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Whole-plant and organ-level nitrogen isotope discrimination indicates modification of partitioning of assimilation, fluxes and allocation of nitrogen in knockout lines of *Arabidopsis thaliana*

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The nitrogen isotope composition ($\delta^{15}N$) of plants has potential to provide time-integrated information on nitrogen uptake, assimilation and allocation. Here, we take advantage of existing T-DNA and γ-ray mutant lines of *Arabidopsis thaliana* to modify whole-plant and organ-level nitrogen isotope composition. Nitrate reductase 2 (*nia2*), nitrate reductase 1 (*nia1*) and nitrate transporter (*nrt2*) mutant lines and the Col-0 wild type were grown hydroponically under steady-state NO_3^- conditions at either 100 or 1000 μM NO_3^- for 35 days. There were no significant effects on whole-plant discrimination and growth in the assimilatory mutants (*nia2* and *nia1*). Pronounced root vs leaf differences in $\delta^{15}N$, however, indicated that *nia2* had an increased proportion of nitrogen assimilation of NO_3^- in leaves while *nia1* had an increased proportion of assimilation in roots. These observations are consistent with reported ratios of *nia1* and *nia2* gene expression levels in leaves and roots. Greater whole-plant discrimination in *nrt2* indicated an increase in efflux of unassimilated NO_3^- back to the rooting medium. This phenotype was associated with an overall reduction in NO_3^- uptake, assimilation and decreased partitioning of NO_3^- assimilation to the leaves, presumably because of decreased symplastic intercellular movement of NO_3^- in the root. Although the results were more varied than expected, they are interpretable within the context of expected mechanisms of whole-plant and organ-level nitrogen isotope discrimination that indicate variation in nitrogen fluxes, assimilation and allocation between lines.

Introduction

Reducing the need for nitrogen fertilizer in global agricultural production represents one of the major challenges in the plant sciences. Nitrogen uptake and assimilation is a complex process that is a function of internal demand interacting with external supply. Although the localization and functional role of genes related to nitrogen uptake and assimilation have been well constrained, an integrated method of measuring nitrogen use during the complete plant life cycle still needs to be developed (Hirel et al. 2007). Traditional measures of nitrogen uptake and assimilation are often not conducive to an integrated view of nitrogen use without intensive and careful sampling to reflect temporal and spatial variability in expression, activity and regulation. Using nitrogen isotope composition of plants and plant parts has potential to provide

Abbreviation – GFP, green fluorescent protein; PPFD, photosynthetic photon flux density.

time-integrated information on nitrogen use at the whole plant and organ level.

Nitrogen isotope discrimination is a function of nitrogen use and provides an integrated picture of nitrogen uptake and assimilation that cannot be provided by traditional nitrogen-use assays (Evans, 2001). Abiotic stress (Yousfi et al. 2009, 2012), nitrogen availability (Buschhaus et al. unpublished), source (Evans et al. 1996) and CO_2 enrichment (Buschhaus et al. unpublished) can all impact whole-plant and organ-level nitrogen isotope discrimination. Environmental effects on nitrogen isotope composition have been reported suggesting a relationship between nitrogen isotope discrimination and external supply and internal demand. Although intraspecific variation in nitrogen isotope discrimination has been reported (Robinson et al. 1998, Pritchard and Guy 2005, Yousfi et al. 2009), the underlying reasons for this remain unclear. Several theoretical models have suggested physiological mechanisms responsible for plant and organ-level variations in nitrogen isotope composition (Comstock 2001, Evans, 2001, Robinson 2001). However, to date few studies have used empirical data to test these theoretical models of discrimination. As the understanding of nitrogen isotope discrimination becomes more refined, more accurately constrained parameters contributing to this variation can be estimated.

Any change to nitrogen supply or demand should affect nitrogen isotope discrimination. Changes in whole-plant $\delta^{15}N$ relative to source nitrogen $\delta^{15}N$ (fractionation) arise through isotopic discrimination (Comstock 2001, Evans 2001, Robinson 2001). Discrimination is the kinetically determined process whereby the heavier isotope (^{15}N) is 'discriminated' against causing a relatively greater fraction of the lighter isotope (^{14}N) to be incorporated into plant tissues (Handley and Raven 1992, Hayes 2001). Isotopic composition is expressed as $\delta^{15}N$ (‰):

$$\delta^{15}\mathrm{N}\,(‰) = \left(\frac{R_{\mathrm{sample}}}{R_{\mathrm{standard}}} - 1\right) \times 1000 \qquad (1)$$

where R_{sample} is the $^{15}N/^{14}N$ isotope ratio of the sample and $R_{standard}$ is the isotope ratio of a known standard ($^{15}N/^{14}N$ ratio of air = 0.00365). Therefore, when discrimination against ^{15}N occurs, the $^{15}N/^{14}N$ ratio for R_{sample} decreases and $\delta^{15}N$ becomes negative. The instantaneous discrimination factor for nitrate reductase has been best estimated at about 15‰ (Ledgard et al. 1985) where, in that work, the product of the reaction was −15‰ relative to the reaction substrate. However, recent work has suggested discrimination by nitrate reductase is >15‰ and is closer to 22‰ (Needoba et al. 2004, Tcherkez and Farquhar 2006) or 26‰ (Karsh et al. 2012). The instantaneous discrimination factor determines the maximum fractionation that can occur if cytoplasmic inorganic nitrogen $\delta^{15}N$ is equal to substrate $\delta^{15}N$. If total consumption of the substrate pool occurs, product $\delta^{15}N$ must be equal to source $\delta^{15}N$. Isotope fractionation is only observed when there is partial consumption of a substrate pool (Mariotti et al. 1981, Comstock, 2001). Organ-level isotope fractionation occurs at branch points where there is either loss of substrate or two or more pathways competing for the same substrate (Macko et al. 1987, Comstock 2001, Hayes 2001, Werner and Schmidt 2002, Tcherkez 2011, Gauthier et al. 2012).

