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## RESEARCH PAPER

# Exploring nitrogen remobilization for seed filling using natural variation in *Arabidopsis thaliana*

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## Abstract

Nineteen *Arabidopsis* accessions grown at low (LOW N) and high (HIGH N) nitrate supplies were labelled using  $^{15}\text{N}$  to trace nitrogen remobilization to the seeds. Effects of genotype and nutrition were examined. Nitrate availability affected biomass and yield, and highly modified the nitrogen concentration in the dry remains. Surprisingly, variations of one-seed dry weight ( $\text{DW}_{1\text{S}}$ ) and harvest index (HI) were poorly affected by nutrition. Nitrogen harvest index (NHI) was highly correlated with HI and showed that nitrogen use efficiency (NUE) was increased at LOW N. Nitrogen remobilization efficiency (NRE), as  $^{15}\text{N}$  partitioning in seeds ( $^{15}\text{NHI}$ ), was also higher at LOW N. The relative specific abundance (RSA) in seeds and whole plants indicated that the  $^{14}\text{NO}_3$  absorbed post-labelling was mainly allocated to the seeds (SEEDS) at LOW N, but to the dry remains (DR) at HIGH N. Nitrogen concentration (N%) in the DR was then 4-fold higher at HIGH N compared with LOW N, whilst N% in seeds was poorly modified. Although NHI and  $^{15}\text{NHI}$  were highly correlated to HI, significant variations in NUE and NRE were identified using normalization to HI. New insights provided in this report are helpful for the comprehension of NUE and NRE concepts in *Arabidopsis* as well as in crops and especially in *Brassica napus*.

**Key words:**  $^{15}\text{N}$ , harvest index, nitrogen use efficiency, remobilization, yield.

## Introduction

A major challenge of modern agriculture is to reduce the excessive input of fertilizer and at the same time to improve grain quality without affecting yield. Yield and seed quality are important for human and animal food consumption. The percentage of protein contained in cereal grains determines their nutritive and commercial values and both nitrogen harvest index (NHI) as [grain N/whole plant N] and seeds nitrogen concentration ( $\text{N}\%_{\text{SEEDS}}$ ) are major indicators of nitrogen use efficiency and seed nutritional quality (see Masclaux-Daubresse *et al.*, 2010, for a review).

Until now, most plant cultivars have been selected under non-limiting nitrogen conditions (Bänziger *et al.*, 1997; Presterl *et al.*, 2003) for productivity and grain yield. Under such condition, it was shown that the improvement of seed

dry weight and plant yield was associated with the increase in leaf longevity and with the prolongation of photosynthesis that contributes to carbon filling into seeds (Duvick, 1992; Rajcan and Tollenaar, 1999; Borrel *et al.*, 2001; Gregersen *et al.*, 2008). Because delaying leaf senescence was also shown to delay nitrogen remobilization (Diaz *et al.*, 2008), negative correlations between grain protein concentrations and yield were also observed (Beninati and Busch, 1992). Therefore, it is suspected that breeding plants with delayed leaf senescence has to cope with the dilemma of getting higher yield but lower grain protein content. One way to solve the yield versus seed protein content dilemma and to decrease fertilizer use is to improve plant nitrogen management through manipulating nitrogen recycling and

Abbreviations: LOW N, low nitrogen; HIGH N, high nitrogen; DR, dry remains after seed harvest; SEEDS, total seeds of one plant; DW, dry weight;  $\text{DW}_{\text{DR}}$ , dry weight of dry remains;  $\text{DW}_{\text{SEEDS}}$ , dry weight of total seeds;  $\text{DW}_{1\text{S}}$ , dry weight of one seed; E%,  $^{15}\text{N}$  enrichment; N%, nitrogen concentration as  $\text{g}\cdot(100\text{g DW})^{-1}$ ; C%, carbon concentration as  $\text{g}\cdot100\text{g}^{-1}\text{DW}$ ; DAS, days after sowing; HI, harvest index; NHI, nitrogen harvest index;  $^{15}\text{NHI}$ ,  $^{15}\text{N}$  partitioning in seeds; RSA, relative specific abundance;  $\Delta\text{Rem}$ , indicator for extra nitrogen remobilization; NUE, nitrogen use efficiency; NRE, nitrogen remobilization efficiency.

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especially nitrogen remobilization from vegetative plant organs to the seeds. Improving nitrogen remobilization efficiency (NRE) from vegetative tissues has the advantage of improving grain filling while limiting exogenous nitrogen demand after flowering and to decrease the amount of unused nitrogen in the dry remains after harvest.

The main control for nitrogen filling in seeds is located in the source leaves and for some plants in the stems (Good *et al.*, 2004; Zhang *et al.*, 2007). In contrast to carbon, the nitrogen imported into the developing seeds is largely derived from the recycling of nitrogen sources assimilated before the onset of the reproductive phase (Cliquet *et al.*, 1990; Patrick and Offler, 2001). Depending on the species, it appears that nitrogen uptake is down-regulated and, for some, totally inhibited during seed filling. Studies using  $^{15}\text{N}$  tracing on cereals, oilseed rape, and legumes showed that the onset of grain filling was a critical phase for seed production, because N uptake and  $\text{N}_2$  fixation declined after flowering and during seeds maturation (Christensen *et al.*, 1981; Salon *et al.*, 2001; Rossato *et al.*, 2002). In *Arabidopsis*, it is shown that, when plants are grown under non-limiting N supply conditions, nitrate uptake is lower during seed maturation than in the vegetative stage, although it still operates (Masclaux-Daubresse *et al.*, 2010).

To study nitrogen remobilization, special tools and skills have been developed and adapted to different plant species. In crops, the total nitrogen amount at anthesis and at harvest time was determined to give an estimation of the amount of nitrogen remobilized or absorbed after anthesis (Masclaux-Daubresse *et al.*, 2008, and references therein). This method, called the 'apparent remobilization' method, was, however, subject to large experimental errors. Despite such inconvenience, the mapping of QTL of nitrogen remobilization was possible using this method in barley and durum wheat (Joppa *et al.*, 1997; Mickelson *et al.*, 2003).

The use of  $^{15}\text{N}$  tracing, also named  $^{15}\text{N}$  long-term labelling, is a good alternative that allows the determination of fluxes. Long-term labelling was usually achieved by applying  $^{15}\text{N}$  nitrate to plants grown either under controlled conditions in hydroponics, sand or compost (Diaz *et al.*, 2008) or in the field (Gallais *et al.*, 2006). Recently using such  $^{15}\text{N}$  long-term labelling in maize, several QTL of N uptake and N remobilization were mapped. In this report, traits for NUE were monitored and the coincidence of QTL for agronomical, physiological, and biochemical traits was observed. QTL clustering showed antagonism between N remobilization and N uptake at several loci. Positive coincidences between N uptake, root system architecture and leaf greenness were also found. Most of the N remobilization QTL mapped were co-localizing with leaf senescence QTL. The mapping of QTL for NRE in wheat and maize then confirmed that the contribution of leaf N remobilization to grain N content varied depending on genotype and on environment (Joppa *et al.*, 1997; Coque *et al.*, 2008).

While the approaches commonly used with *Arabidopsis* to explore the network of leaf senescence and nitrogen remobilization processes are mutant analysis and tran-

scriptomic approaches, the recent reports of Diaz *et al.* (2008) and Lemaitre *et al.* (2008) showed that both genetic and environmental variations exist for these traits in *Arabidopsis*. Exploring natural variation of N remobilization and seed filling under different environmental conditions might then be highly informative.

In the present study, a similar plant material to that used by Chardon *et al.* (2010) was used to evaluate the proportion of nitrogen remobilized from rosette leaves to the seeds using the  $^{15}\text{N}$ -labelling procedure already experimented in our laboratory (Diaz *et al.*, 2008). From the agronomic traits related to biomass, yield, carbon, and nitrogen concentrations in seeds and dry remains, the nitrogen use efficiency of accessions for seed production was estimated. From  $^{15}\text{N}$  partitioning in seeds nitrogen remobilization efficiency (NRE) was estimated for all the accessions. All the data collected allow us to understand the physiology of N remobilization for seed production in *Arabidopsis* in response to nitrate availability and to detect extreme or uncommon accessions with regard to the global behaviour of the *Arabidopsis thaliana* core collection. In addition, while these data do not provide any mechanistic information about N-remobilization, they demonstrate the physiological links existing between traits and provide new indicators that will be useful for further studies in *Arabidopsis* and crops, such as rapeseed.

