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ORIGINAL ARTICLE

Genetic diversity for nitrogen use efficiency in *Arabidopsis thaliana*

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Abstract

Main conclusion **The plasticity of plant growth response to differing nitrate availability renders the identification of biomarkers difficult, but allows access to genetic factors as tools to modulate root systems to a wide range of soil conditions.**

Nitrogen availability is a major determinant of crop yield. While the application of fertiliser substantially increases the yield on poor soils, it also causes nitrate pollution of water resources and high costs for farmers. Increasing nitrogen use efficiency in crop plants is a necessary step to implement low-input agricultural systems. We exploited the genetic diversity present in the worldwide *Arabidopsis thaliana* population to study adaptive growth patterns and changes in gene expression associated with chronic low nitrate stress, to identify biomarkers associated with good plant performance under low nitrate availability. *Arabidopsis* accessions were grown on agar plates with limited and sufficient supply of nitrate to measure root system architecture as well as shoot and root fresh weight. Differential gene expression was determined using Affymetrix ATH1 arrays. We show that the response to differing nitrate availability is highly variable in Arabidopsis accessions. Analyses of vegetative shoot growth and root system architecture identified accession-specific reaction modes to cope with limited nitrate availability. Transcription and epigenetic factors were identified as important players in the adaption to limited nitrogen in a global gene expression analysis. Five nitrate-responsive genes emerged as possible biomarkers for NUE in Arabidopsis. The plasticity of plant growth in response to differing nitrate availability in the substrate renders the identification of morphological and molecular features as biomarkers difficult, but at the same time allows access to a multitude of genetic factors which can be used as tools to modulate and adjust root systems to a wide range of soil conditions.

Keywords Biomarkers · Gene expression · Long-term nitrogen limitation · Nitrate · Plasticity · Root growth · Root system architecture

Abbreviations

NUE	Nitrogen use efficiency
(m)RIL	(Mixed) recombinant inbred line

Introduction

Nitrogen (N) is an integral component of nucleic acids and amino acids, and as such an essential macronutrient for living organisms. Plants take up inorganic N mainly as nitrate from the soil through the roots, and transform it into organic N in roots and leaves. Consequently, N availability in the soil influences plant development and morphology, and is

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a major determinant of crop yield (Frink et al. 1999). Substantial yield increases have been obtained through application of fertiliser, but at high environmental costs, such as nitrate pollution of water resources (Sutton et al. 2011). The extensive use of N fertilisers in crop production also causes high costs for farmers (Robertson and Vitousek 2009). To implement low-input regimes, increasing the nitrogen use efficiency (NUE) of crop plants is of paramount importance (Gutiérrez 2012). NUE is a complex trait that emerges from integration of metabolic, physiological and developmental processes, and depends mainly on two factors: N uptake efficiency and N utilisation efficiency (Masclaux-Daubresse et al. 2010; Xu et al. 2012). A large proportion of the total N in the plant is bound in photosynthetic proteins; a crucial component of N utilisation efficiency is, therefore, likely to be the ability to detect changes in light intensity and quality, and reallocate N to zones with the most favourable light regimes (Hirose and Bazzaz 1998).

Quantitative trait loci (QTL) for NUE were identified in maize (Agrama et al. 1999), barley (Kindu et al. 2014), rice (Wei et al. 2012) and Arabidopsis (Loudet et al. 2003). Molecular analyses of NUE have focused on individual genes whose properties or annotation suggested a role in the regulation of processes related to nitrogen uptake, assimilation or use, such as members of the NRT1 and NRT2 nitrate transporter families (reviewed in Krapp et al. 2014; O'Brien et al. 2016). Their relation to NUE, however, remains unclear.

In general, high nitrate supply promotes shoot growth (Scheible et al. 2004), while low nitrate availability induces enhanced root growth for foraging (Walch-Liu et al. 2006; Gruber et al. 2013). Moreover, the effect of nitrate on root morphology is independent of its assimilation (Forde and Lorenzo 2001). Therefore, nitrate has been considered not only as a major macronutrient, but also as a powerful signalling molecule. The nitrate signal has both systemic morphological effects, inhibiting the lateral root development when provided at a uniform high concentration, and local morphological effects, enhancing local lateral root elongation when provided in a restricted area of the root (Zhang and Forde 2000). More recently, natural variation in the nitrogen response of different Arabidopsis accessions has been described (North et al. 2009; De Pessemier et al. 2013). Several studies pointed out the interdependence between nitrogen and carbohydrate metabolism (Martin et al. 2002; Scheible et al. 2004; Tschoep et al. 2009; Huarancca Reyes et al. 2016).

The world-wide Arabidopsis population contains a diverse range of genotypes, which have adapted to many different growth conditions and show large variation in the expression of numerous traits including morphology, biotic and abiotic stress tolerance, flowering time, seed composition, water use, and nutrient efficiency (Koornneef et al. 2004; Weigel 2012). They show considerable variation of NUE (Chardon et al. 2010; Ikram et al. 2012). In the Bay x Sha RIL population, QTL were detected that affect NUE and other traits related to nitrogen metabolism and uptake (Loudet et al. 2003).

Many molecular studies of the transcriptomic response to N stress address the primary nitrogen response using either very short exposures to stress (2–30 min), or resupplying N after a longer stress (Kiba et al. 2012; Marchive et al. 2013; Vidal et al. 2013). These studies have shown that plants react within minutes to changes in the nitrate/ammonium level in their environment. The primary nitrogen response leads to transcriptomic reprogramming of up to several thousand genes. Fewer studies are concerned with morphological and transcriptomic adaptations to sustained nitrogen stress. Several differentially expressed genes were identified under mild or severe chronic N stress in Arabidopsis (Bi et al. 2007). Nitrogen remobilisation was identified as a means to cope with long-term N limitation (Masclaux-Daubresse et al. 2010).

We performed deep phenotyping and gene expression analyses on Arabidopsis accessions and mixed recombinant inbred lines to explore ways to detect morphological traits that could be used as biomarkers allowing identification of plants with good NUE at an early developmental stage, and to elucidate the genetic factors associated with good NUE. We show that the (growth) response to differing nitrate availability is highly diverse in Arabidopsis accessions. Transcription and epigenetic factors were identified as important players in the adaption to limited nitrogen in a global gene expression analysis. Five nitrate-responsive genes emerged as possible biomarkers for NUE in Arabidopsis.

Materials and methods

Biological materials

Two complementary populations of *Arabidopsis thaliana* that sample existing natural diversity were used. One population consisted of 101 Arabidopsis accessions selected based on genetic diversity estimated using 149 SNPs (Platt et al. 2010) and different geographical origins (Table S1). A mixed population of 123 recombinant inbred lines (mRILs) was assembled from 9 crosses involving 18 different parental accessions from various sources (Table S2). As mRILs originate from recent crosses, they offer the chance to uncover natural variation hidden in the accessions.

Plant culture

Population screens

All accessions and mixed RILs were screened for biomass production in an agar plate-based high-throughput

procedure with limited supply of nitrogen. The cultivation medium was adapted from Estelle and Somerville (1987). Severe nitrogen stress was applied for the selection. For sowing, the seeds, which had been stored in the refrigerator at − 20 °C, were removed from the Eppendorf tubes with a toothpick and transferred onto agar plates, with limited supply of nitrogen (0.05 mM KNO_3). In total, approximately, 15–30 seeds were distributed horizontally on each plate (12 × 12 cm). After sowing, plates were subjected to stratification for 2–4 days in the dark at 4 °C. After stratification, the plates were transferred into growth cabinets and plants were grown under a 16-h-light, 8-h-dark rhythm at 20 °C. The daytime light intensity was set to 100 µmol PAR m^{-2} s^{-1} (Philips TL5 54 W/840 HO). Excessive plants were removed from the plate 10–11 days after sowing, leaving up to 8 plants per plate for further screening and biomass assessment. Plant fresh biomass was assessed by weighing of shoots and roots 23 days after stratification.

In a complementary approach, analysis of shoot growth of the populations on low N soil was performed according to Tschoep et al. (2009). Shoot fresh weight was determined 35 days after germination.

Validation of candidate biomarker genes was performed using independent accessions with contrasting growth on low nitrogen, with Ak-1, Cvi-0, Gy-0 showing low NUE, and El-0, Col-0, Appt-1 demonstrating good NUE. Plants were grown for 40 days on a mixture of 70% vermiculite (Kakteen Schwarz, Nürnberg, Germany) and 30% soil substrate 2 (Klasmann-Deilmann GmbH, Geeste, Germany) and irrigated every day with liquid Estelle and Somerville solution containing 0.4 mM nitrate.

Assessment of morphological traits

Thirteen lines with contrasting NUE in the population screens were selected for deep phenotyping in vertical square petri dishes (120 × 120 × 17 mm). The medium (Estelle and Somerville 1987) was supplemented with 8% agar and 1% sucrose. The control medium (9 mM nitrate) contained 5 mM KNO_3 and 2 mM $Ca(NO_3)_2$, the low nitrogen medium (0.4 mM nitrate) contained 0.2 mM $Ca(NO_3)_2$ and 5 mM KCl. The nitrogen stress applied, less severe than in the population screen, balances the need for a strong selection with the need to produce enough material for molecular analyses. Seeds were surface-sterilised for 1 min in 70% ethanol followed by 1 min in 2% NaClO and four washing steps in sterile distilled water. 12 or 6 seeds were regularly spaced 1 cm from the upper border of the dish. Seeds were stratified for 24 h at 4 °C, and then transferred to a growth cabinet set to 16-h light (100 µmol PAR m^{-2} s^{-1})/8-h dark, 20°/18 °C for 21 days.

Plants were scanned on an Epson Expression 10000XL scanner 14 days after sowing. Background and contrast of the images were adjusted in Adobe Photoshop CS3. Root architecture traits, namely number of roots, main root length in mm, and total lateral root length in mm, were extracted using WinRhizo Pro with root morphology and link analysis in developmental mode. Lateral root density was calculated as number of roots/main root length, lateral root ratio as total lateral root length/(total lateral root length + main root length).

After 21 days, plants were harvested, separated into root and shoot, weighed and immediately quenched in liquid nitrogen. The plant material was stored at − 80 °C until further use. Shoot and root fresh weight, in mg $plant^{-1}$, were determined using pools of 3–10 plants per plate, and fresh weight was calculated per plant. The shoot–root ratio was derived from the calculated values as shoot fresh weigh/root fresh weight.

Gene expression analysis

Six lines showing contrasting growth characteristics on low N in the deep phenotyping screen were selected for gene expression analysis, the accessions El-0 and Fei-0, and the mRILs KB006, NG064, NG087, SG063. For each line, root and shoot samples were taken from plants grown on 0.4 mM and 9 mM nitrate. Three biological replicates were used per organ and treatment, in total 72 samples. One replicate consisted of a pool of 30 plants harvested from three plates. Total RNA was isolated using the adapted hot-borate method (Wan and Wilkins 1994). Hybridisation to the Affymetrix ATH1 array and readout of data in the form of CEL files were performed by Atlas Biolabs (Berlin, Germany). The ATH1 gene expression datasets generated were submitted to the Gene Expression Omnibus (GEO) repository (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE110171).

