A proteomic and targeted metabolomic approach to investigate change in Lolium perenne roots when challenged with glycine

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Received 27 July 2006; Revised 17 November 2006; Accepted 27 November 2006

Abstract
A combined proteomic and isotope tracer approach was used to investigate the impact of supplying N as glycine to roots of Lolium perenne. Initially, ammonium nitrate was supplied to all plants, after which half received glycine as their sole N source, while the remainder continued to receive ammonium nitrate. Plants supplied with glycine acquired less N than those receiving the mineral source, resulting in reduced root nitrate concentrations. The amino acid complement of roots was also strongly affected by the form of N supplied, and 15N labelling indicated that the biochemical fate of acquired N in roots was dependent on the form of N available for uptake. Proteomic analysis of Lolium roots indicated that 6% of 627 root proteins resolved on 2D gels changed in abundance in response to the form of N applied. Multivariate analysis of protein abundance clearly discriminated the proteomes of Lolium perenne roots as a function of treatment applied. Seven affected proteins were identified (mostly by protein homology with sequenced species), including methionine adenosyltransferase, an enzyme involved in glycine metabolism. Although some changes in root amino acid and protein complement were due to responses to reduced N supply, both the distinct fate of 15N tracers and the abundances of identified proteins could be attributed specifically to the form of N available to roots. The results demonstrate the potential of targeted proteomic approaches to identify functioning of plants where more traditional methods cannot resolve multiple, co-incident biological interactions and element fluxes.

Key words: Amino acids, glycine, Lolium perenne, metabolomic, proteomic, roots.

Introduction
Despite the recognition that plant roots have the potential to acquire amino acids directly as a source of N (Näsholm et al., 1998) and that soluble organic N is a quantitatively important form of N in some soils (Kielland, 1995), the contribution that amino acid uptake makes to plant N nutrition is uncertain (Jones et al., 2005). The traditional view of N cycling in soil is that organic forms of N in the soil are first mineralized by microbes before uptake of mineral forms by plants. Since microbes generally have high substrate affinities for amino acids (Vinolas et al., 2001), are distributed throughout the soil matrix, and can rapidly modify their growth rates in response to availability of substrate, it is unclear to what extent plant roots are able to compete for and acquire amino acids, despite their potential to do so. The fact that molecular pathways for uptake and assimilation of nitrate, ammonium, and amino acids in plant roots are distinct (Miller and Cramer, 2004) provides an opportunity to identify which pathways are active by assessing the abundance of proteins (enzymes and transporters) that are integral to these pathways.

The chemical composition of soil solutions is highly dynamic, varying both temporally and spatially (Farley and Fitter, 1999). In particular, the different forms of N...
change markedly in concentration and relative abundance (Schmidt and Stewart, 1997; Henry and Jefferies, 2002). A change in the forms of N available to roots would be expected to alter the abundance of enzymes and transporters required to utilize these forms of N, where their gene expression is inducible. Such changes would also affect the abundance of metabolites contributing to the respective pathways of assimilation. For example, plant exploitation of an increased abundance of an amino acid may require synthesis of a specific transporter protein. If the amino acid was not one synthesized by the initial metabolism of inorganic N in the GOGAT cycle, then it is probable that proteins required for its further metabolism would also be synthesized. In addition, the amino acid pool in plant roots has important regulatory control over synthesis of transporters and enzymes involved in the metabolism of inorganic N (Chevalier et al., 1996; Oliveira and Coruzzi, 1999; Kawachi et al., 2002; Loque and von Wiren, 2004). Therefore, the uptake of amino acids by roots may affect the uptake and biochemical fate of inorganic N.

