Plasticity of rosette size in response to nitrogen availability is controlled by an RCC1-family protein

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Abstract

Nitrogen (N) is fundamental to plant growth, development, and yield. Genes underlying N utilization and assimilation are well characterized, but mechanisms underpinning plasticity of different phenotypes to varying amounts of N in the soil remain elusive. Here, using *Arabidopsis thaliana* accessions, we dissected the genetic architecture of plasticity in early and late rosette diameter, flowering time and yield in response to three levels of N in soil. Genome-wide association analysis identified three significant associations for phenotypic plasticity, one for early rosette diameter and two for flowering time. We confirmed that the gene *At1g19880*, hereafter named as *PLASTICITY OF ROSETTE TO NITROGEN 1* (PROTON1), encoding for a regulator of chromatin condensation 1 (RCC1) family protein, conferred plasticity of rosette diameter in response to changes in N availability. The altered plasticities were a result of faster development under limiting N, and correlated with the plasticity in the levels of primary metabolites. By using different growth conditions for a subset of accessions, we showed that plasticities of growth and flowering-related traits in response to N availability differed between the environmental cues, indicating decoupled genetic programs regulating these traits. Our findings provide a prospective for identification of genes that stabilize performance under fluctuating environments.

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Keywords

Arabidopsis thaliana , natural variation, plasticity, nitrogen, GWA

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Conflict of Interest

The authors declare no conflict of interest.

Summary statement

Genome-wide and mutant analysis revealed that a RCC1-family protein controls plasticity of rosette diameter in response to N in soil. Furthermore, our results showed that plasticity in traits in response to different environments are decoupled.

Author contributions

GTD did all data analysis, ran GWAS, identified and characterized T-DNA lines for the candidate genes, planned and performed the adaptability experiment, planned and conducted the qRT-PCR. All figures were made by GTD. PP conducted the initial screening and quantifications and extraction of metabolites. NV measured the early rosette diameter and helped PP with the screening. SA measured metabolite levels. ARF supervised metabolomics measurements. RAEL and ZN planned the experiments, RAEL supervised the experiments and ZN supervised data analysis. RAEL, GTD and ZN wrote the manuscript. All authors have read the final version of the manuscript.

Introduction

Plasticity is the ability of an organism to produce diverse phenotypes in response to changes in the environment. For sessile organisms such as plants, plasticity is particularly advantageous as it allows rapid adjustment to different environments. Nitrogen (N) is an essential component for the production of amino acids, nucleic acids, and chlorophyll, and thus directly affects plant growth and development (Guignard et al., 2017; Oldroyd & Leyser, 2020). Plants take up N from the soil primarily as ammonium and nitrate. N distribution in soil varies both in time and in space resulting in uneven growth among individuals, populations and species (Lark et al., 2004; Pandey et al., 2019). In agriculture, stable crop growth and yield are ensured by applying fertilizers that contain N. However, losses of the large amount of N supplied via fertilization to the environment negatively impact entire ecosystems (Guignard et al., 2017; McAllister, Beatty, & Good, 2012). One route towards improved yield without addition of fertilizers is to understand the mechanisms underlying plant plasticity responses to N availability. This will allow the development of crop lines with stable growth under varying and unpredictable N availability.

