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Enzymatic and Metabolic Diagnostic of Nitrogen Deficiency in *Arabidopsis thaliana Wassileskija* Accession

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Adaptation to steady-state low-nutrient availability was investigated by comparing the Wassileskija (WS) accession of *Arabidopsis thaliana* grown on 2 or 10 mM nitrate. Low nitrogen conditions led to a limited rosette biomass and seed yield. The latter was mainly due to reduced seed number, while seed weight was less affected. However, harvest index was lower in high nitrate compared with limited nitrate conditions. Under nitrogen-limiting conditions, nitrate reductase activity was decreased while glutamine synthetase activity was increased due to a higher accumulation of the cytosolic enzyme. The level of nitrogen remobilization to the seeds was higher under low nitrogen, and the vegetative parts of the plants remaining after seed production stored very low residual nitrogen. Through promoting nitrogen remobilization and recycling pathways, nitrogen limitation modified plant and seed compositions. Rosette leaves contained more sugars and less free amino acids when grown under nitrogen-limiting conditions. Compared with high nitrogen, the levels of proline, asparagine and glutamine were decreased. The seed amino acid composition reflected that of the rosette leaves, thus suggesting that phloem loading for seed filling was poorly selective. The major finding of this report was that together with decreasing biomass and yield, nitrogen limitation triggers large modifications in vegetative products and seed quality.

Keywords: Asparagine — Glutamine synthetase — Nitrate reductase — Nitrogen limitation — Nitrogen remobilization — Seed composition.

Abbreviations: DAS, days after sowing; GABA, γ-aminobutyric acid; GS, glutamine synthetase; HN, high nitrogen nutrition; LN, low nitrogen nutrition; NHI, nitrgen harvest index; NR, nitrate reductase; NRE, nitrogen remobilization efficiency; %P, partitioning; RSA, relative specific allocation.

Introduction

Nitrogen and water availablity are two major limiting factors in plant growth (Lea and Azevedo 2006). Plants have a fundamental dependence on inorganic nitrogenous fertilizers, and their quantity, added to soil worldwide annually, is increasing every year (Good et al. 2004). Nitrogen has a major impact on seed and forage production, while the quality of these products is strongly influenced by nitrogen availability. Together with yield, one important agronomical trait is the percentage of proteins contained in cereal grains that determines seed nutritional quality, and is defined by the N harvest index (NHI, as N in grain/total N uptake).

To date, most plant cultivars bred for crops have been selected under non-limiting conditions (Bänziger et al. 1997, Presterl et al. 2003) for productivity and seed yield. Numerous studies have reported a negative correlation between grain protein concentration and yield (Beninati and Busch 1992, and references therein), thus suggesting that breeding cultivars for a higher yield has resulted in a decrease in the NHI.

Nitrogen, as a commercial fertilizer, is one of the most expensive nutrients to supply and it represents the major cost in plant production (Singh 2005). Moreover, crop plants are only able to convert 30–40% of this applied nitrogen to useful food products. This has led to an important ecological problem due to lost nitrogen polluting soil and water. The possibility of lowering fertilizer input and breeding plants with better nitrogen use efficiency is a major aim (Lea and Azevedo 2006, Hirel et al. 2007). Indeed, improving nitrogen use efficiency, defined as the yield of grain per unit of available nitrogen in the soil, is now the central point of many physiological and agronomic studies. However, limiting nitrogen input in the field will certainly have effects other than limiting dry matter production and yield, and will probably also modify the quality of useful food products.

The effect of low nitrogen availability on plant biomass, nitrate uptake and root architecture has already been widely studied (Brouwer 1962, Drew and Saker 1976, Van der Werf and Nagel 1996, Lea and Azevedo 2007). We know that plants modify their root architecture, changing the lateral/primary root ratio and decreasing the shoot/root ratio at the same time, to forage the soil for nutrients. The influence of nitrogen limitation on the fine-tuning of plant metabolism is less well documented. Attention has been mainly paid to plant responses to nitrogen starvation, an abiotic stress that can be encountered by all plants, even

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in the field, several times during their development cycles (Wang et al. 2000, Scheible et al. 2004). On the other hand, adaptation to steady-state low-nutrient availability is poorly documented (for a review, see Forde and Lea 2007). The available results on the comparison of plant metabolism under low and high nitrogen nutrition are often fragmentary and difficult to compare and analyze since they have been obtained on different plant species or tissues, or by using different growth conditions (Tercé-Laforgue et al. 2004, Fritz et al. 2006).

It would be useful to obtain a complete picture of the modifications in plant metabolism when nitrogen is poor or abundant. Transcriptomics is a powerful tool to help analyze and compare the phenotypes of plants, and it has been used with success to detect changes and adaptations brought about in mutants and stressed plants, such as phosphate-starved, nitrogen-starved or sugar-induced plants. Enzymatic or metabolic analyses can be used as a diagnostic tool for nutritional problems and to help decipher the effects of a mutation on the ability of plants to use nutrients, such as nitrogen, for example. In this report, we have compared the metabolism traits of the rosette leaves of *Arabidospsis thaliana* plants grown under limiting or under full nitrate nutrition, and characterized the metabolic pathways favored by an adaptation of plants to low nitrogen conditions. It was found that nitrate nutrition changed metabolite levels and enzyme activities considerably. The data show that the fine-tuning of plant metabolism is sensitive to nitrate levels and that the quality of plant material depends on nitrogen availability. This report provides a metabolic diagnostic of nitrogen deficiency in *A. thaliana* that should facilitate the detection or analysis of nitrogen-related problems.