Recently developed methodologies such as metabolomics, proteomics, and DNA microarray analysis, which can be used to characterize simultaneously many responses of biological systems, have been applied to identify processes in plants that are regulated in response to variations in availability of inorganic N to roots (Wang et al., 2000, 2001; Bahrmann et al., 2004, 2005; Scheible et al., 2004; Bölling and Fiehn, 2005; Flæte et al., 2005). The aim of this study was to assess the potential of proteomic methods to identify changes in protein abundance of Lolium perenne roots that are characteristic of plant responses required for utilization of an organic form of N (glycine). Lolium perenne was selected as it is a species that is subject to rapid changes in the abundance of forms of N available for uptake and has been shown to be capable of direct uptake of glycine (Thornton, 2001). It is a common pasture species, and in grazed systems glycine is an important N input to soil via urine deposition. Glycine is a useful model organic N form to study due to (i) its high relative abundance in many ecosystems (Kielland, 1995; Schmidt and Stewart, 1997); (ii) the fact that it is less readily utilized by soil microbes than other amino acids (Lipson et al., 1999; Paterson et al., 2006) favouring acquisition by plants; and (iii) the fact that the initial metabolism of inorganic N in roots does not produce glycine, allowing distinct pathways of metabolism to be traced. That L. perenne is not fully sequenced means that identification of specific proteins which change in abundance during utilization of glycine was likely to be problematic and based on protein homologies with better characterized species. However, it is hypothesized (i) that challenging plants with glycine will result in changes in protein abundance that can be assessed by multivariate statistics at the level of the whole proteome; and (ii) that these changes will also be apparent in distinct biochemical partitioning of acquired glycine N, relative to plants utilizing mineral N only.

Materials and methods

Growth of plant material

Seeds of L. perenne L. (Emorsgate Seeds, King’s Lynn, UK) were surface-sterilized as described in Thornton (2004). Seeds were then transferred onto grids of Tygan mesh over sterile deionized water within covered crystallizing dishes and maintained in the dark at 20 °C. After 6 d, seedlings were transferred aseptically onto new grids (20 per grid). Each grid was then placed within sterile culture vessels containing 950 ml of a complete nutrient solution. The nutrient solution, pH 6.0, was as described by Thornton and Bausenwein (2000) except that all N was supplied as 1 mol m⁻³ ammonium nitrate (2 mol m⁻³ N). The nutrient solution within each vessel was aerated by bubbling 1.0 l min⁻¹ sterile air through it. The grids and culture vessels have been described previously (Thornton, 2001).

The culture vessels were arranged in a replicate block design within a controlled environment room (Conviron, Winnipeg, Canada). The room was set with a 16 h photoperiod with 400 µmol m⁻² s⁻¹ photosynthetically active radiation (PAR) at plant height. Within the culture vessels, plants experienced a constant temperature of 20 °C. Plants were grown in these vessels for a further 20 d, during which time the nutrient solution was replaced after 7 d and 14 d. After the 20 d growth period, the nutrient solutions were again replaced; plants either continued to receive the solution containing 1 mol m⁻³ ammonium nitrate or received an identical solution except that all N was supplied as 2 mol m⁻³ glycine (2 mol m⁻³ N).

For plants allocated to proteomic analysis, the N in these final nutrient solutions was not enriched with ¹⁵N; plants were harvested 1 d and 3 d after the final solution change. For plants allocated to N uptake and amino acid studies, three groups of plants were used. In the first group, all N in the final nutrient solutions was enriched with ¹⁵N. The nutrient solution containing ammonium nitrate was dual labelled with ¹⁵N to an enrichment of 5.14 atom%, and the solution containing glycine enriched to 4.96 atom%. Plants were harvested after 1 d in the ¹⁵N-enriched solutions. In the second group, the nutrient solutions containing ammonium nitrate and glycine were also ¹⁵N enriched as described above; plants remained in the enriched solutions for 2 d, after which they were removed from the culture vessels and discarded. The ¹⁵N-enriched nutrient solutions in the vessels, now nutrient depleted to some extent, were retained and referred to as ‘nutrient-depleted ¹⁵N-enriched’ solutions. In the third group, plants received solutions containing ammonium nitrate and glycine not enriched with ¹⁵N for the first 2 d. At this point, the plants were then transferred into the equivalent (ammonium nitrate or glycine) ‘nutrient-depleted ¹⁵N-enriched’ solutions, where they remained for a further 1 d, after which they were harvested. This ensured for plants harvested after both 1 d and 3 d that they were only ¹⁵N labelled over a 1 d period immediately prior to being harvested and that they experienced the same nutrient depletion (due to the culture vessels having a finite volume of 950 ml) as plants used for proteomics.