Whole-plant nitrogen isotope discrimination is thought to be mainly a function of one inward and three outward fluxes: (1) gross influx from the rooting medium, (2) partial assimilation by root nitrate reductase, (3) efflux of some unassimilated nitrate back to the medium and (4) export of nitrate to the leaves (Evans 2001). The fourth flux, together with the xylem export of root-assimilated nitrogen, is thought to be the main determinant of root–shoot differences in $\delta^{15}N$ (Evans 2001). This comes about because partial assimilation enriches the root cytosolic nitrate pool (Robinson et al. 1998). ^{15}N enriched cytosolic nitrate is then transported to the shoot and assimilated in the leaf. Because all nitrate exported to leaves must eventually be consumed, no fractionation will be observed in leaves. Thus, leaf nitrogen is a mix of root- and leaf-derived organic nitrogen with identifiable $\delta^{15}N$ signatures.

Changes to genes controlling nitrogen fluxes and assimilation should yield differences in nitrogen isotope composition. Nitrate reductase is active in both roots and shoots and has two different genes responsible for nitrate reductase activity in Arabidopsis (Wilkinson and Crawford 1993). When both genes are disrupted, Arabidopsis is unable to grow solely on NO_3^- (Wilkinson and Crawford 1993). Nitrate reductase 1 (NIA1) is responsible for 10% of total plant nitrate reductase activity (Wilkinson and Crawford 1993). NIA1 is expressed in both roots and leaves. However, it is expressed in a greater proportion in the leaves than NIA2 (Winter et al. 2007). NIA2 is responsible for the other 90% of nitrate reductase activity. NIA2 is expressed in both roots and leaves but expression is proportionately higher in roots than NIA1 (Winter et al. 2007).

Nitrate transporters are a group of plasma membrane-bound proteins that are responsible for nitrate uptake from the substrate and movement of nitrate within the plant (Glass et al. 2002). NRT2.1 and NRT2.2 are considered to be responsible for approximately 60–70% of nitrate inducible uptake in *Arabidopsis thaliana* (Arabidopsis) at low to moderate nitrate conditions

(Cerezo et al. 2001, Li et al. 2007). NRT2.1 and NRT2.2 are highly expressed throughout the root including the epidermis, cortex and endodermis (Winter et al. 2007). NRT2.1 and NRT2.2 have been shown to have a large role in nitrate uptake. However, there are other nitrate transporters that contribute to overall nitrate uptake including the entire NRT1 transporter family and other NRT2 genes (Glass et al. 2002). Although the description of NRT2.1 and NRT2.2 and its role in controlling nitrate movement within the root has been well defined, the contributions of this group of nitrate transporters to cytosolic nitrate homeostasis and nitrate dynamics in roots remain mostly unresolved. Environmental, temporal and intraspecific variation can all impact nitrogen-use measurements limiting the ability to accurately describe the contributions of specific transport genes to complex nitrogen transport dynamics over time.

Arabidopsis is a suitable system to modify plant nitrogen isotope discrimination because of the accessibility to multiple homozygous knockout lines for nitrogen uptake and assimilation genes. Here, we take advantage of existing T-DNA and γ-ray mutant lines of *Arabidopsis thaliana* to evaluate a model explaining whole-plant and organ-level fractionation of nitrogen isotopes. Knockout mutant lines of *A. thaliana* that had modified nitrate reductase or nitrate transporter activity were used to determine whether whole-plant and organ-level nitrogen isotope fractionation is predictably modified by changing internal or external nitrogen supply or nitrogen demand. Differences in nitrogen isotope fractionation among lines may also provide new information on nitrate uptake and assimilation dynamics in the lines compared to the wild type.

Materials and methods

Plant material and growth conditions

Homozygous lines of *nia1* (CS879617), *nia2* (CS2355), *nrt2* (SALK_035429C) and a wild type Col-0 (CS6673) were obtained from the Arabidopsis Biological Research Centre (http://abrc.osu.edu/). T-DNA insertions or γ-ray knockouts were confirmed using PCR to detect the location of the insertion and homozygocity of the line. Stratified seeds were sown onto Rockwool placed in plastic microcentrifuge tubes (N = 10) with bottoms removed. Tubes were inserted into predrilled lids for 70 l hydroponics containers filled with a modified 1/10th Johnson's solution containing either 100 (low NO_3^-) or 1000 μM (high NO_3^-) NO_3^-. Final nutrient composition, excluding $Ca(NO_3)_2$, was: 200 μM KH_2PO_4, 200 μM K_2SO_4, 100 μM $MgSO_4$, 100 μM $CaSO_4$, and micronutrients: 5 μM Cl, 2.5 μM B, 0.2 μM Mn, 0.2 μM Zn, 0.1 μM Mo, 0.05 μM Cu and 50 μM Fe^{2+}-EDTA. Once seeds had germinated and roots began to emerge from the bottom of the Rockwool, plugs were thinned to one plant per plug.

Plants were grown at 21°C and ambient humidity with a 12 h photoperiod providing a photosynthetic photon flux density (PPFD) of 150 $\mu mol\ m^{-2}\ s^{-1}$. Nitrate concentrations were assayed every second day using the perchloric acid method (Cawse 1967). When the NO_3^- concentration approached a 10% reduction from starting, the solution was completely replaced using $Ca(NO_3)_2$ from the same premixed source ($\delta^{15}N = 2.78 \pm 0.23‰$ [N = 3]). Growth on a homogenous, steady-state nitrogen source is required for the expression of isotope effects that reflect plant physiology rather than the spatial and/or temporal isotopic vagaries typical of natural soils and pot experiments. Frequent measurements of NO_3^- concentration ensured that increased growth rates over time were matched by an increasing frequency of complete nutrient replacement sufficient to avoid any significant isotopic change in the substrate. Plants were harvested when they were 35-days-old. Roots and shoots were placed into plastic vials, flash frozen in liquid nitrogen and stored at −80°C until samples could be freeze-dried at −50°C for 2 days. The plants had not yet bolted so the rosette shoot consisted almost entirely of leaves and is hereafter treated as such. Once dried, roots and leaves were weighed for dry weight. Thirty-five days was identified as the end of the vegetative period from a previous experiment where plants grown under the same conditions started to flower at approximately 40 days. Plants were harvested prior to flowering to avoid significant remobilization of root and leaf nitrogen into the developing inflorescence. The use of older but still vegetative plants also provided maximum dilution of the small amounts of nitrogen originating from the seed, minimized potential effects of changes in biomass partitioning that occur over the course of plant development (Gedroc et al. 1996), and ensured measurements would reflect the assimilation-averaged nitrogen use over the full vegetative phase of plant development.