Results and Discussion

Development, biomass and yield are affected by low nitrogen nutrition

Many Arabidopsis genotypes show a large variation in traits related to development and fitness between low (2 mM nitrate; LN) and high (10 mM nitrate; HN) nitrogen growth conditions (Loudet et al. 2003). We observed that nitrogen limitation delayed flowering time from 60 days after sowing (DAS) to 66 DAS in the Wassileskija (WS) accession analyzed in this work. At 43 DAS, the FW of the rosette was significantly higher under HN than under LN, and this difference increased to 6-fold at flowering time (Fig. 1A). The DW of the seeds and of the dried harvested vegetative remains (rosette, flowering stems and silique envelopes) that correspond to the stover in crop plants were 2.3 and 2.9 times higher, respectively, for HN-grown plants than for LN-grown plants (Fig. 1B). Therefore, HN nutrition appeared to be more valuable for the vegetative

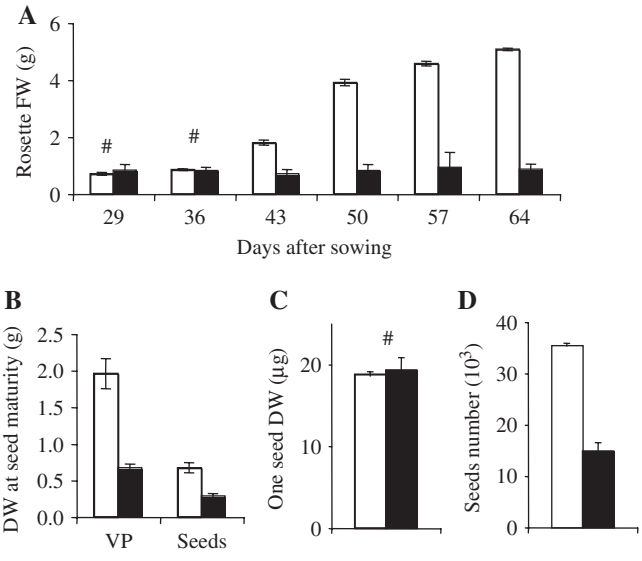

Fig. 1 Comparison of biomass (A–C) and seed production (D) between plants grown under nitrate-limiting (black bars) or abundant (white bars) conditions. Means and SE of four individual plants are presented. # indicates a non-significant difference between white and black bars with $P \geq 0.05$.

organs compared with seed production, and NHI [seed DW · (whole plant DW)$^{-1}$] was slightly lower in HN than in LN conditions. Nitrogen availability did not influence single-seed DW (Fig. 1C) but it increased seed yield >2-fold, as a result of higher seed number under HN conditions (Fig. 1D). This also suggests that plant fitness is better under HN conditions, whereas single-seed biomass is not affected.

Nitrogen nutrition modifies the activities of nitrogen assimilation and nitrogen recycling enzymes

Since nitrogen limitation did not affect the total soluble protein concentration in rosette leaves (Fig. 2A), measured enzyme activities were expressed with respect to protein content (nmol · min^{-1} · mg^{-1} protein).

Nitrogen allocation in the plant depends on several steps of plant metabolism that determine the physiology of a plant. In *Arabidopsis* it is assumed that most of the nitrate, taken up via transporters in the roots, is reduced and assimilated in the leaves. Beside nitrate uptake efficiency, nitrate reduction is the main limiting point for nitrogen assimilation. Nitrate reductase (NR, EC.1.7.1.1) is known to be positively regulated by nitrate availability (Vincentz et al. 1993). Indeed, the total NR activity was higher in HN- than in LN-grown plants during the rosette lifespan, except at 29 DAS when rosette biomass was similar for HN and LN plants, and at 64 DAS when leaf senescence of HN-grown plants had reduced NR activity to the same level as LN plants (Fig. 2B). NR activity decreased with plant aging under both conditions;

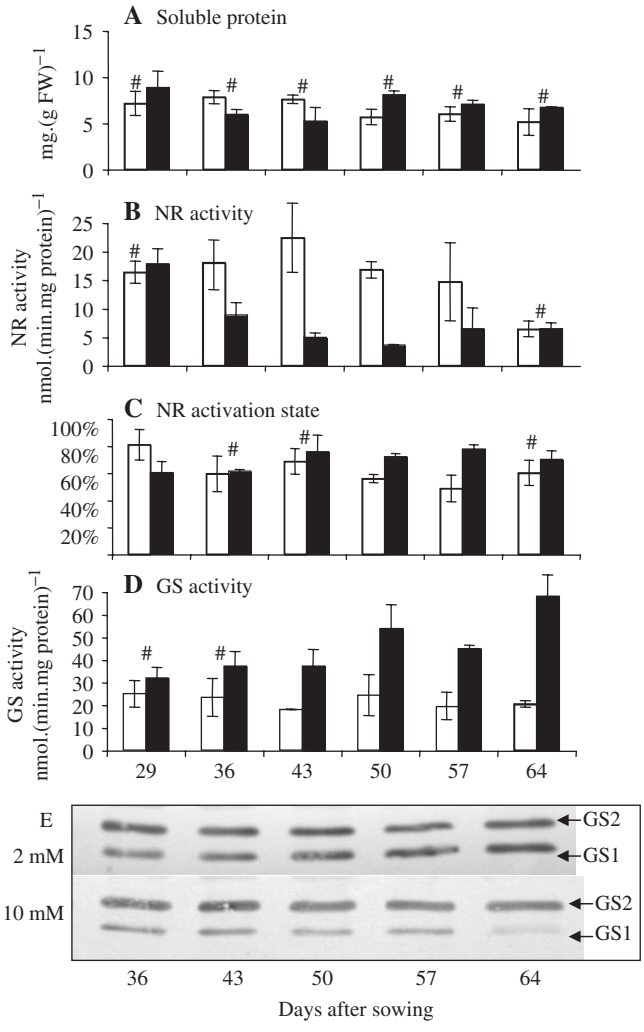

Fig. 2 Comparison of protein concentration (A), nitrate reductase activity (NR; B), nitrate reductase activation state (C), glutamine synthetase activity (GS; D) and GS protein content (E) in rosettes of plants grown in low nitrate (black bars) or in high nitrate (white bars) conditions. Means and SE of four individual plants are presented. # indicates a non-significant difference between white and black bars with $P \geq 0.05$. For protein separation before Western blot, an equal amount of soluble protein was loaded in each lane (10 µg).