## Materials and methods

### *Plant material and growth conditions*

Seeds of the *Arabidopsis thaliana* Akita, BI-1, Bur-0, Col-0, Ct-1, Edi-0, Ge-0, Kn-0, Mh-1, Mr-0, Mt-0, N13, Oy-0, Sakata, Shahdara, St-0, Stw-0, Tsu-0, and WS have been provided by the Versailles Resource Centre (INRA Versailles France, <http://dbsgap.versailles.inra.fr/vnat/>) and the same batches of seeds (except WS) were used by Chardon *et al.* (2010) to study natural variation of nitrate uptake. Sixteen of these accessions are part of the core collection of 24 accessions selected by McKhann *et al.* (2004) on the basis of genetic variability only. Then the 16 accessions retained from the core collection of 24 lines were selected on the basis of their genetic variability and on their close flowering time in short days (see Supplementary Table S1 at *JXB* online). Accessions that presented very early flowering or very late flowering times have been eliminated. Col-0 and WS, which are parental lines for most of the recombinant inbred lines and mutant populations available at Versailles Resource Center were added. Mr-0 was also added.

Seeds were sown in small pots filled with medium particle size sand topped with a thin layer of small particle size sand at the same time and in the same conditions as performed by Chardon *et al.* (2010). One week after sowing, a single seedling was retained in each pot, and black sand poured to avoid algal proliferation. Two independent biological repeats were carried out in two consecutive culture cycles in the same growth chamber. Each biological repeat included four accession repetitions (four plants per genotype). Plants were grown in short days (8 h light) with 21 °C day and 17 °C night temperatures. Photon flux density was 160  $\mu\text{mol m}^{-2} \text{s}^{-1}$  until they reached 56 DAS (days after sowing). At 56 DAS, plants were transferred to long day conditions (16 h light), maintaining similar day/night temperatures and light intensity in order to induce flowering and to reduce flowering variation between accessions. Plants were cultivated on sand under

low nitrogen nutrition (LOW N, 2 mM nitrate) or under high nitrogen nutrition (HIGH N, 10 mM nitrate) as described in Chardon *et al.* (2010).

#### <sup>15</sup>N labelling, flowering induction, and harvest

At the two <sup>15</sup>N-uptake time points 40 DAS and 42 DAS, the unlabelled watering solution was replaced by a solution that had the same nutrient composition except that <sup>14</sup>NO<sub>3</sub> was replaced by <sup>15</sup>NO<sub>3</sub> 10% enrichment (see Supplementary Fig. S1 at *JXB* online). After labelling, plant roots and sand were rinsed with deionized water. Unlabelled nutrient solution was then used for the rest of the culture cycle. Plants were grown in short days until 56 DAS. Afterwards, plants were transferred to long day conditions (light/dark cycle 16 h/8 h) to induce flowering. Plants were harvested at the end of their cycle when all seeds were matured and the rosette dry. Samples were separated as (i) dry remains (DR=rosette+stem+cauline-leaves+empty-dry-silicles) and (ii) total seeds (SEEDS). It has to be noted that roots were included in the DR and were not used for measurements because a large part of the root system was lost in the sand at harvest. The dry weight (DW) of the DR and SEEDS were determined. Four replicates (plants) were harvested and the experiment was carried out twice.

#### Determination of total nitrogen content and <sup>15</sup>N abundance

For all the experiments, unlabelled samples were harvested in order to determine the <sup>15</sup>N natural abundance. After drying and weighing each plant, material was ground to obtain a homogenous fine powder. A subsample of 1000–2000 µg was carefully weighed in tin capsules (Sartorius MP2P) to determine the total N content and <sup>15</sup>N abundance using an elemental analyser (roboprep CN, SERCON Europa Scientific Ltd, Crewe, UK) coupled to an isotope ratio mass spectrometer (Tracermass, PDZ Europa Scientific Ltd, Crewe, UK) calibrated using natural abundance. The <sup>15</sup>N abundance was calculated as atom per cent and defined as  $A\% = 100 \times ({}^{15}\text{N}) / ({}^{15}\text{N} + {}^{14}\text{N})$  for labelled plant samples and for unlabelled plant controls ( $A\%$ control was *c.* 0.3660). The <sup>15</sup>N enrichment (E%) of the plant material was then defined as  $(A\%_{\text{sample}} - A\%_{\text{control}}) / (A\%_{\text{nutritive\_solution}} - A\%_{\text{control}})$ , with  $A\%_{\text{nutritive\_solution}} = 0.1$ . The  $RSA_{\text{SEEDS}} / RSA_{\text{SEEDS+DR}}$  ratio previously described by Gallais *et al.* (2006) was calculated as  $(E\%_{\text{SEEDS}}) / [(E\%_{\text{SEEDS}} \times N\%_{\text{SEEDS}} \times DW_{\text{SEEDS}} + E\%_{\text{DR}} \times N\%_{\text{DR}} \times DW_{\text{DR}})] / (N\%_{\text{SEEDS}} \times DW_{\text{SEEDS}} + N\%_{\text{DR}} \times DW_{\text{DR}})$ .

#### Flowering time

Flowering time was recorded as the number of days between seed germination and bolting (i.e. the time when the principal bud starts to emerge). Values obtained on the four plant repeats were averaged.

#### Statistical analysis

Statistical analysis of phenotypic data was carried out by analysis of variance, using the GLM procedure of SAS using Supplementary Table S0 (which can be found at *JXB* online) as the data source. The adjusted means for each accession was estimated with the LSMEANS option. Genetic variances at HIGH N and LOW N were then estimated with the VARCOMP option of SAS. Heritability was estimated:  $h^2 = \sigma^2_g / (\sigma^2_g + [\sigma^2_e / r])$ , with  $\sigma^2_g$  being the genetic variance,  $\sigma^2_e$  the residual variance, and  $r$  the number of replicates. Correlations between traits within nitrogen conditions were computed using the Proc CORR procedure of SAS. Normal

distribution of measured and calculated traits was verified using Kolmogorov–Smirnov test using XLSTAT.

## Results and discussion

### *Nitrogen limitation affects plant biomass, yield, and harvest index but does not change one-seed dry weight*

Several traits related to yield and nitrogen use efficiency (NUE) have been measured or calculated on the 19 accessions of *Arabidopsis* cultivated under low (LOW N; 2 mM nitrate) and high (HIGH N, 10 mM nitrate) nitrogen nutrition (see Supplementary Fig. S1 at *JXB* online). The dry weights (DW, as g plant<sup>-1</sup>), nitrogen concentration (N%, mg 100 mg<sup>-1</sup> DW), carbon concentration (C%, mg 100 mg<sup>-1</sup> DW), and <sup>15</sup>N enrichment (E%, <sup>15</sup>N as % of total N) were measured on the dry remains (DR) and seeds (SEEDS) after harvest. The dry weight of one-seed (DW<sub>1S</sub>) was also determined.

From the measured trait data, key indicators for yield and NUE were calculated as follows. From the dry weights (DW) harvest index (HI) was estimated as the  $(DW_{\text{SEEDS}}) / (DW_{\text{DR}} + DW_{\text{SEEDS}})$  ratio which is a key indicator for individual plant yield. Then DW and nitrogen concentrations (N%) were combined to determine the nitrogen harvest index (NHI) as  $(N\%_{\text{SEEDS}} \times DW_{\text{SEEDS}}) / (N\%_{\text{DR}} \times DW_{\text{DR}} + N\%_{\text{SEEDS}} \times DW_{\text{SEEDS}})$  which is a key indicator of seed filling with nitrogen. Since plant morphology and harvest index varied depending on accessions, the ratio NHI/HI (named  $\Delta$ NHI when the ratio is normalized to the mean) was monitored in order to compare nitrogen use efficiency (NUE) performances between accessions. In a similar manner, DW, N%, and <sup>15</sup>N enrichments (E%) were combined to determine the partition of <sup>15</sup>N in seeds, named here as <sup>15</sup>N-Harvest-Index (<sup>15</sup>NHI), which is the proportion of <sup>15</sup>N absorbed at the vegetative stage and remobilized to the seeds at the reproductive stage. <sup>15</sup>NHI calculated as  $E\%_{\text{SEEDS}} \times N\%_{\text{SEEDS}} \times DW_{\text{SEEDS}} / (E\%_{\text{DR}} \times N\%_{\text{DR}} \times DW_{\text{DR}} + E\%_{\text{SEEDS}} \times N\%_{\text{SEEDS}} \times DW_{\text{SEEDS}})$  is an indicator for nitrogen remobilization efficiency to the seeds. Again, due to the plant morphology and harvest index genetic variations, the ratio <sup>15</sup>NHI/HI (named  $\Delta$ Rem when the ratio is normalized to the mean) was estimated to compare nitrogen remobilization efficiency (NRE) performances between accessions. C/N<sub>SEEDS</sub> is a key indicator for seed nutritive value. The relative specific abundance (RSA, see the Materials and methods) is an indicator of the <sup>15</sup>N enrichment of SEEDS and DR that can be combined to estimate the  $RSA_{\text{SEEDS}} / RSA_{\text{SEEDS+DR}}$  ratio and nitrogen dilutions.

