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Arabidopsis and *Lobelia anceps* access small peptides as a nitrogen source for growth

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Abstract. While importance of amino acids as a nitrogen source for plants is increasingly recognised, other organic N sources including small peptides have received less attention. We assessed the capacity of functionally different species, annual and nonmycorrhizal *Arabidopsis thaliana* (L.) Heynh. (Brassicaceae) and perennial *Lobelia anceps* L.f. (Campanulaceae), to acquire, metabolise and use small peptides as a N source independent of symbionts. Plants were grown axenically on media supplemented with small peptides (2–4 amino acids), amino acids or inorganic N. In *A. thaliana*, peptides of up to four amino acid residues sustained growth and supported up to 74% of the maximum biomass accumulation achieved with inorganic N. Peptides also supported growth of *L. anceps*, but to a lesser extent. Using metabolite analysis, a proportion of the peptides supplied in the medium were detected intact in root and shoot tissue together with their metabolic products. Nitrogen source preferences, growth responses and shoot–root biomass allocation were species-specific and suggest caution in the use of *Arabidopsis* as the sole plant model. In particular, glycine peptides of increasing length induced effects ranging from complete inhibition to marked stimulation of root growth. This study contributes to emerging evidence that plants can acquire and metabolise organic N beyond amino acids.

Additional keywords: amino acids, organic nitrogen, plant nutrition.

Introduction

While many plants can access organic nitrogen (ON) in the form of amino acids, the extent to which ON contributes to plant N nutrition remains debated (reviewed by Näsholm *et al.* 2009). The wide-ranging ability of plants to acquire and use amino acids as a source of N for growth has been demonstrated on many levels; evidence spans from the presence of amino acids in soil (Abuarghub and Read 1988; Kielland 1995; Jones and Kielland 2002; Jämtgård *et al.* 2010), to characterisation of amino acid transporters in plant membranes (Hirner *et al.* 2006; Lee *et al.* 2007; Svennerstam *et al.* 2008) and uptake of amino acids under field conditions (Näsholm *et al.* 2000; McKane *et al.* 2002; McFarland *et al.* 2010). Only recently has the focus on amino acids expanded to consider more complex ON sources, although this research has been primarily restricted to proteins or peptides with molecular mass greater than 3 kDa (Paungfoo-Lonhienne *et al.* 2008; Adamczyk *et al.* 2009) and little consideration has been given to intermediate compounds such as small peptides. Extending our understanding of plant ON use is crucial to addressing concerns over increases in reactive N in the biosphere, considered a problem of major global significance that is caused, to a large extent, by inefficient use of man-made inorganic N (IN) fertilisers in bioproduction systems (Gruber and Galloway 2008; Rockström *et al.* 2009). Defining the role of ON in plant nutrition will inform the design of N-sustainable bioproduction systems with more efficient N use than in current IN-dominated systems characterised by high N losses.

Though proteinaceous ON dominates the soil N pool (Schulten and Schnitzer 1997), the soluble ON fraction of intermediate mass remains poorly characterised due to technical difficulties associated with isolating and analysing N compounds larger than single amino acids. Low molecular mass peptides (<5 kD, < ~43 amino acids) can represent 4–15% of the total N pool in some soils (Warman and Isnor 1989; Isnor and Warman 1990), and soluble peptides of undefined mass occur in the same concentration range as free amino acids or IN in temperate forest soil (Rothstein 2009). The spatial heterogeneity of ON distribution in soil is well recognised: although bulk soil concentrations of small ON compounds such as amino acids may be in the micromolar range, Jones *et al.* (2005) point out that concentrations in plant and animal cells occur in the millimolar range and argue that cell lysis is thus expected to create transient patches of high-concentration ON. A similar scenario is likely to exist for oligopeptides (2–10 amino acids) originating from both from cellular material and from the breakdown of complex ON compounds in decaying residues, though prevalence and turnover rates remain to be established.

Ecto- and ericoid-mycorrhizal symbionts enable plant access to ON of high molecular mass (Read 1991), but this ability is not restricted to plants with mycorrhizal symbionts. Nonmycorrhizal species including *Hakea actites* W. R. Barker (Proteaceae) and *Arabidopsis thaliana* (L.) Heynh. use di- and tripeptides and protein for growth (Schmidt *et al.* 2003; Komarova *et al.* 2008; Paungfoo-Lonhienne *et al.* 2008). Internal peptide transport is a

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well recognised process in plants (Stacey *et al*. 2002; Tegeder and Rentsch 2010) and multiple peptide transporters (PTRs) and oligopeptide transporters (OPTs) have been identified in several plant species (Miranda *et al*. 2003; Zhao *et al*. 2010). Such transporters may play various roles in seed germination, source–sink translocation, development and uptake of peptides from the soil. *Arabidopsis* mutants overexpressing di- and tripeptide transporters had significantly greater biomass and N content than wild-type plants and *atptr1* mutants when grown with peptides as a sole N source (Komarova *et al*. 2008). The increased growth of overexpressing lines with dipeptides versus amino acids supports the notion that peptides are taken up intact and demonstrates a role for peptide transport in utilisation of peptide N for growth (Komarova *et al*. 2008). This process is confirmed in *H. actites*, where dipeptide glycine (Gly)–Gly enters roots intact and is subsequently transported and metabolised in shoots (Paungfoo-Lonhienne *et al*. 2009); tripeptide uptake has been observed in wheat (*Triticum aestivum* L.) (Hill *et al*. 2011).

