RESEARCH PAPER

Plant response to nitrate starvation is determined by N storage capacity matched by nitrate uptake capacity in two Arabidopsis genotypes

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Abstract

In a low-input agricultural context, plants facing temporal nutrient deficiencies need to be efficient. By comparing the effects of NO₃⁻-starvation in two lines of Arabidopsis thaliana (RIL282 and 432 from the Bay-0×Shahdara population), this study aimed to screen the physiological mechanisms allowing one genotype to withstand NO₃⁻-deprivation better than another and to rate the relative importance of processes such as nitrate uptake, storage, and recycling. These two lines, chosen because of their contrasted shoot N contents for identical shoot biomass under N-replete conditions, underwent a 10 d nitrate starvation after 28 d of culture at 5 mM NO₃⁻. It was demonstrated that line 432 coped better with NO₃⁻-starvation, producing higher shoot and root biomass and sustaining maximal growth for a longer time. However, both lines exhibited similar features under NO₃⁻-starvation conditions. In particular, the nitrate pool underwent the same drastic and early depletion, whereas the protein pool was increased to a similar extent. Nitrate remobilization rate was identical too. It was proportional to nitrate content in both shoots and roots, but it was higher in roots. One difference emerged: line 432 had a higher nitrate content at the beginning of the starvation phase. This suggests that to overcome NO₃⁻-starvation, line 432 did not directly rely on the N pool composition, nor on nitrate remobilization efficiency, but on higher nitrate storage capacities prior to NO₃⁻-starvation. Moreover, the higher resistance of 432 corresponded to a higher nitrate uptake capacity and a 2–9-fold higher expression of AtNRT1.1, AtNRT2.1, and AtNRT2.4 genes, suggesting that the corresponding nitrate transporters may be preferentially involved under fluctuating N supply conditions.

Key words: Arabidopsis thaliana, genetic variability, N partitioning, N recycling, N use efficiency, nitrate deficiency, nitrate remobilization rate, nitrate transporter gene expression, nitrogen reserves, plant development.

Introduction

Due to their immobility, plants have no choice but to cope with environmental fluctuations that may be temporary yet sudden. Competition for nitrogen resources in a crop population often leads to nutrient deficiencies in the root medium, especially during the interval between fertilizations. In agronomy, situations of sub- versus supra-optimal N supply are usually assessed using the nitrogen nutrition index (NNI). The latter is based on the concept of critical N concentration, defined as the minimum shoot N content allowing maximum shoot growth rate (Gastal and Lemaire, 2002). However, the NNI does not allow the singling out of the physiological processes that enable plants to withstand periods of N shortage while limiting consequences for subsequent growth (Jeuffroy et al., 2001). Understanding these processes would help in the selection of cultivars more tolerant to temporary N deficiency and in the production of crops more environmentally sound that sustainable agriculture requires.

Faced with temporary N starvation in the root medium, plants display a two-step response to cope with it. In the...
first step, like plants submitted to low but steady N concentrations, N-starved plants reduce leaf elongation rate which causes a decrease in shoot growth rate without affecting photosynthesis at first (Chapin et al., 1988b; McDonald and Davies, 1996; Anandacoomaraswamy et al., 2002). The carbon produced is mainly directed toward the subterranean part of the plant so that root elongation can be maintained or even stimulated (Smolders and Merckx, 1992). This results in a decrease in the shoot to root biomass ratio (Jarvis and Macduff, 1989; Scheible et al., 1997; Anandacoomaraswamy et al., 2002). Nitrogen content in plant tissues also decreases markedly during the early period of N starvation (Macduff et al., 1989; Walker et al., 2001). Among the N compounds, nitrate disappears the most quickly, evidence that plants are able to mobilize their reserves to maintain N metabolism, at least for a short period of time (Mackown, 1987; Macduff et al., 1989; van der Leij et al., 1998). Concomitantly, the capacity of plants to take up nitrate from the root medium usually increases during the first two days of N starvation, and then decreases (Chapin et al., 1988a; Jarvis and Macduff, 1989; Siddiqi et al., 1989). All these reactions indicate that plants are able to cope with a short period of N deficiency without serious consequences on growth.

In the second step, when N starvation does persist, a breakdown of leaf nucleic acids and enzymes occurs, a physiological process usually associated with leaf senescence (Crafts-Brandner et al., 1996, 1998; Hortensteiner and Feller, 2002; Mae, 2004). N deficiency is known to hasten senescence, due to a high sugar:nitrogen ratio (Wingler et al., 2004; Gombert et al., 2006). The breakdown of Rubisco late in the nitrogen starvation period leads to a decrease in the maximum photosynthetic capacity of the plant, ultimately inhibiting whole plant growth (Chapin et al., 1988b; Walker et al., 2001). This breakdown is relatively irreversible because regreening of senescent leaves is only possible for a very short period of time (Zavaleta-Mancera et al., 1999a, b). If plants are to sustain temporary N shortage, one of the key factors is therefore the ability to lengthen the first step, i.e. the recycling of N to fulfill growth requirements without affecting photosynthetic capacity.