Two independent experiments (biological repeats R1 and R2) were carried out, at two different dates and in identical growth conditions. Each biological repeat contained four plant replicates per genotype. ANOVA was performed on the whole set of data from R1 (and R2 at HIGH N and LOW N, in order to determine the effect of nutrition, genotype, and repeat treatments on the variations of traits. For most of the traits, the effect of nutrition on their variations was higher than the effect of genotype, repeat or

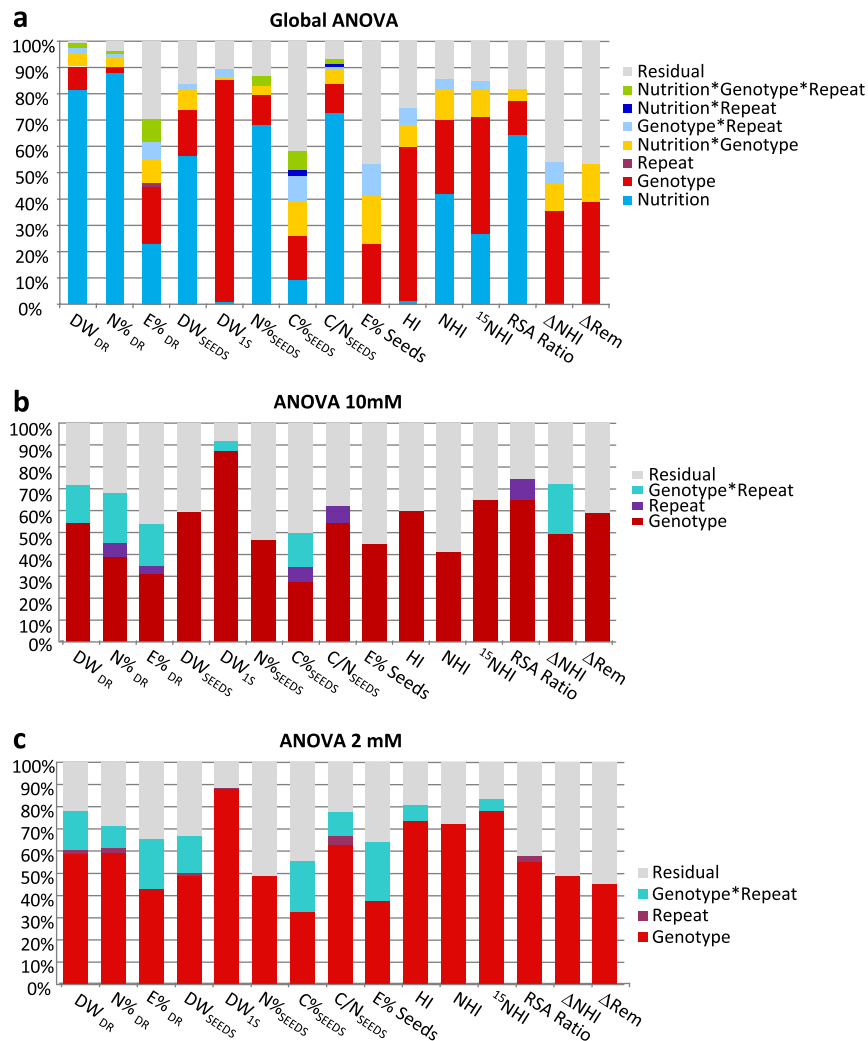
interactions (Fig. 1a; see Supplementary Table S2 at *JXB* online). Surprisingly, the variation of  $DW_{1S}$ ,  $E\%_{SEEDS}$  and HI were poorly affected by plant nutrition or repeats showing that seed size and sink-to-source ratio are robust traits in *Arabidopsis*. For all the traits, the effects of genotype and of nutrition  $\times$  genotype interaction were significant, revealing heritable differences between genotypes whatever the plant nutrition conditions, as well as a differential response to nutrition depending on genotype. It can be noticed that repeat effects were minor for DR traits and not significant for SEEDS traits (Fig. 1a; see Supplementary Table S2 at *JXB* online), allowing us to use R1 and R2 as biological repeats.

Performed independently on data obtained at LOW N and at HIGH N (Fig. 1b, c; see Supplementary Table S3,S4 at *JXB* online), ANOVA analyses confirmed that

behind nutrition, the main effect on trait variations was due to genotype. One important result of ANOVA on HIGH N and LOW N traits was that genetic effects were still high for the calculated traits, such as HI, NHI,  $^{15}NHI$ , RSA, and  $\Delta Rem$ . The genetic part of trait variation led us to estimate heritabilities (Table 1). Except for  $C\%_{SEEDS}$ ,  $E\%_{DR}$  at HIGH N, and  $C\%_{DR}$  at LOW N heritabilities higher than 0.50 were estimated for all the traits.

#### Characterization of plant behaviour with regards to yield

Harvest index [ $HI = DW_{SEED} / (DW_{SEEDS} + DW_{DR})$ ] was very variable depending on genotypes, but globally unchanged between HIGH N and LOW N for most of the accessions (Fig. 2a). The comparison of the mean ( $\pm SD$ ) of each



**Fig. 1.** Schematic representation of ANOVA of dry weight (DW, g), N concentration ( $N\%$ ,  $mg\ 100\ mg^{-1}\ DW$ ), C concentration ( $C\%$ ,  $mg\ 100\ mg^{-1}\ DW$ ), C/N ratio,  $^{15}N$  enrichment ( $E\%$ ;  $^{15}N/N$  as %), HI (harvest index), NHI (nitrogen harvest index),  $^{15}NHI$  (partition of  $^{15}N$  in seeds), RSA ratio, and extra remobilization ( $\Delta Rem$ ) in the 19 *Arabidopsis* accessions grown under HIGH N and LOW N nutrition. DR, Dry remains; SEEDS, all seeds; 1S: one seed. Results of ANOVA are fully presented in Supplementary Table S2,S3,S4 at *JXB* online. (a) Global ANOVA using data obtained at HIGH N and LOW N. (b) ANOVA 10 mM using data obtained at HIGH N. (c) ANOVA 2 mM using data obtained at LOW N. Histograms show the effects due to nutrition, genotype, repeat and interactions as a percentage of the variation explained.