Analysis of metabolite pools to trace assimilation of ON compounds has occurred predominantly for amino acids supplied in short-term uptake studies. Experiments demonstrate that the rate of amino acid uptake and metabolism varies based on plant species, plant internal N status and amino acid source (Schmidt and Stewart 1999; Thornton 2001; Thornton and Robinson 2005; Persson *et al*. 2006; Forsum *et al*. 2008). Additionally, although several studies have examined plant uptake preferences for IN versus ON (usually using the amino acid glycine as the ON source) (Miller and Bowman 2003; Thornton and Robinson 2005; Ashton *et al*. 2010), comparisons of preferences for different ON compounds across species are less common and are limited to a small range of single amino acids (Turnbull *et al*. 1995; Harrison *et al*. 2007; Andresen *et al*. 2008).

Knowledge gaps include the (i) composition of soluble ON of low to intermediate molecular mass in soils; (ii) innate ability of plants to access ON larger than single amino acids for growth; and (iii) effects of ON on plant development. Here, the two latter issues were addressed by comparing the capacity of two functionally different herbaceous species to take up, metabolise and use peptides as an N source for growth. Nonmycorrhizal annual *A. thaliana* (Brassicaceae), the most widely used model plant for studying N relations (Vidal and Gutiérrez 2008), was compared with *Lobelia anceps* L.f. (Campanulaceae;, formerly *L. alata*), a previously unstudied species that can be cultivated under the same experimental conditions. *L. anceps* is a widely distributed southern hemisphere perennial with very small seeds, which, like those of *A. thaliana*, have minimal N stores. *L. anceps* forms vesicular arbuscular mycorrhizal associations (Warcup 1988) but was cultivated here without mycorrhizal symbionts to assess its intrinsic ability to use different N sources.

Materials and methods

Plant material and growth conditions

Seeds of *Arabidopsis thaliana* (L.) Heynh. (ecotype Columbia [Col-0]) and *Lobelia anceps* L.f. (formerly *Lobelia alata*; N. Walsh and D. Albrecht, Western Australian Herbarium, pers. comm.) collected at Boddington, Western Australia (Nindethana Seed Service, Albany, WA, Australia) were sterilised, germinated and grown axenically on the following media: Medium A (no N; control): N-free MS medium (Murashige and Skoog 1962) composed of MS basal salt micronutrient solution (Sigma-Aldrich, Sydney, NSW, Australia) supplemented with 3 mM $CaCl_2$, 1.5 mM $MgSO_4$ and either 1.25 mM KH_2PO_4 (*A. thaliana*) or 0.625 mM KH_2PO_4 (*L. anceps*). pH was adjusted to 5.3 using KOH and media supplemented with 1% sucrose and 0.3% Phytagel (Sigma-Aldrich). Medium B: as Medium A, but supplemented with 10 mM N as either NH_4NO_3, leucine (L)–alanine (Ala), L–glutamine (Gln), L–Gly, L–phenylalanine (Phe), Gly–Ala, Ala–Gln, Ala–Ala, Gly–Phe, Gly–Gly, Gly–Gly–Gly or Gly–Gly–Gly–Gly (Sigma-Aldrich). Sources were supplied so that each treatment received the same quantity of N (1.4 mg N mL^{-1}). N sources were filter-sterilised using 0.22-µm syringe filters (Millipore, Bedford, MA, USA) and added to sterile medium. ON sources were selected to represent the most abundant amino acids in soil solution (Rothstein 2009; Jämtgård *et al*. 2010).

Twenty-five millilitres of medium was used per Petri dish (85 mm diameter) and seeds were sown evenly spaced at a density of ~60 per plate for *A. thaliana* and ~100 per plate for *L. anceps* (due to a lower germination rate that resulted in an average of 35 germinated seeds per plate (Soper, unpubl. data)). Plates were sealed with gas-permeable Micropore tape (3M, St. Paul, MN, USA) and cultivated in a growth room at 21°C with 16 h : 8 h day : night cycle and a uniform light intensity of ~150 µmol m^{-2} s^{-1} at plant level. *A. thaliana* seedlings were cultivated for 20 days and *L. anceps* for 35 days, owing to the slower growth rate of this species.

Upon harvesting, plants were separated into root and shoot tissue, and washed with 0.5 mM $CaCl_2$ to remove N from plant surfaces. A sample of 6–10 plants (depending on individual plant size) from each replicate was frozen immediately in liquid N_2 for ultra performance liquid chromatography (UPLC) analysis. Four to seven independent replicates (individual plates) per species for each treatment were analysed for biomass, after visibly contaminated plates were discarded over the course of the experiment. Tissue was oven-dried for 48 h at 60°C and weighed.

UPLC analysis

Amino acid, ammonium and supplied peptide concentrations in root and shoot tissue were quantified using an Acquity Ultra Performance Liquid Chromatograph equipped with Bridged Ethyl Hybrid C_{18} 1.7 µm 2.1 × 100 mm column and tuneable UV detector at 254 nm (Waters, Milford, MA, USA) (n = 3–6 tissue samples for each species–tissue–treatment combination). Frozen tissue was homogenised and extracted in 250 µL 20% methanol. Samples were centrifuged at 13 000*g* for 10 min at 4°C. For analysis, 40 µL of sample extract was mixed with 120 µL borate buffer and 40 µL AccQ-Tag reagent (AccQ-Tag derivatisation kit, Waters, Milford, MA, USA). Tissue pellets were dried overnight at 55°C and weighed.