however, this decrease occurred earlier in LN plants compared with HN plants. In vivo NR activity depends on the activation state of the enzyme, which is controlled through NR phosphorylation. The activation state (see Materials and Methods) was high (>50%) during the time course of our experiments in both LN and HN rosettes (Fig. 2C). At 43 and 48 DAS, the activation state was significantly higher in LN rosettes compared with HN rosettes. This suggests that the lower NR activity of LN rosettes was due to a lower NR protein content rather than to an inhibition of the enzyme by phosphorylation.

When nitrate and nitrite are reduced, the ammonium produced is assimilated by glutamine synthetase (GS, EC.6.3.1.2) isoforms. Surprisingly the in vitro GS activity was significantly higher in the LN rosettes compared with those from HN plants (this was seen since 43 DAS, Fig. 2D). Antibodies raised against a conserved peptide domain of GS1 and GS2 allowed us to detect both isoezymes using Western blots and protein extracts from rosettes grown under HN or LN conditions (Fig. 2E). GS1 was clearly detected in all the LN rosette extracts, and the amount of GS1 was increased with rosette aging. At 64 DAS, the amount of GS1 in LN rosettes appeared as high as the amount of GS2. This was not the case for the HN rosette extracts. In HN rosettes, the GS1 content detected was clearly lower than the GS2 content. Moreover, GS1 decreased with leaf aging and was almost undetectable at 64 DAS in HN rosettes. Loading HN and LN extracts on the same gel (data not shown) confirmed that the amount of GS2 was similar in HN and LN rosettes and did not change with aging. It also confirmed that the amount of GS1 was higher in LN rosettes than in HN rosettes. The increase of GS1 in LN rosettes with aging and the decrease of GS1 in the HN rosette with aging could explain why higher GS activity was measured in LN rosettes.

GS1 isoforms are well known markers of N remobilization and senescence (Masclaux et al. 2000, Martin et al. 2005). Five genes in Arabidopsis that are differentially regulated encode GS1. The different GS1 isoforms certainly have different locations and roles in nitrogen assimilation and recycling (for a review, see Masclaux-Daubresse et al. 2008). In other plant species, several GS1 isoforms have indeed been detected in different plant tissues such as phloem or mesophyll cells depending on leaf development (Brugière et al. 2000, Masclaux et al. 2000). The low level and decrease of GS1 in HN rosette extracts suggest that the GS1 isoforms present in young HN rosettes are mainly dedicated to nitrogen primary assimilation. The increase of GS1 proteins during LN rosette aging confirms GS1 as a senescence-related marker in *Arabidopsis* when nitrogen is limited (Diaz et al. 2008). Together with the early decrease of NR activity in the LN rosettes, the high amount of GS1 suggests that LN rosettes exhibit senescence-related symptoms early during their development and mobilize nitrogen efficiently. This is consistent with the idea that N limitation is an exogenous senescence-triggering factor that facilitates N remobilization at the whole plant level (Wingler et al. 2004).

Nitrogen deficiency stimulates nitrogen remobilization to the seeds

Since GS1 proteins were more abundant and appeared earlier in LN rosettes compared with HN rosettes, the effect of LN on N remobilization was analyzed by ^{15}N tracing experiments to measure the amount of nitrogen mobilized

from the rosettes to the seeds. Labeling of the rosettes was performed at the vegetative stage (40 and 42 DAS), 20 d before flower bud emergence and 80 d before harvest of seeds and vegetative parts. This allowed us to assume that all of the provided $^{15}NO_3^-$ had been assimilated and incorporated into organic molecules, before remobilization took place.

At seed maturity, the dry vegetative remains and the total seeds were harvested for analysis. ^{15}N enrichment, total nitrogen concentration and dry matter of the vegetative remains and seeds compartments were measured, allowing the calculation of the ^{15}N enrichment, total nitrogen concentration and dry matter in the whole plant (vegetative remains + seeds). The partitioning of ^{15}N (P%^{15}N), nitrogen (P%N) and dry matter (P%DM) in the seeds was calculated as the ratio [seeds/(seeds + vegetative remains)]. The relative specific allocation (RSA) of ^{15}N in the different compartments (seeds, vegetative remains and whole plants) was also calculated, and it is a representation of tissue enrichment (Table 2; Deléens et al. 1994).