Harvesting

The 20 plants within a single culture vessel were bulked together to provide one biological replicate. Plants were removed from the culture vessels and their roots were dipped in 1 mol m⁻³ CaSO₄ at 4 °C for 1 min to remove nutrients from the water and Donnan free
spaces of the roots. The plants were then separated into root and shoot material, discarding any remaining seed material, weighed fresh, and immediately frozen at −80 ºC.

**Determination of N uptake, nitrate, and amino acids**

The frozen samples were freeze-dried (Supermodulyo, Edwards High Vacuum International, Crawley, UK), reweighed, then ball milled (Retsch MM2000, Haan, Germany). The total N and 15N concentrations of the milled plant materials were determined using a TracerMAT continuous flow isotope ratio mass spectrometer (Finnigan MAT, Hemel Hempstead, UK). The uptake of the 15N-labelled compounds was determined using the equations of Millard and Nielsen (1989).

Aliquots of 5 mg of milled root material were placed in 2 ml microfuge tubes with pierced lids, 1.5 ml of deionized water was added, and the tubes were briefly vortexed, before heating in an oven at 105 ºC for 1 h. Tubes were then centrifuged for 10 min at 16 000 g and the supernatant retained. The water extraction was repeated on the pellet remaining after centrifugation; the supernatants were combined and diluted to 10 ml with deionized water. Nitrate was determined in the water extracts using a Skalar SAN++ nitrate kit (Skalar, The Netherlands) according to the manufacturer’s instructions.

Protein extraction, 2D-electrophoresis, and identification

A 1 g aliquot of frozen root material was ground to a fine powder using liquid nitrogen with a mortar and pestle. 2 g of polyvinylpyrrolidone was added, and the mixture was ground further. Extraction buffer (20 ml), modified from Wetzel et al. (1989) [50 mol m−3 ascorbic acid, 50 mol m−3 sodium borate, 1% dithiothreitol (DTT), and 1% broad specificity protease inhibitor cocktail for plant cell extracts (Product No P9599, Sigma, Dorset, UK)], was added and the mixture incubated at 4 ºC for 30 min, then centrifuged at 100 000 g at 4 ºC for 1 h. The protein concentration of an aliquot of the supernatant was determined using a microcuvette reader (Bio-Rad Laboratories Ltd) according to the manufacturer’s instructions. Purification of amino acids in the ball-milled root samples and determination of their concentrations and 15N enrichments by gas chromatography–mass spectrometry (GC-MS) was as described by Thornton and Robinson (2005).

Calculation of protein concentration

Protein concentrations per unit fresh mass were converted to a per unit dry mass basis using the fresh mass:dry mass ratio of plants in the equivalent treatment used for the determination of N uptake. The amount of N in root protein was calculated assuming the N content of protein was 16.7% (Brouquisse et al., 1998). Analysis of variance (ANOVA) and principal component analysis (PCA) were carried out using Genstat 7th Edition, Release 7.1© Lawes Agricultural Trust (IACR-Rothamsted, UK). Results of normalized spot volume and the percentage contribution of each individual amino acid to the total free amino acid N concentration were subject to angular arc-sine transformation prior to analysis by ANOVA. As transformation did not alter the interpretation of results, untransformed data are presented for clarity.

**Results**

**N uptake and root N concentrations**

Plants supplied ammonium nitrate had, on average, rates of N uptake >3 times greater than those supplied glycine (P <0.001, Table 1). The rate of N uptake for those plants receiving ammonium nitrate decreased from day 1 to day 3; this reduction was most probably caused by nutrient depletion of the solution in the culture vessels. The lesser N uptake of the plants receiving glycine resulted in these plants having a lower concentration of total N in their roots compared with plants receiving ammonium nitrate (P <0.001, Table 1). Additionally, plants receiving glycine showed a reduction in the total N concentration in roots.
from day 1 to day 3 ($P < 0.001$). These changes in the root total N concentrations were the result of impacts of treatments on the root nitrate N pool ($P < 0.001$ in each case), as the root concentrations of protein N and amino acid N were unaffected by either the form of N supplied or the harvest date ($P > 0.05$ in each case, Table 1).