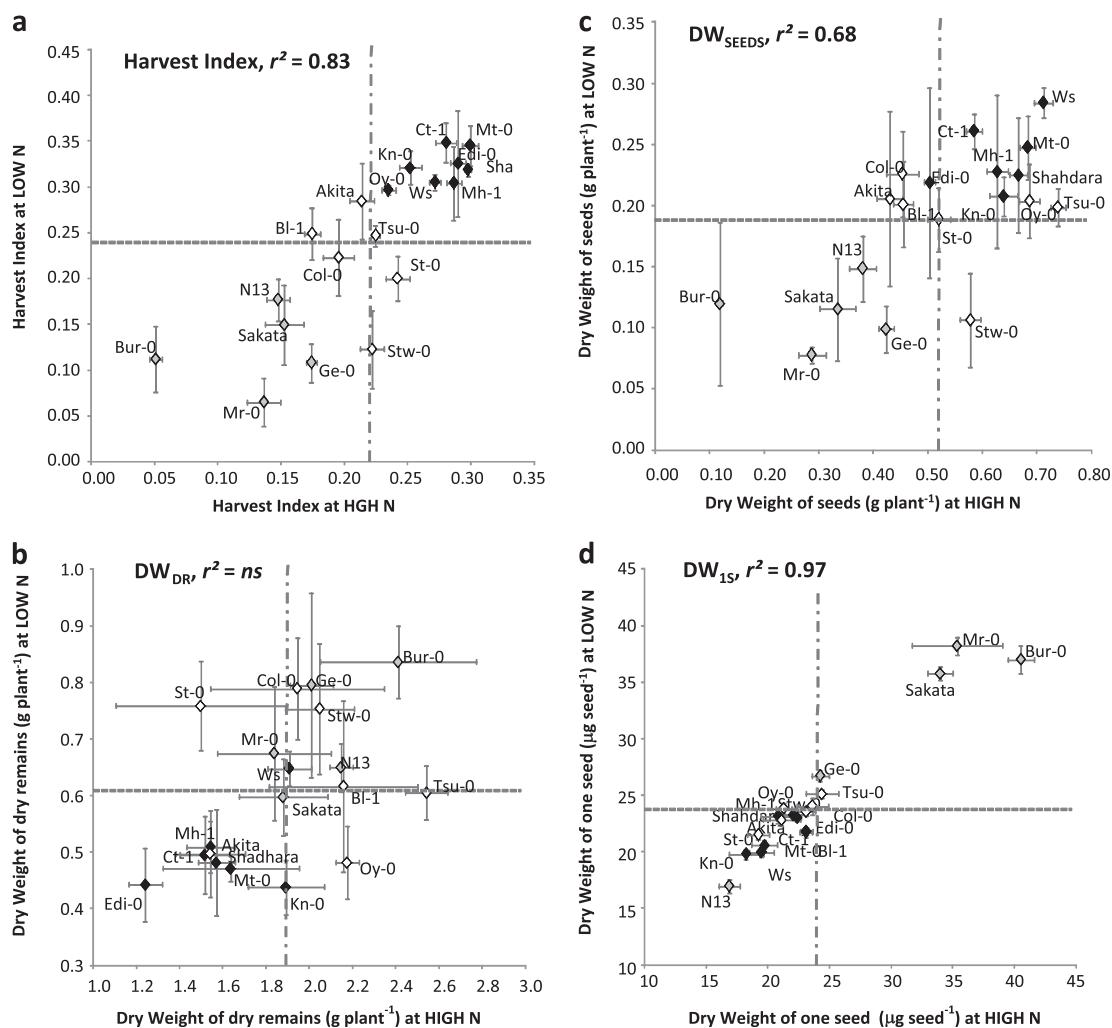
accession with the mean value of the core collection facilitated the classification of accessions into three groups: (L) low HI (significantly lower than the mean at LOW N and HIGH N), (M) medium HI (not significantly different from the mean at LOW N or HIGH N), and (H) high HI (significantly higher than the mean at LOW N and HIGH

N). It is noticeable that the HI of Bur-0 and Mr-0 were the lowest at HIGH N and LOW N, respectively, while HI of Mt-0 was the highest at both HIGH N and LOW N. The accessions belonging to the L group were mostly characterized by a lower  $DW_{SEEDS}$  (Fig. 2c) whereas the accessions belonging to the H group were mostly characterized by

**Table 1.** Heritability of dry weight ( $DW$ , g), N concentration ( $N\%$ , mg 100 mg<sup>-1</sup> DW), C concentration in seeds ( $C\%$ , mg 100 mg<sup>-1</sup> DW), C/N ratio in seeds, <sup>15</sup>N enrichment ( $E\%$ ; <sup>15</sup>N/N as %), HI (harvest index), NHI (nitrogen harvest index), <sup>15</sup>NHI (partition of <sup>15</sup>N in seeds), RSA ratio,  $\Delta NHI$ , and  $\Delta Rem$  at HIGH N (10 mM) or at LOW N (2 mM)

Two independent biological repeats containing four individual plants have been done.

	$DW_{DR}$	$N\%_{DR}$	$E\%_{DR}$	$DW_{SEEDS}$	$DW_{1S}$	$N\%_{SEEDS}$	$C\%_{SEEDS}$	$C/N_{SEEDS}$	$E\%_{SEEDS}$	HI	NHI	<sup>15</sup> NHI	RSA ratio	$\Delta NHI$	$\Delta Rem$
HIGH N	0.74	0.60	0.38	0.68	0.93	0.57	0.33	0.71	0.56	0.69	0.51	0.75	0.81	0.66	0.68
LOW N	0.79	0.75	0.62	0.64	0.92	0.60	0.38	0.80	0.57	0.84	0.82	0.86	0.68	0.61	0.57



**Fig. 2.** Natural variation in harvest index (HI, a), dry weight of dry remains ( $DW_{DR}$ , g plant<sup>-1</sup>, b), dry weight of seeds ( $DW_{SEEDS}$ , g plant<sup>-1</sup>, c), and dry weight of one seed ( $DW_{1S}$ , mg seed<sup>-1</sup>, d) at high (HIGH N) and low (LOW N) nitrate supplies. Values are adjusted means from two biological repeats with four individual plants each,  $\pm$ SD. Accessions belonging to HI clusters L (Low HI), M (Medium HI), and H (High HI) are represented by diamonds with light grey, white, and black colours, respectively. The horizontal hashed grey line represents the mean of the core collection at HIGH N and the vertical hashed grey line represents the mean at LOW N. Correlations between trait at LOW N and HIGH N are presented in the upper left square.  $r^2$ , Determination coefficient; ns, not significant.

a lower DW<sub>DR</sub> (Fig. 2b). At HIGH N or LOW N, HI was indeed correlated with the dry weight of seeds (DW<sub>SEEDS</sub>) and with the dry weight of dry remains (DW<sub>DR</sub>) (Table 2). The DW<sub>DR</sub> and DW<sub>SEEDS</sub> were increased by approximately 3-fold under ample nitrate supply (HIGH N) compared to nitrate limitation (LOW N), (Fig. 2b, c, respectively). This fold-range was, however, different depending on the accessions, with some like Tsu-0 showing higher increases in DW<sub>SEEDS</sub> and DW<sub>DR</sub> at HIGH N compared with LOW N, contrasting with some like Bur-0 with lower DW<sub>SEEDS</sub> at HIGH N compared with LOW N, for example (Fig. 2b, c).

Yield, as DW<sub>SEEDS</sub> or HI, might depend on the number of seeds produced by each plant as well as on the dry weight of each seed (DW<sub>IS</sub>). Most of the accessions presented a DW<sub>IS</sub> close to 23 µg (Fig. 2d) and the range of variation for DW<sub>IS</sub> was too narrow to explain the range of HI and DW<sub>SEEDS</sub> variations, showing that the main source of variation for yield was the number of seeds. However, a few heavy-seed accessions (Bur-0, Mr-0, and Sakata) and light-seed accessions (N13 and Kn-0) (Fig. 2d) were noted. Despite the weak genotype variation for DW<sub>IS</sub>, the negative correlation observed between DW<sub>SEEDS</sub> and DW<sub>IS</sub> at HIGH N and LOW N might reveal that plants producing heavier seeds also produced fewer seeds (Table 2). HI, DW<sub>DR</sub>, DW<sub>SEEDS</sub>, and DW<sub>IS</sub> were correlated to flowering time (Table 2; see Supplementary Fig. S2 at JXB online) showing that plants flowering late had a lower yield and a higher vegetative biomass compared with early flowering accessions.

*N and C concentrations in seeds are differentially sensitive to nutrition depending on accessions*