The results showed that nitrogen limitation had no significant effect on P%DM and that seeds represented about 28% of the whole plant dry matter in both 2 and 10 mM nitrate-grown plants. However, nitrogen limitation strongly modified nitrogen allocation to seeds (P%N) since >65% of the total nitrogen absorbed by the plant during its lifespan was located to the seed compartment of the LN plants, vs. 25% for HN plants. This result is in agreement with the very low N% remaining in the vegetative remains of LN plants compared with HN plants, whereas the seed N% is similar in LN and HN seeds. The P%N and %P%^{15}N were similar for LN plants, thus resulting in an RSA_{seeds} : $RSA_{whole\ plant}$ ratio close to 1. This underpins a close relationship between $RSA_{vegetative\ remains}$ and RSA_{seeds}, suggesting that when *Arabidopsis* plants are cultivated with 2 mM nitrate nutrition, N remobilization occurred in a way that seems to follow the model described by Gallais et al. (2006) for maize. This model proposes that quasi-equality to 1 observed for the ratio RSA_{seeds} : $RSA_{whole\ plant}$ would have existed very early during development when N remobilization and N uptake were both active. There would be no dilution or concentration of ^{15}N in the seeds because the amino acids from newly assimilated N are mixed with amino acids from proteolysis for synthesis of new proteins that will be further hydrolyzed to support protein neosynthesis. This recycling process would be maintained until amino acid flux to the seeds. Under nitrogen limitation, proteolysis supporting rapid protein turnover and continuous N recycling for protein neosynthesis, together with a rapid and efficient nitrogen remobilization to the seeds after flowering, could explain the observed RSA_{seeds} : $RSA_{whole\ plant}$ close to 1. However, whereas this equality was independent of environmental conditions in maize (Gallais et al. 2006), this does not appear to be the case in *Arabidopsis* since we observed $RSA_{seeds} > RSA_{whole\ plant}$ for HN-grown plants (Table 2). This suggests that in the seeds of HN plants, the amount of nitrogen coming from N remobilization is higher than the amount of unlabeled nitrogen assimilated after flowering and during grain maturation. This is also consistent with the fact that P%N is lower than P%^{15}N in HN plant seeds, whereas P%N equals P%^{15}N in LN seeds. The comparison of P%^{15}N/P%DM between plants is a good way to estimate and compare the nitrogen remobilization efficiency (NRE) when plants do not show the same biomass or sink–source development. Indeed this ratio normalizes ^{15}N flux to seeds taking into account the respective sizes of sink and source compartments. The P%^{15}N/P%DM ratio shows that NRE in LN plants is 1.7-fold that of HN plants, and it confirms that nitrogen deficiency stimulates plant nitrogen remobilization (Schulze et al. 1994). Altogether these results shows that whereas NRE is higher in LN plants than in HN plants, the amount of nitrogen coming from N remobilization is higher in the HN seeds than in the LN seeds. This surprising feature might be due to HN plants absorbing high quantities of unlabeled nitrogen, before flowering, after flowering and during seed filling, that was stored in leaf rosettes, possibly as nitrate, rather than incorporated into organic compounds. This unlabeled and non-remobilized nitrogen would dilute the rosette ^{15}N pool, thus decreasing the RSA of the vegetative remains and at the same time increasing the ratio RSA_{seeds} : $RSA_{whole\ plant}$.

Nitrogen nutrition modifies metabolite concentrations in rosette leaves

Since the nitrogen pools in rosettes and seeds seem to be differentially influenced by N assimilation and N remobilization depending on HN and LN growth conditions, it was hypothesized that metabolite quality and quantity would be modified. As expected, the total nitrogen concentration was higher (2–4 times) in the rosettes of HN-grown plants (Fig. 3A). This difference was mainly due to a higher absorption of nitrate by the HN plants that resulted in the accumulation of nitrate and amino acids, since the ammonium and soluble protein concentrations were similar in rosettes of HN and LN plants (Figs. 3B, 2A). The total ^{15}N remaining in the whole plant is related to plant nitrate uptake and transport if we assume that no efflux has occurred. This showed that rosette $^{15}NO_3^-$ absorption at 40 and 42 DAS was 10 times higher under HN (0.2451 ± 0.0730 mg per plant, $n = 4$) than under LN nutrition (0.0219 ± 0.0036 mg per plant, $n = 4$). Nitrate content in HN rosettes was high (50–100 nmol mg^{-1} FW) and increased with rosette aging (Fig. 3C). On the other hand, nitrate content in LN rosettes was very low (around 5 nmol mg^{-1} FW) during plant

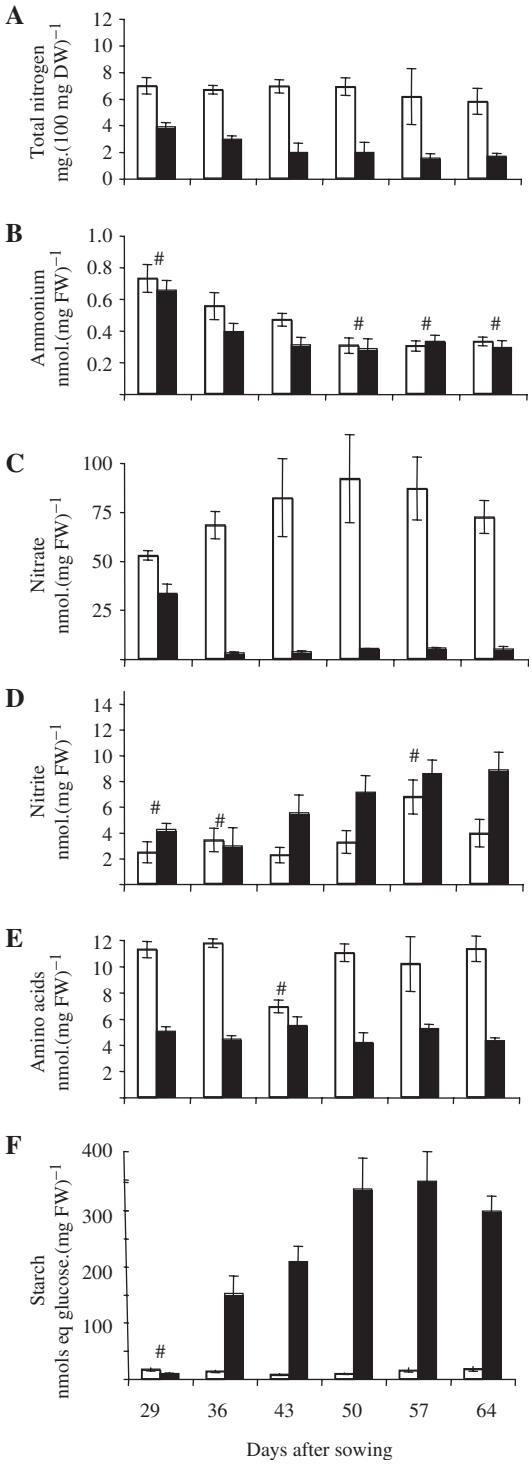

Fig. 3 Comparison of total nitrogen content [mg · (100 mg DW)$^{-1}$; A], ammonium concentration [nmol · (mg FW)$^{-1}$; B], nitrate [nmol · (mg FW)$^{-1}$; C], nitrite [nmol · (mg FW)$^{-1}$; D], free amino acids [nmol · (mg FW)$^{-1}$; E] and starch [nmol · (mg FW)$^{-1}$; F] in the rosettes of plants grown under nitrate-limiting (black bars) or abundant (white bars) conditions. Means and SE of four individual plants are presented. # indicates a non-significant difference between white and black bars with $P \geq 0.05$.