Candidate biomarker genes were analysed in six accessions with four biological and three technical replicates using quantitative real-time PCR. RNA was extracted from shoots using the Spectrum Plant Total RNA Kit (Sigma–Aldrich, Munich, Germany) according to the manufacturer's instructions. cDNA was synthesised using the RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific). *AtEF1α* (AT1G07940) was used as housekeeping reference gene. The following primer pairs were designed using Primer3web version 4.1.0 (Untergasser et al. 2012) (http://bioinfo.ut.ee/primer3): *AtAMT1;2* (5′-tgg gtg acg gta act atg gg-3′, 5′-gtg tca tgt cca ttc ctg cc-3′), *AtGATA21* (5′-tgt aac acc acc aag act cc-3, 5′-aga cac gag cca aac aca-3); *AtGLU1* (5′-tgc tcg ata ttg gcg gac ta-3′, 5′-act ttt gcc tca tca act ggt -3′); *AtNPF6.4* (5′-gac gct aaa ctc ggt cgc ta-3′, 5-tgg ctc cat gag atg atg aca-3′); *AtEF1α* (5′-tga gca cgc tct tct tgc ttt ca-3′, 5′-ggt ggt ggc atc cat ctt gtt aca-3′). qPCR

was performed on a Quantstudio 6 Real-Time PCR System (Applied Biosystems) using the SYBR Green PCR Master Mix (Thermo Fisher Scientific). ΔC_T were calculated as C_T (marker gene) − C_T (EF1alpha reference gene) and compared by ANOVA using Fisher's LSD (alpha = 0.05) with Bonferroni correction.

Statistical analyses

The biomass data were log10-transformed before further analysis to achieve an approximate normal distribution. Univariate statistics of the log10-transformed data were calculated by ANOVA with a linear model in SAS (proc mixed) with line (accession/mixed RIL) as a fixed effect. Each line was tested against the remaining population. Significance level was set to an alpha-error of 5%.

Further statistical analyses were performed using GenSTAT 17th edition (VSNi, Hempstead, UK). Adjusted mean values and associated standard errors for fresh weight, root architecture traits were estimated by ANOVA. The variation explained was extracted from sums of squares. Significant differences between lines within treatments were determined by least significant difference (LSD at 5%) with Bonferroni correction. Significant differences between the treatments within lines were analysed by Mann–Whitney *U* test with Bonferroni correction. Pearson correlations were performed using the procedure FCORRELATION, with FDRMIXTURES for correlations between genes and morphological traits, and FDRBONFERRONI for correlations among morphological traits. PCA was performed in R using package pcaMethods (Stacklies et al. 2007) on scaled and centred data.

Microarrays were analysed using the R packages affy (Gautier et al. 2004), limma (Ritchie et al. 2015), simpleaffy (Wilson and Miller 2005), and affyPLM (Bolstad et al. 2005). Quality was controlled using fitPLM (affyPLM package) and qc (simpleaffy package). Data were normalised with GC-RMA algorithm (affy package). Differential expression was detected by fitting linear models using lmFit (limma package) to the contrasts between plus and minus nitrogen samples. Resulting *p* values were controlled for multiple error correction using false discovery rate (FDR).

Mean transcript levels were used for a pattern matching search using Pavlidis Template Matching embedded in the MultiExperiment Viewer 4.9 (Saeed et al. 2006) (http://www.tm4.org/mev.html). The patterns followed the distribution of the morphological traits (shoot fresh weight, root fresh weight) across the lines. VENN diagrams were constructed with InterActiVenn (Heberle et al. 2015). GO term overrepresentation analyses were performed using the web-based application GOrilla (http://cbl-gorilla.cs.technion.ac.il; Eden et al. 2009).

Results

Large-scale phenotypic screening for plant lines with contrasting NUE

Good NUE in the context of the population screens was defined as higher than average aerial biomass (fresh weight) on N depleted synthetic media, and determined in two complementary populations. The first population consisted of a collection of 123 mixed recombinant inbred lines (mRILs) and their 18 parental lines. Adjusted mean fresh weight was estimated using ANOVA with line as fixed effect (Table S3). 55 mRILs from the first screen with high, average or low biomass were selected for two further rounds of screening. To identify lines with reproducible growth characteristics on depleted N for deep phenotyping in synthetic media, a reproducibility measure based on rank categories was implemented (see below). From this set, three top-ranking mRILs (KB006, SG063, KT130) and three low-ranking mRILs (NG064, NG087, WC056) were selected for deep phenotyping. Transgressive segregation was observed in most mRIL combinations, with individual RILs exceeding the fresh weight of the worst or best parent by 38% and 118%, respectively (Table 1). The extent of transgression is negatively correlated with the phenotypic difference between the parental lines ($r = -0.733$, $P = 0.006$ and $r = -0.817$, $P = 0.002$, respectively, for lines larger than the high parent or smaller than the low parent), confirming the segregation of 'hidden' alleles.

Phenotypic screening was also performed using a population of 101 Arabidopsis accessions. Growth of the accessions was assayed in two consecutive experiments in synthetic (agar) media, and furthermore in two

Table 1 Transgressive segregation for shoot fresh weight in mRILs

Population	% < Low *P*	% > High *P*	Abs. diff *P*
CA	16.60	55.20	1.10
DE	26.38	118.34	0.69
KB	38.00	105.30	1.93
KT	27.04	48.95	0.13
LF	8.63	22.89	2.52
NG	14.88	11.07	2.39
SG	9.30	13.73	5.27
TM	26.38	102.61	0.75
WC	8.53	− 35.66	9.32

The extent of transgressive segregation is illustrated by % difference in shoot fresh weight (SFW) between extreme RILs compared to their respective parents. % < low *P* (% > high *P*) indicates the SFW of the extreme RILs of each population as percentage of the SFW of the respective parent with the lower (higher) SFW. Abs. diff *P* gives the difference in SFW between the parental lines of each population

consecutive experiments in soil. Adjusted mean fresh weight was estimated using ANOVA (line = fixed effect). Mean fresh weight ranged between 0.55–1.67 (experiment 1) and 0.51–1.50 (experiment 2) mg plant^{-1} in agar, and between 27.08–152.50 and 37.41–106.63 mg plant^{-1} in soil (Table S4), respectively, revealing considerable (genetic) variation. For each experiment, lines were assigned the classes A, B, C, or D, according to their ranking in the upper, second, third or lower quartile. Lines that did only switch between adjacent classes were considered to exhibit reproducible behaviour on low N, and therefore suitable for further analyses. From this set, three top-ranking accessions (El-0, Rak-2, Lu) and three low-ranking accessions (Fei-0, Ts-1, C24) were selected for deep phenotyping, supplemented by the reference accession Col-0. Correlations between the different experiments were significant ($P < 0.05$) within growth conditions, with $r = 0.537$ for soil and $r = 0.337$ for agar media experiments, but no significant correlation between growth in agar and in soil was found. Twenty-one accessions showed similar behaviour in agar and in soil on limited N (Fig. 1a), whereas 14 accessions showed contrasting behaviour (Fig. 1b).

Deep phenotyping

The 13 lines with contrasting NUE selected from the population screens underwent deep phenotyping in agar plates on low (0.4 mM) and sufficient (9 mM) nitrate (Table 2). ANOVA revealed significant effects ($P < 0.001$) in shoot and root fresh weight and shoot–root ratio for line (genotype), treatment (nitrogen) and the interaction term, indicating that lines reacted differently to changes in the nitrogen supply (Table S5). The 13 lines tested differed in their growth at sufficient nitrogen, e.g. KB006 produced three times more shoot material than Col-0 (Table 2). However, the shoot growth behaviour at sufficient N did not correlate with growth at low N; the correlation of the respective ranks, $r = -0.11$, is not significant. There is a significant correlation between shoot and root growth at sufficient N ($r = 0.835$, $P = 0.0004$), but this link is lost on low N ($r = 0.106$, $P = 0.7304$). The root response was more variable than the shoot response, with a CV of 39.7% vs. 21.1%. This is also reflected in the respective portions of variance explained by genotype (line), treatment and the interaction between genotype and treatment. The highest portion of the variation can be attributed to nitrogen treatment for all three traits (69.0, 50.3. 27.4%

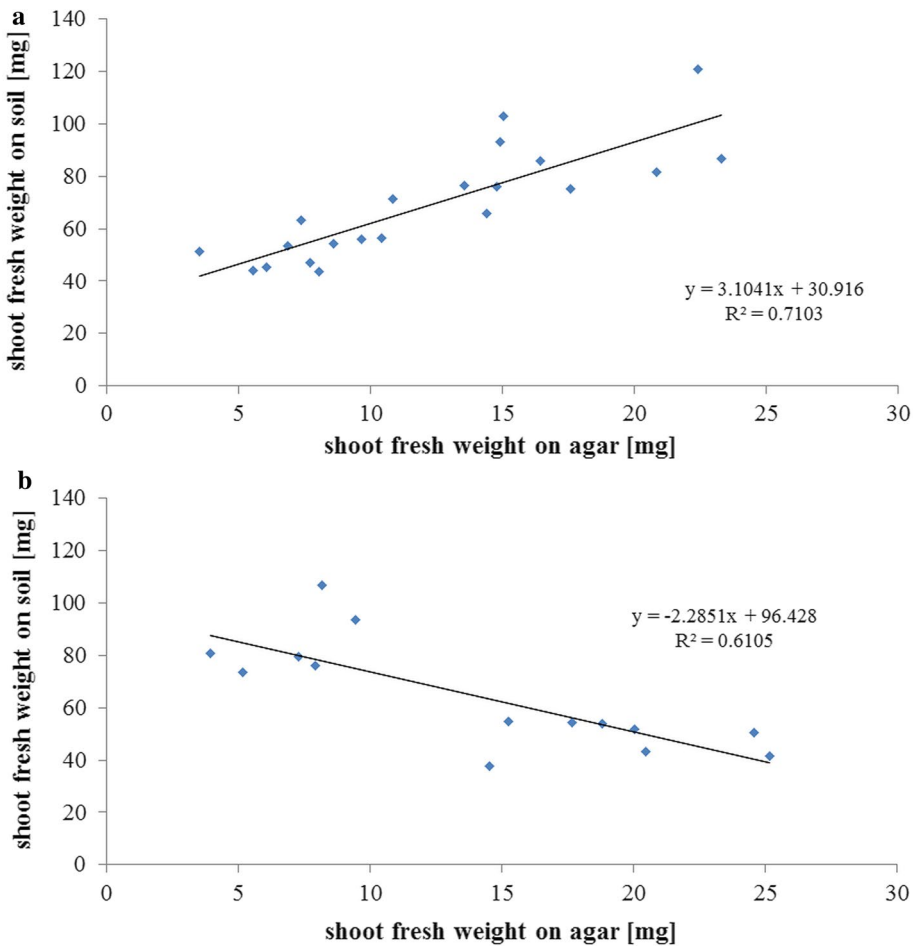

Fig. 1 Accessions with similar **a** and contrasting **b** growth behaviour on agar and soil on low N during population screens. Shown are scatterplots of adjusted mean shoot fresh weight of accessions grown for 21 days on synthetic agar medium (x-axis) or for 35 days on soil (y-axis). The corresponding linear regression is indicated by the black line

Table 2 Fresh weights and ratios of 13 Arabidopsis accessions and lines grown on differing N regimes