The length of the first step partly relies on plant nitrogen use efficiency, i.e. the nitrogen to ensure maximal growth. Among the various plant species, some are less nutrient-demanding than others, those growing in very poor soils. The genetic diversity of Arabidopsis thaliana makes it a suitable model for studying genetic variability of plant adaptation to nutrient deficiency since ecotypes have been found in a wide range of habitats differing notably in soil richness (see, for example, http://dbsgap.versailles.inra.fr/vnat). This species is also an effective means to identify the particular genes underlying QTLs, thanks to the functional genomic and high-throughput genetic tools available for A. thaliana (Koornneef et al., 2004). In a previous study, the genetic basis (QTLs) determining nitrogen use efficiency were identified in a recombinant inbred line population derived from the cross of the Bay-0 and Shahdara ecotypes, which are genetically and ecologically distant (Loudet et al., 2002, 2003).

The duration of plant survival may also depend on differential C and N management in the whole plant, such as radiation use efficiency, N remobilization and reassimilation capacities, the proportion and quantity of N reserve compounds, the extent of the leaf elongation rate decrease, and the balance between shoot versus root biomass allocation. Most of the studies carried out on N deprivaton have compared N-starved plants with control ones, which allows for a detailed description of these processes but not for determining the importance of the role they play. On the other hand, to pinpoint key determinants of plant adaptation to N availability, numerous studies have used mutants, transformants or ecotypes, cultivated under limiting versus non-limiting N conditions (see references in Stitt and Krapp, 1999; Walch-Liu et al., 2005; Sakakibara et al., 2006). However, the limit of this approach is that N supply, even if low, is known to interfere with C and N assimilation and recycling (Millard et al., 1989). Comparing the reactions of contrasting genotypes placed under the same conditions of total nitrate starvation ensures (i) obtaining a true starvation based on the management of nitrate availability, (ii) the time-decorrelation of N uptake or storage and remobilization, and (iii) the comparative rating of processes. It seems therefore an appropriate way to separate the processes involved in resistance to NO₃⁻-starvation and thereby have a clear-cut base to evaluate their relative importance.

Such was our approach in the present work. The study focused on two lines of A. thaliana (numbered 282 and 432) chosen among the Bay-0 × Shahdara RIL population and exhibiting contrasted shoot N content (6.2% versus 7.6%, respectively) for the same shoot biomass (21 mg dry matter per plant) after 35 d of non-limiting N nutrition (Loudet et al., 2003). They were submitted to a 10 d NO₃⁻-starvation period in order to verify whether one of the lines would withstand nitrogen stress better than the other and to screen the physiological mechanisms by which it would manage to do so, with a particular focus on nitrate uptake, storage, and recycling. The main aim was to rate the relative importance of the processes potentially involved, such as N storage capacity, the type of N reserves stored, nitrate remobilization rate and/or duration, nitrate uptake capacity, but also light interception, radiation use efficiency, and C partitioning. It was demonstrated that line 432 coped better with nitrate starvation. More specifically, it was found that under these experimental conditions, many of the factors under consideration played no direct role in the resistance to
nitrate starvation. However, one factor emerged as the determining one: a higher storage capacity for nitrate, with a higher nitrate uptake capacity and a higher expression of nitrate transporter genes to match.

Materials and methods

Plant material and culture conditions

The Arabidopsis thaliana lines 282 and 432 belong to the Bay-0×Shahdara recombinant inbred line (RIL) population (Loudet et al., 2002). F2 seeds of the two lines were supplied by the Institut Jean-Pierre Bourgin stock centre (INRA Versailles, France). They were sterilized in ethanol-bayrochlor’ (95–5% v/v) prior to stratification in water at 4 ºC for 4 d. Each seed was sown on the top of one cut Eppendorf tube filled with half-strength nutrient solution (see below for composition) containing 0.7% agar. Tubes were inserted into a plate, resulting in an overall plant population of 676 plants m−2, and placed over a 6.0 l plastic tank filled with nutrient solution. The plants were cultivated hydroponically for 38 d (entire vegetative growth) in a Phytotron (Sanyo, France) equipped with fluorescent lights providing a Photosynthetic Photon Flux Density (PPFD) of 300 μmol m−2 s−1 for 8/16 h day/night. The short photoperiod was chosen to prevent early flowering. The level of irradiance used corresponds to 70–75% of the maximal CO2 assimilation rate (Toquin and Perilleux, 2004). The day/night temperature was 21/18 ºC, respectively, and relative humidity was 80%.