development except at 29 DAS when no difference in biomass could be detected between HN and LN rosettes. However, the nitrate concentration was significantly higher in HN rosettes than in LN rosettes at all time points, 29 DAS included. Whereas nitrate concentration was lower in LN rosettes, the nitrite content appeared slightly higher than in HN rosettes. This difference was significant at 43, 50 and 64 DAS (Fig. 3D). Ammonium concentrations were found to be similar in HN and LN rosettes, thus suggesting an efficient assimilation into amino acids in both LN and HN plants. However, the total free amino acid concentration was 2-fold higher in HN rosettes (Fig. 3E). Sugar accumulation in plant tissues is a well-known consequence of nitrogen deficiency (Tercé-Laforgue et al. 2004). Hexoses and sucrose were almost undetectable in the HN rosettes, whereas their concentration increased steadily from 10 and 2 nmol · mg^{-1} FW at 29 DAS, up to 50 and 10 nmol · mg^{-1} FW, respectively, at 64 DAS in LN rosettes. Starch was detected in both LN and HN plants (Fig. 3F). At 29 DAS, starch levels were not different in LN and HN rosettes, after which starch dramatically increased in LN rosettes, peaking at 50–57 DAS, just before flowering (Fig. 3F). On the other hand, HN rosettes did not show an increase in starch levels during the development of the plants. Under low nitrogen nutrition conditions, LN plants become nitrogen deficient, and the imbalance between carbon fixation and nitrogen assimilation leads to starch accumulation. The carbohydrate accumulation in LN rosettes could contribute to the promotion of 'senescence-like' symptoms such as early induction of GS1 (Masclaux-Daubresse et al. 2005).

Individual amino acid proportions are modified by nitrogen availability in a similar manner in the seeds and in the rosette leaves

Individual amino acid contents were determined in rosettes and seeds of LN- and HN-grown plants using anion exchange chromatography. All of the individual amino acid levels were higher in HN-grown rosettes (Table 1) when compared with LN-grown rosettes; glutamine, proline and aspartate were particularly higher. However, since the total free amino acid concentration was higher in the HN-grown rosettes (Fig. 3E), individual amino acid concentrations mainly reflected the global increase in total amino acid content. Glutamate occupies a central position in amino acid metabolism since this is the main α-amino group donor for the biosynthesis of glutamine through the GS pathway and for the biosynthesis of other amino acids through the action of a wide variety of aminotransferases (Forde and Lea 2007). A good picture of the α-amino transfer network is given by the analysis of the proportion of each amino acid among the total amino acid pool. The relative amount (as a percentage of the total amino acid content) allowed us to determine amino acid pools favored by plant metabolism

Table 1 Individual amino acid composition in the rosettes of plants grown on 10 and 2 mM nitrate for 29, 36, 50, 57 and 64 DAS

Amino acid nmol · (mg FW)$^{-1}$	29 DAS		36 DAS		50 DAS		57 DAS		64 DAS	
	10 mM	2 mM	10 mM	2 mM	10 mM	2 mM	10 mM	2 mM	10 mM	2 mM
Aspartate	1.672	1.133	1.210	0.403	2.177	1.039	1.830	0.969	1.385	1.010
Threonine	0.376	0.249	0.232	0.107	0.591	0.248	0.831	0.216	0.778	0.194
Serine	0.584	0.510	0.406	0.353	0.906	0.873	1.148	0.867	1.260	0.757
Asparagine	0.596	0.336	0.559	0.087	0.930	0.081	0.772	0.093	1.048	0.102
Glutamate	3.270	2.188	2.071	0.732	3.167	2.539	3.130	2.413	2.855	2.509
Glutamine	1.919	1.108	1.631	0.328	2.978	0.483	2.348	0.685	2.115	0.908
Glycine	0.371	0.183	0.300	0.027	0.176	0.080	0.134	0.131	0.179	0.116
Alanine	1.167	0.919	0.610	0.211	0.814	0.390	0.832	0.529	0.556	0.509
Valine	0.180	0.143	0.139	0.099	0.253	0.328	0.256	0.378	0.254	0.370
Arginine	0.026	0.026	0.026	0.027	0.118	0.038	0.203	0.028	0.525	0.025
Proline	0.393	0.169	0.328	0.112	1.342	0.211	1.588	0.198	1.441	0.189

Free amino acids were separated by chromatography and determined as nmol · (mg FW)$^{-1}$. Results were obtained after pooling the amino acid samples from four plants on the basis of equal FW used in the extraction.