Determination of nitrate

Assays of nitrate content in root and shoot extracts was performed using a microtitre plate spectrophotometer method; 40 µL of

65 mM $VaCl_3$ in 1 M HCl was added to 40 µL of sample (same extract as for UPLC analysis, $n = 3–6$) to reduce nitrate to nitrite (Miranda *et al*. 2001) and 20 µL each of 2% w/v sulfanilamide in 5% HCl and 0.1% w/v N-(1-naphthyl) ethylenediamine dihydrochoride in H_2O were added. Samples were incubated in the dark at room temperature for 60 min and absorbance was measured at 540 nm using a plate reader (Powerwave XS, Bio-Tek, Winooski, VT, USA). Replicate subsamples were incubated without addition of $VaCl_3$ and absorbance values were subtracted from the sample values to correct for interference from amino acids (Miranda *et al*. 2001).

Total N analysis

Root and shoot dry tissue was combined and homogenised, and total N was determined using a PDZ Europa ANCA-GSL elemental analyser interfaced with a PDZ Europa 20–20 isotope ratio mass spectrometer (Sercon Ltd, Cheshire, UK) at the UC Davis Stable Isotope Facility (Davis, CA, USA). Very low total biomass in the control and Gly–Phe (*A. thaliana*) treatments necessitated pooling of replicates for %N analysis ($n = 2$); for all other treatments, $n = 4–5$.

Statistical analysis

Data were analysed using GraphPad Prism (GraphPad Software, San Diego, CA, USA). Significant differences between treatments were determined by log-transforming data to correct for unequal variance and then analysing with one-way ANOVA followed by Tukey's *post hoc* test. For analysis of proportion data the dataset was first subjected to arcsine transformation.

Results

Peptides support growth of A. thaliana *and* L. anceps

To determine whether plants can use peptides as a sole N source, *A. thaliana* and *L. anceps* were grown with 10 mM N in the form of di-, tri- or tetrapeptides, or their constituent amino acids, or NH_4NO_3. In both species, all supplied peptide sources supported significantly greater biomass production than the control (no N) (Fig. 1*a*, *b*). Control plants germinated but showed no subsequent growth. Plants supplied with Phe failed to germinate in both species (not shown) and were excluded from analysis.

In *A. thaliana*, mean biomass production was greatest in plants supplied with NH_4NO_3, but did not differ significantly from that produced by Gln, Gly–Ala and Ala–Gln (Fig. 1*a*). Biomass achieved with peptides, on average, ranged from 34 (Gly–Gly) to 74% (Ala–Gln) of that produced by NH_4NO_3, with the exception of putatively toxic peptide Gly–Phe, where this value was ~5% (Fig. 1*a*). Root growth of *A. thaliana* was almost completely inhibited by 10 mM Gly, and total growth in Gly and Ala treatments was poor compared with Gln (Fig. 1*a*). Biomass produced in Gly–Gly, Gly–Gly–Gly and Gly–Gly–Gly–Gly treatments was 230%, 460% and 320% of the Gly treatment, respectively, and dipeptide Ala–Ala produced 310% of the biomass of Ala.

L. anceps showed a lesser capacity to use supplied peptides for growth relative to the maximal growth seen with Gln (Fig. 1*b*). NH_4NO_3 supplied at 10 mM N almost completely inhibited root growth of *L. anceps*; as with no-N control plants, roots were too small to be separated from shoot and combined biomass is shown. Relative to Gln, peptides produced only between 19% (Ala–Ala and Gly–Phe) and 37% (Gly–Gly–Gly–Gly) as much biomass overall, though all ON forms promoted growth significantly above the no-N control (Fig. 1*b*). Unlike *A. thaliana*, *L. anceps* Ala, Gly and Gly–Phe treatments supported growth comparable to other peptide sources and no peptides supported higher growth than their constituent amino acids.

Glycine peptide length affects root growth and shoot : root ratio

A. thaliana and *L. anceps* exhibited differing root growth and biomass allocation responses when supplied with Gly peptides of increasing length (Table 1, Figs 1*a*, *b*, 2). *A. thaliana* plants grown on Gly media showed near complete inhibition of root growth, while the peptides Gly–Gly and Gly–Gly–Gly promoted the greatest root biomass response (Figs 1*a*, 2). In *A. thaliana*, relative allocation of biomass to root tissue, as expressed by shoot : root ratio, decreased with increasing Gly peptide length (Table 1). Plants supplied with Gly–Gly–Gly–Gly displayed a shoot : root DW ratio of 6.6 ± 0.7; a 3-fold increase over those grown with Gly–Gly, despite the total plant biomass being similar between the two treatments (Table 1, Fig. 1*a*).

In *L. anceps*, Gly–Gly–Gly–Gly induced growth of very long and unbranched roots, which differed morphologically from those observed in other treatments (Fig. 2). A significant increase in root biomass was observed in plants supplied with Gly–Gly–Gly–Gly, at least 3-fold greater on average than was observed in other Gly and Gly peptide treatments, despite total plant biomass remaining constant (Figs 1*b*, 2). This pattern was also reflected by a substantially reduced shoot : root ratio in the Gly–Gly–Gly–Gly treatment (Table 1).

N source influences the magnitude of the soluble N pool

All *A. thaliana* peptide treatments (excluding Gly–Phe, which was not analysed due to inadequate weight) accumulated as much or significantly more total N (µg N g DW^{-1}) than plants supplied with NH_4NO_3, including treatments where total biomass was significantly lower (e.g. Gly–Gly, Fig. 1*a*, *c*). In *A, thaliana*, the percentage of measured soluble N (NH_4^+, NO_3^-, amino acids and supplied peptides) over total N was greatest in the Ala (soluble N averaged 96% of total N) and Gly (79%) treatments (Fig. 1*c*). Other treatments which supported significantly greater biomass than Ala and Gly comprised <61% average soluble N (Fig. 1*a*, *c*).