**Amino acid N concentrations**

In order to present equivalent PCA data for the proteins and free amino acids, PCA was performed on the percentage contribution of each individual amino acid to the total free amino acid N concentration. Changing the form of N supplied to plants resulted in distinct complements of root amino acids (Fig. 1A). The first principal component explained the majority of the variability, accounting for 96.3% of the total; the second component accounted for a further 2.7%. On changing the form of N supplied from ammonium nitrate to glycine, the contribution of asparagine fell from 72% to 35% ($P < 0.001$) whilst the contribution of serine rose from 6% to 40% ($P < 0.001$). For glycine-supplied plants, there is evidence that the complement of amino acids continued to change between the harvests on days 1 and 3; in contrast, for plants supplied ammonium nitrate, the amino acid complement showed no separation between days 1 and 3 (Fig. 1A).

In common with the PCA data, the absolute concentrations of amino acids showed greater change in response to the form of N supplied than to the date of harvest (Table 2). Twelve of the 16 amino acids showed change with the form of N supplied. The concentrations of asparagine, aspartate, glutamate, valine, and methionine were all greater when N was supplied as ammonium nitrate, whilst the concentrations of leucine, isoleucine, proline, threonine, phenylalanine, glycine, and serine were greater when N was supplied as glycine ($P < 0.05$ in all cases, Table 2). In contrast, the concentrations of only two amino acids showed change with harvest date. The concentration of threonine was greater on day 3 than day 1 ($P < 0.05$), whilst the concentration of proline was also greater on day 3 than day 1, but only when plants were supplied N as glycine ($P < 0.05$, Table 2).

### Table 1. The specific N uptake (mg N g$^{-1}$ root DW) over the 24 h period immediately prior to harvest and the root concentrations of total N, protein N, amino acid N, and nitrate N (mg N g$^{-1}$ root DW) of L. perenne plants at harvest

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Specific N uptake</th>
<th>Total N concentration</th>
<th>Protein N concentration</th>
<th>Amino acid N concentration</th>
<th>Nitrate N concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>A1</td>
<td>29.1 ± 5.6</td>
<td>37.6 ± 0.5</td>
<td>16.6 ± 2.4</td>
<td>1.9 ± 0.4</td>
<td>10.5 ± 0.6</td>
</tr>
<tr>
<td>A3</td>
<td>17.1 ± 2.1</td>
<td>37.9 ± 0.5</td>
<td>13.0 ± 1.6</td>
<td>2.7 ± 0.9</td>
<td>10.1 ± 0.2</td>
</tr>
<tr>
<td>G1</td>
<td>6.3 ± 0.3</td>
<td>34.6 ± 0.4</td>
<td>12.7 ± 1.6</td>
<td>1.6 ± 0.2</td>
<td>7.2 ± 0.3</td>
</tr>
<tr>
<td>G3</td>
<td>6.4 ± 0.5</td>
<td>30.1 ± 0.4</td>
<td>14.4 ± 2.5</td>
<td>1.7 ± 0.3</td>
<td>3.2 ± 0.3</td>
</tr>
</tbody>
</table>

* A1, plants which received N as ammonium nitrate harvested on day 1; A3, plants which received N as ammonium nitrate harvested on day 3; G1, plants which received N as glycine harvested on day 1; G3, plants which received N as glycine harvested on day 3.
Table 2. The total free amino acid N concentrations (μg N g⁻¹ root DW) and ¹⁵N-labelled free amino acid N concentrations (μg N g⁻¹ root DW) in the roots of L. perenne at harvest

Values are mean ±SE of five replicates. nd, not detectable. A1, A3, G1, and G3 are as defined in Table 1.