The gene regulatory and metabolic networks for N uptake, assimilation and utilization are well characterized and have been used to improve nitrogen use efficiency (NUE) in plants (Arsova, Kierszniowska, & Schulze, 2012; Fredes, Moreno, Diaz, & Gutierrez, 2019; Gutierrez, 2012; Krapp et al., 2011; Li, Hu, & Chu, 2017; Meyer et al., 2019; Vidal & Gutierrez, 2008). For instance, modifying the transport of amino acids has been employed to improve NUE in pea (Perchlik & Tegeder, 2017). Similarly, DNA methylation and epigenetic mechanisms were found to contribute to the modulation of NUE (Kuhlmann et al., 2020). Given the tight coordination between carbon and N metabolism, genetically improving photosynthesis may also increase NUE and reduce the necessity of fertilizers (Evans & Clarke, 2018). It was also demonstrated that the plasticity in growth-related traits in response to N availability varies between local populations of *Arabidopsis thaliana* (Arabidopsis) (Pandey et al., 2019). Further, Arabidopsis accessions cope with differences in N availability by modifying the root and shoot architecture, growth, and biomass (de Jong et al., 2019; Ikram, Bedu, Daniel-Vedele, Chaillou, & Chardon, 2012; Masclaux-Daubresse & Chardon, 2011; Meyer et al., 2019; North, Ehlting, Koprivova, Rennenberg, & Kopriva, 2009). However, the question of whether or not there are genes that control plasticity of different focal traits to N availability remains open. The availability of accessions, as genetically homozygous lines (Kramer, 2015; Weigel, 2012), along with a large repertoire of genetic and molecular tools, renders Arabidopsis an excellent model system to address this question.

Despite the high potential of genome-wide association (GWA) in identifying genetic basis of phenotypic variation, only a few studies have used this approach to investigate genotype variation in plasticity among Arabidopsis accessions (Brachi, Faure, Bergelson, Cuguen, & Roux, 2013; de Jong et al., 2019; Sasaki, Zhang, Atwell, Meng, & Nordborg, 2015). A challenge for mapping genes that control plasticity, hereafter referred to as plasticity genes, lies in the quantification of plasticity as a trait. Plasticity can be quantified by different approaches, including, but not limited to linear regression of the reaction norms, the coefficient of variation (CV), plasticity index, and fold change (FC) (Laitinen & Nikoloski, 2019; Pennacchi et al., 2020).

Here we focus on dissecting the genetic architecture of plasticity of growth- and flowering- related traits in response to the availability of N in the soil in a panel of Arabidopsis accessions. Since growth and development are tightly linked to metabolism, we also asked if the plasticity of growth- and flowering-related traits could be explained by the plasticity of primary metabolites. Finally, we investigated if the studied plasticities were specific for different environmental cues, including light and day length. Our GWA study revealed that the genetic architecture of the studied plasticities differed. We identified that *At1g19880* , gene encoding for a RCC1 family protein, is involved in controlling the plasticity of rosette size in the beginning of the vegetative growth in response to N. Additionally, our results indicated that the mechanisms controlling the plasticity of plant size and flowering time to N availability are independent from other growth-limiting environmental cues, such as light.

Materials and methods

Plant Growth Conditions

For the phenotyping, 190 *Arabidopsis thaliana* accessions were grown on soil containing limiting, intermediate

and optimal amounts of nitrogen and as described in Pandey et al. 2019. The accessions that were obtained from the Nottingham Arabidopsis stock centre (NASC) and are listed in Table S1. The plants were grown on pots ([?] = 6 cm, h = 5.5 cm) containing the same mass of adjusted white peat soil (Gramoflor GmbH). The seeds were germinated for one day under short day (8h/16h) condition at 20degC/16degC and 150 µE $m^{-2}s^{-1}$ of light intensity. To ensure flowering, all accessions were vernalized for eight weeks at 4°C under long days and light intensity of 7 µE m^{-2} s^{-1}. After this, plants were transferred to new pots. Four plants in two pots (n=4) were grown under long days at 21°C/17°C (day/night) and 150 µE m^{-2} s^{-1} of light intensity. Early rosette (ERD) diameter was the rosette diameter ten days after pricking and the final rosette diameter (FRD) at the time of flowering. Rosette diameters were measured from pictures taken at these stages using ImageJ (Schneider, Rasband, & Eliceiri, 2012). Flowering time (FT) was scored at the number of days from pricking of the plants to the day of first open flower. Seed yield (YIE) was quantified from two plants together by dividing the total weight of the seeds with the weight of hundred seeds and multiplying by 100. In all experiments, to minimize stochastic variation the replicates were distributed across different trays and the trays were periodically rotated in the growth chamber.