The *L. anceps* Gly treatment also displayed a high percentage of soluble N in tissue (an average 91% of total N), but was not associated with low biomass production (Fig. 1*b*, *d*). The NH_4NO_3 treatment accumulated 88% soluble N and displayed inhibition of root growth (Fig. 1*b*, *d*).

Supplied peptides can be taken up intact

Some peptides supplied in the growth media could be detected in root and shoot tissue, but accounted for <6% of the measured soluble N pool in any peptide treatment (Table 2). To adjust for large variations in the size of the measured soluble N pool between treatments (Fig. 1*c*, *d*) and to allow comparison, individual peptide concentrations were expressed as a percentage of the total measured soluble N concentration. Percentage values for each peptide are displayed for only the

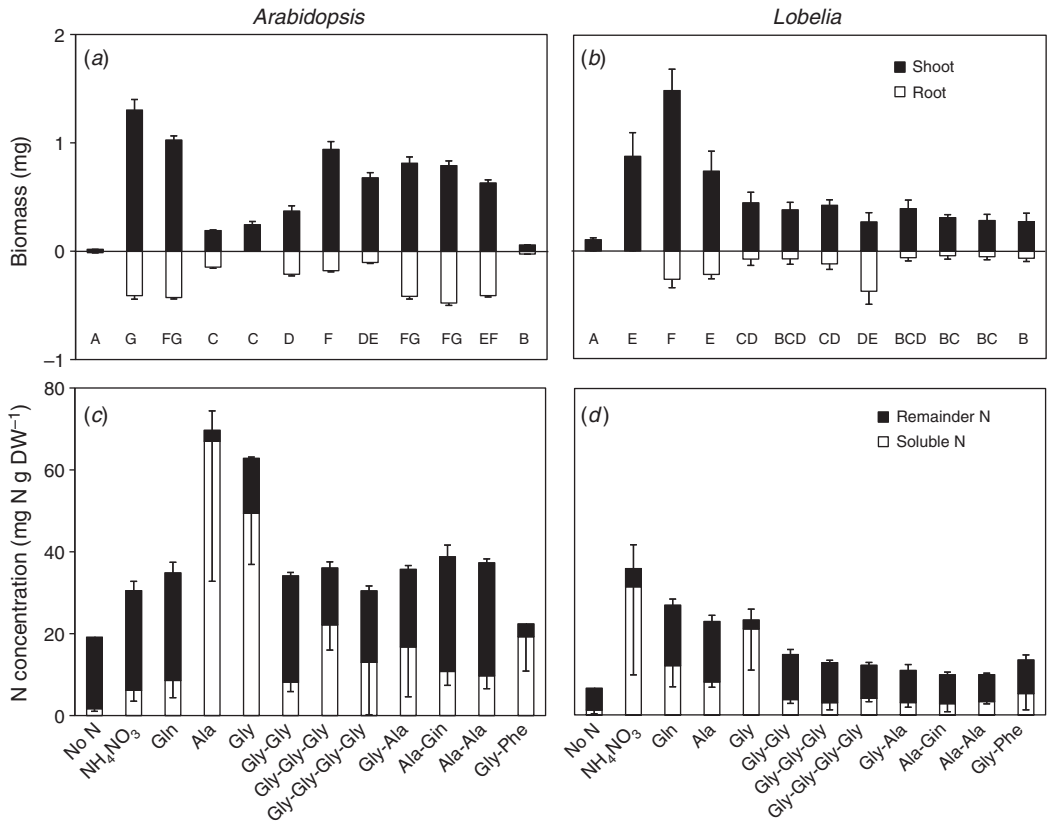

Fig. 1. (*a*, *b*) Biomass (mg DW per plant) and (*c*, *d*) tissue N concentration (mg N g DW^{-1}) of (*a*, *c*) *Arabidopsis thaliana* and (*b*, *d*) *Lobelia anceps*. (*a*, *b*) Root (open bars) and shoot (closed bars). (*c*, *d*) Soluble N (open bars, sum of free amino acids, measured peptides, ammonium and nitrate) and 'remainder N' (closed bars, equal to total N minus measured free amino acids, measured peptides, ammonium and nitrate). Total root and shoot DW per plate was divided by the number of plants per plate to obtain average biomass values for each plate. Bars represent the mean of 4–7 replicates (plates) ± s.d. Values associated with the same letter indicate non-significant differences within species ($P < 0.05$). Roots of *L. anceps* no-N (control) seedlings were too small to be separated from shoots and total biomass is displayed. Values represent the mean of 2–5 replicates for 'remainder N' and 3–6 replicates for soluble N ± s.d.

Table 1. Shoot : root DW ratio of *Arabidopsis thaliana* and *Lobelia anceps* grown with no N, or 10 mM N NH_4NO_3, Gly or Gly peptides
Values represent the mean of 4–7 replicates ± s.d. n.d., not determined. Values associated with the same letter indicate nonsignificant differences within each column ($P < 0.05$).

N source	Shoot : root ratio	
	Arabidopsis thaliana	*Lobelia anceps*
No N	1.3 ± 0.4^{a}	n.d.
NH_4NO_3	3.2 ± 0.2^{d}	n.d.
Gly	n.d.	3.8 ± 1.1^{bc}
Gly–Gly	1.7 ± 0.3^{ac}	4.9 ± 2.0^{bc}
Gly–Gly–Gly	5.2 ± 0.8^{e}	4.2 ± 2.6^{bc}
Gly–Gly–Gly–Gly	6.6 ± 0.7^{f}	0.7 ± 0.2^{a}

treatment where that peptide was the supplied N source (Table 2). Uptake of intact peptide was considered to be significant only in cases where a given peptide was detected at a significantly higher concentration in the treatments where it was directly supplied, compared with treatments where it was not supplied. This criterion controls for the possibility that some peptides may occur endogenously in plant tissue. Ala–Ala, for example, was detected at low levels in multiple tissues and treatments of both species (data not shown) and its stereoisomer D-alanyl-L-alanine (D–Ala–L–Ala) can be synthesised endogenously in wild rice, *Oryza sativa* L. (Manabe 1992).