<table>
<thead>
<tr>
<th>Amino acids</th>
<th>A1</th>
<th>A3</th>
<th>G1</th>
<th>G3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total amino acid N concentrations</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Asparagine</td>
<td>1417 ± 312</td>
<td>2003 ± 717</td>
<td>660 ± 148</td>
<td>557 ± 149</td>
</tr>
<tr>
<td>Glutamine</td>
<td>13 ± 5</td>
<td>5 ± 2</td>
<td>3 ± 1</td>
<td>5 ± 2</td>
</tr>
<tr>
<td>Aspartate</td>
<td>46 ± 5</td>
<td>41 ± 4</td>
<td>32 ± 3</td>
<td>32 ± 4</td>
</tr>
<tr>
<td>Glutamate</td>
<td>136 ± 20</td>
<td>183 ± 66</td>
<td>37 ± 7</td>
<td>27 ± 2</td>
</tr>
<tr>
<td>Alanine</td>
<td>22 ± 1</td>
<td>20 ± 1</td>
<td>19 ± 1</td>
<td>21 ± 2</td>
</tr>
<tr>
<td>Valine</td>
<td>17 ± 2</td>
<td>20 ± 2</td>
<td>3 ± 0</td>
<td>3 ± 0</td>
</tr>
<tr>
<td>Leucine</td>
<td>9 ± 1</td>
<td>12 ± 2</td>
<td>17 ± 1</td>
<td>16 ± 1</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>8 ± 2</td>
<td>10 ± 1</td>
<td>17 ± 2</td>
<td>17 ± 2</td>
</tr>
<tr>
<td>GABA</td>
<td>16 ± 2</td>
<td>14 ± 2</td>
<td>9 ± 1</td>
<td>15 ± 1</td>
</tr>
<tr>
<td>Proline</td>
<td>3 ± 0</td>
<td>4 ± 0</td>
<td>6 ± 0</td>
<td>9 ± 1</td>
</tr>
<tr>
<td>Threonine</td>
<td>23 ± 6</td>
<td>31 ± 3</td>
<td>63 ± 5</td>
<td>90 ± 11</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>5 ± 1</td>
<td>5 ± 1</td>
<td>13 ± 0</td>
<td>13 ± 1</td>
</tr>
<tr>
<td>Glycine</td>
<td>20 ± 4</td>
<td>26 ± 5</td>
<td>69 ± 5</td>
<td>84 ± 18</td>
</tr>
<tr>
<td>Serine</td>
<td>116 ± 32</td>
<td>173 ± 44</td>
<td>543 ± 64</td>
<td>765 ± 115</td>
</tr>
<tr>
<td>Cysteine</td>
<td>67 ± 17</td>
<td>128 ± 48</td>
<td>83 ± 31</td>
<td>60 ± 6</td>
</tr>
<tr>
<td>Methionine</td>
<td>3 ± 1</td>
<td>5 ± 1</td>
<td>2 ± 0</td>
<td>1 ± 0</td>
</tr>
</tbody>
</table>

¹⁵N-labelled amino acid N concentrations

When plants were supplied with dual ¹⁵N-labelled ammonium nitrate, within the root amino acid pool the ¹⁵N was primarily incorporated into asparagine, with progressively smaller amounts into glutamate, aspartate, alanine, glutamine, and GABA (Table 2). When plants were supplied ¹⁵N-labelled glycine, the majority of ¹⁵N was again incorporated into asparagine; however, in this instance the other amino acids into which ¹⁵N was incorporated were serine, cysteine, and glycine (Table 2).

Protein concentrations

Despite the total concentrations of root protein N being unaffected by treatment (Table 1), PCA demonstrated that the relative protein abundances for each treatment were sufficiently distinct to discriminate them from each other at the level of the whole protein complement (Fig. 1B). On the PCA plot, the direction of change from roots harvested on day 1 to day 3 differed with the form of N supplied (Fig. 1B). This indicates that switching the N supply to glycine caused changes in the protein complement other than those due to reduced N uptake alone. The first and second principal components accounted for 28.2% and 17.3%, respectively, of the total variability in protein complement between the gels.

Across all treatments, 627 protein spots were observed on the 2D gels. A total of 39 protein spots, representing 6.5% of the total, were affected (P < 0.05) by the form of N supplied to the plants, expression of 25 spots being up-regulated and 14 down-regulated by the presence of glycine. Forty-eight spots changed significantly with harvest date (P < 0.05), with 24 spots being up-regulated and 24 spots down-regulated in roots on day 1 compared with those on day 3. Of the protein spots showing a response, 15 responded both to the form of N supplied and to the harvest date.

Of the subset of 22 spots chosen for peptide mass mapping, the MOWSE scores (Pappin et al., 1993) indicated a significant (P < 0.05) match for seven proteins (Table 3). For these identified proteins, the number of independent matching peptides ranged from six to eight and their sequence coverage ranged from 25% for malate dehydrogenase to 34% for nucleoside-diphosphate kinase. The other spots remained unidentified due to a lack of matching sequence data, rather than any problems with the peptide profiles. Only nucleoside-diphosphate kinase was identified as being a protein from L. perenne. The other six proteins were identified using sequence homology to proteins isolated from other plant species; for all but malate dehydrogenase this was with members of the Poaceae family. The predicted mass and isoelectric point (pI) of most of the proteins identified by peptide mass mapping (Table 3) were in good agreement with their actual positions on the 2D gel (Fig. 2).