Lines	SFW 0.4 mM N	SFW 9 mM N	*U*	RFW 0.4 mM N	RFW 9 mM N	*U*	SRR 0.4 mM N	SRR 9 mM N	*U*
Col-0	9.23 ± 2.112^{d}	11.97 ± 2.112^{a}	ns	4.12 ± 1.048^{abc}	3.67 ± 1.048^{a}	ns	2.397 ± 0.2405^{cd}	3.384 ± 0.2405^{cd}	ns
Lu	7.36 ± 1.316^{cd}	34.90 ± 1.670^{cd}	*	3.42 ± 0.653^{abc}	11.58 ± 0.829^{bcd}	*	2.336 ± 0.1498^{cd}	3.098 ± 0.1902^{bcd}	ns
NG087	6.09 ± 1.574^{abc}	17.52 ± 1.674^{ab}	*	4.34 ± 0.781^{abc}	5.19 ± 0.831^{ab}	ns	1.528 ± 0.1792^{abc}	3.520 ± 0.1907^{d}	*
Rak-2	6.26 ± 1.323^{abc}	$32.8\ 0 \pm 1.676^{bcd}$	*	5.01 ± 0.657^{c}	14.89 ± 0.831^{d}	*	1.262 ± 0.1507^{ab}	2.204 ± 0.1908^{abc}	*
NG064	6.00 ± 1.497^{abc}	20.49 ± 1.497^{ab}	*	4.89 ± 0.743^{bc}	7.33 ± 0.743^{abc}	*	1.294 ± 0.1704^{ab}	2.916 ± 0.1704^{abcd}	*
WC056	5.85 ± 1.497^{abc}	25.44 ± 1.580^{abc}	*	5.29 ± 0.743^{c}	10.53 ± 0.784^{abcd}	*	1.224 ± 0.1704^{ab}	2.393 ± 0.1799^{abcd}	*
KB006	5.65 ± 1.497^{abc}	39.71 ± 1.497^{d}	*	2.09 ± 0.743^{a}	13.13 ± 0.743^{cd}	*	2.858 ± 0.1704^{d}	3.261 ± 0.1704^{cd}	ns
Fei-0	5.10 ± 1.428^{ab}	27.38 ± 1.796^{abc}	*	5.34 ± 0.708^{c}	10.68 ± 0.891^{abcd}	*	0.950 ± 0.1626^{a}	2.752 ± 0.2045^{abcd}	*
Ts-1	4.98 ± 1.384^{ab}	21.27 ± 1.591^{abc}	*	2.64 ± 0.686^{ab}	11.29 ± 0.789^{abcd}	*	1.992 ± 0.1575^{bcd}	1.844 ± 0.1811^{a}	ns
El-0	4.87 ± 1.429^{ab}	25.96 ± 1.583^{abc}	*	4.20 ± 0.709^{abc}	10.49 ± 0.785^{abcd}	*	1.253 ± 0.1627^{ab}	2.534 ± 0.1803^{abcd}	*
C24	4.80 ± 1.370^{ab}	22.40 ± 1.796^{abc}	*	2.61 ± 0.680^{ab}	9.69 ± 0.891^{abcd}	*	1.948 ± 0.1560^{bcd}	2.333 ± 0.2045^{abcd}	ns
KT130	4.15 ± 1.497^{ab}	31.03 ± 1.797^{bcd}	*	2.56 ± 0.743^{ab}	13.67 ± 0.892^{cd}	*	1.881 ± 0.1704^{abc}	2.232 ± 0.2046^{abc}	ns
SG063	3.68 ± 1.681^{a}	30.24 ± 1.497^{bcd}	*	5.83 ± 0.834^{c}	14.96 ± 0.743^{d}	*	0.886 ± 0.1914^{a}	2.084 ± 0.1704^{ab}	*

SFW and RFW represent shoot and root fresh weight, respectively, in mg plant^{-1}. SRR is shoot–root ratio calculated as SFW/RFW. Significant ($P < 0.05$) differences between lines within treatments are indicated by different letters. Significant ($P < 0.05$) differences between treatments within lines are marked by an asterisk in the column *U* (Mann–Whitney *U* test). Shown are adjusted mean values and associated standard errors ($n > 5$)

ns not significant

for shoot and root fresh weight and shoot–root ratio, respectively; Table S5).

Root biomass on low N was significantly and strongly reduced in three lines, Ts-1, KB006, KT130, and moderately in four lines, C24, El-0, Lu, SG063 (Table 2). The two lines KB006 and KT130 showing the strongest reduction in shoot growth also had the strongest reduction in root growth. At sufficient N, shoot–root ratio is greater than 1 in all lines, reflecting a greater investment in shoot growth. At low N, the ratio is generally lower, and a significant reduction was detected in seven lines (El-0, Fei-0, NG064, NG087, SG063, WC056), indicating a shift to increased root growth. Only Ts-1 displays a slight increase in shoot–root ratio, while the shoot–root ratio of KB006 remains virtually unchanged on low N. Interestingly, Fei-0 and SG063 on low N display a ratio below 1 (0.950 and 0.886, respectively); a significant decrease from 2.752 and 2.084, respectively, on sufficient N. Twelve lines reacted to low N with very strongly reduced shoot growth (average $19.9 \pm 4.2\%$ of biomass at sufficient N, ranging from 13.3 to 24.8%; Table 3), and significant differences between shoot biomass at low and sufficient nitrogen. The notable exception is Col-0 whose growth was only reduced to 77.2% (Table 3), leading to no significant differences in fresh shoot weight.

The analysis of biomass was accompanied by a deep phenotyping of root system architecture. Only lines with consistent shoot fresh weight were analysed in detail in these experiments. Col-0, Lu, NG087, NG064 were defined as 'good' NUE lines, and Ts-1, Fei-0, El-0 and KT130 as 'bad' NUE lines, based on the shoot weight at low N (Table 2). Six

Table 3 Differences in plant growth normalised to performance at sufficient N

Line	% Sugar	SFW % N9	RFW % N9	SRR % N9
C24	2	22.9	25.5	88.3
Col-0	2	77.2	112.1	70.8
El-0	2	17.9	39.0	49.8
Fei-0	2	16.9	48.6	33.7
KB006	2	14.9	14.4	97.8
KT130	2	13.3	15.4	86.0
Lu	2	20.8	28.6	76.3
NG064	2	22.8	47.0	46.4
NG087	2	26.7	52.9	47.7
Rak-2	2	19.1	32.7	60.1
SG063	2	15.8	38.1	52.0
Ts-1	2	22.3	21.2	110.0
WC056	2	24.8	51.0	52.5

% N9 corresponds to performance at 0.4 mM N expressed as % of the performance at 9 mM N

SFW shoot fresh weight, *RFW* root fresh weight, *SRR* shoot–root ratio

plants were grown per plate, and scanned after 14 days in a growth cabinet. Root morphology, i.e. number of roots, main root length, total lateral root length and lateral root density were determined using WinRHIZO Pro and subjected to the same analyses as the biomass traits described above. Results are summarised in Table 4. ANOVA (Table S5) revealed significant effects ($P < 0.001$) in all root system architecture traits for line (genotype) and the interaction term, indicating

Table 4 Root system architecture traits at low and sufficient N

Trait	Main root length							Number of lateral roots							Total lateral root length							Lateral root density						
treatment	N04			N9				N04			N9				N04			N9				N04			N9			
line	M	SE	Sig	M	SE	Sig	*U*	M	SE	Sig	M	SE	Sig	*U*	M	SE	Sig	M	SE	Sig	*U*	M	SE	Sig	M	SE	Sig	*U*
C24	1.225	0.1492	a	2.846	0.190	a	*	4.323	0.5271	a	8.296	0.6777	a	*	0.698	0.188	a	2.159	0.237	a	*	3.545	0.120	c	2.767	0.156	bcd	*
El-0	1.682	0.1450	a	3.689	0.169	ab	*	5.163	0.5053	ab	9.349	0.5948	ab	*	1.297	0.178	b	3.135	0.211	ab	*	2.931	0.111	b	2.338	0.132	ab	*
Fei	3.036	0.1466	b	3.718	0.244	abc	ns	8.145	0.5195	de	9.155	0.8631	ab	ns	1.559	0.186	b	2.857	0.310	ab	*	2.502	0.114	ab	2.286	0.195	ab	ns
KB006	1.413	0.1658	a	4.916	0.233	cd	*	6.647	0.5881	bcd	14.091	0.8265	cd	*	1.242	0.205	ab	4.681	0.285	cd	*	4.746	0.131	d	2.993	0.176	cd	*
Lu	2.975	0.1329	b	3.509	0.205	ab	ns	7.243	0.4663	cd	9.869	0.7199	ab	ns	1.733	0.170	b	2.281	0.252	a	ns	2.224	0.104	a	2.686	0.162	bcd	ns
NG064	4.158	0.2340	c	6.875	0.285	e	*	9.621	0.8297	e	17.165	1.0313	d	*	3.485	0.292	d	5.954	0.366	d	*	2.296	0.177	a	2.443	0.233	abc	ns
NG087	3.071	0.1749	b	5.500	0.225	d	*	6.326	0.6075	bcd	11.898	0.7852	bc	*	1.448	0.216	b	3.423	0.275	abc	*	2.085	0.136	a	2.393	0.179	abc	ns
Rak-2	3.130	0.1272	b	4.289	0.158	bc	*	8.21	0.4434	de	8.816	0.5564	ab	ns	2.395	0.159	c	3.203	0.197	ab	*	2.545	0.097	ab	2.197	0.124	a	ns
SG063	3.862	0.1967	c	4.869	0.264	cd	ns	7.591	0.6758	cde	13.68	0.9116	bcd	*	2.922	0.245	cd	4.422	0.315	bcd	ns	2.158	0.144	a	3.241	0.213	d	*
Ts-1	2.713	0.1319	b	3.933	0.157	bc	*	6.175	0.4632	bc	9.981	0.5523	ab	*	1.412	0.167	b	2.584	0.199	a	*	2.201	0.102	a	2.663	0.127	bcd	*

Shown are means (M) and standard errors (SE) estimated by ANOVA. *Sig* different letters indicate significant differences between lines after Bonferroni correction. *U* asterisk gives significant differences between low and sufficient N within a line, estimated by Mann–Whitney *U* test with Bonferroni correction

ns not significant

that lines reacted differently to changes in the nitrogen supply. The nitrogen treatment effect was significant ($P < 0.001$) for main root length, number of roots and total lateral root length, but not lateral root density ($P = 0.06$). The genotype explained 22.2, 8.3, 15.2 and 20.9% of the variation, respectively, the treatment 16.8, 11.7, 13.8 and 0.3%, and the interaction 5.6, 4.2, 4.0 and 8.8%. Overall, the percentage variation explained by these factors is lower than for the biomass traits. As for biomass, root architecture traits are generally reduced on low N compared to sufficient N. Main and total lateral root length are reduced by 60% and 52% on low N compared to sufficient N, respectively. KB006 has the strongest reduction in main and lateral root length and number of roots, but the strongest induction in lateral root density (Table 4). In contrast to root length and number, the lateral root density, i.e. the number of lateral roots per cm main root, is significantly increased in C24, El-0, and KB006, but decreased in SG063 and Ts-1.

Differentially expressed genes

Six lines with contrasting NUE selected in the deep phenotyping screen were used for expression analysis. NG087, NG064 and KB006 were defined as 'good' NUE lines, and Fei-0, El-0 and SG063 as 'bad' NUE lines, based on the shoot weight at low N. Three biological replicates (30–50 plants each) were grown on agar plates. Shoot and root material was harvested separately after 21 days. Signal and fold change (logFC) data are available in Table S6.

As a first step, we looked at the expression of genes known to be involved in nitrogen metabolism to verify the experimental set-up and detect possible differences between lines (Table S6). On low N compared to sufficient N, the expression of *AtNRT2.4* (AT5G60770), encoding a high-affinity nitrate transporter, is increased in roots in all lines; while the expression of *AtNRT2.5* (AT1G12940), also encoding a high-affinity nitrate transporter, is significantly and substantially increased in the shoot. Its expression level differs between the lines grown on low N, with a fold change of − 1.25 between the shoots of El-0 and NG087, and a twofold difference in the roots of El-0, Fei-0, SG063 vs. KB006, NG087. The expression of high-affinity nitrate transporter *AtNRT2.6* (AT3G45060) is decreased in the root in low N. The expression of *AtCLCa* (AT5G40890) is significantly down-regulated in both shoot and root. The transcription factors (TF) LBD37/38/39 (AT5G67420, AT3G49940, AT4G37540) are significantly down-regulated in limited N in shoots of all lines, and LBD38/39 are also significantly lower expressed in roots of all lines (Table S6). The bZIP transcription factor gene *TGA1* (AT5G65210*),* but not *TGA4 (*AT5G10030*),* is up-regulated in the shoots of all lines but NG087, and down-regulated in the roots of El-0 only. *TGA4* is only up-regulated in the shoot of KB006.

