounted for by the activity of the known endophyte alkaloids in these associations (Ball et al. 2006). Likewise, the chemical basis of resistance of endophyte-infected perennial ryegrass to pasture mealybug (Balanococcus poae) (Pennell et al. 2005) remains to be identified. Elucidation of the chemistry underlying these defensive mechanisms offers the prospect of new opportunities for developing endophyte-grass associations with improved characteristics for agriculture.

These examples focus on insect herbivores. While there is strong indicative evidence that several of the known endophyte alkaloids play a defensive role against mammalian herbivores (Siegel & Bush 1996), as with insects, there is certainly the possibility that other endophyte metabolites as yet undiscovered affect livestock performance.

The new technologies and approaches to investigating plant and fungal metabolites now being developed in metabolomics provide new opportunities for uncovering novel defensive chemistry in grass-endophyte symbioses. Metabolomics has been developed as part of the suite of tools of functional genomics to reveal the links between genes and metabolic phenotypes. As originally defined it is comprehensive analysis in which all the metabolites of an organism are identified and quantified (Fiehn 2002). Given the chemical diversity and wide dynamic range of metabolites this remains an elusive goal. In current practice metabolomics involves the application of broad-ranging analytical methods to provide insights into gene function. The ambition of metabolic

Figure 1 Scheme outlining potential applications of metabolomics research tools to the discovery of novel anti-herbivore factors.

![Diagram](image_url)

Figure 2 Dendrogram of correlations between relative levels of known and putative novel indoleterpenoid metabolites detected by LC-MS/MS analysis in ryegrass plants infected with wild-type Epichloë festucae F1 or with either of two strains with deletions in the sidF gene. Metabolites highly correlated with lolitrem B show a similar pattern of elevated accumulation in plants infected with the sidF deletion strains.
The pioneering investigations of the chemistry of grass endophytes described four classes of alkaloids biosynthesised by Neotyphodium spp. fungi in their grass hosts: pyrrolizidine alkaloids of the loline family (Bush et al. 1993); ergot alkaloids, particularly ergovaline (Garner et al. 1993); indolediterpenoids, particularly lolitrem B (Gallagher et al. 1981; Munday-Finch et al. 1998 and references cited); and the pyrrolopyrazine peramine (Rowan & Gaynor 1986). For each of these classes evidence was obtained suggesting they play a role in defending the symbiosis against herbivores whether vertebrate (Siegel & Bush 1996) or invertebrate (Popay & Bonos 2004).

In the case of loline alkaloids, peramine and ergot alkaloids, genetic evidence of their significance as defensive compounds is now available. The asexual nature of Neotyphodium endophytes has limited conventional genetic studies. However, direct evidence for the defensive significance of loline alkaloids has been provided by Mendelian genetic analysis of progeny of loline alkaloid expressing and non-expressing chemotypes of the sexual endophyte Epichloë festucae (Wilkinson et al. 2000). Loline alkaloid expression was found to segregate 1:1 between progeny and co-segregate with activity against two aphid species, and in planta levels of the alkaloids were correlated with anti-aphid activity.

With recent progress in dissecting the molecular genetics of endophyte alkaloid biosynthesis, genetic analysis is now being applied to the elucidation of defensive mechanisms of Neotyphodium endophytes. The defensive role of peramine against adult Argentine stem weevils (Listronotus bonariensis) has recently been directly confirmed by gene deletion studies. A non-ribosomal peptide synthetase gene, perA was identified in Epichloë festucae strain Fl1 as essential for the synthesis of peramine in planta (Tanaka et al. 2005). In a feeding choice assay, feeding by adult weevils was much higher on leaf blades of perennial ryegrass infected with a perA deletion mutant strain than on leaf blades from plants infected with the wild-type strain, and comparable to that on blades from uninfected plants.

Similarly, direct evidence of the effects of endophyte ergot alkaloids on a mammalian herbivore (rabbits) has been provided by studies with two mutant endophyte strains with gene deletions early and late in the ergot alkaloid pathway (Panaccione et al. 2006a). While the ingestion of ergovaline reduced appetite, the production of ergot alkaloids earlier in the pathway (clavines) reduced preference. However, when the entire alkaloid pathway was eliminated, rabbits unexpectedly preferred endophyte-infected plants, and the basis for this remains unknown. Conversely, experiments with these deletion mutants have eliminated ergot alkaloids as a factor responsible for the suppression of infection of endophyte-infected perennial ryegrass by the lesion nematode Pratylenchus scribneri (Panaccione et al. 2006b). While ergovaline suppressed nematode motility in a laboratory bioassay, nematode infection was inhibited in endophyte-infected plants whether the endophyte strain was the wild-type, or a mutant with a gene deletion early or late in the ergot alkaloid pathway. Thus gene deletion studies can expose evidence of as yet uncharacterised defensive factors as well as better defining the role of known ones.

Clearly there are opportunities for extending these studies to a wider range of herbivores, and to other pathways, notably indolediterpene biosynthesis. There is also the potential for the identification of novel defensive metabolites by the combination of gene deletion experiments and metabolomics (below).

Attempts to define the defensive role of particular metabolites or classes of metabolites by comparative studies of grass-endophyte associations using selected fungal strains from nature...
which produce or do not produce them have been less conclusive as they are likely to be confounded by variations in other endophyte (or plant) metabolites. In practice this has proved to be a valuable route for discovering new variants of endophyte alkaloid biosynthesis. Thus, the unexpected insect resistance and performance in the field of ryegrass associations with the AR37 endophyte (Popay & Wyatt 2005) led to the discovery of a new class of endophyte indolediterpenoids, the epoxy-janthitrems (Tapper & Lane 2004). Similarly, comparisons of the effects of high ergovaline and low ergovaline strains of Neotyphodium sp. endophytes in tall fescue drew attention to the clavine alkaloids, particularly setoclavine and isosetoclavine present in the “Claviplus” endophytes (Tapper et al. 2002). Further investigation of grass associations with Neotyphodium endophyte strains that do not produce appreciable concentrations of known defensive chemicals is likely to uncover further components of their defensive armoury.