Table 2 Comparison of RSA and P% of samples from plants grown on 10 and 2 mM nitrate

	LN (2 mM nitrate)	HN (10 mM nitrate)
P%DM	30.1% ± 2.2%	25.4% ± 1.9%
P%N	65.5% ± 3.2%	25.3% ± 3.0%
P%^{15}N	68.7% ± 1.8%	35.8% ± 7.8%
P%^{15}N / P%DM	2.29 ± 0.17	1.40 ± 0.22
RSA_{seeds}	0.90 ± 0.10	0.83 ± 0.07
RSA_{seeds} / $RSA_{whole\ plant}$	1.03 ± 0.03	1.19 ± 0.09
N% seeds [mg (100 mg · DW)$^{-1}$]	3.19 ± 0.06	4.34 ± 0.11
N% vegetative remains [mg · (100 mg DW)$^{-1}$]	0.72 ± 0.04	4.39 ± 0.23

Partitioning P% is the ratio between the total seed compartment and the whole plant. P%^{15}N = [g of ^{15}N in seeds · (g of ^{15}N in the whole plant)$^{-1}$], P%N = [g of N in seeds · (g of N in the whole plant)$^{-1}$] and P%DM = [g of dry matter in seeds · (g of dry matter in the whole plant). P% and RSA are the mean of four plants, and standard errors are presented. The experiment was repeated twice giving similar results and differences between 10 and 2 mM. A single experiment is presented.

when plants have been cultivated under LN or HN conditions (Fig. 4). Except at 29 DAS (Fig. 4A), the relative amounts of serine, glutamate, alanine, valine and γ-aminobutyric acid (GABA) were higher in the rosettes of LN-grown plants, whereas the relative amounts of threonine, asparagine, glutamine, arginine and proline were higher in the rosettes of plants grown in nitrate-rich conditions. These trends and the difference between LN and HN were exacerbated with aging (Fig. 4A–F). Under HN growing conditions, rosettes preferentially accumulated glutamine, asparagine, arginine and proline. Glutamine is the first product obtained from ammonium assimilation. Glutamine is dedicated to transport in the phloem sap and can accumulate to buffer glutamate concentration and detoxify ammonium. The argine molecule contains four nitrogen atoms and acts as a nitrogen reservoir. Proline which is preferentially synthesized in young leaf tissues can also accumulate to high concentration, especially when nitrogen assimilation is high. Asparagine build-up was observed in several water-stressed plants and has been proposed as a transient nitrogen storage metabolite in sugar-starved maize roots, and in seeds during dehydration and germination (Martinelli et al. 2007, and references therein). In high nitrogen conditions, the increase of asparagine that parallels the increase of glutamine stresses the importance of this molecule as a temporary N storage compound in annuals (Lea et al. 2007).

Since the relative amount of each amino acid was different between LN and HN rosettes, a possible influence on seed composition was investigated. Amino acid levels were 2-fold higher in HN seeds than in LN seeds (30 ± 5 vs. 14 ± 2 nmol · (mg FW)$^{-1}$; $n = 4$). The total nitrogen concentration and the total protein content were also significantly higher in HN seeds than in LN seeds [4.46 ± 0.17 vs, 3.31 ± 0.13, $n = 6$, (mg N · (100 mg DW)$^{-1}$) and 220 ± 8 vs. 171 ± 16, $n = 3$, (μg protein · (mg FW)$^{-1}$), respectively].

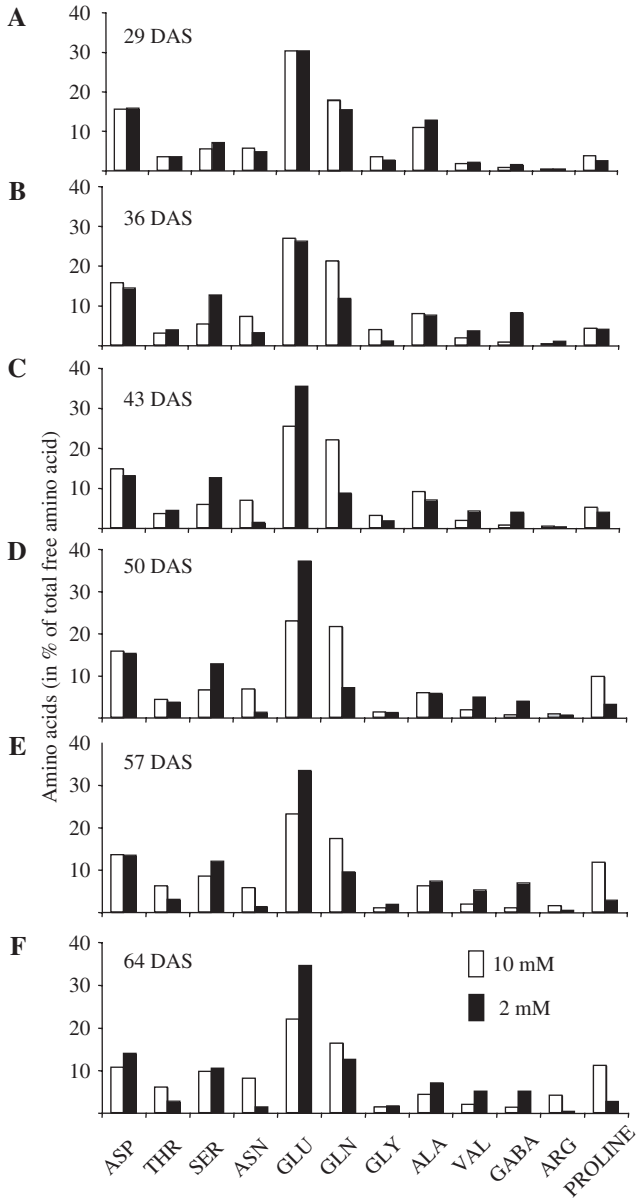

Fig. 4 Comparison of individual amino acid proportions (as a percentage of total amino acids) between extracts of rosettes grown under nitrate-limiting (black bars) or abundant (white bars) conditions for 29, 36, 43, 50, 57 and 64 d. Individual amino acids were quantified using ion-exchange chromatography of pooled samples extracted from four plants and mixed on the basis of equal total free amino acid content.