In total, 12428 loci displayed at least one significant logFC. Of these loci, 18.6% were detected in both shoot and root with the majority of genes (87.4%) behaving similarly in shoot and root, and only 12.6% showing contrasting behaviour in the two organs. Overall, 5374 genes showed a higher expression (FC > 1, $P < 0.01$) in shoots on low nitrogen, 4748 genes a lower expression. The number of differentially expressed genes was lower in the roots, with 2428 genes up-regulated on low nitrogen, and 1950 genes down-regulated in low nitrogen. The overlap between these groups is low (Fig. 2). Overlaps between 'up' and 'down' within an organ may indicate genotypic differences, i.e. the lines may react differently to the nitrogen regimes. In all lines, more genes are up-regulated on low nitrogen in the shoot; while in the root, in all lines but KB006, more genes are down-regulated on low nitrogen. No significant GO term enrichments were found in the different classes.

Linking phenotype and gene expression

A Principal Component Analysis (PCA) of the Affymetrix signal data from 12428 genes with significant expression changes between low and sufficient N was performed. The first principal component (PC1) accounted for 40% of the variation and separated shoot and root, while PC2 explained 14% of the variation and separated low and sufficient N (Fig. 3a). PC4 accounts for 4.1% variance and partially separates accessions and mRILs, PC5 accounts for 3.2% and partially separates the lines (Fig. 3b), with the related mRILs NG064 and NG087, exhibiting 'good' NUE, clustering together as expected, as do the accessions El-0 and Fei-0 with 'bad' NUE. The unrelated mRILs KB006 ('good' NUE) and SG063 ('bad' NUE) are distinguished by PC5. We used the 30 highest and 30 lowest loadings of each PC (Table 5) and performed a GO term overrepresentation analysis (Table 6). As expected, genes associated with chloroplasts are significantly overrepresented in PC1. A total of 23 genes (38.3%) present in the top loadings are located in

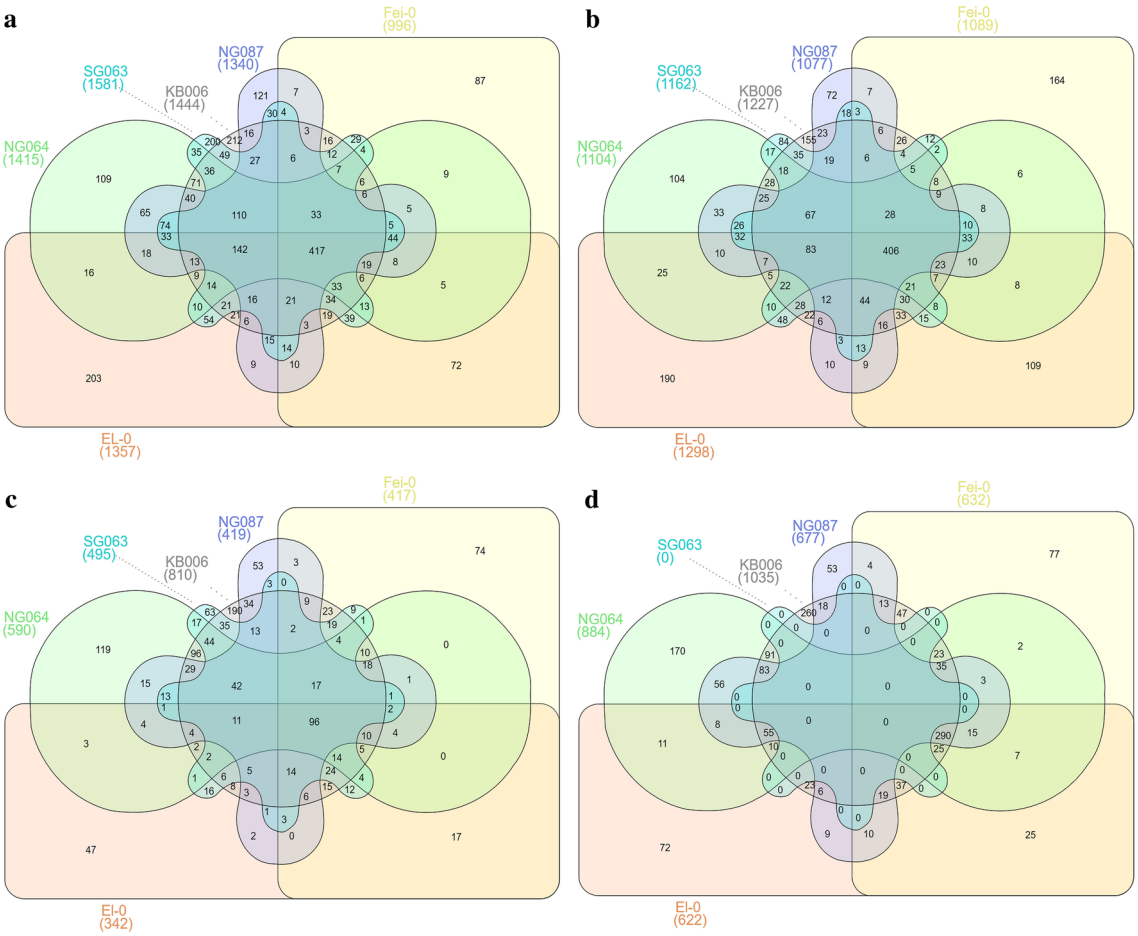

Fig. 2 VENN diagrams of gene expression on low N using two accessions and four mixed RILs selected for contrasting and stable growth during deep phenotyping. Depicted are four conditions: expression increased on low N in shoot **a** or root **c**, and expression decreased on low N in shoot **b** or root **d**

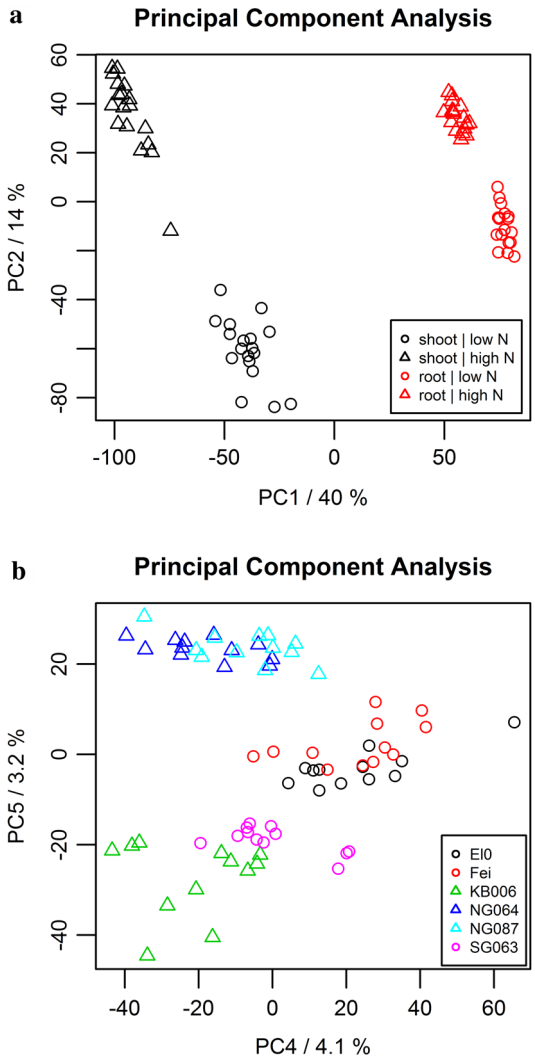

Fig. 3 Principal Component Analysis (PCA) of differentially expressed genes. **a** Scatterplot of PC1 and PC2, explaining 40% and 14% of the variation, respectively, and separating the samples according to organ and nitrogen treatment. The colour of the data points indicates the organ (black = shoot; red = root); the shape indicates the nitrogen treatment (circle = low N, triangle = high N). **b** Scatterplot of PC4 and PC5, explaining 4.1% and 3.2% of the variation, respectively, which separate the lines, as indicated by the colour code. Good NUE lines are represented by a triangle, low NUE lines by a circle

the chloroplast. Surprisingly, the only GO term associated with PC2 is phosphate ion homeostasis, represented by two genes (Table 6). Inspecting the loci contributing to PC2 in more detail, six genes linked to nitrogen metabolism or signalling are found in the top loadings (Table 5).

Pearson correlations between morphological traits of plants grown on low or sufficient N (Table 7) reveal different correlation patterns between the nitrogen treatments. On low N, root fresh weight is negatively correlated with shoot–root ratio and lateral root density, but positively with total fresh weight, while lateral root density is positively correlated with shoot–root ratio. On sufficient N, only positive significant correlations are detected, between total fresh weight and root fresh weight, between number of roots and main root length, and between number of roots and lateral root length. Shoot fresh weight, the criterion used for classification of the lines, does not show a significant correlation with any other trait on low N. We also determined Pearson correlations between transcript levels and morphological traits in shoot (Table S7) and in root (Table S8). For shoot, 13590 significant correlations were detected, ranging from 5428 (53.6% of tested genes) for shoot fresh weight to 83 (0.82%) for main root length (Table 8). For root, 4208 significant correlations were detected, ranging from 1303 (27.0%) for shoot fresh weight to 54 (1.1%) for shoot/root ratio (Table 8). No significant correlations were found for lateral root density in neither shoot nor root. No significant GO term enrichment was found.

Using Pavlidis Template matching in Mev4.9, transcript levels were clustered according to the pattern observed for shoot growth on low nitrogen. The respective transcript levels of 1068 and 436 genes expressed in shoot and root, respectively, corresponded to the growth pattern. Genes involved in translation are overrepresented in these sets (Fig. S1). Looking at genes significantly changed in expression in relation to their occurrence in lines with low (Fei-0, El-0) or high (NG087, NG064) shoot fresh weight on low N, 3187 genes are expressed in the shoot unique to the low-weight class vs. 986 genes in the high-weight class. The inverse ratio is observed in the root, with 457 genes unique to low shoot weight, and 1067 genes to high shoot weight. The expression of AT3G21670, encoding the NPF6.4/NRT1.4 low-affinity nitrate transporter, is lower in roots of lines with high shoot fresh weight. The transcription factor GATA21 (AT5G56860) shows higher expression in shoots of lines with high shoot weight, while one of its targets, *GLU1* (AT5G04140), shows the opposite expression pattern. Similarly, the expression of the ammonium transporter gene *AMT1;2* (AT1G64780) and the aspartate amino transferase gene *ASP3* (AT5G11520) is lower in shoots of lines with high fresh weight.