For the adaptability experiments, 19 accessions were selected based on their ERD and FT plasticity to N (Table S8) and were germinated and vernalized as described above. For the experiments under short days, long days (control condition), and low light, five pots with two plants each were prepared for every accession and condition (n = 10). For the experiments under high light, five plants were transferred to big pots ([?] = 13 cm, h = 10 cm) and in the polytunnel (natural light and temperature), three plants were transferred to big pots ([?] = 13 cm, h = 10 cm) in both conditions resulting in n=15. For the experiments under short day (8h/16h) and the long day control (16h/8h), the temperature was 20°C/16°C (day/night) and light intensity 120 µE m^{-2} s^{-1}. In the low and high light the plants were grown at long-day 16h/8h with temperature 21°C/17°C day/night cycle with light intensity of 20 µE $m^{-2}s^{-1}$ (low light) and 750 µE $m^{-2}s^{-1}$ (high light). The experiment in the polytunnel under natural conditions was performed during summer 2020.

PROTON1 promoter sequencing

The promoter region of *At1g19880* was amplified by PCR with At1g19880_Prom-Fw and At1g19880_Prom-Rv primers (Table S9) for 90 accessions that were part of the initial screen but not the 1001 Genomes dataset (Table S5). For each accession, the fragment of approximately 1500 bp was purified from 0.9 % agarose gel using NucleoSpin Gel and PCR Clean-up columns (Macherey-Nagel GmbH & Co KG, catalog 740609), and Sanger-sequenced using the same primers (LGC, Biosearch Technologies). Sequencing files were evaluated using Chromas v.2.6.6 (Technelysium Pty Ltd).

Metabolite profiling

For the primary metabolites quantification, four plants of the selected set of accessions (Tables S6 and S7) were grown on the same conditions used for the N screening. When the plants reached the ten-leaf stage, they were collected and frozen in liquid nitrogen. Another four plants in two pots were harvested and frozen to liquid nitrogen at mid-day for metabolites analysis at ten-leaf stage. Extraction and analysis by gas chromatography–mass spectrometry (GC-MS) was performed as described in (Lisec, Schauer, Kopka, Willmitzer, & Fernie, 2006). Briefly, frozen ground material was homogenized in 300 µl of methanol at 70 °C for 15 min and 200 µl of chloroform followed by 300 µl of water was added. The polar fraction was dried under vacuum, and the residue was derivatized for 120 min at 37 °C (in 40 µl of 20 mg ml^{-1}methoxyamine hydrochloride (Sigma-Aldrich, cat. no. 593-56-6) in pyridine followed by a 30 min treatment at 37 °C with 70 µl of N-methyl-N (trimethylsilyl)trifluoroacetamide (MSTFA reagent; Macherey-Nagel, cat. no. 24589-78-4). The GC-MS system used was a gas chromatograph coupled to a time-of-flight mass spectrometer (Leco Pegasus HT TOF-MS). An auto sampler Gerstel MultiPurpose system injected the samples. Chromatograms and mass spectra were evaluated by using Chroma TOF 4.5 (Leco) and Xcalibur 2.1 software, peak area was normalized by comparison to an internal standard (ribitol; CAS488-81-3) and the fresh weight of the sample used for extraction.