Gly–Gly, Gly–Gly–Gly and Gly–Phe peptides were detected at significant levels in the root tissue of *A. thaliana* plants supplied with those sources (corresponding to absolute concentrations of 5.28 ± 5.14, 3.53 ± 3.65 and 18.5 ± 20.94 µmol mg DW^{-1}, respectively); similarly, Gly–Gly was detected in shoot tissue (2.68 ± 1.26 µmol mg DW^{-1}; data not shown). In *L. anceps*, Gly–Phe and Gly–Gly were detected at a significant level in the root tissue of those treatments (3.80 ± 4.92 and 14.06 ± 5.86 µmol mg DW^{-1}) and Gly–Phe was detected in shoot tissue (2.38 ± 5.06 µmol mg DW^{-1}; data not shown).

Peptide metabolites can be traced in tissue

The amino acid composition of the soluble N pool was analysed to discern uptake and assimilation of N sources. A metabolic 'signature' of Gly-based peptides was evident in both species. In

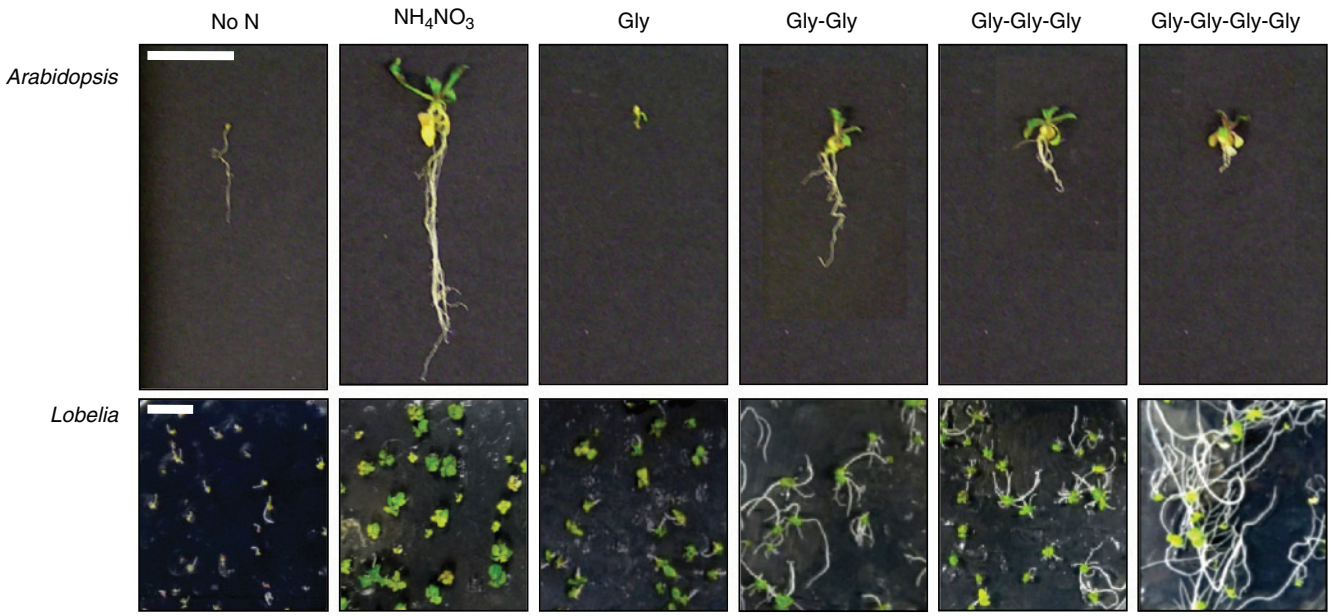

Fig. 2. Root morphology of *Arabidopsis thaliana* and *Lobelia anceps* grown with no N, or 10 mM N NH_4NO_3, Gly or Gly peptides. Photos show representative individuals of *A. thaliana* and plates of *L. anceps*. Scale bar = 1 cm.

Table 2. Intact source peptides in root and shoot tissue of *Arabidopsis thaliana* and *Lobelia anceps* grown with 10 mM N peptide sources
Values are expressed as a percentage of the concentration of the total measured soluble N in each tissue and represent the mean of 3–6 replicates ± s.d. n.d., not determined; d.l., detection limit.* indicates that a value differs significantly ($P < 0.05$) from the percentage of the indicated peptide detected in plants not supplied with that peptide (10 treatments, data not shown) based on ANOVA analysis of arcsine transformed proportion data.