Isotope analysis

Freeze-dried root and shoot tissue were ground to a fine powder using a mortar and pestle in liquid nitrogen. From ground leaf and root samples, 3 ± 0.1 mg of each sample was weighed into tin capsules (Elemental Microanalysis Ltd., 8 × 5 mm, D1008) and analyzed for $\delta^{15}N$ and nitrogen concentration on either a Europa ANCA-SL preparation module and a Europa Hydra 20/20 isotope ratio mass spectrometer (University of California Stable Isotope Facility, Davis, CA) or an Isoprime (GV Instruments) Isotope Ratio Mass Spectrometer (IRMS) coupled with an

Elementar Vario EL Cube Elemental Analyzer (EA) (UBC Faculty of Forestry Stable Isotope Facility). Isotopic composition of a plant nitrogen pool was calculated using Eqn 1. Whole-plant and organ-level isotope composition is expressed here as the difference from the source salt as $\Delta\delta^{15}N$ (i.e. $\Delta\delta^{15}N_{plant} = \delta^{15}N_{plant} - \delta^{15}N_{source}$) (Evans 2001, Pritchard and Guy 2005).

Mass balance calculations

An isotope mass balance model was used to obtain several nitrogen-use traits from measurements of nitrogen isotope composition, nitrogen concentration and biomass of roots and leaves. The proportion of total plant nitrogen found in the leaf pool (N_{leaf}/N_{total}) was calculated from plant tissue nitrogen concentration and tissue mass:

$$\frac{N_{leaf}}{N_{total}} = \frac{Biomass_{leaf} \times [N]_{leaf}}{Biomass_{plant} \times [N]_{plant}} \quad (2)$$

where $[N]_i$ is the nitrogen concentration in the plant expressed as a fraction of total dry mass. The assimilation-averaged net flux of inorganic nitrogen across the root is equal to the total plant nitrogen divided by the root biomass:

$$\text{Net root uptake } (\mu\text{mol N mg}^{-1}\text{ dw}) = \frac{N_{total}}{Biomass_{root}} \quad (3)$$

All inorganic nitrogen translocated to the shoot (Ti) by way of the xylem is assumed to be in isotopic equilibrium with the root cytoplasmic NO_3^- pool. Root-assimilated NO_3^- is depleted in ^{15}N relative to the cytoplasmic pool by the absolute difference in $\delta^{15}N$ caused by nitrate reductase ($\Delta\delta^{15}N_{enz}$), taken here to be 15‰. Organic nitrogen delivered to the shoot (T_o) is considered to be isotopically equal to root-assimilated organic nitrogen. The proportion of the total leaf nitrogen pool translocated to the leaves as inorganic NO_3^- (Ti/Tt) is then calculated from the difference between leaf and root $\delta^{15}N$:

$$\frac{Ti}{Tt} = \frac{(\Delta\delta^{15}N_{leaf} - \Delta\delta^{15}N_{root})}{\Delta\delta^{15}N_{enz}} \quad (4)$$

The proportion of total plant nitrogen that was assimilated in the leaves is simply a product of N_{leaf}/N_{total} and Ti/Tt. The remaining fraction of plant nitrogen is assimilated in the roots (P_{root}):

$$P_{root} = 1 - \left(\left(\frac{N_{leaf}}{N_{total}}\right) \times \frac{Ti}{Tt}\right) \quad (5)$$

The isotopic composition of the whole-plant relative to the external source ($\Delta\delta^{15}N_{plant}$) is proportional to efflux over influx (E/I) and P_{root}:

$$\Delta\delta^{15}N_{plant} = -\Delta\delta^{15}N_{enz} \times \frac{E}{I} \times P_{root} \quad (6)$$

where the maximum depletion in $\delta^{15}N$ relative to the substrate is equal to $-\Delta\delta^{15}N_{enz}$. Rearrangement of Eqn 6 yields an estimate of E/I:

$$\frac{\text{Efflux}}{\text{Influx}} = \frac{\Delta\delta^{15}N_{plant}}{-\Delta\delta^{15}N_{enz} \times P_{root}} \quad (7)$$

Root and leaf assimilation activities are obtained by calculating the amount of plant nitrogen derived from either roots or leaves and expressing it as a function of the biomass of either roots or leaves:

$$\text{Root Assimilation Activity } (\mu\text{mol N mg}^{-1}\text{ dw}) = \frac{N_{total} \times P_{root}}{Biomass_{root}} \quad (8)$$

$$\text{Leaf Assimilation Activity } (\mu\text{mol N mg}^{-1}\text{ dw}) = \frac{N_{total} \times (1-P_{root})}{Biomass_{leaf}} \quad (9)$$

Statistical analysis

Analysis of variance with unequal observations per treatment was used to compare treatment means of biomass, root: shoot ratio, $\delta^{15}N$, nitrogen concentration and total nitrogen of leaf and root tissues. The statistical model was as follows:

$$Y_{ij} = \mu + \alpha_i + \tau_j + \beta_{ij} \quad (10)$$

where μ is the overall mean response, α_i is the effect due to the seed line, τ_j is the effect due to the j-th level of NO_3^- and β_{ij} is the effect due to any interaction between the seed line concentration and NO_3^- concentration. Analysis of variance procedure was carried out using GRAPHPAD PRISM 6 (La Jolla, CA) to obtain estimates of the means followed by Tukey's multiple comparison tests to separate means. Where necessary, data were log transformed to meet assumptions of homogeneity of variance and normality. Differences between treatments described as significant are those where $P < 0.05$.