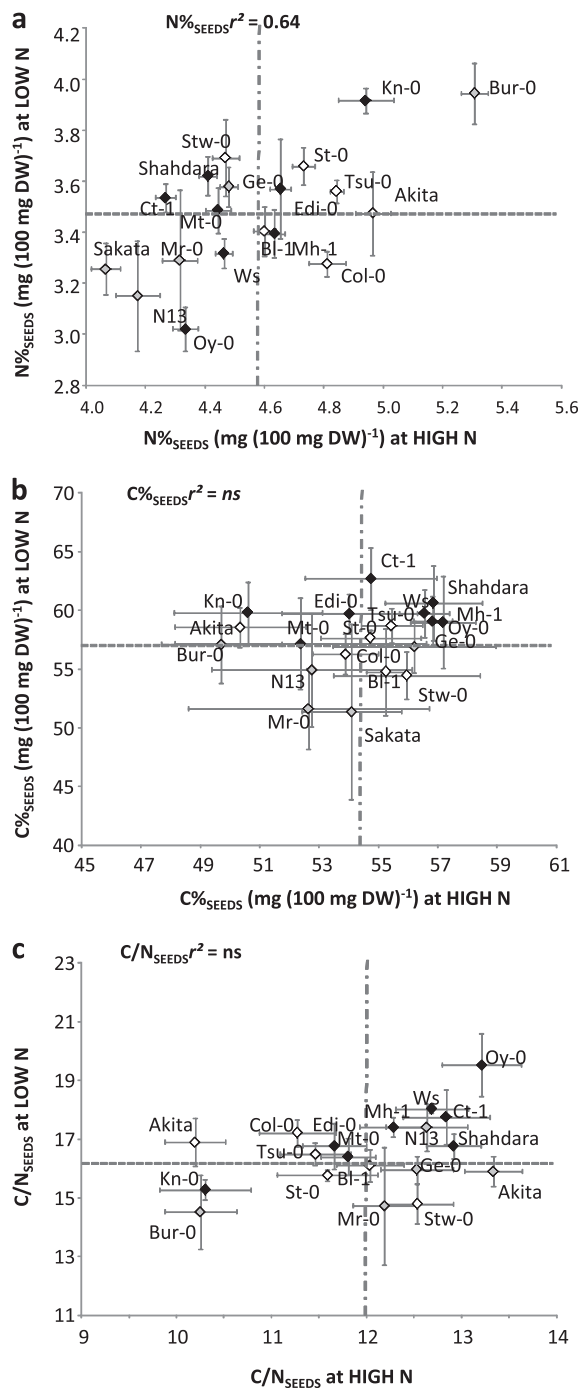
Seed quality in crops depends on lipid or protein contents as for rapeseed or wheat, for example. The nitrogen concentration in seeds (N%<sub>SEEDS</sub>) was significantly higher at HIGH N than at LOW N (Fig. 3a). At HIGH N, N%<sub>SEEDS</sub> ranged between 4% and 5.5% while at LOW N, N%<sub>SEEDS</sub> ranged between 3% and 4%. Globally, N% in seeds at HIGH N was 1.3-fold that measured at LOW N. N%<sub>SEEDS</sub> at HIGH N and LOW N were significantly correlated ( $r^2=0.64$ ,  $P < 0.05$ ) showing that plants with high (Bur-0 and Kn-0) or with low (N13, Oy-0, and Sakata) nitrogen concentrations in seeds were the same at HIGH N and LOW N. Some accessions were better for N%<sub>SEEDS</sub> at HIGH N than at LOW N, like Col-0, others were relatively better at LOW N, like Shahdara and Stw-0.

As another component of seed quality, C% is a potential indicator of oil or starch amount in seeds. At the opposite of N%<sub>SEEDS</sub>, C%<sub>SEEDS</sub> was globally higher at LOW N than at HIGH N (Fig. 3b) and there was also a large variation depending on genotype. It is noticeable that the C%<sub>SEEDS</sub> was highly affected by HIGH N environment in Bur-0, Kn-0, and Akita. Independently of some individual behaviour, there was globally a positive correlation between C%<sub>SEEDS</sub> and DW<sub>SEEDS</sub> (Table 2) at LOW N and HIGH N showing that, in *Arabidopsis*, as in many plants, yield is correlated with carbon filling in seeds. C%<sub>SEEDS</sub> was correlated with N%<sub>SEEDS</sub> only at HIGH N, suggesting that nitrogen in seeds was diluted by carbon when plants were growing under ample nitrogen nutrition.

**Table 2.** Correlations between measured and calculated traits at HIGH N (upper right, normal) or at LOW N (lower left, *italic*) in the 19 different *Arabidopsis* accessions

Correlations were computed using all the means calculated for each accession. Only significant correlations ( $P < 0.05$ ) are presented. Light grey boxes underpin similarities in correlations found at HIGH N and at LOW N. (FT: flowering time). Dark grey boxes separate values of correlations at HIGH N from correlations at LOW N.

HIGH N 10mM	DRY REMAINS HIGH N			SEEDS HIGH N						YIELD HIGH N						FT
	DW <sub>DR</sub>	N% <sub>DR</sub>	E% <sub>DR</sub>	DW	DW <sub>IS</sub>	N%	C%	ΔRem	E%	HI	NHI	<sup>15</sup> NHI	RSA ratio	ΔNHI	ΔRem	
DW <sub>DR</sub>		-0.52							-0.65	-0.63	0.69	-0.63	0.55			
N% <sub>DR</sub>	0.61		-0.47					0.50	0.61		-0.55		-0.70	-0.88	0.50	
E% <sub>DR</sub>				-0.66	0.64					-0.63	0.65	-0.63	0.52			0.59
DW <sub>SEEDS</sub>	-0.58	-0.91			-0.71		0.56			0.86	-0.76	0.82				-0.71
DW <sub>IS</sub>		0.63		-0.67						-0.71	0.63	-0.65				0.51
N% <sub>SEEDS</sub>							-0.50	0.55								0.55
C% <sub>SEEDS</sub>	-0.50	-0.79		0.74	-0.54							0.46				
C/N <sub>SEEDS</sub>		-0.70		0.64	-0.52	0.68	0.51									
E% <sub>SEEDS</sub>			0.60					0.64			-0.55	0.55	-0.83	-0.60	0.64	
HI	-0.81	-0.90		0.93	-0.66				0.77		-0.97	0.97	-0.57			-0.76
NHI	0.83	0.77		-0.87	0.65		-0.64			-0.96		-0.93	0.66			0.73
<sup>15</sup> NHI	-0.71	-0.96		0.95	-0.63		0.82			0.97	-0.88		-0.70			-0.78
RSA ratio		0.50		-0.51				0.72				-0.52		-0.58	0.72	0.57
ΔNHI	0.76			-0.55	0.49			-0.51		-0.69	-0.56	-0.56				
ΔRem													0.61	0.50		
FT	0.58	0.54	0.80	-0.56	0.45		-0.51			-0.64	0.57	-0.65	0.47			
LOW N 2 mM	DW <sub>DR</sub>	N% <sub>DR</sub>	E% <sub>DR</sub>	DW	DW <sub>IS</sub>	N%	C%	C/N	E%	HI	NHI	<sup>15</sup> NHI	RSA ratio	ΔNHI	ΔRem	FT
	DRY REMAINS LOW N			SEEDS LOW N						YIELD LOW N						



**Fig. 3.** Natural variation in nitrogen concentration in seeds ( $N\%_{SEEDS}$ ,  $\text{mg } 100 \text{ mg}^{-1} \text{ DW}$ , a), carbon concentration in seeds ( $C\%_{SEEDS}$ ,  $\text{mg } 100 \text{ mg}^{-1} \text{ DW}$ , b), and carbon to nitrogen ratio in seeds ( $C/N_{SEEDS}$ , c) at high (HIGH N) and low (LOW N) nitrate supplies. Values are adjusted means from two biological repeats with four individual plants each,  $\pm$ SD. Accessions belonging to HI clusters L (Low HI), M (Medium HI), and H (High HI) are represented by diamonds with light grey, white, and black colours respectively. The horizontal hashed grey line represents the mean of the core collection at HIGH N and the vertical hashed grey line represents the mean at LOW N. Correlations between trait at LOW N and HIGH N are presented in the upper left square.  $r^2$ , Determination coefficient; ns, not significant.

The  $C/N_{SEEDS}$  ratio of seeds can be used as a global indicator of lipid/starch to protein relative contents.  $C/N_{SEEDS}$  was correlated with  $C_{SEEDS}$  and  $N_{SEEDS}$  at HIGH N and LOW N, and with HI and  $^{15}\text{NHI}$  at LOW N, showing the effect of nitrogen remobilization on C and N relative contents in seeds (Table 2).  $C/N_{SEEDS}$  was the highest in Oy-0 at HIGH N and LOW N and the lowest in Bur-0 at HIGH N and LOW N and in Akita and Kn-0 at HIGH N (Fig. 3c).