Four of the potential biomarkers could also be analysed using real-time quantitative PCR in two independent experiments using six different Arabidopsis accessions with contrasting growth behaviour on low nitrate. Only shoot material from plants grown on limiting nitrogen was included in the analysis. The resulting ΔC_T values (C_T values normalised to the housekeeping gene *AtEF1α*) are listed in Table 8. The expression of *AMT1;2, GATA21* and *GLU1* shows highly significant differences between accessions. *AMT1;2* and *GATA21* differentiate good and bad NUE lines to a limited extent, as accession Ak-1 is misclassified in both cases. Only the expression of *GLU1* completely follows the

Table 5 Top loadings of the principal components PC1, PC2, PC4 and PC5

PC1		PC2		PC4		PC5	
Loading	Locus (AGI code)	Loading	Locus (AGI code)	Loading	Locus (AGI code)	Loading	Locus (AGI code)
0.0141168	AT1G24180	0.0228877	AT1G67910	0.0339877	AT3G20100	0.0441140	AT2G21130
0.0140690	AT1G71780	0.0228353	AT4G37610	0.0337770	AT5G55960	0.0436720	AT1G65370
0.0140466	AT3G15300	0.0225893	AT1G49500	0.0311642	**AT3G03990**	0.0430234	AT4G19600
0.0140099	AT1G04850	0.0225434	AT4G28050	0.0310574	AT2G20340	0.0415123	AT3G43740
0.0140065	AT4G23885	0.0225328	AT5G44020	0.0310318	*AT3G30720*	0.0411115	AT1G65980
0.0139782	AT2G27510	0.0224660	*AT5G40890*	0.0301800	AT2G34810	0.0404602	AT1G24822
0.0139563	AT3G27190	0.0223910	**AT1G68670**	0.0301452	AT5G27350	0.0397495	AT1G30940
0.0139488	AT5G54800	0.0222322	AT1G03870	0.0294866	**AT1G65960**	0.0397414	AT1G23970
0.0139456	AT5G10830	0.0222209	AT5G13100	0.0293765	AT2G27360	0.0386280	AT2G41650
0.0139353	AT2G17130	0.0221225	AT2G34590	0.0293429	AT1G17370	0.0383072	AT2G07140
0.0139286	AT1G76760	0.0220487	AT1G01620	0.0292190	AT4G20480	0.0382104	AT1G27580
0.0139274	AT1G79530	0.0217858	AT1G55810	0.0289912	AT1G49480	0.0380114	AT5G47760
0.0139263	**AT2G01140**	0.0216315	AT5G15350	0.0282819	AT2G24540	0.0378161	AT1G29410
0.0139235	AT2G29750	0.0214804	*AT4G12420*	0.0282168	**AT3G01120**	0.0376158	AT5G44780
0.0139158	AT1G54540	0.0214586	AT3G48360	0.0278150	AT5G23920	0.0375174	AT3G53710
0.0139147	AT5G40760	0.0213495	AT4G17890	0.0277773	AT2G15320	0.0371740	AT4G15420
0.0139073	AT1G24430	0.0213441	AT1G11380	0.0277514	AT5G18670	0.0359639	AT4G21650
0.0139044	AT2G31390	0.0213384	AT1G58080	0.0273009	AT3G07650	0.0347176	AT1G60710
0.0139013	**AT3G02360**	0.0212566	AT3G17440	0.0271342	AT3G23530	0.0346396	AT3G14350
0.0138986	AT5G67590	0.0212535	AT3G60130	0.0269052	AT1G79970	0.0342158	AT4G20880
0.0138966	**AT2G45290**	0.0211902	AT5G24580	0.0267629	AT1G63770	0.0333249	AT3G25070
0.0138950	AT5G66985	0.0211774	*AT4G37540*	0.0266513	AT5G13930	0.0326457	AT3G47290
0.0138921	AT1G02530	0.0211578	AT5G12250	0.0266274	AT1G60270	0.0324836	AT3G02900
0.0138921	AT2G22560	0.0210845	AT3G16690	0.0265241	AT5G24770	0.0322721	AT5G40610
0.0138865	AT3G21230	0.0210706	AT4G33960	0.0263977	AT4G37150	0.0321548	AT1G44414
0.0138847	AT1G06120	0.0209889	*AT5G57440*	0.0263470	**AT1G71090**	0.0321003	AT5G45050
0.0138736	AT3G22240	0.0209572	AT2G26980	0.0262762	AT5G37260	0.0318554	*AT3G17590*
0.0138654	AT3G26770	0.0209304	AT1G14290	0.0260732	AT5G23730	0.0318343	AT3G46110
0.0138652	AT4G15093	0.0209213	AT1G75680	0.0258610	AT2G40460	0.0316635	AT1G61500
0.0138638	AT2G42350	0.0209021	**AT5G41080**	0.0256625	AT5G17700	0.0315869	AT4G13360
− 0.0140400	**AT3G09050**	− 0.0212620	AT5G53420	− 0.0283779	AT5G25920	− 0.0329764	AT2G25250
− 0.0140402	AT3G14110	− 0.0212856	AT3G09100	− 0.0284585	AT1G09980	− 0.0330181	AT3G18270
− 0.0140405	AT3G53130	− 0.0212938	AT3G26820	− 0.0286033	AT1G05090	− 0.0330629	AT3G26420
− 0.0140440	AT1G23400	− 0.0212948	AT1G43910	− 0.0287127	AT4G17420	− 0.0333992	AT5G45300
− 0.0140466	**AT5G55710**	− 0.0213362	AT3G51430	− 0.0287355	AT2G40010	− 0.0336536	AT3G58470
− 0.0140491	AT2G48070	− 0.0213408	AT3G14020	− 0.0289301	AT1G12650	− 0.0338641	AT3G03360
− 0.0140535	AT2G01590	− 0.0213911	AT3G08690	− 0.0291592	ATMG00640	− 0.0341454	AT2G42070
− 0.0140559	AT4G01690	− 0.0213957	AT1G66880	− 0.0291689	AT3G50670	− 0.0343426	AT1G69750
− 0.0140614	AT5G13510	− 0.0214008	AT1G09070	− 0.0293311	AT2G25920	− 0.0344936	AT1G78670
− 0.0140634	**AT2G33800**	− 0.0214270	AT2G04040	− 0.0293477	AT3G56460	− 0.0350539	AT5G19910
− 0.0140654	AT5G42070	− 0.0214692	AT5G08170	− 0.0293818	AT4G27040	− 0.0352147	AT3G18070
− 0.0140661	**AT2G33450**	− 0.0214726	AT5G59050	− 0.0294921	ATMG01200	-0.0353632	AT3G53668
− 0.0140681	AT3G14930	− 0.0215334	AT1G10690	− 0.0296206	AT5G44200	− 0.0360550	AT3G53210
− 0.0140702	AT1G14150	− 0.0216234	AT1G20100	− 0.0296274	AT5G28350	− 0.0361688	AT3G10630
− 0.0140711	**AT3G09580**	− 0.0216382	AT3G21690	− 0.0299066	AT4G32240	− 0.0362009	AT5G23460
− 0.0140730	AT3G61080	− 0.0216425	AT1G12940	− 0.0303298	AT2G01630	− 0.0368671	AT3G43520
− 0.0140733	AT3G23760	− 0.0216566	AT4G00500	− 0.0306554	AT5G44150	− 0.0368761	AT4G20060
− 0.0140739	AT2G29290	− 0.0216583	AT5G06510	− 0.0306721	AT1G06070	− 0.0380558	AT5G24670

Table 5 (continued)

PC1		PC2		PC4		PC5	
Loading	Locus (AGI code)	Loading	Locus (AGI code)	Loading	Locus (AGI code)	Loading	Locus (AGI code)
− 0.0140745	AT1G15180	− 0.0217424	AT1G56170	− 0.0307813	AT4G11640	− 0.0381999	AT1G16900
− 0.0140772	**AT3G15520**	− 0.0217591	AT4G27830	− 0.0309831	AT3G62140	− 0.0382732	AT3G23570
− 0.0140792	AT5G45680	− 0.0218176	AT4G33040	− 0.0313262	AT1G01290	− 0.0382926	AT1G11270
− 0.0140886	**AT5G53490**	− 0.0218203	AT1G03040	− 0.0315495	ATMG00210	− 0.0393270	AT1G33520
− 0.0140943	AT3G15850	− 0.0218716	*AT2G43500*	− 0.0318215	ATMG01190	− 0.0394409	AT3G17890
− 0.0140946	**AT5G14260**	− 0.0218884	AT3G11580	− 0.0318515	AT4G14385	− 0.0395201	AT5G27990
− 0.0140986	**AT3G56650**	− 0.0218921	AT3G59140	− 0.0319357	AT4G19350	− 0.0395688	AT3G18880
− 0.0141024	AT1G77490	− 0.0219197	AT2G46400	− 0.0321648	AT4G31980	− 0.0405422	AT2G27285
− 0.0141037	**AT5G38520**	− 0.0221123	AT1G02850	− 0.0325856	AT2G19830	− 0.0425340	AT4G16770
− 0.0141080	**AT1G20810**	− 0.0225630	AT4G27900	− 0.0340950	AT3G06110	− 0.0427171	AT1G35612
− 0.0141105	AT3G44890	− 0.0226562	AT5G23850	− 0.0341533	ATMG01360	− 0.0433049	AT5G44510
− 0.0141143	AT4G30950	− 0.0229095	*AT5G16570*	− 0.0356838	ATMG01280	− 0.0442104	AT4G26870

Genes highlighted by GO term enrichment analysis are shown in bold print; genes associated with nitrogen metabolism are identified by cursive and underlined print

pattern predicted from the array data (the same is true for *NPF6.4*, but the differences between the accessions are not significant).

Discussion

Validation of experimental setup using nitrogen metabolism marker genes

Several markers genes for nitrogen metabolism were analysed to verify the experimental set-up. The results demonstrate the expected overall nitrate response of gene expression, thus validating our experimental set-up. As expected, the expression of the high-affinity nitrate transporter genes *AtNRT2.4* (AT5G60770) and *AtNRT2.5* (AT1G12940) is increased under limiting nitrogen. A similar increase in transcript abundance upon exposure to N limitation for both genes has been reported by Lezhneva et al. (2014). AtNRT2.4 is induced in long-term starvation and has been linked to nitrate uptake in the roots at very low nitrate concentration (Kiba et al. 2012), which fits to our findings of a significant and substantial increase of *AtNRT2.4* expression in the root in all lines after 21-day growth on low N. The expression in the shoot, while slightly increased, is not significantly changed. The opposite situation is found with *AtNRT2.5*, whose expression is significantly and substantially increased in the shoot. AtNRT2.5 is required to support the growth of nitrogen-starved adult plants by ensuring the efficient nitrate uptake in the root and efficient remobilisation of nitrogen in the leaves (Lezhneva et al. 2014). In contrast to the other high-affinity transporters, expression of *AtNRT2.6* (AT3G45060) is decreased in the root in low N. *AtNRT2.6* expression has been described to be induced by high nitrogen levels (Dechorgnat et al. 2012). Its overexpression failed to complement the nitrate uptake defect of an nrt2.1-nrt2.2 double mutant. *AtCLCa* (AT5G40890) encodes a nitrate/proton exchanger that transports excess nitrate into the vacuole for storage and maintenance of nitrate homeostasis in the cytosol (De Angeli et al. 2006), and consequently its expression is down-regulated on low N, consistent with our findings. Three members, LBD37/38/39 (AT5G67420, AT3G49940, AT4G37540), of the LATERAL ORGAN BOUNDARY DOMAIN transcription factor gene family are induced by nitrate and suppress N starvation responses such as anthocyanin biosynthesis and key genes required for NO_3- uptake and assimilation (Rubin et al. 2009). As expected, their expression was down-regulated under our low N condition. The bZIP transcription factor gene *TGA1* (AT5G65210), but not *TGA4* (AT5G10030), is up-regulated in the shoots of all lines but NG087, and down-regulated in the roots of El-0 only. *TGA4* is only up-regulated in the shoot of KB006. Expression of *TGA1* and *TGA4* was shown to be induced by nitrate in roots of Col-0, to regulate the expression of *NRT2.1* (AT1G08090) and *NRT2.2* (AT1G08100) and to affect lateral root formation in response to nitrate (Alvarez et al. 2014). We did detect expression differences between Arabidopsis accessions, demonstrating the diversity of the N stress response.