For 28 d plants were fed a complete nutrient solution containing 5 mM nitrate as nitrogen source (4 mM KNO3, 0.5 mM Ca(NO3)2, 0.25 mM KH2PO4, 0.25 mM MgSO4, 0.2 mM NaCl). Then, for 10 d, plants were fed a 0 mM nitrogen solution (0.25 mM KH2PO4, 1.5 mM K2SO4, 0.25 mM MgSO4, 0.5 mM CaCl2, 1 mM KCl, 0.2 mM NaCl). A set of control plants was maintained on the complete nutrient solution. All nutrient solutions contained oligoelements (10 μM MnSO4, 24 μM H3BO3, 3 μM ZnSO4, 0.9 μM CuSO4, 0.04 μM (NH4)6MoO4·3H2O), and iron-EDTA 10 mg l−1 (Sequestrene, Ciba-Geigy, Switzerland). They were renewed once during the first week of culture, three times a week during the two following weeks, and thereafter every day until the end of the experiment.

Plant sampling and measurements

A first harvest of plants was carried out at 28 d after sowing (DAS), 3.5 h prior to the beginning of the NO3− starvation period. N-starved plants were then harvested at 1, 2, 4, 7, and 10 d of NO3− starvation (DNS), i.e. at 29, 30, 32, 35, and 38 DAS, respectively. For control plants, a sole harvest was carried out at 35 DAS. At each date, five replicates of 1–3 plants were sampled. Each replicate was placed on a 98% 15N labelled nutrient solution (0.2 mM 15NO3−, with 4/5 K15NO3 and 1/5 Ca(15NO3)2 added to the N-free solution) for 5 min in order to measure NO3− influx. Before and after the uptake period, roots were rinsed for 1 min in a 0.1 mM CaSO4 solution.

Roots of each replicate were patted dry with a paper towel and rapidly separated from the shoots. After weighing, roots were frozen and ground in liquid nitrogen. An aliquot of the powder was dried for 2 d at 80 ºC in order to determine root dry matter content (% of fresh weight) from which total root dry mass was calculated. At the same time, leaves of one plant per replicate were separated and leaves larger than 2 mm2 were counted. In addition, total leaf area was measured by scanning each and every leaf regardless of size, and by analysing images using the WINRHIZO PRO software package (Regent Instruments, Inc., Canada). This software was also used to calculate photosynthetic leaf area (PLA) from photos of growing plants taken in the lowest direction prior to each harvest. Shoots of each replicate were then lyophilized, weighed, and ground into a fine powder.

Total C, total N and 15N determination

Total N and C contents as well as 15N proportion in roots were determined from an aliquot (1–2 mg) of dried powder using a flash combustion technique based on the Dumas principle and involving an automatic element analyser coupled with a mass spectrometer (Fisons Instruments NA 1500, Thermoelectron, Courtaboeuf, France).

Metabolite analysis

An aliquot of powder was weighed and extracted in a four-step ethanol–water procedure as described in Orsel et al. (2004). For nitrate content determination, extracts were evaporated and diluted in water before analysing by HPLC using a DX-120 instrument (Dionex, Sunnyvale, CA, USA). The same extracts were subjected to an evaluation of free amino acid content using glutamine as a standard (Rosen, 1957). For roots, results were expressed in μmol g−1 dry weight by multiplying the data obtained (μmol g−1 fresh weight) by the dry matter content (% of the fresh weight).

Soluble proteins were extracted from an aliquot of lyophilized powder with 0.1 M H3PO4, 0.1 M NaCl, 0.1 mM EDTA, 1% β-mercaptoethanol (v/v), pH 7.5 in presence of insoluble polyvinylpolypyrrolidone. After agitation and sonication for 20 min, extracts were centrifuged (4000 g, 10 min, 4 ºC). Soluble proteins were quantified from supernatants of samples from three harvest dates (Lowry et al., 1951) using bovine serum albumin as a standard.

Gene expression

The expression of several genes thought to be involved in nitrate transport was measured in roots just prior to NO3−-starvation (at 28 DAS), and after 2 DNS and 7 DNS in NO3−-starved plants, using real-time PCR after total RNA extraction from the frozen powder and reverse transcription according to the procedure described by Orsel et al. (2004). Specific primer sets were used for AtNRT2.1, AtNRT2.4, and AtNRT2.5 genes as previously described (Orsel et al., 2002, 2004). The AtNAR2.1 cDNA was amplified between the 5′-CCA GAA GAT CCT TCT TGC TTC ACT forward primer and the 5′-CCC AAT CGA GCT TAG CTT GCA reverse primer. For the AtNRT1.1 gene, the forward and reverse primers were, respectively, 5′-AGA CGG AAC CAA AAG AAC GA and 5′-CCA GGA TAA CCG CAG CAA CC. Results were expressed as a percentage of the constitutive AteF1A4α gene expression level (Orsel et al., 2004). To normalize the differences in the gene expression level and to compare both lines more easily, the ratio between expression in line 432 to expression in line 282 for each gene was calculated.