#### *Variation of the residual nitrogen in the dry remains is much larger than variation of nitrogen contents in seeds*

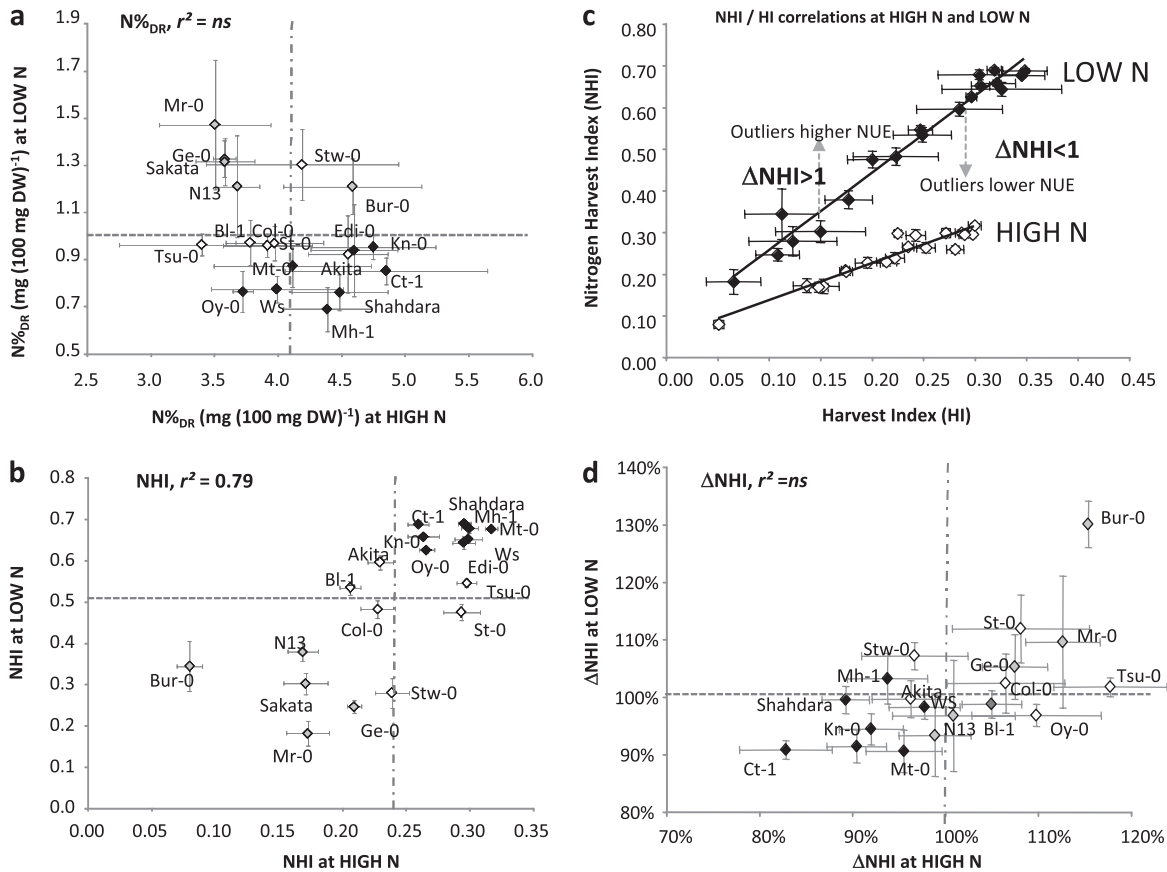
In agriculture, an essential component of nitrogen use efficiency is the residual nitrogen remaining in the dry parts of the plant that is left in the field after harvest. Indeed it can be considered that the nitrogen of the dry remains was not utilized for plant production. Therefore, two indicators were examined: the nitrogen concentration in the dry remains ( $N\%_{DR}$ ) and the nitrogen harvest index (NHI) that is the proportion of nitrogen contained in the seeds compared with the whole plant nitrogen content ( $\text{mg N in seeds}/\text{mg N in whole plant}$ ).

The large increase of  $N\%_{DR}$  at HIGH N compared to LOW N showed that the amount of nitrogen in dry remains after harvest increased when plants were not nitrate limited while the nitrogen concentration in their seeds was poorly modified (Fig. 4a).  $N\%_{DR}$  was also variable depending on the accessions. The careless accessions, with high  $N\%_{DR}$ , and the careful accessions, with low  $N\%_{DR}$ , were not the same at HIGH N and LOW N (Fig. 4a), showing that genotype performances were dependent on environmental conditions. Negative and positive correlations between  $N\%_{DR}$  and  $\text{DW}_{DR}$  were found at HIGH N and LOW N, respectively (Table 2). At HIGH N, negative correlation again reveals the dilution effects of nitrogen due to plant growth and biomass increase. At LOW N, it shows the limiting effect of nitrogen availability on biomass production.

NHI of accessions growing at LOW N and at HIGH N are presented in Fig. 4b. Due to the low genetic variation of  $N\%_{SEEDS}$  and  $N\%_{DR}$  at both HIGH N and at LOW N, NHI was highly correlated to HI (Table 2). Therefore, HI seems to be a good estimate of NHI in *Arabidopsis*. However, in contrast to HI, the NHI values were significantly higher at LOW N than at HIGH N (Fig. 4b). This is mainly the result of the significantly higher values of  $N\%_{DR}$  at HIGH N compared with LOW N (4-fold; Fig. 4a). The higher NHI values found at LOW N compared with HIGH N show that nitrogen use efficiency was better under nitrate-limiting conditions than under plentiful conditions.

#### *Nitrogen use efficiency is higher at LOW N than at HIGH N and presents genetic variation*

The accessions clustered in the H, M, and L groups on the basis of their HI values (Fig. 2a) were grouped in a similar way for NHI (Fig. 4b) thus revealing strong



**Fig. 4.** Natural variation in nitrogen concentration in dry remains ( $N\%_{DR}$ ,  $\text{mg } 100 \text{ mg}^{-1} \text{ DW}$ , a) and total nitrogen index harvest (NHI, b) and  $\Delta NHI$  (d) at high (HIGH N) and low (LOW N) nitrate supplies. Values are adjusted means from two biological repeats with four individual plants each,  $\pm$ SD. Accessions belonging to HI clusters L (Low HI), M (Medium HI), and H (High HI) are represented by diamonds with light grey, white, and black colours, respectively. The horizontal hashed grey line represents the mean of the core collection at HIGH N and the vertical hashed grey line represents the mean at LOW N. Correlations between traits at LOW N and HIGH N are presented in (c). Correlations between HI and NHI are presented at HIGH N (white) and LOW N (black) and outliers with higher NUE ( $\Delta NHI > 1$ ) or lower NUE ( $\Delta NHI < 1$ ) are shown.  $r^2$ , Determination coefficient; ns, not significant.

correlations between HI and NHI at both LOW N and HIGH N (Fig. 4c; Table 2). While NHI was mainly dependent on HI and on the sink-to-source ratio, outliers from the regression line were also identified (Fig. 4c). To characterize them, for each individual plant (p) the  $\Delta NHI$  indicator was computed as:  $\Delta NHI = (NHI_p / HI_p) / (NHI / HI \text{ mean})$ .  $\Delta NHI$  was representative of the relative nitrogen use efficiency (NUE) performance of each accession and was independent of HI. Accessions with  $\Delta NHI > 100\%$  were high-performing whereas accessions with  $\Delta NHI < 100\%$  were low-performing, compared with the middle range of the core collection. It can be noted that, at both LOW N and HIGH N, Bur-0 had the highest  $\Delta NHI$  while Shahdara and Ct-1 had the lowest (Fig. 4d). Few accessions, like Oy-0, were more efficient at HIGH N than at LOW N and, by contrast, opposite accessions like Stw-0 or Mh-1 were more efficient at LOW N than at HIGH N.