There is also considerable scope for expanding knowledge of the range of antiherbivory factors of Neotyphodium spp. and related endophytes by applying more research effort to exploring associations with grasses beyond the predominant commercially important forage species. Thus, metabolite profiles from associations with Echinopogon ovatus, Melica decumbens, and Poa huecu (Miles et al. 1998; Towers 1997) remain to be elucidated.

Potential Role of Metabolomics

There are several routes by which metabolomics with the combination of comprehensive approaches to analysis and multivariate statistical tools can potentially aid in the discovery of new anti-herbivore metabolites and indeed classes of metabolites (Fig. 1). These include gene-focussed approaches such as comprehensive metabolic analysis of gene knockouts, and the combination of metabolomics with transcriptome (microarray) analysis, and also approaches where comprehensive and sensitive analytical technologies are to the forefront. One of the key roles of metabolomics in functional genomics from its inception has been in elucidating the metabolic outcome of gene deletions (e.g. Raamsdonk et al. 2001). The comparative examination of metabolite profiles of host grass associations with wild type and mutant endophyte strains with deletions in putative secondary metabolite biosynthesis genes is a potential route to the discovery of new metabolites. Such an approach could also provide the experimental material for unambiguous evaluation of defensive function as in the examples above. However, its efficacy will be entirely dependent on the prior identification of a key regulatory or biosynthetic gene for such a metabolite. The investigation of genes of unknown role has provided new insights into the grass-fungus symbiosis through the construction and introduction to plants of mutant endophytes with deletions in genes which are evidently vital to the stability of the symbiosis, namely noxA, a NADPH oxidase gene (Tanaka et al. 2006) and sidF, a gene for a non-ribosomal peptide synthetase involved in the biosynthesis of a novel siderophore (Johnson et al. 2007). These studies have revealed the importance of reactive oxygen species (ROS) and Fe regulation, respectively, to the symbiosis, and also the vital role bioactives produced by the endophyte may play apart from plant defence (Johnson et al. 2007). Reports of the discovery of new defensive metabolites identified by this route are awaited. However, new putative defence factors may also be revealed by the unexpected effects of gene deletions. Thus a novel clavine, 6,7-secolysergine was found to accumulate to unusually high levels in a ryegrass plants infected with mutant strain of
Investigations of a range of grass-endophyte associations, both in planta and co-workers in (Zhang et al. 2006). An early outcome of the acquisition of the powerful and sensitive analysis tools required for metabolomics has been expansion of knowledge of known classes of endophyte metabolites. HPLC-Mass Spectrometry /Mass Spectrometry using ion-trap detection has proved a powerful tool for the analysis of indolederipterpenoids providing high sensitivity and selectivity for a wide range of structural types, and valuable characterising data in the patterns of collision-induced fragmentation in the ion trap (or source). Investigations of a range of grass-endophyte associations, both lolitrem B producers and non-producers have revealed that the “minor” indolederipterpenoids previously reported from seed of perennial ryegrass infected with common strains of \( N. \text{lollii} \) (Munday-Finch et al. 1998; Gatenby et al. 1999), in particular the terpendoles, are fairly widely distributed, providing further
evidence that the absence of lolitrem B does not imply the absence of indoleterpenoids (B.A. Tapper unpublished). The defensive role of these compounds remains to be clarified, and gene deletion technology is probably the most powerful option now available.

The sensitivity of HPLC-MS/MS is also providing new insights into the distribution of endophyte alkaloids within the grass plant. The recent detection in gutation fluid from endophyte-infected grass plants, of peramine from perennial ryegrass plants and also ergovaline and lolines in the case of tall fescue plants (Koulman et al. 2006a; Koulman et al. 2007) opens up interesting questions about the significance of this process to plant defence. This technology may also be valuable in elucidating the mechanisms by which endophytes afford protection against root-feeding insects.

There are further opportunities as yet unexplored for applying metabolomics methodologies to the discovery of new anti-herbivore factors in grass-endophyte associations. Bioassay-directed discovery is a powerful but laborious approach to the identification of unknown bioactives. By introducing methodology from metabolomics it may be possible to accelerate the process by comparative metabolite profiling and bioassay of fractions, for example, with ELISA assays for compound classes, applying multivariate statistics to identify the features associated with high activity early in the separation process. Using MS/MS ion-mapping methodologies it may be possible to discover highly active minor constituents within complex fractions. However, no equivalent short-cuts are evident to the laborious task of isolating active compounds to verify their activity.

Despite more than 20 years of research into and exploitation of the defensive chemistry of associations of endophytic Neotyphodium spp. fungi more opportunities remain. With new analytical approaches from metabolomics, new advances in functional genomics, and the energies of a new generation of analytical approaches from metabolomics it may be possible to accelerate the identification of unknown bioactives. By introducing directed discovery is a powerful but laborious approach to herbivore factors in grass-endophyte associations. Bioassay-directed discovery is a powerful but laborious approach to the identification of unknown bioactives. By introducing methodology from metabolomics it may be possible to accelerate the process by comparative metabolite profiling and bioassay of fractions, for example, with ELISA assays for compound classes, applying multivariate statistics to identify the features associated with high activity early in the separation process. Using MS/MS ion-mapping methodologies it may be possible to discover highly active minor constituents within complex fractions. However, no equivalent short-cuts are evident to the laborious task of isolating active compounds to verify their activity.

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