Statistical analysis and calculations

The experiment was conducted twice. Because data from both experiments were highly similar, results from both experiments were combined in this article. For each date, results are the mean of 2 to 8 according to the variable considered. Significance of the differences between genotypes and/or N-starved versus control plants was estimated using analyses of covariance (ANCOVA) carried out with XLStat 2007 (Addinsoft SARL, France), using the Bonferroni test with α=5%. The model Yi j =Di +Gj +Nk +eijk was used, in which Di was the date effect, Gj the genotype effect, and Nk the nutrient effect.

NNI was calculated for each date by dividing the total N content measured in the shoots by the critical N content, calculated for the
measured shoot dry matter using the critical dilution curve of C3 plants established by Gastal and Lemaire (2002) and converted to Arabidopsis plants (%Nc=13–DW–0.32), in which DW corresponded to the shoot dry matter in mg per plant and %Nc was the critical N content (data not shown).

Partitioning of C between shoots, roots, and reserves was evaluated as follows. The proportion of root allocation to structural root growth was estimated to be the carbon quantity in roots multiplied by the lowest root carbon content observed in control plants, and then divided by the whole plant C quantity. The same calculation was performed to estimate the proportion of shoot structural C. The underlying hypothesis of this calculation is that stored C quantity is negligible in Arabidopsis plants grown under non-limiting N conditions (Orsel et al., 2004). In our case, the possible discrepancy between whole plant C quantity and the one used for structural growth constituted a potential C reserve compartment.

The amount of N in amino acids and proteins was calculated given that 1 mole of these compounds contains about 17 g of N (i.e. 12.2% of dry mass) at the beginning of NO3-starvation and 16 g (11.4%) and 18 g (13%) at 7 DNS in shoots and roots, respectively. Those values were derived from data obtained on ecotype WS during a N-starvation experiment, in which variation of each individual amino acid was quantified (M Orsel, unpublished data).

Results

Assessment of NO3-starvation effects

Firstly, the effect of nitrate starvation in A. thaliana was evaluated by comparing N-starved and control plants after 7 d of nitrate starvation (DNS). To appraise the sensitivity of the plants to NO3-starvation, we relied on the nitrogen nutrition index (NNI), calculated with a critical dilution curve of C3 plants (Gastal and Lemaire, 2002). As expected, the NNI was markedly lower (3.6 times) in the N-starved plants (Table 1). They exhibited a \(<1\) NNI as of the second or the third DNS, inferring an early plant growth limitation (Fig. 1). At 7 DNS, shoot dry mass was 1.6 times less than in control plants (Table 1). The shoot growth inhibition was accompanied by a marked reduction in total leaf area and, to a lesser extent, in photosynthetic leaf area. By contrast, neither leaf number nor root growth significantly differed from control plants at 7 DNS. As a result, the root:shoot ratio was two times higher than in the control plants (Table 1).

As NNI depends on shoot N content, it was not surprising that total N content was divided by 3 in shoots and by 2 in roots compared with control plants (Table 1). This was related to a fall in total amino acid content and mostly to a fall in nitrate content, which underwent an up to 120-fold reduction compared with control plants. By contrast, soluble protein content was not different from control plants in shoots and in roots (Table 1). Nitrate starvation did not affect either the total amino acid-N net amount or the proportion in the whole plant. A very small depletion of the total amino acid-N net amount was observed in shoots, fully compensated by an equal increase in roots (Table 2). This contrasted with nitrate-N
amount, which was fully depleted during NO$_3^-$-starvation (Table 2). Nitrate accounted for 35–40% of total N at the onset of NO$_3^-$-starvation and dropped to 0–2% after 7 DNS. By contrast, an increase was observed in the net amount of soluble proteins in shoot and in other N-compounds (assumed to be mainly insoluble proteins) in roots. As a result, the proportion of soluble proteins accounted for up to 70% of shoot total N at 7 DNS (Table 2).

Taken together, these results indicate that nitrate starvation greatly affected most of the components of plant development and C and N management, and it induced a large reorganization of the nitrogen internal pool by depleting the nitrate pool in favour of proteins. However, nitrate starvation did not impair root growth, visible leaf appearance rate or soluble protein content.