N source	Percentage of source peptide in soluble N pool (%)			
	Arabidopsis thaliana		*Lobelia anceps*	
	Root	Shoot	Root	Shoot
Gly–Gly	0.8 ± 0.7*	1.3 ± 0.5*	5.1 ± 2.5*	1.3 ± 1.7
Gly–Gly–Gly	0.6 ± 0.6*	0.3 ± 0.4	1.3 ± 0.1	< d.l.
Gly–Gly–Gly–Gly	n.d.	1.2 ± 1.4	4.3 ± 4.0	3.0 ± 2.6
Gly–Ala	0.1 ± 0.2	<d.l.	0.9 ± 2.1	<d.l.
Ala–Gln	0.2 ± 0.1	<d.l.	<d.l.	<d.l.
Ala–Ala	0.1 ± 0.1	0.2 ± 0.3	0.8 ± 0.9	1.5 ± 1.8
Gly–Phe	3.1 ± 3.8*	1.0 ± 0.8	2.5 ± 1.9*	2.6 ± 2.1*

A. thaliana, serine (Ser) (the immediate metabolic product of Gly) accumulation was evident in root and shoot tissues of Gly-based peptide treatments, significantly elevated above both the no N control and NH_4NO_3 treatments (Fig. 3*a*, *c*; for absolute concentrations of metabolites see Table S1, available as an Accessory Publication to this paper). In *L. anceps*, Ser accumulated significantly in roots of Gly–Gly, Gly–Gly–Gly and Gly–Gly–Gly–Gly treatments, but not in the Gly–Ala or Gly–Phe treatments (Fig. 3*d*) or in shoot tissue (Fig. 3*b*).

Ala-based peptide treatments in *A. thaliana* (Ala–Ala, Ala–Gln and Ala–Gly) showed significant accumulation of Ala in the soluble root N pool compared with NH_4NO_3 and the no N control. In *L. anceps*, Ala accumulated in roots of plants supplied with single Ala but not in Ala-based peptide treatments (Fig. 3*d*).

Gln was prominent in *A. thaliana* shoots of all peptide treatments, except Gly–Phe, which supported minimal growth (Figs 1*a*, 3*a*). Particularly high accumulation of Gln occurred in *A. thaliana* shoots of the Ala and Gly treatments, showing a 10-fold higher concentration than observed in any other treatment (Table S1). In *L. anceps* peptide treatments (with the exception of Gly–Gly) proportionally more N occurred as NH_4^+ (15–42%) than as Gln (11–30%) in shoot tissue, though these differences between treatments did not follow the same pattern as overall growth (Figs 1*b*, 3*b*). Absolute concentrations of NH_4^+ in *L. anceps* peptide treatments were in the same order of magnitude as for *A. thaliana*, despite comprising a significantly greater percentage of the soluble pool (Table S1, Fig. 3*b*, *d*).

Phe accumulated in roots and shoots of plants of both species treated with Gly–Phe in a significantly greater proportion than in any other treatment, up to a maximum of 64% of soluble N in *A. thaliana* shoots (Fig. 3). Absolute and proportional accumulation of Phe was at least 3-fold or greater in *A. thaliana* than in *L. anceps* (Table S1, Fig. 3). *L. anceps* displayed greater accumulation of N storage compound arginine (Arg) in shoots, comprising 3–23% of the soluble N pool in *L. anceps* but only <1–7% in *A. thaliana* (Fig. 3*a*, *b*).

Discussion

This study provides evidence in support of the notion that plants access small peptides as an N source (Schmidt *et al.* 2003; Komarova *et al.* 2008) and demonstrates that (i) exogenously supplied peptides of up to four amino acids are acquired and support growth; (ii) peptides and peptide metabolites can be traced in plant tissue; (iii) certain peptides have pronounced effects on root growth and shoot : root ratio; and