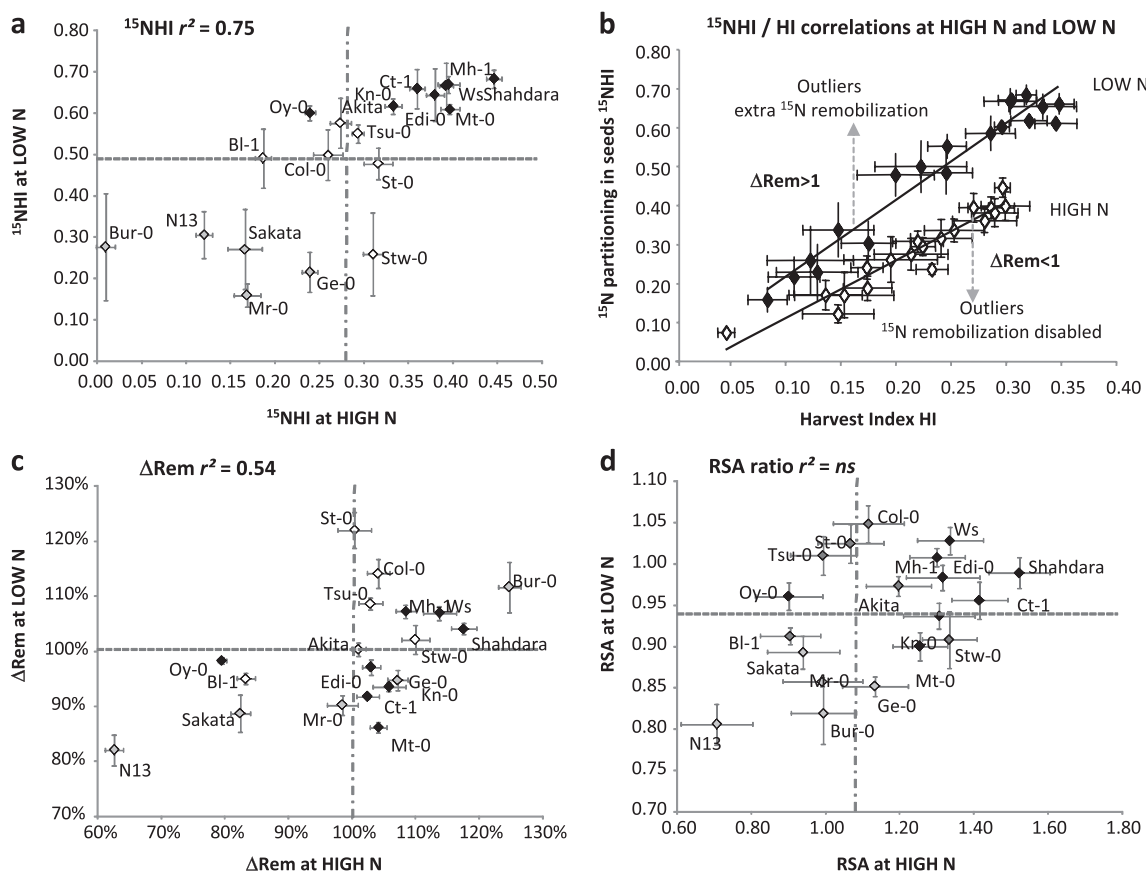
*Nitrogen remobilization to the seeds is higher at LOW N compared to HIGH N and more variable at HIGH N than at LOW N*

Nitrogen remobilization was investigated using  $^{15}\text{N}$  tracing experiments as done on crops in fields (Coque and Gallais, 2007; Coque *et al.*, 2008). Labelled  $^{15}\text{NO}_3$  was provided twice at the vegetative stage, a long time before flowering (15–45 d depending on accessions; see Supplementary Table S2 at *JXB* online), when rosette growth rate and leaf emergence were optimum. This allowed it to be assumed that the entire  $^{15}\text{N}$  nitrate absorbed was used for assimilation and protein synthesis and not for storage into the vacuole. After labelling, sand on which the plants were grown was carefully rinsed, thus avoiding further  $^{15}\text{N}$  isotope residual uptake. As a prerequisite of data meaning, the total  $^{15}\text{N}$  contained in the whole plant [DR+SEEDS] at harvest was considered to be equal to the total quantity of  $^{15}\text{N}$  absorbed by the plant at labelling time. Therefore the partition of  $^{15}\text{N}$  in the seeds, computed

as the ratio [mg  $^{15}\text{N}$  in the SEEDS/mg  $^{15}\text{N}$  in the (DR+SEEDS)], and named  $^{15}\text{NHI}$  by reference to the HI and NHI abbreviations used below, is assumed to be representative of the efficiency of  $^{15}\text{N}$  remobilization to the seeds.

Globally, the  $^{15}\text{NHI}$  values at LOW N were significantly higher than at HIGH N (Fig. 5a). This trend was similar to that found for NHI and showed that N remobilization to the seeds was 2-fold higher at LOW N compared with HIGH N. As observed for NHI, the relative  $^{15}\text{NHI}$  variations between accessions were similar to those observed for HI, and the accessions from the clusters H, M, and L previously defined (Fig. 2a) were grouped in a similar way. Therefore, at HIGH N and LOW N, HI and  $^{15}\text{NHI}$  values were significantly and strongly correlated to each other (Table 2) indicating that N remobilization to the seeds was significantly linked to yield and sink-to-source ratio. When the regression lines of the HI to  $^{15}\text{NHI}$  correlations were observed, although most of the accession means were plotted on the regression line (Fig. 5b), outliers were significantly identified. Accessions significantly above the regression line were more efficient at  $^{15}\text{N}$  remobilization.

Those significantly located below the line were less efficient (Fig. 5c). To facilitate the identification of outliers, for each individual plant (p) the distance from their point to the regression line was computed, calculating the relative slope  $\Delta\text{Rem}$  as:  $\Delta\text{Rem} = ({}^{15}\text{NHI}_p/\text{HI}_p)/(\text{mean of } {}^{15}\text{NHI}/\text{HI})$ .  $\Delta\text{Rem}$  was then expressed as a % of the mean value of the whole population and was representative of the relative nitrogen remobilization efficiency (NRE). Then, a 100%  $\Delta\text{Rem}$  value represents points located on the regression line (Fig. 5b) and  $\Delta\text{Rem}$  can be understood as an 'extra-remobilization' indicator when higher than 100% and as a 'N remobilization disabled' indicator when lower than 100%.  $\Delta\text{Rem}$  showed that N13, Sakata, Bl-1, and Oy-0 were significantly less efficient at  $^{15}\text{N}$  extra-remobilization, whereas Bur-0, Col-0, Ws, Mh-1, and Shahdara were performing significant extra-remobilization (Fig. 5c). It can be noted that, for most of the lines, their extra or disabled remobilization tendencies were independent of nutrition (Fig. 1). A few accessions, such as Mt-0, Ge-0, and Stw-0, presented extra-remobilizing performances at HIGH N but were remobilization disabled at LOW N. There was no accession with extra-remobilization potential at LOW N but



**Fig. 5.** Natural variation in  $^{15}\text{N}$  partitioning in seeds ( $^{15}\text{NHI}$ , a), extra remobilization ( $\Delta\text{Rem}$ , c), and relative specific abundance ratio (RSA ratio, d) at high (HIGH N) and low (LOW N) nitrate supplies. Values are adjusted means from two biological repeats with four individual plants each,  $\pm\text{SD}$ . Accessions belonging to HI clusters L (Low HI), M (Medium HI), and H (High HI) are represented by diamonds with light grey, white, and black colours, respectively. The horizontal hashed grey line represents the mean of the core collection at HIGH N and the vertical hashed grey line represents the mean at LOW N. Correlations between traits at LOW N and HIGH N are presented in (b). Correlations between HI and  $^{15}\text{NHI}$  are presented at HIGH N (white) and LOW N (black) and outliers with extra remobilization ( $\Delta\text{Rem} > 1$ ) or remobilization disabled performances ( $\Delta\text{Rem} < 1$ ) are shown.  $r^2$ , Determination coefficient; ns, not significant.

remobilization deficiency at HIGH N. A striking result was that accessions with higher or lower  $\Delta\text{Rem}$  were not always the same as those with higher or lower  $\Delta\text{NHI}$ . For example,  $\Delta\text{NHI}$  value of Shahdara and Ct-1 ranked among the lowest at HIGH N while their  $\Delta\text{Rem}$  values were the best at HIGH N. This indicated that better nitrogen filling into the seeds was not obligatory due to higher nitrogen remobilization performance. Nitrogen uptake after labelling and during seed filling might also be an important process for NHI.

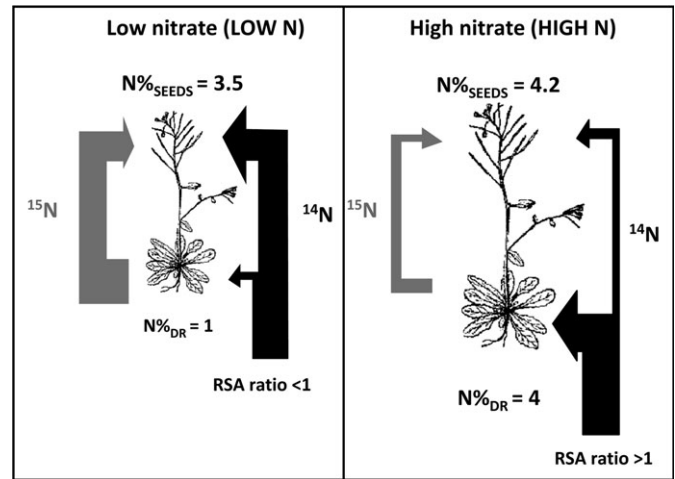
*Relative specific abundance (RSA) ratio as indicator of the allocation of the nitrogen absorbed during seed filling*

RSA is related to  $^{15}\text{N}$  enrichment and is an indicator of  $^{15}\text{N}$  dilution by the natural  $^{14}\text{N}$  absorbed by roots after labelling and until harvest.  $\text{RSA}_{\text{SEEDS}}/\text{RSA}_{\text{DR+SEEDS}}$  ratio, previously used by Gallais *et al.* (2006) in maize, can be (i) equal to 1 when  $^{15}\text{N}$  dilution is similar in SEEDS and in DR, (ii) lower than 1 when the  $^{14}\text{N}$  absorbed after labelling is routed to the seeds, while  $^{15}\text{N}$  is retained in DR, and (iii) higher than 1 when seeds are preferentially loaded with  $^{15}\text{N}$  remobilized while the  $^{14}\text{N}$  absorbed post-labelling is allocated to the vegetative parts of the plant. It was found that, except in a few accessions, the  $\text{RSA}_{\text{SEEDS}}/\text{RSA}_{\text{DR+SEEDS}}$  ratio was higher than 1 at HIGH N and lower than 1 at LOW N (Fig. 5d).