Phenotypic plasticity

The phenotypic analysis of two complementary populations of genetically diverse Arabidopsis lines highlighted the plasticity of plant growth in response to differing nitrate availability in the substrate. Plasticity of root morphology

Table 6 GO term enrichment analysis of Principal Component Analysis (PCA) top loadings

PC	distinction	%Var expl.	Categorie	GO term	Description	*P* value	q-value	Enrichment	*N*	*B*	*n*	*b*
PC1	shoot/root	40%	Component	GO:0044434	Chloroplast part	1.92E−14	1.18E−11	12.3	12417	475	34	16
				GO:0044434	Chloroplast part	2.92E−12	1.58E−09	8.81	6868	367	34	16
PC2	N04/N9	14%	Process	GO:0055062	Phosphate ion homeostasis	9.50E−05	1.11E−01	131.4	12471	7	27	2
				GO:0055062	Phosphate ion homeostasis	1.48E−04	1.12E−01	101.75	6868	5	27	2
PC4	Acc/mRIL	4.10%	Process	GO:1905393	Plant organ formation	5.04E−04	1.00E+00	59.13	12471	14	30	2
				GO:1905393	Plant organ formation	2.42E−04	7.31E−01	83.25	6868	11	15	2
PC5	Lines	3.20%		No GO term enrichment found								

N the total number of genes, *B* the total number of genes associated with a specific GO term, *n* the number of genes in the target set, *b* the number of genes in the intersection, Enrichment = (*b*/*n*)/(*B*/*N*). *N* = 12417: tested against all Affymetrix Chip genes, *N* = 6868: tested against total DEGs

in response to abiotic factors has been described on the inter- and intra-species level (Forde and Lorenzo 2001; Gruber et al. 2013), and natural variation for foraging under nutrient-deficient conditions has been observed (North et al. 2009; Chardon et al. 2010; Ikram et al. 2012). However, these studies did not analyse natural variation of the growth response to long-term limiting nitrogen. The lack of correlation between growth in two complementary experimental setups, synthetic agar medium and soil, respectively, indicates that distinct processes contribute to NUE in the two different growth conditions. This could be due to the difference in plant age at the time of sampling (23 days in agar vs. 35 days in soil). Differential stress responses during development have been described for drought tolerance in barley (Szira et al. 2008).

The detailed characterisation of a selected set of lines provided clear evidence that different genotypes (lines) follow very different adjustment/survival strategies to cope with long-term nitrogen limitation. Both biomass and root architecture traits are generally reduced on low N compared to sufficient N. ANOVA revealed that the highest portion of the variation for the biomass traits shoot and root fresh weight and shoot–root ratio can be attributed to nitrogen treatment. Similar results were reported from a study of 22 Arabidopsis accessions grown hydroponically (Ikram et al. 2012). 'Genotype' explains almost as much variance as nitrogen treatment for shoot–root ratio (24.6%), but considerably less for shoot and root fresh weight (6.1 and 11.2%). This is in agreement with reports describing the shoot–root ratio as the trait most suitable to analyse natural variation in plant nitrogen response (Scheible et al. 1997; Ågren and Franklin 2003). Overall, the percentage phenotypic variation explained by the root architecture factors is lower than for the biomass traits. The experimental error is expected to be higher in the more complex measurements of the root architecture. Interestingly, the genotypic component is more important than the treatment, in relative terms, for the root architecture traits. To be useful as biomarkers, morphological traits which are influenced by both genotype and treatment seem the most promising, but overall, the respective contributions appear rather low for reliable indicators. In line with this observation, no clear pattern in root architecture can be distinguished between lines with good NUE (Lu, NG087, NG064; shoot fresh weight ≥ upper quartile) and bad NUE (Ts-1, Fei-0, El-0; shoot fresh weight ≤ lower quartile).

Many plant species show increased root growth at the expense of shoot growth under N limiting conditions, thus increasing their chances to reach additional sources of nitrogen to sustain their growth (grapevine (Grechi et al. 2007); maize (Brouwer 1962); tobacco (Scheible et al. 1997); Arabidopsis (Ikram et al. 2012)). On the other hand, plants need carbon substrates from photosynthesis to be able to fix the available nitrogen, and therefore, they must balance

Table 7 Pearson correlation between morphological traits

	SRR	RFW	SFW	TFW	LRD	LRL	MRL	NR
SRR	*	− 0.71844	− 0.25076	− 0.63051	− 0.00178	0.08122	0.26364	0.17037
RFW	**− 0.97941**	*	0.82512	**0.99289**	0.26937	0.04616	− 0.34300	− 0.05923
SFW	0.30190	− 0.33187	*	0.87779	0.18382	0.12670	− 0.32369	− 0.02873
TFW	− 0.87310	**0.95355**	− 0.35098	*	0.30027	0.06541	− 0.33762	− 0.03693
LRD	**0.97696**	**− 0.94087**	0.17439	− 0.81440	*	0.66921	0.61165	0.81015
LRL	− 0.28071	0.43152	− 0.11445	0.62326	-0.13818	*	0.88797	**0.95256**
MRL	− 0.60428	0.73741	− 0.03349	0.87916	− 0.54845	0.81243	*	**0.94033**
NR	− 0.02128	0.18473	0.20598	0.41438	0.05046	0.81285	0.78373	*

Given are the coefficients of correlation for each N treatment, with values corresponding to low N on the lower triangular matrix, and sufficient N on the upper triangular matrix. Bold print indicates significant correlation after FDR correction for multiple testing (FDR = 0.05)

SRR shoot–root ratio, *RFW* root fresh weight, *SFW* shoot fresh weight, *TFW* total fresh weight, *LRD* lateral root density, *LRL* total lateral root length, *MRL* main root length, *NR* number of roots

Table 8 Real-time qPCR analysis of biomarker candidate genes

Gene	Accession	NUE measured	NUE predicted	dCT	Fisher's LSD 5%	P_{ANOVA}
AMT1;2	Ak-1	Low	Low	5.127	a	< 0.001
	Col-0	Good	Low	5.204	a	
	Appt-1	Good	Intermediate	5.718	ab	
	El-0	Good	Low	6.292	b	
	Gy-0	Low	High	7.208	c	
	Cvi-0	Low	High	7.438	c	
GATA21	Gy-0	Low	Good	10.26	a	0.001
	Cvi-0	Low	Good	11.43	ab	
	Appt-1	Good	Intermediate	12.59	bc	
	Col-0	Good	Low	13.01	c	
	Ak-1	Low	Low	13.36	c	
	El-0	Good	Low	14.77	d	
GLU1	Gy-0	Low	Low	3.488	a	0.008
	Ak-1	Low	Low	3.586	a	
	Cvi-0	Low	Low	3.666	a	
	Col-0	Good	Good	4.333	b	
	Appt-1	Good	Good	4.445	c	
	El-0	Good	Good	5.217	b	
NPF6.4	Ak-1	Low	Low	10.92		0.486
	Gy-0	Low	Low	11.14		
	Cvi-0	Low	Low	12.24		
	Col-0	Good	Good	12.85		
	Appt-1	Good	Good	12.99		
	El-0	Good	Good	15.37		

High ΔC_T values (C_T values normalised to the housekeeping gene *AtEF1α*) correspond to low gene expression, low ΔC_T values correspond to high gene expression. Significant differences between accessions, identified with Fisher's least significant difference with Bonferroni correction when $P_{ANOVA} < 0.05$, are indicated by different letters. 'NUE measured' is based on shoot fresh weight on low nitrogen, NUE predicted corresponds to the expected gene expression from the array data

both demands. Several studies identified the C/N balance in the shoot as a trigger for changes in the biomass allocation (Stitt 1999; Martin et al. 2002), while sucrose, as indicator of the nutritional status, acts as a signal for increased root growth (Gibson 2005). Our findings expose the bandwidth of adaptive reactions to limiting N conditions within the species *Arabidopsis thaliana*. Different lines, with different genetic backgrounds, follow different ways of achieving a

balance between growth reduction due to N limitation and foraging to detect nitrogen sources. We observed several genotype-specific modes of action to achieve growth under N deficiency: modification of root system architecture, general root growth reduction, maintenance of root system, or use of nutrients primarily for shoot growth. Fei-0 builds an overall increased root system on low N, while KB006 produces a very dense root system with a short main root and many short lateral roots. These findings support the notion that lines with different genetic backgrounds adopt different adjustment strategies to low nitrogen (Chardon et al. 2010; Ikram et al. 2012; De Pessemier et al. 2013). From an agricultural point of view, this means that there is not one optimal way to increase growth and yield under limiting conditions, which broadens the basis for selection, but also render the selection of optimal lines more difficult.

This diversity of reaction modes causes on the one hand difficulties to identify certain morphological and molecular features of root systems for good nitrogen use efficiency. On the other hand, it opens access to a multitude of genetic factors which can be used as tools to modulate and adjust root systems to a wide range of environmental (soil) conditions. It will, thus, be of great interest to identify the molecular genetic determinants of various responses to low N supply. The large diversity of response modes observed in the Arabidopsis population may preclude the use of the frequently applied Genome Wide Association Study (GWAS) approach for identification of causal genetic loci and may rather ask for the use of the more classical linkage mapping approach using biparental populations, e.g. recombinant inbred line (RIL) or introgression line (IL) populations, each of which would segregate for a specific set of NUE-related traits.

Linking phenotype and gene expression

Several avenues were explored to link phenotype and gene expression. Using a principal component analysis, we subjected the 30 highest and 30 lowest loadings of each PC to a GO term overrepresentation analysis. As expected, genes associated with chloroplasts are significantly overrepresented in PC1, which separated shoot and root samples. Surprisingly, the only significant GO term associated with PC2, which separated low and sufficient N samples, is phosphate ion homeostasis, represented by two genes. However, six genes linked to nitrogen metabolism or signalling are found in the top loadings of PC2. These include genes encoding the nitrate transporter NRT2.5 (At1g12940), the glutamine synthase GLN1;4 (At5g16570), the chloride channel CLC-A (At5g40890), the nitrate-responsive transcriptional regulator LBD39 (At4g37540), the NIN-like transcription factor NLP8 (At2g43500), and *GS1* (At5g57440) encoding a HAD superfamily protein. *SKU5* (At4g12420) encodes a cupredoxin superfamily protein involved in root tip growth, and differences in gene expression might contribute to different root architecture.

We also determined Pearson correlations between transcript levels and morphological traits in shoot and in root. Significant correlations between genes expressed in the root with shoot fresh weight indicate that gene expression in the root is associated with the growth above-ground, and that crosstalk between the organs is likely bidirectional. Among the significantly correlated genes are several encoding nitrate transporters (*NRT1.1, NRT1.6, NRT2.2, NRT2.5*), several genes related to RNA regulation and stress or nutrient signalling, and the *RDR2* gene encoding the RNA-dependent RNA polymerase 2, which is involved in the generation of endogenous siRNAs (Jia et al. 2009). Post-transcriptional and epigenetic regulation mechanisms have been proposed for several enzymes related to nitrate transport or nitrogen metabolism. The switch between modes of the dual affinity nitrate transporter NPF6.3/NRT1.1 (AT1G12110) is achieved by phosphorylation (Liu and Tsay 2003). Protein structure analyses suggest that the increase in transport rate is the result of altering the efficiency in the formation (Parker and Newstead 2014). Nitrate-responsive miRNAs have been identified in Arabidopsis (Vidal et al. 2010; Zhao et al. 2011) and several crop species (Zhao et al. 2012; Paul et al. 2015).

In a complementary approach, transcript levels were clustered according to the pattern observed for shoot growth on low nitrogen. A GO term analysis revealed that genes involved in translation/organonitrogen compound biosynthetic processes are overrepresented in these sets. This is consistent with a remodelling of the metabolism during severe nitrogen stress, such as down-regulation of genes involved in photosynthesis and of ribosomal genes involved in protein biosynthesis; while genes encoding enzymes involved in starch biosynthesis and protein degradation are up-regulated (Bi et al. 2007). These events correspond to nitrogen remobilization from senescing tissues (Havé et al. 2017) also described for Arabidopsis under low N conditions (Diaz et al. 2008). Protein degradation serves as source for amino acids which are transported into active tissues via the phloem.