Results

There were morphological differences between lines that were not differentially affected by nitrate availability

There were no significant differences in root, shoot or total dry mass between the wild type and *nia2*, *nia1* or *nrt2* transformants (Fig. 1A). As expected, biomass was greater when grown with 1000 µ*M* NO_3^- than 100 µ*M* NO_3^-. Mean total dry mass was 12.6 and 25.3 mg for plants grown with 100 µ*M* NO_3^- and 1000 µ*M* NO_3^-, respectively. Although shoot and root dry masses were

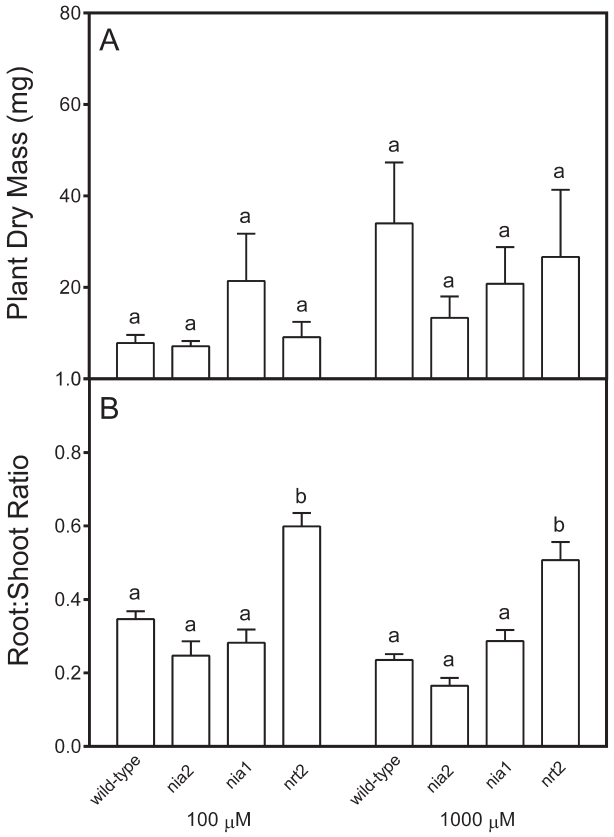

Fig. 1. Total dry mass (A) (mg) and root:shoot ratio (B) for Col-0 wild type and *nia2*, *nia1* or *nrt2* lines of *Arabidopsis thaliana* grown hydroponically in 100 μ*M* or 1000 μ*M* NO_3^- (N = 10). Letters denote statistical significance determined by a Tukey's multiple comparison test ($P < 0.05$).

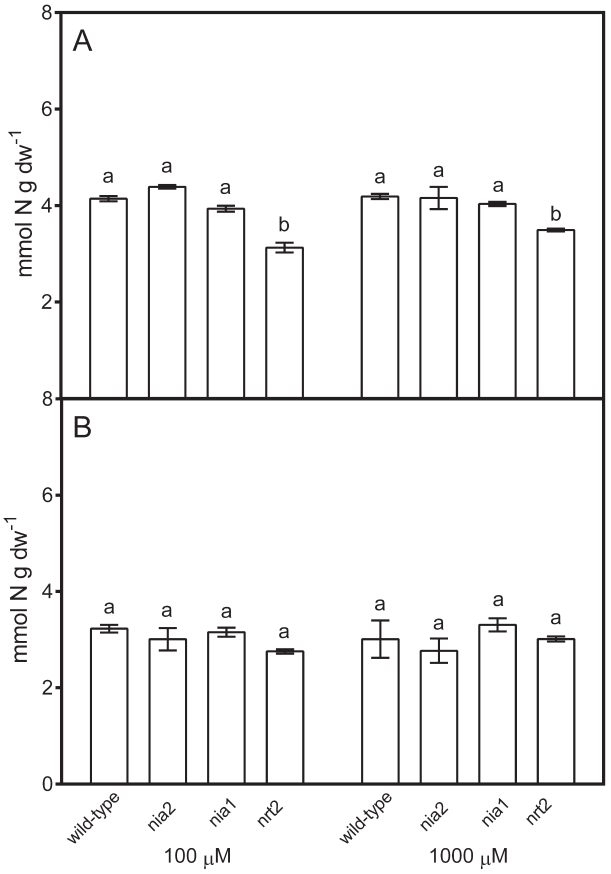

Fig. 2. Leaf (A) and root (B) nitrogen concentration (mmol N g^{-1} dw) for Col-0 wild type and *nia2*, *nia1* or *nrt2* lines of *Arabidopsis thaliana* grown hydroponically in 100 μ*M* or 1000 μ*M* NO_3^- (N = 10). Letters denote statistical significance determined by a Tukey's multiple comparison test ($P < 0.05$).

both greater on high NO_3^- than low NO_3^-, the effects on shoot mass were greater. Consequently, there was an overall decrease in root:shoot ratio in plants grown with 1000 μ*M* NO_3^- (Fig. 1B). Root:shoot ratios for *nia1* was unchanged relative to the wild type. However, for nia2, the root:shoot ratio was less than the wild type and for *nrt2*, the root:shoot ratio was greater than the wild type.