Two protein spots were identified as being methionine adenosyltransferase (Table 3). Good evidence that these protein spots were isozymes was that on the 2D gel each had the same mass and differed only in pI (Fig. 2). Additionally, the changes in mean abundances of these proteins in response to treatments were found to be correlated (Fig. 3), with a positive linear relationship between the two proteins also apparent on individual gels (Fig. 4A). Despite this relationship, only the expression of the second isozyme responded to the form of N supplied, being greater when plants were supplied glycine (P < 0.05).

Two additional identified root proteins also responded to the form of N supplied: expression of adenosine kinase was greater when supplied glycine (P < 0.001) whilst glyceraldehyde-3-phosphate dehydrogenase was expressed to a greater extent (P < 0.05) when plants were supplied with ammonium nitrate (Fig. 3). Adenosine kinase and isozyme 2 of methionine adenosyltransferase both showed greater expression on day 3 than on day 1 (P < 0.05 in
each case), Considering individual gels, a linear relationship between these two proteins was confirmed (Fig. 4B).

The expression of proteins reported above (Figs 1, 3, and 4) is based on normalized spot volumes, as such they represent the percentage contribution of individual proteins to the protein complement recovered from roots. However, as the total concentration of root protein N was unaffected by either the form of N supplied or the harvest date, the reported changes in relative protein abundance would also be expected to indicate changes in absolute protein abundances.

**Discussion**

*N uptake and initial metabolism*

The rates of glycine uptake during the 24 h periods prior to harvests on days 1 and 3 were not significantly different. This indicates that either glycine uptake was not up-regulated in response to the increased availability of glycine (and removal of mineral N), or that up-regulation was rapid such that the rate of uptake integrated over the 24 h prior to harvest on day 1 reflected up-regulated glycine uptake. This is consistent with the findings of Schobert and Komor (1987) who reported that there was no evidence of up-regulation of proline uptake following a 19 h pre-incubation of *Ricinus communis* with proline. However, a rapid up-regulation of amino acid uptake cannot be discounted in that study either.

On supplying the plants with dual $^{15}$N-labelled ammonium nitrate, subsequent incorporation of $^{15}$N primarily into asparagine but also into glutamine, aspartate, and glutamate was consistent with the nitrate being first reduced to ammonium and subsequently the $[^{15}$N]ammonium being incorporated into the root free amino acid pool via the GOGAT cycle (Lea and Ireland, 1999). For...
 assimilation of glycine, the initial products of glycine metabolism are, in sequence, serine, cysteine, and methionine. In the roots of plants supplied with $^{15}$N-labelled glycine, the appearance of $^{15}$N in serine and cysteine proved that this pathway was operating at least up to cysteine. No evidence of $^{15}$N derived from glycine was found in methionine; however, it is still likely that cysteine was further metabolized to methionine. In this metabolic step, cysteine initially reacts with $O$-phospho-homoserine (OPH) and it is the N donated by OPH which goes on to be present in methionine. In the sterile solution culture used, microbial transformation of $[^{15}$N]glycine to $[^{15}$N]ammonium prior to uptake can be discounted. There are at least two in planta reactions by which N derived from $[^{15}$N]glycine could have been assimilated in this manner. Hartung and Ratcliffe (2002) using nuclear magnetic resonance (NMR) spectroscopy concluded that the metabolism of glycine to serine in the roots of Zea mays mainly involved the combined action of

Fig. 3. The response of the mean normalized spot volumes (%) of the seven identified proteins to treatment, values are mean $\pm$ SE, $n$=3. Treatments A1, A3, G1, and G3 are as defined in Fig. 1. (A) MAT 1, methionine adenosyltransferase isozyme 1; (B) MAT 2, methionine adenosyltransferase isozyme 2; (C) GPDH, glyceraldehyde-3-phosphate dehydrogenase; (D) MDH, malate dehydrogenase; (E) NDPK, nucleoside-diphosphate kinase; (F) APX, ascorbate peroxidase; and (G) ADK, adenosine kinase.