Comparison of susceptibility to NO$_3^-$-starvation

The nitrogen nutrition index (NNI) was calculated for both lines in order to compare their susceptibility to NO$_3^-$-starvation. Despite a similar progressive decrease in the NNI for both lines, line 432 exhibited a significantly higher NNI than line 282 throughout the 10 days of starvation (Fig. 1). It was evaluated that NNI became inferior to 1 from 2.6 DNS for 432 and from 1 DNS for 282, indicating that 432 maintained an optimal growth for 1.5 d longer. In addition, despite similar biomasses at the beginning of the experiment and under non-limiting N conditions, line 432 produced more biomass than line 282 throughout NO$_3^-$-starvation (Fig. 2A, B; Table 1). By the end of the experiment, this led to a gain of 34% in dry weight in shoots and 42% in roots for 432. Taken

Table 2. Quantitative changes and distribution of the nitrogen compounds between 0 d and 7 d of NO$_3^-$-starvation for lines 282 and 432

Results are expressed as N quantity (mg) per plant present in each type of nitrogen compound (upper half) and as participation of each compound to total N in percentage (lower half) for which a comparison with control plants (CT) is given.

<table>
<thead>
<tr>
<th></th>
<th>282 Shoots</th>
<th>282 Roots</th>
<th>282 Whole plant</th>
<th>432 Shoots</th>
<th>432 Roots</th>
<th>432 Whole plant</th>
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<tbody>
<tr>
<td>DNS 0</td>
<td>DNS 7</td>
<td>Delta</td>
<td>DNS 0</td>
<td>DNS 7</td>
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<td>DNS 0</td>
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<td>0.11</td>
<td>0.00</td>
<td>-0.11</td>
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<tr>
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<td>Soluble proteins</td>
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<tr>
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<td><strong>Per cent of total N</strong></td>
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<tr>
<td>Nitrate</td>
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<td>0%</td>
<td>39%</td>
<td>0%</td>
<td>0%</td>
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<tr>
<td>Amino acids</td>
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<td>4%</td>
<td>6%</td>
<td>6%</td>
<td>7%</td>
<td>5%</td>
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<td>Nitrate</td>
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<td>Amino acids</td>
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<td>6%</td>
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together, these results indicate that line 432 was less susceptible to nitrate starvation than line 282.

**Investigation of aerial development and C production variability**

To keep growing under N-shortage conditions implies a C residue supply and that led us to investigate if light interception, C production, and C partitioning differed between the two lines. The number of leaves was significantly higher for 282 than for 432, but this was also the case in control plants (Fig. 2D; Table 1). This had no impact on light interception because total and photosynthetic leaf area did not differ between the two lines (Fig. 2E, F). The capacity to intercept light was therefore similar in the two lines.

Carbon partitioning between shoots and roots did not differ either, since the root:shoot ratio was similar in both lines (Fig. 2C). NO$_3^-$-starvation induced carbon storing in the plant, as indicated by the increase of carbon content in root (from 38% to 40% DW) and mostly in shoots (from 34% to 39% DW) over the first few days (Fig. 3A, B). Shoot carbon content was 1% higher in 432 than in 282 due to an initial difference between the two lines, the time-course of C content during NO$_3^-$-starvation being

**Fig. 2.** Developmental response of both lines to NO$_3^-$-starvation, estimated via the evolution of shoot dry mass (A), root dry mass (B), partitioning of total dry mass between aerial and subterranean compartments (C), number of visible leaves per plant (D), photosynthetic leaf area (E), and total leaf area (F). Leaves were counted when their size was at least 2 mm². Cotyledons were considered. Error bars indicate standard deviation of mean ($n=2–8$). Using the Bonferroni test with $\alpha=0.05$, the letters $a$ and $b$ indicate significant difference between lines.
exactly the same for both lines. Nonetheless, the partitioning of C between reserves and the structural growth of shoots and roots was the same in both lines (Fig. 3C).

Investigation of the variability of N metabolites in response to nitrate starvation

N-starvation has already been shown to strongly alter plant N contents. The variation of N metabolites was analysed in both lines to investigate whether variability in susceptibility to NO$_3^-$-starvation may arise from differences in N metabolite content, quantity or relative proportion.

Basically, both lines exhibited the same dynamics of N metabolite changes in response to nitrate starvation (Fig. 4; Table 2). They underwent a 2- and 3-fold decline in total N content between 0 DNS and 10 DNS in roots and shoots, respectively, due to a decrease to the same extent in amino acid content, and mostly to a sharp and early drop in nitrate content. The latter lost 90% and 75% of its initial value within 2 d in roots and in shoots, respectively, to reach about zero at 4 DNS in roots and 7 DNS in shoots (Fig. 4). In addition, N partitioning among the metabolites and changes in the proportion of these N metabolites during NO$_3^-$-starvation were similar for both lines (Table 2).