However, it can be noted that HN seeds preferentially accumulated free amino acids rather than proteins, compared with LN seeds. Indeed, the concentration of each individual amino acid, except glutamate, was higher in HN seeds compared with LN seeds. The glutamate content was similar in LN and HN seeds, whereas glutamine and asparagine levels were 19- and 6-fold higher in HN seeds. Arginine, proline and alanine were 3-fold higher (Fig. 5A).

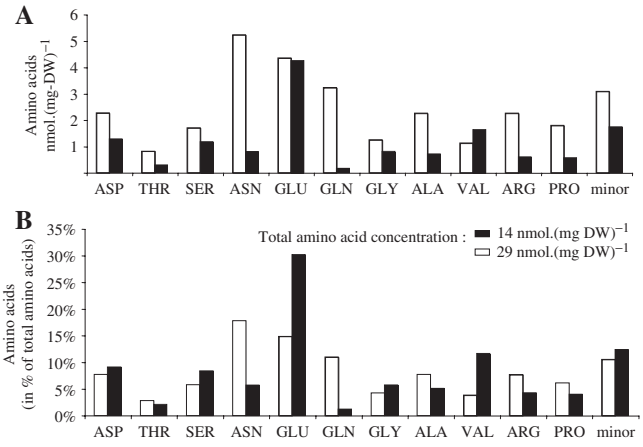

Fig. 5 Comparison of individual amino acid concentrations (A. nmol · (mg DW)$^{-1}$) and proportions (B, as a percentage of total amino acids) between seeds of plants grown under nitrate-limiting (black bars) or abundant (white bars) conditions. Individual amino acids were quantified using ion exchange chromatography with pooled samples extracted from seeds of four plants and mixed on the basis of equal total free amino acid content.

The relative individual amino acid amounts were calculated for HN and LN seeds (Fig. 5B). HN seeds preferentially accumulated asparagine, glutamine, alanine, arginine and proline, thus showing similarities between the relative amino acid amounts found in HN rosettes. Our results suggest that the individual amino acid proportions in the seeds are directly related to the individual amino acid proportions in the rosette leaves.

Diagnostic of nitrogen deficiency main effects on Arabidopsis thaliana metabolism and physiology

It has been known for a long time that nitrogen availability strongly influences plant yield and biomass. However, comparisons of nutrition effects on plant physiology and metabolism have remained fragmentary and poorly documented.

The most striking results showed that limiting nitrogen nutrition not only influenced plant development, yield and biomass, but also modified nitrogen metabolism and metabolite composition. N-limiting conditions promoted early nitrogen remobilization and recycling, allowing per se efficient plant economy. More than 68% of the rosette nitrogen was remobilized to the seeds under LN conditions, vs. 36% under HN conditions. Since at the same time as nitrogen was efficiently remobilized from LN rosettes, N assimilation was limited by poor nitrogen availability, the total nitrogen remaining in the LN vegetative remains at the end of the plant cycle was very low (six times less than in the HN vegetative remains). In addition, seed %N (mg N · (100 mg DW)$^{-1}$) was less affected and only 1.4 fold lower in LN seeds compared with HN seeds. Therefore,

the main problematic effect of LN nutrition is the large decrease of yield.

In accordance with a positive effect of nitrogen limitation on N recycling and remobilization to the seeds, N limitation has been seen to control senescence-related enzyme activities by inducing the early synthesis of GS1 and a premature decrease of NR activity (Masclaux et al. 2000). However, other premature senescence symptoms such as leaf yellowing had not been reported; in contrast, leaf redness was increased by LN as a result of anthocyanin accumulation (data not shown). As a result of the changes in N assimilation/N remobilization balance and in N allocation, it was found that the quality and the quantity of nitrogenous compounds was modified in both seeds and vegetative organs. The HN rosettes accumulated large amounts of nitrate and amino acids, but the protein concentration was similar to that of LN rosettes. Accumulation of amino acids in HN rosettes compared with LN rosettes resulted in a preferential increase in storage molecules such as amides, arginine and proline. Seed composition was also modified, with HN seeds accumulating 2-fold more amino acids than LN seeds. However, in contrast to HN rosettes, HN seeds also accumulated proteins. The nature of the amino acids stored in seeds was similar to that of those stored in the old rosettes (at 50 DAS), and HN seeds preferentially accumulated amides, arginine and proline compared with LN seeds. For both LN and HN plants, the seed amino acid composition paralleled the rosette amino acid composition. These observations led to questions concerning the mechanisms involved in amino acid flux to the seeds (van der Graaff et al. 2006, Masclaux-Daubresse et al. 2008). The fact that amino acid patterns in the phloem sap of several species resemble those in the cytosol of source cells indicates that the phloem loading process would be mainly unspecific (Lohaus and Moellers 2000). This could explain why loading seeds with phloem amino acid results in similar amino acid compositions in both source and sink compartments.

An important feature of this study is that a modification of fertilizer quantity will result in major changes in plant product quantities and yields, as well as in their nutritional quality.

Materials and Methods

Plant material

Seeds of *A. thaliana* accession WS were stratified for 48 h in 0.1% agar solution (in water) in the dark at 4°C before sowing. Homogeneous germination occurred 2 DAS. Three times per week, the pots were watered (by immersion of their base) in a solution containing 2 or 10 mM nitrate. Phosphate and sulfate were present at the same concentration (0.25 mM), as well as magnesium (0.25 mM) and sodium (0.20 mM) ions. The watering solution also contained 2.75 mM potassium, 0.5 mM calcium and 0.7 mM chloride ions. The pH of the watering solutions remained between 5.1 and 5.5. The homogeneous vegetative plant material was grown in a growth chamber under controlled conditions (Loudet et al. 2002). The experimental unit was a small pot (length = 60 mm, width = 65 mm, height = 60 mm) containing six plants positioned in a circle for the nitrate-limiting condition (2 mM nitrate) or a single plant per pot for the nitrate-abundant condition (10 mM). The plants were maintained under short days with a photoperiod of 8 h throughout the culture. Day and night temperatures were regulated at 21 and 17°C, respectively. Light was provided by 20 mercury-vapor bulbs, ensuring a photosynthetic photon flux density of approximately 160 μmol $m^{-2} s^{-1}$.