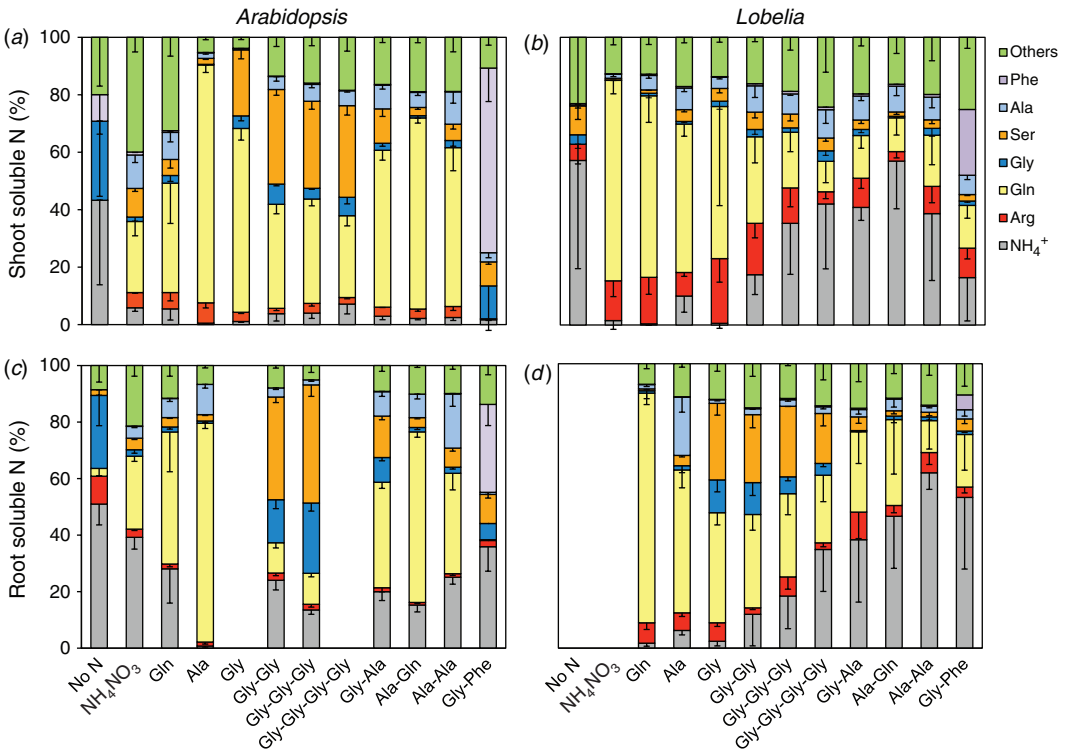

Fig. 3. Proportion of ammonium and selected amino acids in (*a*, *b*) shoot and (*c*, *d*) root tissue of (*a*, *c*) *Arabidopsis thaliana* and (*b*, *d*) *Lobelia anceps* plants grown with no N or 10 mM N of 11 N sources, expressed as a percentage of the total measured soluble N pool. Plants were germinated and grown axenically for 20 days (*A. thaliana*) or 35 days (*L. anceps*) on N-free MS media with no N or supplemented with 10 mM N of indicated N sources. Missing root tissue values occur where insufficient tissue was available to perform UPLC analysis, or where root and shoot tissues could not be separated adequately. 'Other' represents the sum of all other free amino acids plus nitrate. Bars represent the mean of 3–6 replicates ± s.d.

(iv) *A. thaliana* and *L. anceps* differ in their capacity to use peptides as an N source for biomass allocation under axenic growth conditions. While plant species from many functional groups show the capacity to take up amino acids, few studies have traced the subsequent metabolic fate of ON (Näsholm *et al.* 2009). The experimental system used in this study allows concurrent examination of acquisition, metabolism and transport of ON as well as determining effects on biomass allocation. Nitrogen was supplied as single source at a relatively high concentration (10 mM), as the aim of this study was to identify the innate capacity of species to access peptide N and gain insights into the mechanisms of interspecies variation in peptide N use, rather than to mimic field conditions. These artificial experimental conditions are necessary in the absence of continued N input into the axenic growth system and are common to studies of this type (e.g. Komarova *et al.* 2008). Axenic experiments, unlike the more commonly used ^{15}N–^{13}C label soil injection technique, eliminate the uncertainty associated with symbiont-mediated versus direct plant uptake and exclude microbial processing of supplied ON before plant uptake.

Both *A. thaliana* and *L. anceps* used the supplied di-, tri- and tetrapeptides as a sole N source. *A. thaliana* differed in its capacity to use different peptide sources, with growth varying from 34–74% of maximal biomass achieved with IN. In the nonmycorrhizal state tested here, *L. anceps* displayed a lesser ability overall to use peptide N than *A. thaliana*, with biomass not exceeding 36% of the maximum growth achieved with Gln. Relative growth responses to specific peptides, such as Gly–Phe, were distinct between species and support the notion that taxa differ in the capacity to access ON sources (McKane *et al.* 2002; Miller and Bowman 2003).

In *A. thaliana*, the disparity between poor growth on single amino acids Gly and Ala, and significantly greater biomass production when Gly and Ala are supplied in their peptide form may be explained by concentration effects. High concentrations of certain amino acids are known to inhibit plant growth (Voll *et al.* 2004; Forsum *et al.* 2008; Näsholm *et al.* 2009), and the accumulation of soluble N (in particular high concentrations of Gln) in *A. thaliana* tissue supplied with Gly and Ala suggests a bottleneck in downstream metabolism. At 3 mM N, Gly and Ala are taken up more effectively than Gln in *A. thaliana*, but produce substantially less biomass (Forsum *et al.* 2008). Supplying concentrations of 10 mM N in this experiment may have further exacerbated the disparity between uptake and efficient metabolism for growth. When supplied with peptides, uptake and breakdown processes may expose plants to a lower internal physiological concentration of free amino acids, so that concentration-dependent inhibition of N metabolism (reflected by soluble N accumulation) and subsequent growth inhibition are not observed. Clearly, further research needs to examine the ability of plants to acquire and metabolise the complex ON mixtures characteristic of soils.

Some di- and tripeptides supplied in the growth media were detected in root tissues of *A. thaliana* or *L. anceps*, suggesting that peptides rather than component amino acids were taken up. The possibility that a proportion of the supplied peptides are hydrolysed before uptake cannot be excluded, as several species including *A. thaliana*, *H. actites*, wheat and leek (*Allium porrum* L.) exude proteases that are capable of degrading proteins (Adamczyk *et al*. 2008, 2009; Paungfoo-Lonhienne *et al*. 2008). Arguably, if the supplied peptides were hydrolysed to free amino acids before uptake, the resultant growth or growth inhibition responses in peptide treatments would resemble those seen in plants supplied with the constituent amino acids, and the metabolic responses to amino acids and peptides would be more congruent. For example, *A. thaliana* supplied with Ala grew poorly compared with other ON sources, and tissue-soluble N pools were heavily dominated by Gln (77–83% of soluble N); by contrase, plants supplied with Ala–Ala showed lower Gln accumulation (36–55%) and produced 3-fold greater biomass. This is consistent with evidence from other species and experimental approaches that peptides are taken up directly rather than being completely broken down first (Komarova *et al*. 2008; Paungfoo-Lonhienne *et al*. 2009).

When peptides were not detected in tissue, their metabolic products could be detected. For example, accumulation of free Ala was demonstrated in *A. thaliana* roots supplied with Ala peptides. Metabolic changes were particularly evident with Gly peptides, where free Gly and its' immediate metabolite Ser constituted a significantly greater fraction of the measured soluble N pool than in other treatments. Ser can be synthesised directly from Gly (Keys 1980) and has been traced as the immediate metabolic product, primarily by transamination, in plants supplied with Gly (Schmidt and Stewart 1999; Thornton 2001). Here, Ser accumulation in shoot tissue may have occurred either as a result of root to shoot Ser transport or *in situ* metabolism of Gly. Although *L. anceps* also accumulated Ser in root tissue of Gly treatments, significant transport or accumulation of Ser in shoot tissue was not evident, providing further indication for differential metabolism of Gly and resultant metabolites between the two species.