Glycine-induced change in roots
the glycine decarboxylase complex and serine hydroxymethyltransferase; essentially in this reaction 2 mol of glycine produces 1 mol of serine and 1 mol of ammonia. Indeed, these authors were able to detect 15N-labelled ammonium in the root tips of *Z. mays* following incubation with [15N]glycine (Hartung and Ratcliffe, 2002). A second source of 15N-labelled ammonium in plants supplied [15N]glycine would have been when N present in cysteine was released as ammonium during the metabolism of cysteine to methionine as described above. However, following the application of [15N]glycine to roots of plants growing in non-sterile systems such as soil, whilst the initial appearance of [15N] in the root pools of glycine, serine, and cysteine can be considered indicative of intact glycine uptake, the appearance of [15N] in asparagine is ambiguous as it can also occur through mineral N assimilation.

**Change in root amino acids**

Challenging the roots of *L. perenne* with glycine resulted in a distinct complement of free amino acids in this tissue. Changes in the concentrations of many amino acids in the roots of *L. perenne* plants supplied with ammonium nitrate compared with plants supplied with glycine were strikingly similar to the changes observed in 9-d-old whole *Arabidopsis thaliana* plants 24 h after reintroducing nitrate to previously N-starved plants (Scheible et al., 2004). In the present study and that of Scheible et al. (2004), the concentrations of the amino acids asparagine, aspartate, and glutamate plus methionine increased, the concentration of alanine remained unchanged, while concentrations of the minor amino acids leucine, isoleucine, threonine, and phenylalanine decreased. The similarity between our results and those of Scheible et al. (2004) suggests that the changes in many of the root amino acids when challenged with glycine may be a response to change in the root nitrate concentration rather than to change in the form of N supplied per se. However, Scheible et al. (2004) did not measure glycine, serine, and cysteine, the amino acids most likely to be specifically affected by switching the form of N from ammonium nitrate to glycine, and in the present study the concentrations of glycine and serine in roots changed significantly when challenged with glycine. Therefore, the distinct amino acid complement that developed during utilization of glycine can be considered to reflect both reduced activity of metabolic pathways involved in inorganic N assimilation and specific changes in root glycine and serine pools in response to availability of glycine as an N source to *L. perenne*.

Scheible et al. (2004) interpreted their results as indicating that on reintroducing nitrate, the rate of protein synthesis was increased to a greater extent than the rate of minor amino acid synthesis. On switching the form of N supplied to plants from ammonium nitrate to glycine, the reverse situation must be considered, the rate of protein synthesis being reduced to a greater extent than the rate of minor amino acid synthesis. This imbalance in the synthesis of protein and minor amino acids was still apparent 3 d after switching the form of N supplied. Despite any reduction in protein synthesis on switching plants to glycine, it was shown that 1 d was sufficient for a distinct protein complement to have developed.

**Change in root protein abundances**

The 6.2% of proteins which responded to the form of N supplied was sufficient to discriminate protein complements on the basis of multivariate analysis of relative protein abundances, and is of similar magnitude to the 8.5% of the *Triticum aestivum* root proteome found to change in response to magnitude of N supply (Bahrman et al., 2005). Clearly, multivariate statistical approaches are a powerful means of dealing with proteomic data where the number of variables (proteins) is large and the likelihood of making ‘type 1’ statistical errors (i.e. false positives) in a comparison of individual protein abundances is great. In this study, the approach gives confidence that there were significant differences in protein

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**Fig. 4.** Correlation of the normalized spot volumes for selected identified proteins: (A) MAT 1 versus MAT 2 and (B) ADK versus MAT 2. Protein abbreviations are as in Fig. 3. Symbols are individual values from the 12 gels in all four treatments.
complements and justifies more detailed investigations of the sources of these differences.