At LOW N, most of the nitrogen assimilated before and post-flowering was then allocated to the seeds, thus increasing the nitrogen use efficiency for seed filling and decreasing the amount of nitrogen lost in the dry remains (Fig. 6). By contrast, at HIGH N, most of the accessions were routing the nitrogen taken up from the substrate ‘post-labelling’ to their rosettes where it was stored and subsequently lost in the dry remains (Fig. 6). Some exceptions to this HIGH N feature were, however, detected in N13, Sakata, Bl-1, and Oy-0 (Fig. 5c). These four accessions used their post-flowering nitrogen absorption to the benefit of seed filling. This was consistent with the fact that they exhibited a lower  $\text{N}\%_{\text{DR}}$  than other lines and also a  $\Delta\text{Rem}$  below 100% confirming that their strategy was not N remobilization but post-flowering N uptake to ensure seed filling.  $^{15}\text{N}$  labelling was performed at 40 DAS and 42 DAS for all the accessions; it was then possible that flowering time and plant lifespan have differentially modified the  $^{15}\text{N}$  dilution with the  $^{14}\text{N}$  absorbed afterwards. The  $\text{RSA}_{\text{SEEDS}}/\text{RSA}_{\text{DR+SEEDS}}$  ratio was indeed positively correlated with flowering time (Table 2).

## Conclusion

Nitrogen is one among a long list of environmental factors affecting plant development. Adaptation to variable nitrogen availability differs between *Arabidopsis* accessions and several previous studies have demonstrated that there are significant effects of the genotype  $\times$  nitrogen environment



**Fig. 6.** Schematic interpretation of results of N remobilization ( $^{15}\text{NHI}$ ), RSA ratio,  $\text{N}\%_{\text{DR}}$  (mg  $100\text{ mg}^{-1}$  DW) and of seeds ( $\text{N}\%_{\text{SEEDS}}$ , mg  $100\text{ mg}^{-1}$  DW) in plants grown at low (LOW N) and high (HIGH N) nitrate supplies. The allocation of unlabelled  $^{14}\text{N}$  absorbed at reproductive stage is indicated by dark grey arrows. The RSA ratio  $< 1$  observed at LOW N indicates that plants have preferentially allocated unlabelled  $^{14}\text{N}$  absorbed at the reproductive stage to the seeds. The RSA ratio  $> 1$  observed at HIGH N indicates that plants have preferentially allocated unlabelled  $^{14}\text{N}$  absorbed at the reproductive stage to the vegetative tissues (rosette or stems). The remobilization of  $^{15}\text{N}$  from rosette to seeds represented by a light grey arrow is higher at LOW N than at HIGH N. The high  $^{15}\text{N}$  remobilization and the low  $^{14}\text{N}$  post-labelling uptake in rosettes of plants grown at LOW N compared to plants at HIGH N explain the low  $\text{N}\%_{\text{DR}}$  at LOW N compared to HIGH N. The high  $^{15}\text{N}$  remobilization and the high post-labelling translocation of  $^{14}\text{N}$  into seeds of plants grown at LOW N explain that the difference of  $\text{N}\%_{\text{SEEDS}}$  at HIGH N and LOW N is lower than the difference found between  $\text{N}\%_{\text{DR}}$  at HIGH N and LOW N.

interaction for biomass, nitrogen contents, and nitrogen uptake in *Arabidopsis* (Rauh *et al.*, 2002; Loudet *et al.*, 2003; Chardon *et al.*, 2010).

In the present study, it is shown that the dry weight of one seed ( $\text{DW}_{1\text{S}}$ ), the proportion of the seeds biomass compared with whole plant biomass (HI), and the proportion of nitrogen remobilized to the seeds ( $^{15}\text{NHI}$ ) are robust traits, highly heritable, while other traits are more influenced by environment. This suggests that the different accessions are able to make use of the extra N supplied at HIGH N to increase biomass and/or N storage, but that subsequently they allocated a relatively defined proportion of their nitrogen to the seeds. Consistent with this, it was observed that nitrate availability governs nitrogen concentration in dry remains but not in seeds, and influences vegetative biomass and yield but poorly modifies HI or  $\text{DW}_{1\text{S}}$ . Because most of the accessions have the same  $\text{DW}_{1\text{S}}$  at LOW N and HIGH N, nitrate availability mainly results in modifying seed number. The increase at LOW N of both nitrogen remobilization ( $^{15}\text{NHI}$ ) and post-labelling nitrogen allocation ( $\text{RSA}_{\text{SEEDS}}/\text{RSA}_{\text{DR+SEEDS}} < 1$ ) to the seeds has resulted in a lower range of variation in seed nitrogen

concentrations ( $N\%_{SEEDS}$ ) compared with nitrogen concentration in the dry remains ( $N\%_{SEEDS}$ ). The higher  $C/N_{SEEDS}$  at LOW N compared to HIGH N was then mainly due to the higher  $C\%_{SEEDS}$  recorded at LOW N.

The most striking results are provided by the  $^{15}N$  tracing experiments and by the measurement of nitrogen content in seeds and dry remains. Data show that nitrogen use efficiency (NUE) and nitrogen remobilization efficiency (NRE) are higher at LOW N than at HIGH N. Both are highly correlated to harvest index, suggesting a strong link between nitrogen allocation and yield. However, the computing of  $\Delta NHI$  and  $\Delta Rem$  allow the detection of genetic variations of NUE and NRE independently of HI variations, and to identify accessions with a relatively higher or lower score for NUE or NRE.

By contrast with Gallais *et al.* (2006) on maize, a large variation of the  $RSA_{SEEDS}/RSA_{[DR+SEEDS]}$  ratio was found that was highly informative concerning the sink–source nitrogen management in *Arabidopsis*. This ratio shows that LOW N condition increases both nitrogen remobilization and translocation of the nitrogen absorbed post-flowering to the seeds, whereas under ample nitrate supply, the RSA ratio shows that the nitrogen absorbed post-flowering is mainly driven to the vegetative tissues (Fig. 6). Despite this feature, the significant negative correlation at HIGH N between RSA ratio and  $\Delta NHI$  suggests that post-labelling nitrogen uptake was a limiting factor for seed filling at HIGH N also.

The new insights provided here into the links between nitrogen management at the whole plant level and the control of biomass and yield by nitrate availability, are facilitating the comprehension of the various components of nitrogen use efficiency in *Arabidopsis*. The NUE and NRE indicators proposed in this study will be helpful for comparison with other plant species and also as a basis for further analysis in *Arabidopsis* using mutants or mapping populations.

## Supplementary data

Supplementary data are available at *JXB* online.

**Supplementary Fig. S1.** Design of the  $^{15}N$  labelling procedure for pulse/chase assays.

**Supplementary Fig. S2.** Correlations between harvest index and flowering time at HIGH N (a) and at LOW N (b) show continuous natural variation between accessions.

**Supplementary Table S0.** Whole data set provided as an Excel file.

**Supplementary Table S1.** *Arabidopsis thaliana* accession provided by the Versailles Genetics and Plant Breeding Laboratory *Arabidopsis thaliana* Resource Centre (INRA Versailles France, <http://dbsgap.versailles.inra.fr/vnat/>).

**Supplementary Table S2.** Global ANOVA for all traits at LOW N and HIGH N.

**Supplementary Table S3.** ANOVA for all traits at HIGH N.

**Supplementary Table S4.** ANOVA for all traits at LOW N.

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