Plant growth and development on 10 and 2 mM nitrate was similar until 21 DAS. The first harvesting time was 29 DAS, after which rosettes were harvested every 7 d. Four distinct batches of rosettes constituted four independent biological repeats. At each harvesting time, four different bulks of rosettes (four biological repeats) were harvested between 10:00 and 11:00 am, and stored at –80°C until use.

Metabolite extraction and analysis, total nitrogen and protein content determination

Amino acids and ammonium were determined after extraction in a solution of 2% 5-sulfosalicylic acid (50 mg FW·ml^{-1}). Ammonium was determined according to the Berthelot method (Berthelot 1859). Total amino acid content was assayed by the Rosen colorimetric method using glutamine as a reference (Rosen 1957). The amino acid composition was determined in pooled samples extracted from equal dry weights of three individual plant repeats by ion-exchange chromatography using the AminoTac JLC-500/V amino acid analyzer according to the instructions of the manufacturer [JEOL (Europe), Croissy-sur-Seine, France).

Carbohydrates were extracted from aliquots (60 mg FW) of frozen plant material using a three-step ethanol–water procedure. The first step consisted of a 30 min extraction at 80°C using 600 μl of 80% ethanol, the second step consisted of a 20 min extraction at 80°C using 300 μl of 50% ethanol, and the third step completed the extraction by using 300 μl of water at 80°C for 20 min. The supernatant and pellets of each extraction step were obtained after centrifugation (15 min, 13,000 × *g*, 4°C) and pooled. The sucrose and hexose levels were determined from the ethanolic extract using a commercially available kit (R-Biopharm, Glasgow, UK). Starch content was determined from pellets using the same kit. Nitrate was measured according to the method of Cataldo et al. (1975) in the same extract used to measure the NR activity. Nitrite was determined from the blank of the NR assay (see below).

Total nitrogen was determined using oven-dried plant material and the Dumas combustion method with an NA 1500CN Fisons Instrument (Thermoquest, Runcorn, Cheshire, UK) analyzer.

Protein concentration was determined using a commercially available kit (Coomassie Protein assay reagent, BioRad, Hercules, CA, USA) and bovine serum albumin (BSA) as a standard. Seed protein concentrations were determined by measuring the amino acid levels of NaOH hydolysates according to Baud et al. (2007).

Enzymatic assays and Western blots

Enzymes were extracted from frozen leaf material stored at –80°C. Soluble protein content was determined in crude leaf extracts. NR maximal extractable activity and activation state were measured as described by Ferrario-Méry et al. (1998). The activation state of NR is defined as the ratio of the activity measured in the presence of 10 mM $MgCl_2$ divided by the activity in the presence of

5 mM EDTA, and is expressed as a percentage. GS was measured according to the method of O'Neal and Joy (1973). GS1 and GS2 isoforms were separated by SDS–PAGE according to Masclaux et al. (2000). Antibodies were raised in rabbits against the synthetic peptide AYGEGNERRLTG by Eurogentec (Seraing, Belgium) and they detected both GS1 and GS2 isoenzymes.

^{15}N tracing

Plants were grown in sand under similar conditions to those described previously, and at 56 DAS, plants were transferred to long day conditions (light/dark cycle 16 h/8 h) to induce flowering. At the two ^{15}N uptake time points, 40 and 42 DAS, the normal watering solution was replaced by a solution that had the same nutrient composition except that $^{14}NO_3$ was replaced by $^{15}NO_3$ to give 10% enrichment. After labeling, plant roots and sand were rinsed with deionized water, and unlabeled nutritive solution was used for the rest of the culture cycle. Plants were harvested at the end of their cycle when seeds were mature and the rosette dry. Samples were separated to give (i) 'dry vegetative remains' (rosette + stem + cauline leaves + siliques); and (ii) total seeds. Four replicates (plants) were harvested and the experiment was carried out twice.

For all the experiments, unlabeled samples were harvested in order to determine ^{15}N natural abundance. After the drying and weighing of each plant part, material was ground to obtain homogenous fine powder. A subsample of 1,000–2,000 µg was carefully weighed in tin capsules to determine total N content and ^{15}N abundance using an elemental analyzer (roboprep CN, PDZ Europa Scientific Ltd, Crewe, UK) coupled to an isotoper ratio mass spectrometer (Ttwenty-twenty, PDZ Europa Scientific Ltd, Crewe, UK) calibrated measuring natural abundance. The ^{15}N abundance of samples was calculated as atom percent and defined as $A\% = 100 \cdot (^{15}N)/(^{15}N + ^{14}N)$ for labeled plant samples and for unlabeled plant controls (Acontrol% was approximately 0.3660). The ^{15}N enrichment of the sample i was then defined as (Ai% – Acontrol%). The absolute quantity of ^{15}N contained in the sample i was defined as $Qi = DWi$; $(Ai\% - Acontrol\%) \cdot \%Ni$, with $\%Ni = [mg\ N \cdot (100\,mg\ DW)^{-1}]$ in the sample i. The Q value of the whole plant (wp) can be measured and $Qwp = \Sigma Qi$. The partition P% of ^{15}N in the organ i was calculated as $(\%P^{15}N \text{ in i}) = [(Ai\% - Acontrol\%) \cdot DWi \cdot \%Ni]/[(Awp\% - Acontrol\%) \cdot DWwp \cdot \%Nwp] = Qi/Qwp$. The relative specific allocation of ^{15}N was calculated as (Ai% – Acontrol%)/(Anutritive solution% – Acontrol%).

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