Leaf nitrogen concentration was lower in *nrt2* than the wild type but was not different for *nia2* and *nia1* (Fig. 2A). Leaf nitrogen concentration was 4.17, 4.25 and 3.98 mmol g^{-1} dw for wild type, *nia2* and *nia1*, respectively, and only 3.32 mmol g^{-1} dw for *nrt2*. Unlike leaf nitrogen concentration, root nitrogen concentration was not different between any of the lines but averaged approximately 33% less than shoot nitrogen at 3 mmol g^{-1} dw. Neither root nor leaf nitrogen concentration was affected by NO_3^- availability. Relative to wild type, a combination of low root:shoot ratio and relatively high foliar nitrogen concentration in *nia2* resulted in a greater mass specific net uptake of NO_3^- by roots (Fig. 3). In direct contrast, a high root:shoot ratio and lower foliar nitrogen concentration combined to yield a reduced rate of net uptake in roots of *nrt2*. Net root uptake was not different from wild type in *nia1*. Although not significant, net root uptake was greater in 1000 μ*M* NO_3^- than 100 μ*M* NO_3^- ($P = 0.1004$).

nia2 and *nia1* only show an isotopic phenotype at the organ level while *nrt2* shows an isotopic phenotype at the whole plant and the organ level

Plant $\delta^{15}N$ was more negative than source $\delta^{15}N$ in all lines growing under steady-state NO_3^- conditions (Fig. 4). Whole-plant $\Delta\delta^{15}N$ relative to the source averaged −2.74‰. There were differences in whole-plant and organ-level $\delta^{15}N$ between lines, but lines were similarly ranked at both 100 and 1000 μ*M* NO_3^-. Plant $\Delta\delta^{15}N$ relative to the source $\delta^{15}N$ was more negative

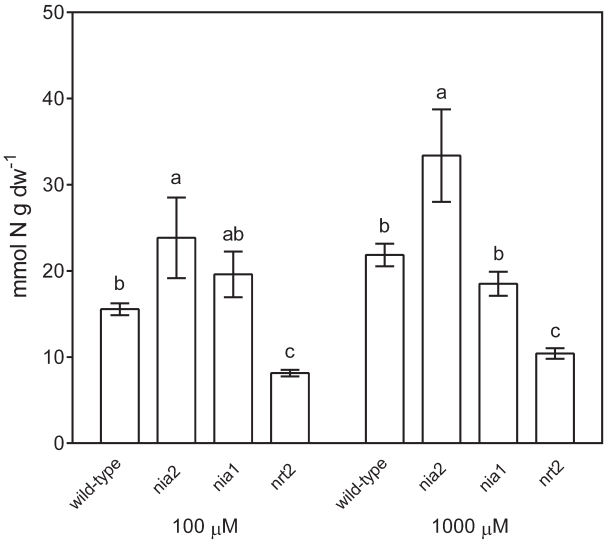

Fig. 3. Net root uptake (mmol N g^{-1} dw) over 35 days for Col-0 wild type and *nia2*, *nia1* or *nrt2* lines of *Arabidopsis thaliana* grown hydroponically in 100 μ*M* or 1000 μ*M* NO_3^- (N = 10). Letters denote statistical significance determined by a Tukey's multiple comparison test ($P < 0.05$).

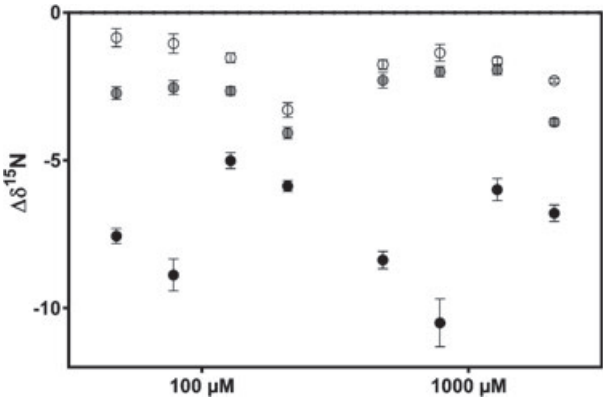

Fig. 4. Whole plant (grey circles), leaf (clear circles) and root (filled circles) $\Delta\delta^{15}N$ for Col-0 wild type and *nia2*, *nia1* or *nrt2* lines of *Arabidopsis thaliana* grown hydroponically in 100 μ*M* or 1000 μ*M* NO_3^- (N = 10).

in *nrt2* than the wild type. Whole-plant discrimination was not different in *nia1* and *nia2* compared to the wild type. Although whole-plant $\Delta\delta^{15}N$ did not indicate differences in nitrogen isotope discrimination for *nia1* and *nia2*, differences in nitrogen isotope discrimination at the organ level were more apparent.

Leaf $\Delta\delta^{15}N$ was less than root $\Delta\delta^{15}N$ for the wild type and all mutant lines (Fig. 4). Mean $\Delta\delta^{15}N$ was −1.70 and −7.43‰ for roots and leaves, respectively. Leaf $\Delta\delta^{15}N$ in *nia1* and *nia2* were not significantly different than the wild type ($P < 0.05$). However, leaf $\Delta\delta^{15}N$ in the *nrt2* line was more negative than the wild type. There was no difference in leaf $\Delta\delta^{15}N$ between plants grown on either 100 or 1000 μ*M* NO_3^-. However, there was an interaction between line and NO_3^- treatment ($P < 0.05$). Plant and leaf $\Delta\delta^{15}N$ were closely related because a greater proportion of plant biomass and nitrogen was allocated to the leaves, particularly under high NO_3^- (Fig. 3). Root $\Delta\delta^{15}N$ was more negative relative to the wild type for *nia2* but more enriched relative to the wild type for *nia1* and *nrt2*. Root $\Delta\delta^{15}N$ was more negative in plants grown at the higher NO_3^- concentration (−7.94 vs −6.92‰ for 1000 and 100 μM NO_3^-, respectively).

Interestingly, whole-plant $\Delta\delta^{15}N$ was more negative at 100 μM NO_3^-. Because the root:shoot ratio was lower in plants grown with high NO_3^-, leaves accounted for a greater proportion of the total nitrogen resulting in an increase in plant $\Delta\delta^{15}N$.