However, beyond this similar pattern, it was observed that line 432 retained a higher N content (10–15%) than 282, especially at the onset of NO$_3^-$-starvation. This advantage resulted from higher contents of nitrate (up to 37% more) and root amino acid (up to 25% more). By contrast, both lines did not differ either in soluble protein or in shoot amino acid contents (Fig. 4G, H).

Characterization of nitrate uptake capacity

Discrepancy in initial N contents led us to believe that nitrate uptake capacities were markedly different in the two lines. Capacity of N-starved plants to take up nitrate was approached by measuring $^{15}$NO$_3^-$ influx throughout the nitrate starvation period. Results showed that line 432 started the NO$_3^-$-starvation period with a nitrate uptake capacity 50% higher than 282 and kept a 25% advantage over 282 during the first 2 d of NO$_3^-$-starvation (Fig. 5A). To explore the molecular bases of this higher performance, the relative expression of five genes thought to be involved in nitrate transport was studied throughout the NO$_3^-$-starvation period. Just before nitrate starvation was
induced, all these genes showed a higher expression in 432 but only three were of significance: two high-affinity transport system genes (AtNRT2.1 and 2.4) and one low-affinity transport system gene (AtNRT1.1) (Fig. 5B). Thus, line 432 showed a 9-fold higher expression of AtNRT2.4 than 282. AtNRT2.1 relative expression was 3-fold higher in 432, which was of great importance considering that this gene bore 99% of the expression of the NRT2 gene family at that time (data not shown). Moreover, expression of AtNRT1.1 was 1.7 times higher in 432. At 2 DNS, the expression superiority of 432 over 282 tended to diminish but was still significant for AtNRT2.4 and AtNRT1.1 (Fig. 5C). At 7 DNS, the relative gene expression of 432 versus 282 was similar for all the genes studied (Fig. 5D), which was in accordance with the absence of difference in $^{15}$NO$_3$ influx at this date.

Fig. 4. Evolution of the nitrogen compound contents in both lines during NO$_3$-starvation, i.e. content of total nitrogen (A, B), nitrate (C, D), amino acids (E, F), and soluble proteins (G, H) in shoots and roots, respectively. Error bars indicate standard deviation of mean ($n$=5). Using the Bonferroni test with $\alpha$=0.05, the letters a and b indicate significant difference between lines.
Therefore, the better nitrate uptake capacity of 432 was found to correlate highly with the higher expression of at least three genes thought to be involved in nitrate transport.

Characterization of N remobilization dynamics associated with nitrate starvation

The ability to withstand N starvation may rely on the quantity of nitrogen stored in the plant as highlighted above, but also on N remobilization capacity, which prompted us to characterize N compound mobilization dynamics. Considering our experimental design, in which no new uptake of nitrate occurred after the beginning of nitrate starvation, it was relevant to characterize the nitrate remobilization rate through the time-dependent decrease in the nitrate content. Interestingly, it was found that the nitrate remobilization rate was proportional to the nitrate content in the whole plant (Fig. 6A) as well as in both shoots (Fig. 6B) and roots (Fig. 6C). The linear relationships were the following: \( \frac{d\%\text{NO}_3}{dt}=a\%\text{NO}_3 \), with \( a=0.66, 0.62, \) and \( 0.89 \) in whole plant, shoots, and roots, respectively. Nitrate remobilization was therefore faster in roots than in shoots.

Nevertheless, the remobilization dynamics of \( \text{NO}_3^{-} \), amino acids, soluble proteins, and total N during \( \text{NO}_3^{-} \)-starvation were the same for both lines, implying that susceptibility to nitrate starvation did not rely on contrasting N remobilization capacity in our lines. Thus, for total N and nitrate contents, compared to the values of 282, those of 432 displayed a time lag of 0.6 d. For root amino acid content, the initial discrepancy held throughout the \( \text{NO}_3^{-} \)-starvation (Fig. 4F).

Discussion

The objective of this study was to screen the determining physiological bases driving susceptibility to nitrate starvation of two contrasting genotypes of \textit{A. thaliana} and to rate their relative importance. Using NNI as an indicator of the N limitation level (Jeuffroy et al., 2001; Gastal and Lemaire, 2002), it was found that line 432 coped better with nitrate starvation than line 282, the former being able
to sustain maximal growth for 1.5 additional days under our conditions. The higher biomass of 432 also indicated a better resistance, both lines showing identical biomasses at the onset of nitrate starvation and under non-limiting NO$_3^-$ conditions.