That *L. perenne* is an unsequenced species, poorly characterized for proteomic studies, made specific identification of treatment-affected proteins difficult. However, through use of protein homology (mainly within the Poaceae family), a number of proteins were successfully identified. Two affected proteins in *L. perenne* roots were identified as isozymes of methionine adenosyltransferase; two isozymes of this enzyme have previously been reported in *Oryza sativa* leaf tissue (Parker et al., 2006). In catalysing the metabolism of methionine to *S*-adenosylmethionine (SAM), methionine adenosyltransferase is directly involved in the further metabolism of glycine. A major metabolic role of SAM is donation of methyl directly involved in the further metabolism of glycine. In catalysing the metabolism of methionine to *S*-adenosylmethionine (SAM), methionine adenosyltransferase is directly involved in the further metabolism of glycine. A major metabolic role of SAM is donation of methyl groups (Rhodes et al., 1999), whilst that of another identified protein adenosine kinase is in recycling of adenosyl and methyl groups (Moffatt et al., 2000; Stasolla et al., 2001). Adenosine kinase can, therefore, also be considered to be involved in the further metabolism of glycine. That protein expression of both an isozyme of methionine adenosyltransferase and adenosine kinase shared a linear relationship, increasing when challenged with glycine, indicates that abundance of individual proteins can be used as an effective measure of current plant utilization of N, particularly where the proteins can be ascribed a specific role within the metabolic pathways for N assimilation. This supports the view that proteomics is a useful tool to study plant responses to their environment, even when the species studied is selected on the basis of its ecological relevance, rather than the extent to which it has been characterized at the molecular level.

Either gene expression or protein abundances of three of the currently identified proteins have been shown to be up-regulated in plants in response to increased nitrate supply. In roots of *Lycopersicon esculentum*, gene expression of methionine adenosyltransferase was upregulated in response to increased nitrate (Wang et al., 2001) and the abundance of the protein itself was greater in *A. thaliana* cell culture when supplied medium containing increased concentrations of nitrate, ammonium, and glutamine (Sarry et al., 2006). Increased nitrate supply increased gene expression of malate dehydrogenase in roots, shoots (Wang et al., 2003), and whole seedlings of *A. thaliana* (Wang et al., 2000); however, in leaves of *T. aestivum*, an interaction between cultivar and N supply was found for the expression of this protein (Bahrman et al., 2004). Gene expression of glyceraldehyde-3-phosphate dehydrogenase was increased in *L. esculentum* roots with increased nitrate supply (Wang et al., 2001); expression of the protein itself was increased in grain of *T. aestivum* which received an increased supply of ammonium nitrate (Flæte et al., 2005).

Based on these previous results, it would be expected that expression of methionine adenosyltransferase, malate dehydrogenase, and glyceraldehyde-3-phosphate dehydrogenase would decrease when challenged with glycine, i.e. conditions where nitrate uptake was zero and root nitrate concentrations were reduced. However, only glyceraldehyde-3-phosphate dehydrogenase followed this pattern. Expression of malate dehydrogenase and an isozyme of methionine adenosyltransferase was unaffected by the form of N supplied, whilst expression of the other isozyme of methionine adenosyltransferase increased when challenged with glycine. Wang et al. (2004), using a nitrate reductase-null mutant of *A. thaliana*, showed that, of these three proteins, only glyceraldehyde-3-phosphate dehydrogenase responded to nitrate itself, the other two proteins responding to some downstream metabolite of nitrate. Therefore, when downstream metabolites of nitrate are involved in feedback regulation of a protein, differing responses to reducing nitrate (i.e. N supply) and replacing ammonium nitrate with glycine (i.e. N form) occur. That the influence of N supply and form of N on *in planta* N metabolism can be separated illustrates the potential of targeted proteomics to disentangle closely related processes, and to provide effective indicators of these processes where their direct measurement is impractical. This highlights the potential of proteomics to support identification of processes occurring in complex environments (i.e. field conditions), where more traditional methods cannot resolve multiple, co-incident biological interactions and element fluxes.

Acknowledgements

We thank MR Tyler (Macaulay Institute), E Argo and EC Stewart (both Aberdeen Proteome Facility) for skilled technical assistance, Y Cook (Macaulay Institute) for nitrate analysis, members of the Macaulay Institute analytical group for IR-MS and GC-MS analyses, and EI Duff and J Potts (Biomathematics and Statistics Scotland) for statistical advice. We thank Professor P Millard and two anonymous referees for their constructive comments on earlier versions of the manuscript. The Scottish Executive Rural Affairs Department funded this work.

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