Morphological changes and partitioning of nitrogen and NO_3^- assimilation between plant organs impact whole-plant and organ-level isotopic composition

The partitioning of nitrogen-reflected changes in biomass allocation and differences in nitrogen concentration between roots and leaves. N_{leaf}/N_{total} was greater for plants grown at high NO_3^- ($P < 0.05$). N_{leaf}/N_{total} was not significantly different in the *nia1* and *nia2* mutant lines compared to the wild type (Table 1) but was lower for *nrt2*. Leaf nitrogen accounted for 82% of total nitrogen in the wild type but only 58% in *nrt2*. Although most of the difference in N_{leaf}/N_{total} can be attributed to the higher root:shoot ratio of *nrt2*, a reduced leaf nitrogen concentration also contributed to its lower N_{leaf}/N_{total}.

Through changes in overall partitioning of nitrogen and the difference in $\Delta\delta^{15}N$ between the leaves and roots, the proportional distribution of assimilatory activity can be estimated (Eqn 5). Approximately 65% of NO_3^- was assimilated in the roots of the wild type (Table 1). The proportion of assimilation occurring in the root was lower for *nia2* (50%) but higher for *nia1* (78%) and *nrt2* (84%). Plants grown with 1000 μ*M* NO_3^- had a lower proportion of assimilation occurring in the root than plants grown with 100 μ*M* NO_3^- (66 and 72% for high and low NO_3^-, respectively).

An estimate of *E*/*I* is obtained by comparing the whole-plant $\Delta\delta^{15}N$ to the maximum fractionation expected from nitrate reductase adjusted for partitioning of assimilation (Eqn 7). *E*/*I* was not different in the *nia1* and *nia2* mutant lines relative to the wild type (Table 1). However, in *nrt2*, *E*/*I* was greater than in the wild type at both NO_3^- concentrations. Likewise, NO_3^- concentration did not significantly affect *E*/*I* in the wild

Table 1. N_{leaf}/N_{total}, *Ti/Tt*, P_{root} and *E/I* for wild type and *nia2*, *nia1* or *nrt2* lines of *Arabidopsis thaliana* grown hydroponically in 100 μ*M* or 1000 μ*M* NO_3^- (N = 10). N_{leaf}/N_{total} = proportion of total plant nitrogen that is leaf nitrogen. *Ti/Tt* = proportion of leaf nitrogen that was transported to the shoot as inorganic nitrogen. P_{root} = proportion of plant nitrogen assimilation occurring in roots. *E/I* = efflux/influx between the substrate and root. Letters denote statistical significance determined by a Tukey's multiple comparison test ($P < 0.05$).

Output variable	Wild type	*nia2*	*nia1*	*nrt2*
100 μ*M*				
N_{leaf}/N_{total}	0.79 ± 0.012a	0.85 ± 0.023a	0.82 ± 0.021a	0.66 ± 0.018b
Ti/Tt	0.44 ± 0.025a	0.55 ± 0.0370a	0.24 ± 0.016b	0.17 ± 0.015b
P_{root}	0.65 ± 0.022a	0.56 ± 0.038a	0.81 ± 0.018b	0.89 ± 0.008b
E/I	0.10 ± 0.012a	0.08 ± 0.013a	0.11 ± 0.011a	0.25 ± 0.012b
1000 μ*M*				
N_{leaf}/N_{total}	0.84 ± 0.011ab	0.90 ± 0.015a	0.81 ± 0.020b	0.70 ± 0.020c
Ti/Tt	0.44 ± 0.020b	0.61 ± 0.066a	0.29 ± 0.020c	0.30 ± 0.019c
P_{root}	0.63 ± 0.016b	0.46 ± 0.059a	0.77 ± 0.016c	0.79 ± 0.016c
E/I	0.12 ± 0.009ab	0.08 ± 0.014a	0.13 ± 0.008b	0.19 ± 0.006c

type or *nia1* and *nia2*, but in *nrt2* there was a decrease in *E/I* from 0.25 at 100 μ*M* NO_3^- to 0.19 at 1000 μ*M* NO_3^-.

The mass-specific root and leaf assimilation activities were determined by accounting for total plant nitrogen and the proportional assimilation occurring in either the root or the shoot (Eqns 8 and 9). Overall mean root assimilation activity was 13.1 mmol g^{-1} dw (Fig. 5). Root assimilation activity was not different compared to the wild type for *nia1* and *nia2* but was lower in *nrt2*. However, leaf assimilation activity was greater in *nia2* (2.47 mmol g^{-1} dw) and lower in *nia1* (1.05 mmol g^{-1} dw) and *nrt2* (0.79 mmol g^{-1} dw) compared to the wild type (1.83 mmol g^{-1} dw). Overall, the mass-specific leaf assimilation activity was approximately 17% greater at high NO_3^- (1.40 mmol g^{-1} dw) than at low NO_3^- (1.68 mmol g^{-1} dw). Although leaf assimilation accounted for about 40% of total nitrate assimilation, root assimilation per unit mass was approximately eight times greater than leaf assimilation because of the low amount of root biomass relative to leaf biomass.

Discussion

The objective was to use Arabidopsis knockout lines with modified nitrate reductase or transporter activity to modify nitrogen isotope composition of plants grown hydroponically under steady-state conditions. Although growth phenotypes were not observed; morphological, isotopic and physiological phenotypes were apparent. We expected that disruption of uptake or assimilatory genes, alone or in combination with changes in substrate concentration, would impact nitrate supply and demand. Variation in supply and demand would subsequently affect whole-plant and organ-level nitrogen isotope composition (Evans, 2001, Pritchard and Guy 2005). At the whole-plant level, a decrease in demand relative to supply should increase *E/I* and thereby increase ^{15}N discrimination if assimilation occurs partly in the root. In contrast, a decrease in *E/I* and a decrease in discrimination are expected if uptake is suppressed, but would be dependent on whether uptake physically precedes assimilation across all tissues. If there are restrictions in transport that are localized beyond the epidermis, or if restrictions occur generally throughout the root, *E/I* could instead increase. Here, we show that disruption to genes responsible for either nitrate reductase activity or nitrate transport affected nitrogen isotope composition that corresponded to reported localized expression within the plant and the known role of the disrupted genes on nitrate uptake and assimilation. Although the results were more varied than expected, they are interpretable within the context of the model and demonstrate its utility in studying nitrogen uptake and assimilation in plants.