**Basically, both lines exhibited similar features when faced with nitrate starvation.** They strongly and similarly reduced shoot elongation, but not the rate of leaf emergence, as also observed in several grasses (Stanford et al., 2005; Bartholomew and Williams, 2006). This was perhaps due to the fact that NO$_3^-$-starvation was induced late in the vegetative growth, at a time when most leaves were already initiated. As a result, shoot growth was limited early on, which contrasted with grasses, lettuce, and tea plants, in which dry matter production was not altered after 8–12 d of N-starvation (Jarvis and Macduff, 1989; Walker et al., 2001; Anandacoomaraswamy et al., 2002). At the same time, carbon allocation within the plant was modified to the same extent in both lines. A preferential allocation of C to the roots was observed, as well as the emergence of a pool of C reserves in response to NO$_3^-$-starvation, suggesting that a decrease in photosynthetic activity and plant growth inhibition may not be necessarily correlated, contrary to what has been proposed by Chapin et al. (1988b). In both lines, the nitrate pool was fully depleted early on, whereas the protein pool was greatly increased, mainly in a soluble form in shoots and in an insoluble form in roots. This indicates that protein synthesis did not come to a full stop during NO$_3^-$-starvation and suggests that proteins represented a sink for the released nitrogen supplied by the previously stored nitrate pool. It has been demonstrated that the nitrate remobilization rate was proportional to the nitrate content at any given time in the shoots as well as in the roots, specifying the dynamics of remobilization already obtained at the whole plant scale by Macduff et al. (1989) in grasses. By quantifying the nitrate remobilization rate, it was shown that nitrate depletion was faster in roots than in shoots. As a result, both lines displayed a root growth similar to the one in control plants, as already observed in several species (Jarvis and Macduff, 1989; Anandacoomaraswamy et al., 2002).

**Differences between the lines arose from contrasted nitrate storage capacities.** NO$_3^-$-starvation is known to induce N reserve use (Chapin et al., 1988b). A close relationship between the relative growth rate of N-starved plants and the N content of tissues has already been observed (Walker et al., 2001), but the relative importance of reserve quantity, mobilization rate and duration, as well as N reserve type to sustain exogenous lack of nitrate
remains unclear. Our study highlighted the key role of the initial size of the N storage pool in the capacity of our genotypes to cope with nitrate starvation, unlike the remobilization dynamics and the composition of the internal N pool. Indeed, it was noticed that the most resistant line, 432, beyond its strong similarities to 282, exhibited a higher N content and quantity than 282 throughout NO3−-starvation. The partitioning of N among N-metabolites was similar in both lines and the decrease of N content followed exactly the same rate and duration in both lines, implying that the differences between the lines relied on contrasting initial N values. In five RILs of A. thaliana, Diaz et al. (2005) also observed that nitrate remobilization from senescing leaves occurred similarly despite contrasted initial nitrate contents.

Within the initial N pool, nitrate on its own accounted for 40% and explained one-half of the difference in the N quantity between the two lines. Moreover, it was the only N compound whose net amount decreased during the 10 d NO3−-starvation period (Table 2) in contrast to the content of N compounds which all decreased gradually (Fig. 4). These facts led us to regard nitrate as the main N storage compound in A. thaliana under our experimental conditions. Soluble proteins also accounted for a large proportion of the plant N pool (one-third) but were regarded as a sink rather than as a source for N during nitrate starvation. Nonetheless, amino acids and protein pools could have played a more significant role, had the initial nitrate reserves been less plentiful. Given these facts, an efficient adaptation to N stress seems to lie, at least partly, in the capacity to increase nitrate storage prior to nitrate starvation. Regulations governing nitrate storage capacity appear rather complex. For example, using Arabidopsis mesophyll cells, Cookson et al. (2005) have shown changes in nitrate cytosolic activity in response to a light–dark transition, which can trigger uptake at the plasma membrane or remobilization from the vacuole. Fan et al. (2006) have shown that, in the case of barley roots, a treatment with glutamine may alter membrane nitrate fluxes, resulting in a transient increase in cytosolic nitrate activity without any change in the vacuolar pool of nitrate. Some authors have suggested that the nitrate storage capacity has an upper limit (Améziane et al., 1997; van der Leij et al., 1998). In our case, the shoot initial NO3− content of 432 was statistically shown to be too low, compared to the value expected from remobilization dynamics data. This suggests either an experimental aberration or more likely that the shoot NO3− content of 432 reached a maximum at about 2 mmol g−1 DW. Compared to other species also grown hydroponically, this value seems high, suggesting that, as other Brassicaceae, A. thaliana has a high nitrate storage capacity (Lainé et al., 1993; Luo et al., 2006), arguing that nitrate would be the main N reserve in this species, at least in the case of nitric N supply.