Data analysis

For each trait and condition, the mean values were obtained from at least three replicates. Any accessions that did not have data for at least three replicates for each condition was removed from the analysis. After filtering the number of accessions used for analysis was 142 for ERD, 109 for FRD, 127 for FT and 102 for yield. For each accession, the trait values were estimated as the average of the replicates in each condition (Tables S2 and S6). Two plasticity measurements were used namely the fold change (FC) between two conditions and the coefficient of variation (CV) across conditions. The CV was calculated as the standard deviation (σ) divided by the mean (μ) (CV $=\sigma$ / μ) of the averages of each condition. All statistical analyses were performed in R (https://www.R-project.org/; (Team, 2020)), using RStudio v. 1.3.1056. The correlations were calculated using the*corr.test* function of *psych* package v. 2.0.9 (https://CRAN.R-project.org/package=psych; (Revelle, 2020)), and plotted using the *corrplot* package v. 0.84 (https://github.com/taiyun/corrplot;(Wei, 2017)). The correlations were calculated using Spearman's rank coefficient and pairwise comparisons, and the p-values were corrected by the Benjamini–Hochberg procedure. The boxplots were generated using the *ggplot* function of*ggplot2* package v. 3.3.2 (http://www.jstatsoft.org/v40/i01/; (Wickham, 2009)), *ddply* function of *plyr* package v. 1.8.6 (Wickham, 2011), and the *pivot_longer* function of *tidyr*package v. 1.1.2 (https://CRAN.R-project.org/package=tidyr; (Wickham, 2020)). K-means clustering was performed with the *kmeans* function of *stats* package integrated in R (v. 4.0.3), using Hartigan and Wong algorithm. The optimal number of clusters was determined with the Silhouette method using the *fviz_nbclust* function of*factoextra* package v. 1.0.7 (https://CRAN.R-project.org/package=factoextra;(Kassambara, 2020)), using 1000 bootstrap samples. Heatmaps were plotted using the*ComplexHeatmap* package v. 2.6.2 (Gu, Eils, & Schlesner, 2016). The graphs were further edited using Microsoft Office.

Genome-wide association analysis

GWA analyses were performed using the easyGWAS tool (Grimm et al., 2017)(https://easygwas.ethz.ch/). For each of the complex traits, the averages under limiting, intermediate, or optimal N, the fold change between limiting to optimal N, and the CV across the three N conditions were used as input for GWAS. The analyses were performed with both the 250K SNPs and the 1001 whole sequence datasets, using TAIR10 gene annotation. The data was transformed using the BoxCox method, and each analysis was corrected for population structure using the FaSTLMM algorithm. The minimum allele frequency (MAF) cut-off was set to 10 %, and only associations with correction significance level $\alpha < 0.1$ using Bonferroni-method were reported (Table 1 and S4).

Mutant analysis

T-DNA lines for candidate genes were obtained from NASC and are listed in Table S4. Genomic DNA was extracted using the Sucrose Prep method (Berendzen et al., 2005), following genotyping by PCR for the respective T-DNA insertion using the primer pairs listed in Table S8. Homozygous mutant lines were tested on the same conditions used for the N screening experiments at which the genome-wide association was identified, using Col-0 wild type as a reference. Two and three independent experiments with 10 replicate plants/line for each condition were performed to confirm the associations to FT and ERD plasticities, respectively.

Gene expression analysis

For each line, five pools each containing two rosette of plants grown under optimal or limiting N were harvested to liquid nitrogen 10 days after pricking. Total RNA was extracted using TRIzol reagent (Invitrogen, catalog 15596026) and DNA was removed using TURBO DNA-free kit (Invitrogen, catalog AM1907). cDNA was synthesized with the ImProm-II Reverse Transcription System (Promega, catalog A3800). Quantitative real-time PCR reactions were performed using Power SYBR Green PCR-Master-Mix (Applied Biosystems, catalog 4367659) and the ABI PRISM 7900HT (Applied Biosystems) system. *EF1ALPHA* (At5g60390),*PP2AA3* (At1g13320), and *SAND* (At2g28390) were used as housekeeping genes (Table S9). For each sample, the target gene levels were normalized by the delta-Ct to the average of the three housekeeping genes. The complete list of primers used for gene expression quantification is provided in Table S9.

Results

Plasticity screening using *Arabidopsis thaliana* accessions in response to N availability

We screened 190 Arabidopsis accessions (Table S1), grown on soil supplemented with three different concentrations of N (Methods), for plasticity of focal traits (*i.e.* phenotypes) including early rosette diameter (ERD), final rosette diameter (FRD), flowering time (FT) and yield (YIE) (Table S2). A pilot experiment with a subset of accessions, following an established protocol (Pandey et al., 2019), was used to determine the N conditions considered as limiting, intermediate and optimal for growth (Methods). Accessions with missing data in at least one N condition were removed from further analysis, resulting in 142 (ERD), 109 (FRD), 127 (FT) and 102 (YIE) accessions to characterize plasticity of the four phenotypes. On average, the accessions were larger, flowered earlier and produced more seeds when grown under optimal N in comparison to intermediate and limiting N (Figure 1a).