Disrupting *nia2* activity decreased root:shoot ratio but does not affect nitrogen concentration, while disrupting *nrt2* activity reduced nitrogen concentration and increases root:shoot ratio

Although not significantly different, mean biomass of *nia2* was lower than the wild type ($P = 0.1574$). High variability in biomass among seedlings within lines contributed to the lack of confidence in the differences in mean biomass between *nia2* and the wild type. Wilkinson and Crawford (1993) reported no differences in growth for the same *nia2* line under NO_3^- nutrition compared to wild type. However, Stitt and Feil (1999) observed reduced growth in tobacco transformants with reduced nitrate reductase activity when grown at multiple NO_3^- concentrations. Although there were no differences in total biomass in our experiments,

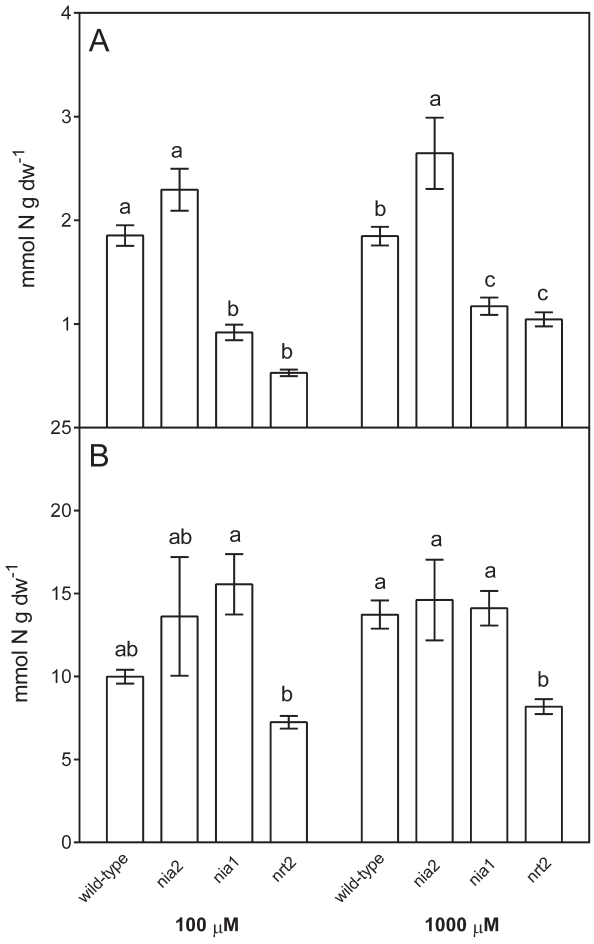

Fig. 5. (A) Leaf and (B) root assimilation (mmol N g^{-1} dw) over 35 days for Col-0 wild type and *nia2*, *nia1* or *nrt2* lines of *Arabidopsis thaliana* grown hydroponically in 100 μ*M* or 1000 μ*M* NO_3^- (N = 10). Letters denote statistical significance determined by a Tukey's multiple comparison test ($P < 0.05$).

root growth was impacted more than shoot growth by disruption of NIA2 activity, causing the root:shoot ratio to decrease (Stitt and Feil 1999). Conversely, there was an increase in root:shoot ratio in *nrt2* grown with either 100 or 1000 μ*M* NO_3^-, as also described by Filleur et al. (2001) and Li et al. (2007). Changes in root:shoot ratio may change the demand for nitrogen from the shoot relative to the amount of root tissue available to take up NO_3^- from the substrate. The reduction in root and leaf nitrogen concentration in *nrt2* (Fig. 2) indicates that net uptake is restricted at either the epidermal and/or intercellular level. On a mass basis, net uptake was reduced in *nrt2*. An approximately 20% reduction in tissue nitrogen concentration in *nrt2* suggests that there is some disruption of supply relative to demand. Although plants had reduced nitrogen levels, they did not show any outward signs of stress or limitation.

Differences in N_{leaf}/N_{total} in mutant lines compared to the wild type demonstrate the impact of changes in biomass partitioning on overall nitrogen allocation (Table 1). Since the root:shoot ratio of *nia2* was low compared to the wild type, a greater portion of overall nitrogen was allocated to the leaf. In essence, *nia2* behaved like a plant growing under a higher nitrate concentration (Gedroc et al. 1996) because supply exceeded demand (reduced by knocking out 90% of total NR activity). On the other hand, *nrt2* behaved like a plant that was nitrogen limited and allocated a greater proportion of overall biomass to the root (Bloom et al. 1985).

The distribution of assimilatory activity indicated by ^{15}N mass balance corresponded to the distribution of nitrate reductase expression in roots and leaves

Nitrate reductase activity is closely linked with substrate availability and assimilatory demand (Campbell 1999). The presence of nitrate has been shown to induce nitrate reductase activity in numerous plant species including Arabidopsis (Crawford et al. 1988) and poplar (Black et al. 2002). Since NIA2 would normally constitute 90% of the nitrate reductase activity, we would expect to see a stronger response in *nia2* than *nia1*. Although not significant, we did see a decrease in growth in *nia2* but not *nia1*. Interestingly, Wilkinson and Crawford (1993) observed no decrease in nitrate reductase activity after disrupting either NIA1 or NIA2. It was suggested that up-regulation of NIA1 offsets the effects of disrupting NIA2 (Wilkinson and Crawford, 1993). It is therefore not surprising that both *nia1* and *nia2* showed no significant growth phenotype when grown under either nitrate concentration. In contrast, there were morphological and physiological phenotypes that indicate differential contributions of NIA1 and NIA2 to whole-plant nitrate assimilation activity.