Looking at genes showing significantly changed expression between lines with low or high shoot fresh weight on low N (low or good NUE), five possible biomarkers emerged from analysis of the array data. The expression of the ammonium transporter gene *AMT1;2* (AT1G64780) and the aspartate amino transferase gene *ASP3* (AT5G11520) is lower in shoots of lines with good NUE. In an independent experiment, qPCR data of *AMT1;2* expression correctly classified four out of six Arabidopsis accessions as low or good NUE lines. *AMT1* family members encode high-affinity ammonium transporters and have also been identified in maize (Gu et al.

2013), poplar (Wu et al. 2015), and rice (Yang et al. 2015). In tomato, Arabidopsis, and rapeseed, several *AMT1* genes are differential regulated by nitrogen supply and time of day (Gazzarrini et al. 1999; von Wirén et al. 2000; Pearson et al. 2002). *AMT1* family members have been linked to N remobilization from old leaves during senescence (Gregersen and Holm 2007; Havé et al. 2017), a similar role under N stress is conceivable. The expression of *AT3G21670*, encoding the NPF6.4/NRT1.3 low-affinity nitrate transporter, is lower in roots of lines with high shoot fresh weight. Up-regulation of NPF6.4 in shoots of Arabidopsis grown under limiting nitrogen supply has been reported (Okamoto et al. 2003; Bi et al. 2007). Similarly, the wheat orthologue TaNPF6.6 was significantly up-regulated by nitrate starvation (Buchner and Hawkesford 2014). The transcription factor GNC (AT5G56860), a nitrate-inducible, carbon metabolism-involved member of the GATA factor family of zinc finger transcription factors, shows higher expression in shoots of lines with high shoot weight. It has been described to modulate chlorophyll biosynthesis and glutamate synthase (GLU1/GOGAT) expression (Hudson et al. 2011) and to be induced by both nitrate and cytokinin (Bi et al. 2005; Kiba et al. 2005). In the validation experiment, four out of six accessions were correctly classified. The glutamate synthase gene *GLU1* (AT5G04140), a target of GATA21, shows lower expression in shoots of lines with high NUE. The array data could be validated using qPCR data, which also associated lower *GLU1* expression with better growth on low N. However, in a global expression study using only the Arabidopsis reference accession Col-0, no fluctuation in the response to differing N supply was detected (Bi et al. 2007). GLU1/GOGAT has been identified in several plant species, including maize, tomato, rice and wheat. GOGAT was identified as a major effector of NUE in wheat (Quraishi et al. 2011) using a 'syntenic' metaQTL approach. A significant positive correlation was found between global expression of the *GoGAT* gene and the Nitrogen Nutrition Index score of leaf samples in two wheat cultivars, indicating that an increased expression of GOGAT is associated with good NUE in wheat, contrary to our findings in Arabidopsis. This difference could be due to the fact that our definition of good NUE is solely based on good shoot growth on low N.

The global expression analysis performed here showed the expected patterns of N and C metabolism-associated genes and pointed to regulatory factors (transcription and epigenetic factors) as important players in the adaption to limited nitrogen. One gene, *GLU1,* emerged as a possible biomarker for NUE in Arabidopsis. These findings need to be validated in different genetic backgrounds of Arabidopsis (and other plant species) with a sequence-independent method such as RNA-Seq and should be deepened towards identification of genes specifically involved in different modes of adjustment to low N conditions.

Author contribution statement TA, RCM, HS and AS designed the research. CG performed the sample collection and RNA extraction, and analysed data. RCM, CN, KWF and MM conducted the deep phenotyping and analysed data. AB processed the gene expression data. RCM performed statistical analyses and drafted the manuscript. TA and AS revised and edited the text. All authors have read and approved the final manuscript.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

References

Agrama HAS, Zakaria AG, Said FB, Tuinstra M (1999) Identification of quantitative trait loci for nitrogen use efficiency in maize. Mol Breed 5(2):187–195. https://doi.org/10.1023/a:1009669507144

Ågren GI, Franklin O (2003) Root: shoot ratios, optimization and nitrogen productivity. Ann Bot 92(6):795–800. https://doi.org/10.1093/aob/mcg203

Alvarez JM, Riveras E, Vidal EA, Gras DE, Contreras-López O, Tamayo KP, Aceituno F, Gómez I, Ruffel S, Lejay L, Jordana X, Gutiérrez RA (2014) Systems approach identifies TGA1 and TGA4 transcription factors as important regulatory components of the nitrate response of *Arabidopsis thaliana* roots. Plant J 80(1):1–13. https://doi.org/10.1111/tpj.12618

Bi YM, Zhang Y, Signorelli T, Zhao R, Zhu T, Rothstein S (2005) Genetic analysis of Arabidopsis GATA transcription factor gene family reveals a nitrate-inducible member important for chlorophyll synthesis and glucose sensitivity. Plant J 44(4):680–692

Bi Y-M, Wang R-L, Zhu T, Rothstein SJ (2007) Global transcription profiling reveals differential responses to chronic nitrogen stress and putative nitrogen regulatory components in Arabidopsis. BMC Genom 8(1):281. https://doi.org/10.1186/1471-2164-8-281

Bolstad BM, Collin F, Brettschneider J, Simpson K, Cope L, Irizarry RA, Speed TP (2005) Quality assessment of Affymetrix GeneChip data. In: Gentleman R, Carey V, Huber W, Irizarry R, Dudoit S

(eds) Bioinformatics and computational biology solutions using R and bioconductor. Springer, New York, pp 33–47
Brouwer R (1962) Nutritive influences on the distribution of dry matter in the plant. Neth J Agric Sci 10(5):399–408
Buchner P, Hawkesford MJ (2014) Complex phylogeny and gene expression patterns of members of the NITRATE TRANSPORTER 1/PEPTIDE TRANSPORTER family (NPF) in wheat. J Exp Bot 65(19):5697–5710
Chardon F, Barthélémy J, Daniel-Vedele F, Masclaux-Daubresse C (2010) Natural variation of nitrate uptake and nitrogen use efficiency in *Arabidopsis thaliana* cultivated with limiting and ample nitrogen supply. J Exp Bot 61(9):2293–2302. https://doi.org/10.1093/jxb/erq059
De Angeli A, Monachello D, Ephritikhine G, Frachisse JM, Thomine S, Gambale F, Barbier-Brygoo H (2006) The nitrate/proton antiporter AtCLCa mediates nitrate accumulation in plant vacuoles. Nature 442(7105):939–942
De Pessemier J, Chardon F, Juraniec M, Delaplace P, Hermans C (2013) Natural variation of the root morphological response to nitrate supply in *Arabidopsis thaliana*. Mech Dev 130(1):45–53. https://doi.org/10.1016/j.mod.2012.05.010
Dechorgnat J, Patrit O, Krapp A, Fagard M, Daniel-Vedele F (2012) Characterization of the *Nrt2.6* gene in *Arabidopsis thaliana*: a link with plant response to biotic and abiotic stress. PloS One 7(8):e42491
Diaz C, Lemaître T, Christ A, Azzopardi M, Kato Y, Sato F, Morot-Gaudry J-F, Le Dily F, Masclaux-Daubresse C (2008) Nitrogen recycling and remobilization are differentially controlled by leaf senescence and development stage in Arabidopsis under low nitrogen nutrition. Plant Physiol 147(3):1437–1449. https://doi.org/10.1104/pp.108.119040
Eden E, Navon R, Steinfeld I, Lipson D, Yakhini Z (2009) GOrilla: a tool for discovery and visualization of enriched GO terms in ranked gene lists. BMC Bioinform 10(1):48
Estelle MA, Somerville C (1987) Auxin-resistant mutants of *Arabidopsis thaliana* with an altered morphology. Mol Gen Genetics 206(2):200–206. https://doi.org/10.1007/bf00333575
Forde B, Lorenzo H (2001) The nutritional control of root development. Plant Soil 232(1):51–68. https://doi.org/10.1023/a:1010329902165
Frink CR, Waggoner PE, Ausubel JH (1999) Nitrogen fertilizer: retrospect and prospect. Proc Natl Acad Sci USA 96(4):1175–1180. https://doi.org/10.1073/pnas.96.4.1175
Gautier L, Cope L, Bolstad BM, Irizarry RA (2004) affy—analysis of *Affymetrix GeneChip* data at the probe level. Bioinformatics 20(3):307–315
Gazzarrini S, Lejay L, Gojon A, Ninnemann O, Frommer WB, von Wirén N (1999) Three functional transporters for constitutive, diurnally regulated, and starvation-induced uptake of ammonium into Arabidopsis roots. Plant Cell 11(5):937. https://doi.org/10.1105/tpc.11.5.937
Gibson SI (2005) Control of plant development and gene expression by sugar signaling. Curr Opin Plant Biol 8(1):93–102
Grechi I, Vivin P, Hilbert G, Milin S, Robert T, Gaudillere J-P (2007) Effect of light and nitrogen supply on internal C: n balance and control of root-to-shoot biomass allocation in grapevine. Environ Exp Bot 59(2):139–149
Gregersen PL, Holm PB (2007) Transcriptome analysis of senescence in the flag leaf of wheat (*Triticum aestivum* L.). Plant Biotechnol J 5(1):192–206. https://doi.org/10.1111/j.1467-7652.2006.00232.x
Gruber BD, Giehl RFH, Friedel S, von Wirén N (2013) Plasticity of the Arabidopsis root system under nutrient deficiencies. Plant Physiol 163(1):161–179. https://doi.org/10.1104/pp.113.218453
Gu R, Duan F, An X, Zhang F, von Wirén N, Yuan L (2013) Characterization of AMT-mediated high-affinity ammonium uptake in roots of maize (*Zea mays* L.). Plant Cell Physiol 54(9):1515–1524
Gutiérrez RA (2012) Systems biology for enhanced plant nitrogen nutrition. Science 336(6089):1673–1675. https://doi.org/10.1126/science.1217620
Havé M, Marmagne A, Chardon F, Masclaux-Daubresse C (2017) Nitrogen remobilization during leaf senescence: lessons from Arabidopsis to crops. J Exp Bot 68(10):2513–2529. https://doi.org/10.1093/jxb/erw365
Heberle H, Meirelles GV, da Silva FR, Telles GP, Minghim R (2015) InteractiVenn: a web-based tool for the analysis of sets through Venn diagrams. BMC Bioinform 16(1):169. https://doi.org/10.1186/s12859-015-0611-3
Hirose T, Bazzaz FA (1998) Trade-off between light-and nitrogen-use efficiency in canopy photosynthesis. Ann Bot 82(2):195–202
Huarancca Reyes T, Scartazza A, Lu Y, Yamaguchi J, Guglielminetti L (2016) Effect of carbon/nitrogen ratio on carbohydrate metabolism and light energy dissipation mechanisms in *Arabidopsis thaliana*. Plant Physiol Bioch 105(Supplement C):195–202. https://doi.org/10.1016/j.plaphy.2016.04.030
Hudson D, Guevara D, Yaish MW, Hannam C, Long N, Clarke JD, Bi Y-M, Rothstein SJ (2011) *GNC* and *CGA1* modulate chlorophyll biosynthesis and glutamate synthase (*GLU1/Fd-GOGAT*) expression in *Arabidopsis*. PLoS One 6(11):e26765
Ikram S, Bedu M, Daniel-Vedele F, Chaillou S, Chardon F (2012) Natural variation of *Arabidopsis* response to nitrogen availability. J Exp Bot 63:91–105
Jia Y, Lisch DR, Ohtsu K, Scanlon MJ, Nettleton D, Schnable PS (2009) Loss of RNA–dependent RNA polymerase 2 (RDR2) function causes widespread and unexpected changes in the expression of transposons, genes, and 24-nt small RNAs. PLoS Genet 5(11):e1000737
Kiba T, Naitou T, Koizumi N, Yamashino T, Sakakibara H, Mizuno T (2005) Combinatorial microarray analysis revealing *Arabidopsis* genes implicated in cytokinin responses through the His→Asp phosphorelay circuitry. Plant Cell Physiol 46(2):339–355
Kiba T, Feria-Bourrellier A-B, Lafouge F, Lezhneva L, Boutet-Mercey S, Orsel M, Bréhaut V, Miller A, Daniel-Vedele F, Sakakibara H (2012) The *Arabidopsis* nitrate transporter *NRT2.4* plays a double role in roots and shoots of nitrogen-starved plants. Plant Cell 24(1):245–258
Kindu GA, Tang J, Yin X, Struik PC (2014) Quantitative trait locus analysis of nitrogen use efficiency in barley (*Hordeum vulgare* L.). Euphytica 199(1–2):207–221
Koornneef M, Alonso-Blanco C, Vreugdenhil D (2004) Naturally occurring genetic variation in *Arabidopsis thaliana*. Annu Rev Plant Biol 55(1):141–172. https://doi.org/10.1146/annurev.arplant.55.031903.141605
Krapp A, David LC, Chardin C, Girin T, Marmagne A, Leprince A-S, Chaillou S, Ferrario-Méry S, Meyer C, Daniel-Vedele F (2014) Nitrate transport and signalling in *Arabidopsis*. J Exp Bot 65(3):789–798. https://doi.org/10.1093/jxb/eru001
Lezhneva L, Kiba T, Feria-Bourrellier A-B, Lafouge F, Boutet-Mercey S, Zoufan P, Sakakibara H, Daniel-Vedele F, Krapp A (2014) The Arabidopsis nitrate transporter NRT2.5 plays a role in nitrate acquisition and remobilization in nitrogen-starved plants. Plant J 80(2):230–241. https://doi.org/10.1111/tpj.12626
Liu KH, Tsay YF (2003) Switching between the two action modes of the dual-affinity nitrate transporter CHL1 by phosphorylation. EMBO J 22(5):1005–1013
Loudet O, Chaillou S, Merigout P, Talbotec J, Daniel-Vedele F (2003) Quantitative trait loci analysis of nitrogen use efficiency in *Arabidopsis*. Plant Physiol 131(1):345–358. https://doi.org/10.1104/pp.102.010785
Marchive C, Roudier F, Castaings L, Bréhaut V, Blondet E, Colot V, Meyer C, Krapp A (2013) Nuclear retention of the transcription factor NLP7 orchestrates the early response to nitrate in plants. Nat Comm 4:1713