Clustering of the accessions according to their reactions norms, representing the phenotype mean as a function of different environments, partitioned the accessions into three groups for ERD and two groups for each of the other complex traits (FRD, FT, and YIE) (k-means clustering, k determined by silhouette index analysis, Figure 1b, Table S2). The differences in response of the studied phenotypes between the clusters of accessions indicated that there is a genetic variation in plasticity of these phenotypes to N availability.

To quantify plasticity of the phenotypes, we calculated the coefficient of variation (CV) for each accession using the mean values in the three N conditions (Methods, Table S3). The average CVs for all traits were smaller than 1, implying a generally low degree of plasticity (Figure 2a). ERD showed higher median plasticity (0.46) than the other phenotypes (Figure 2a). In contrast, FT, measured as the number of days from pricking of the seedlings to the first open flower, had the lowest median CV (0.11) in response to the three N conditions (Figure 2a). We also asked if the accessions differed with respect to the pattern of plasticities for the four phenotypes. To this end, we clustered the 77 accessions for which the CV of all four phenotypes were measured, and found that they can be partitioned into three groups (Figure 2b; Table S3). The accessions in cluster 1 showed lower plasticity in the ERD than those in clusters 2 and 3, while the accessions in cluster 2 showed higher plasticity in the ERD and FRD than those in clusters 1 and 3 (Figure 2b, Table S3). These findings demonstrated that accessions differed with respect to the plasticities of the four investigated phenotypes. Next, using the CVs of the 77 accessions, we tested if the plasticities of the four phenotypes correlated. We found that the CV of the ERD showed significant positive Spearman correlation with the CV of the FRD (0.42, p-value = $4.7\text{x}10^{-5}$) and FT (0.37, p-value = $5.7\text{x}10^{-5}$), indicating that plasticity of the size in the beginning of vegetative growth is moderately associated with plasticity in flowering time (Figure 2c).

To investigate whether the plasticity in the flowering time was due to change in the developmental timing or the growth pattern, we scored the number of rosette leaves until bolting for six accessions with varying plasticities of FT. We noted that under the same N condition, the total number of leaves differed between the selected accessions, but all of them produced approximately twice the number of leaves under optimal N in comparison to the limiting N (Figure S1). This suggests that the earlier flowering under optimal N was due to a faster developmental transition to the reproductive phase.

Genetic basis of plasticity of the growth- and flowering-related phenotypes to N availability

Next, using genome-wide association (GWA) analysis, we investigated the genetic architecture of plasticity of the four studied phenotypes in response to N. To this end, we performed the GWA using the CV over all three N conditions and, in addition, with the fold changes (FC) of the phenotypic means between the optimal and limiting N conditions. GWA analysis for the CVs of the four phenotypes identified only one significant biallelic association for FT at the end of chromosome 1 (p-value = $1.41\text{x}10^{-7}$; Tables 1 and S4, Methods). Interestingly, the GWA with the FC identified four SNPs on the same locus on chromosome 1 that was associated with the CV of FT (Tables 1 and S4). We found no significant association for the other traits.

The low power of our dataset in detecting significant associations could be due to the low heritability (*i.e.* low proportion of genotype x environment variance from the total phenotypic variance) of the scored plasticities (Sasaki et al., 2015; Tam et al., 2019). Another explanation is that the low number of accessions that we used for our study did not suffice to identify the loci with small effects. A third explanation for the missing associations could be the incomplete set of SNPs, since the initial GWA was performed with 250,000 SNPs (Horton et al., 2012). To investigate if the lack of SNPs was the reason for the few detected associations, we took advantage of those accessions with fully sequenced genome, including 58 for ERD, 49 for FRD, 54 for FT and 44 for YIE, and performed GWA for the two plasticity scores. We detected a significant association for the ERD on chromosome 1 to two biallelic SNPs (Figure 3a; Tables 1 and S4).