Our calculations of P_{root} indicate that root assimilation accounted for between 50 and 90% of total nitrate assimilation depending on the mutant line, which is a greater proportion than is generally expected in the literature. Expressed relative to each other at the tissue level, measurements of nitrate reductase activities have been used to provide a first approximation of how assimilatory activity is partitioned between roots and leaves. In most cases, leaf nitrate reductase activities are considerably greater than root nitrate reductase activities (Andrews 1986, Black et al. 2002). Similarly, according to the BAR Arabidopsis expression tool (Winter et al. 2007), nitrate reductase is expressed at higher levels in leaves than roots (Table 2). Similar to nitrate reductase assays,

expression is a measure of enzyme protein levels, not in vivo assimilation activity. Changes in in vitro activity are not always reflective of in vivo nitrate reductase activity. Andrews et al. (1992) estimated partitioning of assimilation in grasses using nitrate reductase activity, tissue nitrate content and reduced nitrogen in the xylem sap and concluded that assimilation is weighted more towards roots, particularly at concentrations below 1 m*M* nitrate. Nitrate reductase can undergo strong substrate (cytoplasmic NO_3^-) limitation (Kaiser and Huber 2001). Cytosolic nitrate concentrations can fluctuate with regulation of assimilatory enzymes (Fan et al. 2006). Root nitrate concentrations are likely more stable than leaf nitrate concentrations given that leaf nitrate concentrations are entirely dependent on xylem transport. Under nonsaturating nitrate conditions, less nitrate may reach the xylem to be transported to the leaf and a greater proportion will be assimilated by root nitrate reductase.

Despite these qualifications, our ^{15}N mass balance approach predicts the same ranking for the partitioning of assimilation as would be expected based on tissue level nitrate reductase expression levels (Table 2). Furthermore, because *nia2* expression is proportionately higher in roots than *nia1*, when *nia2* is disrupted, nitrate reductase expression is likely to be proportionately even higher in leaves, consistent with the decrease in P_{root} indicated by the isotopic mass balance. In contrast, when *nia1* is disrupted, nitrate reductase expression is weighted more towards the roots and we see an increase in the partitioning of assimilation to the roots.

When nitrate transport was disrupted in *nrt2*, we observed an overall decrease in net uptake and assimilation activity accompanied by an increase in the root:shoot ratio. The concomitant changes in ^{15}N mass balance indicate an increase in P_{root}, suggesting that with decreased symplastic movement of nitrate within the root and decreased rate of loading into the xylem, there is less NO_3^- translocated to the leaves. Expression of NRT2.1 and NRT2.2 is localized to not just the plasma membranes of epidermal cells, but also cortical and endodermal cells (Winter et al. 2007, Feng et al. 2011). With assimilatory demand remaining high and movement of nitrate through the root tissue reduced, assimilation would become more weighted to the root since translocation to the leaf would be restricted.

Table 2. Distribution of NIA1 and NIA2 expression between leaves and roots of *Arabidopsis thaliana* compared to observed leaf:root proportioning of nitrate assimilation in Col-0 wild type (NIA1 + NIA2), NIA1 only and NIA2 only. Leaf and root GFP (green fluorescent protein) expression were taken as expression in whole leaf and root cortex tissue under standard conditions. Expression data taken from the Bio-Array Resource for Plant Biology Arabidopsis eFP expression browser (http://bar.utoronto.ca/welcome.htm; Winter et al. 2007).

Gene	Leaf GFP expression	Root GFP expression	Leaf:root expression ratio	Leaf:root proportioning of assimilation
NIA1 + NIA2	2656	1308	2.03:1	0.5:1
NIA1	478	146	3.23:1	1:1
NIA2	2178	1162	1.87:1	0.33:1

Changes in assimilatory demand did not impact efflux/influx but restriction of nitrate transport within root tissue did

For both nitrate reductase mutant lines, our estimates of *E*/*I* remained unchanged compared to the wild type. This was contrary to our initial expectations, as we had predicted that reduced rates of assimilation would allow more substrate to cycle back to the medium. However, as indicated above, nitrate reductase activities would be little impacted in *nia1*, whilst *nia2* had a much reduced root:shoot ratio, placing an increased demand on the roots for xylem transport of inorganic NO_3^- (i.e. in competition with efflux). There is also increasing evidence of homeostatic control of cytoplasmic nitrogen concentrations (Glass et al. 2002, Miller and Smith 2008, Huang et al. 2012) whereby plants can modulate demand (through modifying growth) and supply (through modulating uptake) so that cytosolic nitrogen concentrations are buffered to temporal and spatial changes in supply or demand.

In contrast, when genes associated with NO_3^- transport were disrupted the ^{15}N mass balance was consistent with an increase in *E*/*I*. This result suggests that the sites of root assimilation are morphologically internal to the sites where transport was actually disrupted, which is quite possible given that, as indicated above, NRT2 is expressed throughout the root. Similarly, Bloom et al. (2012) suggested that variation in fluxes between root tissue types influence the sites of assimilation. Although overall uptake into the root was reduced, further movement within the cortex or to the stele may have been more greatly impacted, increasing efflux. Multiple nitrate transporter genes contribute to nitrate uptake and movement within roots (Glass et al. 2002). Although there is a degree of redundancy within the NRT gene family and these genes operate at different NO_3^- concentrations, *NRT2.1* and *NRT2.2* are considered to be two of the primary genes responsible for high affinity uptake in Arabidopsis (Glass et al. 2002, Li et al. 2007). The small differences between leaf and root $\delta^{15}N$ in the double mutant indicate that the leaves rely more heavily on the translocation of organic N from the roots, consistent with a reduced loading of inorganic nitrate into the xylem.

Conclusion

Arabidopsis, with its wide array of described mutant lines, provides an excellent platform to modify nitrogen isotope discrimination by restricting expression of critical genes in the nitrogen uptake and assimilation pathway. Induced variation in nitrogen isotope composition suggests that there is variation in nitrogen uptake or use within plants that affects relative inorganic nitrogen fluxes between the root and substrate as well as between plant parts. We have demonstrated here that whole-plant and organ-level variation in isotopic composition can be modified, in turn providing more detail on overall nitrogen uptake and assimilation.

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