Martin T, Oswald O, Graham IA (2002) Arabidopsis seedling growth, storage lipid mobilization, and photosynthetic gene expression are regulated by carbon:nitrogen availability. Plant Physiol 128(2):472–481

Masclaux-Daubresse C, Daniel-Vedele F, Dechorgnat J, Chardon F, Gaufichon L, Suzuki A (2010) Nitrogen uptake, assimilation and remobilization in plants: challenges for sustainable and productive agriculture. Ann Bot 105(7):1141–1157. https://doi.org/10.1093/aob/mcq028

North KA, Ehlting B, Koprivova A, Rennenberg H, Kopriva S (2009) Natural variation in Arabidopsis adaptation to growth at low nitrogen conditions. Plant Physiol Biochem 47(10):912–918. https://doi.org/10.1016/j.plaphy.2009.06.009

O'Brien JA, Vega A, Bouguyon E, Krouk G, Gojon A, Coruzzi G, Gutiérrez RA (2016) Nitrate transport, sensing, and responses in plants. Mol Plant 9(6):837–856. https://doi.org/10.1016/j.molp.2016.05.004

Okamoto M, Vidmar JJ, Glass ADM (2003) Regulation of *NRT1* and *NRT2* gene families of *Arabidopsis thaliana*: responses to nitrate provision. Plant Cell Physiol 44(3):304–317

Parker JL, Newstead S (2014) Molecular basis of nitrate uptake by the plant nitrate transporter NRT1.1. Nature 507(7490):68–72

Paul S, Datta SK, Datta K (2015) miRNA regulation of nutrient homeostasis in plants. Front Plant Sci 6:232

Pearson JN, Finnemann J, Schjoerring JK (2002) Regulation of the high-affinity ammonium transporter (BnAMT1;2) in the leaves of *Brassica napus* by nitrogen status. Plant Mol Biol 49(5):483–490. https://doi.org/10.1023/A:1015549115471

Platt A, Horton M, Huang YS, Li Y, Anastasio AE, Mulyati NW, Ågren J, Bossdorf O, Byers D, Donohue K (2010) The scale of population structure in *Arabidopsis thaliana*. PLoS Genet 6(2):e1000843

Quraishi UM, Abrouk M, Murat F, Pont C, Foucrier S, Desmaizieres G, Confolent C, Rivière N, Charmet G, Paux E, Murigneux A, Guerreiro L, Lafarge S, Le Gouis J, Feuillet C, Salse J (2011) Cross-genome map based dissection of a nitrogen use efficiency ortho-metaQTL in bread wheat unravels concerted cereal genome evolution. Plant J 65(5):745–756. https://doi.org/10.1111/j.1365-313X.2010.04461.x

Ritchie ME, Phipson B, Wu D, Hu Y, Law CW, Shi W, Smyth GK (2015) limma powers differential expression analyses for RNA-sequencing and microarray studies. Nucleic Acids Res 43(7):e47–e47

Robertson GP, Vitousek PM (2009) Nitrogen in agriculture: balancing the cost of an essential resource. Annu Rev Environ Resour 34(1):97–125. https://doi.org/10.1146/annurev.environ.032108.105046

Rubin G, Tohge T, Matsuda F, Saito K, Scheible W-R (2009) Members of the *LBD* family of transcription factors repress anthocyanin synthesis and affect additional nitrogen responses in *Arabidopsis*. Plant Cell 21(11):3567–3584. https://doi.org/10.1105/tpc.109.067041

Saeed AI, Bhagabati NK, Braisted JC, Liang W, Sharov V, Howe EA, Li J, Thiagarajan M, White JA, Quackenbush J (2006) TM4 microarray software suite. Methods Enzymol 411:134–193. https://doi.org/10.1016/S0076-6879(06)11009-5

Scheible WR, Lauerer M, Schulze ED, Caboche M, Stitt M (1997) Accumulation of nitrate in the shoot acts as a signal to regulate shoot-root allocation in tobacco. Plant J 11(4):671–691

Scheible W-R, Morcuende R, Czechowski T, Fritz C, Osuna D, Palacios-Rojas N, Schindelasch D, Thimm O, Udvardi MK, Stitt M (2004) Genome-wide reprogramming of primary and secondary metabolism, protein synthesis, cellular growth processes, and the regulatory infrastructure of Arabidopsis in response to nitrogen. Plant Physiol 136(1):2483–2499

Stacklies W, Redestig H, Scholz M, Walther D, Selbig J (2007) pcaMethods—a bioconductor package providing PCA methods for incomplete data. Bioinformatics 23(9):1164–1167

Stitt M (1999) Nitrate regulation of metabolism and growth. Curr Opin Plant Biol 2(3):178–186

Sutton MA, Oenema O, Erisman JW, Leip A, van Grinsven H, Winiwarter W (2011) Too much of a good thing. Nature 472(7342):159–161

Szira F, Bálint AF, Börner A, Galiba G (2008) Evaluation of drought-related traits and screening methods at different developmental stages in spring barley. J Agron Crop Sci 194(5):334–342. https://doi.org/10.1111/j.1439-037X.2008.00330.x

Tschoep H, Gibon Y, Carillo P, Armengaud P, Szecowka M, Nunes-Nesi A, Fernie AR, Koehl K, Stitt M (2009) Adjustment of growth and central metabolism to a mild but sustained nitrogen-limitation in *Arabidopsis*. Plant Cell Environ 32(3):300–318. https://doi.org/10.1111/j.1365-3040.2008.01921.x

Untergasser A, Cutcutache I, Koressaar T, Ye J, Faircloth BC, Remm M, Rozen SG (2012) Primer3—new capabilities and interfaces. Nucleic Acids Res 40(15):e115–e115

Vidal EA, Araus V, Lu C, Parry G, Green PJ, Coruzzi GM, Gutiérrez RA (2010) Nitrate-responsive miR393/*AFB3* regulatory module controls root system architecture in *Arabidopsis thaliana*. Proc Natl Acad Sci USA 107(9):4477–4482. https://doi.org/10.1073/pnas.0909571107

Vidal EA, Moyano TC, Krouk G, Katari MS, Tanurdzic M, McCombie WR, Coruzzi GM, Gutiérrez RA (2013) Integrated RNA-seq and sRNA-seq analysis identifies novel nitrate-responsive genes in *Arabidopsis thaliana* roots. BMC Genom 14(1):701

von Wirén N, Lauter F-R, Ninnemann O, Gillissen B, Walch-Liu P, Engels C, Jost W, Frommer WB (2000) Differential regulation of three functional ammonium transporter genes by nitrogen in root hairs and by light in leaves of tomato. Plant J 21(2):167–175. https://doi.org/10.1046/j.1365-313x.2000.00665.x

Walch-Liu P, Ivanov II, Filleur S, Gan Y, Remans T, Forde BG (2006) Nitrogen regulation of root branching. Ann Bot 97(5):875–881. https://doi.org/10.1093/aob/mcj601

Wan CY, Wilkins TA (1994) A modified hot borate method significantly enhances the yield of high-quality RNA from cotton (*Gossypium hirsutum* L.). Anal Biochem 223(1):7–12. https://doi.org/10.1006/abio.1994.1538

Wei D, Cui K, Ye G, Pan J, Xiang J, Huang J, Nie L (2012) QTL mapping for nitrogen-use efficiency and nitrogen-deficiency tolerance traits in rice. Plant Soil 359(1–2):281–295

Weigel D (2012) Natural variation in *Arabidopsis*: from molecular genetics to ecological genomics. Plant Physiol 158(1):2–22

Wu X, Yang H, Qu C, Xu Z, Li W, Hao B, Yang C, Sun G, Liu G (2015) Sequence and expression analysis of the AMT gene family in poplar. Front Plant Sci 6:337

Xu G, Fan X, Miller AJ (2012) Plant nitrogen assimilation and use efficiency. Annu Rev Plant Biol 63(1):153–182. https://doi.org/10.1146/annurev-arplant-042811-105532

Wilson CL, Miller CJ (2005) Simpleaffy: a BioConductor package for Affymetrix Quality Control and data analysis. Bioinformatics 18 (21):3683–3685

Yang S, Hao D, Cong Y, Jin M, Su Y (2015) The rice OsAMT1;1 is a proton-independent feedback regulated ammonium transporter. Plant Cell Rep 34(2):321–330

Zhang H, Forde BG (2000) Regulation of *Arabidopsis* root development by nitrate availability. J Exp Bot 51(342):51–59

Zhao M, Ding H, Zhu JK, Zhang F, Li WX (2011) Involvement of miR169 in the nitrogen-starvation responses in *Arabidopsis*. New Phytol 190(4):906–915

Zhao M, Tai H, Sun S, Zhang F, Xu Y, Li W-X (2012) Cloning and characterization of maize miRNAs involved in responses to nitrogen deficiency. PLoS One 7(1):e29669