Where N sources supported growth to different extents in both species, metabolite analyses pointed to differences in metabolic capacity for some N compounds. Gly–Phe was supplied as a putatively toxic peptide, as Phe and Ala–Phe have been observed to cause toxicity in *A. thaliana*, inhibit growth and cause imbalances in the amino acid pool that perturb homeostasis (Voll *et al*. 2004; Komarova *et al*. 2008). Although Gly–Phe supported only minimal growth in *A. thaliana*, in *Lobelia* biomass was comparable to other peptide sources. Uptake and breakdown of the peptide was evident in *A. thaliana*, where free Phe, Gly and Ser dominated the soluble N pool; in *L. anceps*, these breakdown products did not accumulate to such a degree. Thus, it seems likely that the growth inhibition observed with Gly–Phe in *A. thaliana* compared with *L. anceps* is caused by Phe toxicity resulting from a lower capacity to metabolise the peptide breakdown products. Nitrogen limitation did not appear to be the primary factor constraining growth on peptide sources in *A. thaliana.* All peptides, except Gly–Phe, displayed comparable or higher accumulation of total and soluble N compared with NH_4NO_3, irrespective of total biomass. This suggests that metabolic constraints rather than uptake *per se* may be the factor limiting growth when peptides are supplied as the sole N source.

Supplying Gly peptides of increasing length resulted in markedly different root biomass allocation and morphology in *A. thaliana* and *L. anceps*. When supplied with Gly–Gly–Gly–Gly, *L. anceps* produced 3-fold or greater root biomass than other Gly-based treatments, despite attaining similar total biomass. The Gly–Gly–Gly–Gly treatment in *L. anceps* produced an almost 10-fold lower shoot : root ratio than in *A. thaliana*. However, *L. anceps* supplied with Gly–Gly–Gly–Gly had similar total and soluble N accumulation and amino acid profile to other Gly peptide treatments, suggesting that the tetrapeptide may have a signal-like effect on morphology rather than drastically changing the internal N status of the plants. The addition of sucrose to media is known to change the rooting morphology of *A. thaliana* (Malamy and Ryan 2001; Lee-Ho *et al*. 2007), but consistent sucrose supply across treatments, combined with comparable biomass and N content of plants, suggests that this factor is unlikely to be implicated in the variation of root growth observed in this study. It is known that some N compounds, including nitrate, L-glutamate and, to a lesser extent, L-tryptophan, act as external signals modulating root development (Walch-Liu *et al*. 2006*a*, 2006*b*; Forde and Walch-Liu 2009; Vidal *et al*. 2010) and that exogenous phytosulfokine peptides (4–5 amino acids) stimulate adventitious root formation (Matsubayashi and Sakagami 1996; Yamakawa *et al*. 1998), but much remains to be understood about the signalling mechanisms by which plants sense and respond to N. Exogenously supplied organic phosphorus enhances root growth in *A. thaliana* (Paungfoo-Lonhienne *et al*. 2010), pointing to a general role of organic molecules as signalling compounds to enhance root proliferation in organic matter-rich regions of the soil. To our knowledge, no other small peptides have been investigated for their role as exogenous signals for root development but the results here suggest that such research is warranted. The observation that Gly peptides affect root growth in a size- and species-dependent manner is especially relevant, given that Gly is most commonly used as a model amino acid for uptake studies (Thornton 2001; Näsholm *et al*. 2009).

Though the capacity of plants to access peptide N is established, the relevance of peptides in plant nutrition remains unclear until estimates of actual peptide uptake under field conditions become available. High proportions of ON in soil, spatial heterogeneity of decaying matter and transient localised patches of high-concentration soluble ON (Jones *et al*. 2005) suggest that peptide-N may contribute to the suite of N compounds available to plants. Additionally, considerations of the nutritional role of small peptides must take into account the role of mycorrhizal root associations in accessing ON. The results of this study demonstrate that naturally arbuscular mycorrhizal (AM) species *L. anceps*, like the nonmycorrhizal species *A. thaliana* and *H. actites*, has an inherent ability to use peptides as an N source that is independent of symbioses (Schmidt *et al*. 2003; Komarova *et al*. 2008; Paungfoo-Lonhienne *et al*. 2008, 2009). The majority of terrestrial plants form AM symbioses (Lambers *et al*. 2010), but there is no consensus on the ability of AM fungi to access complex ON (Smith and Read

2008), although there is evidence that AM fungi can take up at least single amino acids (Rains and Bledsoe 2007; Whiteside *et al*. 2009) and transfer amino acids to roots (Hawkins *et al*. 2000). Comparison of mycorrhizal and nonmycorrhizal *L. anceps* would allow assessment of whether symbiosis enhances the plant's innate capacity to access complex ON.

In summary, we demonstrate that two functionally different herbaceous species, *A. thaliana* and *L. anceps*, take up and metabolise small peptides. We confirm observations that di- and tripeptides support growth as a sole plant N source and expand the observed range of plant-accessible N sources to include tetrapeptides. We also find evidence for species-specific growth and metabolic responses to different peptide sources and suggest caution in the use of *Arabidopsis* as the sole plant model. Strongly enhanced root growth in response to some peptides warrants further investigation to determine whether exogenous peptides exert a signalling role. Findings suggest that ON beyond amino acids should be considered as a potential plant N source, and research efforts should be directed at establishing the abundance of peptides in soils and role for plant nutrition.

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