Given our data, we cannot dismiss the hypothesis of qualitative differences in amino acid pool composition as observed by Diaz et al. (2005) in senescing leaves of A. thaliana RILs. If that were the case, the role it played was probably more of a signalling or qualitative rather than of a quantitative significance, considering that amino acid N accounted for only 5% of the total N pool. A signalling role of amino acids in the regulation of nitrate influx and NRT genes has been revealed (Nazoa et al., 2003). Therefore, N content of the plants could ultimately be regulated via signalling by amino acid composition. It was also noticed that 432 had a higher radiation use efficiency than 282, being able to produce larger quantities of C with a photosynthetic leaf area similar to the one of 282, which may also help the plant to withstand starvation. This was most probably due to a better photosynthetic efficiency rather than to a higher Rubisco content, shoot soluble protein content having been similar in both lines. Rubisco is known to accumulate in shoots without necessarily increasing photosynthetic efficiency (Quick et al., 1991). The higher radiation use efficiency of line 432 was, however, not induced by NO3−-starvation but was an intrinsic feature, already observed before NO3−-starvation. Using our experimental approach it was not possible to discriminate between the relative significance of nitrate storage capacity versus radiation use efficiency. Both features were probably implicated, protein synthesis during NO3−-starvation involving N as much as C residues. A means to distinguish which of these two variables is the most highly implicated may be to elaborate a quantitative modelling of C-N interactions during NO3−-starvation and to check the relative sensitivity of each of these parameters. However, looking at the dynamics of C and N accumulation in our study, it may be assumed that, in the case of brief nitrate starvation, the lack of endogenous N would occur before C residues ran out, leading us to regard nitrate storage capacity as the key factor in resistance to nitrate starvation.

Nitrate storage capacity had nitrate uptake capacity and nitrate transporter gene expression to match. To increase plant nitrate storage may rely on a higher nitrate uptake. Our next question was to investigate if NO3− storage capacity was related to nitrate uptake capacity and to explore which nitrate transport genes were involved. With this aim in mind, a short labelling period was chosen to quantify the maximal nitrate uptake capacity of the lines, avoiding perturbations due to 15NO3− efflux or 15NO3− reduction in shoots (Devienne et al., 1994). Although the labelling data may have been underestimated because of possible losses of tracer due to root rinsing and blotting, the results indicated that line 432 exhibited a higher initial nitrate influx than line 282. This superiority held out for 2 d of NO3−-starvation and was in agreement with the nitrate content of the plants. In addition, control plants of line 432 also exhibited a higher nitrate uptake capacity at
7 DNS. The higher nitrate uptake capacity of 432 versus 282 was confirmed by measuring net $^{15}$NO$_3$ uptake for 24 h, which takes nitrate influx, efflux, and metabolization into account in plants grown on soil under high N supply steady-state conditions (data not shown). Thanks to the starvation approach, which allowed us to time-dissociate contribution of nitrate uptake versus recycling to growth, these results suggest that NO$_3$ uptake capacity played a direct role in resistance to NO$_3$-starvation via the establishment of nitrate storage capacity. The genotypic variability found for nitrate storage capacity may also arise from differences in the regulation of nitrate assimilation and/or of nitrate influx into the vacuole. De Angeli et al. (2006) have identified a channel mediating nitrate transport into the vacuole and its expression has been shown to be enhanced by nitrate (Geelen et al., 2000).

Concerning the molecular basis of nitrate influx, it has been shown that the higher resistance to NO$_3$-starvation of 432 was matched by a higher expression of several NRT and NAR genes. The higher expression level of these genes in line 432 may be related to a less efficient regulation of nitrate uptake in this line compared to line 282. Among these genes, AtNRT2.1, AtNRT1.1, and AtNRT2.4 were of particular significance. Interestingly, A. thaliana knock-out mutants of NRT2.1 and NRT2.2 (atnrt2.1-1) or NAR2.1 genes (atnrt2.1-1) displaying a reduced nitrate influx have been shown to have a slowed growth under N-limiting conditions (Filleur et al., 2001; Orsel et al., 2004, 2006). AtNRT1.1 and AtNRT2.1 are thought to be the major contributors to LATS and iHATS root influx, respectively (Cerezo et al., 2001; Guo et al., 2001; Okamoto et al., 2003). Therefore, it may be assumed that these two genes play a main role in the nitrate stockpiling prior to nitrate starvation. Because our results revealed a tight relationship between nitrate storage and nitrate uptake capacities, in future work it would be interesting to investigate the regulation of nitrate uptake in the two lines and especially the role of the two above-mentioned genes in the plant response to fluctuating nitrate supply or to very low external nitrate concentration.

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