Finally, we asked if the plasticity (scored as CV or FC) of the phenotypes is controlled by the same genes that control the differences in the mean trait values in the accessions. To investigate this question, we performed the GWA with the mean value of the traits in the three N conditions. We found a single significant association for ERD at intermediate N on chromosome 1, but to a different SNP than that found for the plasticity of ERD (Methods, Table S1). This implies that the plasticity of the traits in response to N has a different genetic basis than the one controlling the mean trait value in a single condition.

Characterization of the candidate gene using T-DNA lines

Based on the linkage disequilibrium, we considered genes that were located ± 10 Kb and ± 20 Kb of the significantly associated SNPs for ERD and FT, respectively, as candidates. This resulted in eleven candidates around the SNPs associated with the plasticity of FT to N, and six candidate genes for the SNPs associated with the plasticity of ERD to N. To investigate the candidates, we examined 14 and 8 T-DNA mutant lines in Colombia (Col-0) background for the eleven and six candidate genes controlling the plasticity of FT and ERD, respectively (Table S4). After confirming the homozygosity of the T-DNA insertion, the lines were grown under the three N conditions as used for the initial screening, and the plasticity was compared to the Col-0 wild type (WT) in two independent experiments.

We did not detect significantly altered FC nor CV in FT in response to N in any of the tested mutant lines in comparison to Col-0 (Figure S2). We reasoned that the extremely low FT plasticity of Col-0 accession in response to N availability decreases the power for detecting significant differences. Indeed, the CV of FT ranged from 0.01 to 0.05 and the FC of FT ranged from 0.93 to 1.07, reflecting the low plasticity of this trait (Figure S2). For the ERD plasticity, homozygous-T-DNA mutant lines for five of the candidate genes were identified and further characterized. The homozygous SALK_117261 line, with T-DNA insertion in *At1g19880* gene, exhibited approximately 80 % reduced FC in ERD as compared to Col-0 in two independent experiments (Figure 3b, c). Analysis of homology demonstrated that *At1g19880* encodes for an uncharacterized regulator of chromosome condensation (RCC1) family protein, which we further named as *PLASTICITY OF ROSETTE SIZE TO NITROGEN 1* (*PROTON1*). The RCC1 family proteins include one or more RCC1-like domains (RLDs), and are known to have diverse function (Hadjebi, Casas-Terradellas, Garcia-Gonzalo, & Rosa, 2008). In Arabidopsis, there are 24 RCC1 family proteins, from which so far only UV RESISTANCE LOCUS 8 (UVR8) (Christie et al., 2012; Wu et al., 2012), RCC1/UVR8/GEF3-like 3 (RUG3) (Kuhn et al., 2011), TOLERANT TO CHILLING AND FREEZING 1 (TCF1) (Ji et al., 2015) and SENSITIVE TO ABA 1 (SAB1) (Ji et al., 2019) have been characterized.

The role of PROTON1 in controlling the plasticity of ERD in response to nitrogen

The significant association for plasticity of ERD in response to N availability was detected based on the SNPs of 58 fully sequenced accessions, but was not found when using the 250K SNPs due to the absence of the identified SNPs in this dataset. To provide additional support for this association, we sequenced the missing region in the 250K SNP data that contains the biallelic SNP for the 90 accessions that were included in the initial phenotyping panel (Table S5). We then looked at the associations of the sequenced SNP to the plasticity in ERD in response to nitrogen. From all analyzed accessions, 42 carried the minor alleles (A and T, for positions 6904858 and 6904935, respectively), and 106 accessions carried the major alleles (C and G, respectively). On average, the accessions with minor alleles showed 25 % lower FC in ERD in comparison to

the accessions with major alleles (0.55 and 0.41, respectively) (Figure 3d). The difference between the ERD FC of the two haplogroups was significant (p = 0.0016, Mann Whitney U test), giving further support to the association of *PROTON1* to the ERD plasticity in response to N.

Further investigations of growth using the *proton1* T-DNA mutant showed that its smaller FC in ERD was due to a significantly larger early rosette under limiting N, and to a smaller rosette diameter under optimal N in comparison to the WT (Figure 3c). This observation suggests that the reduced plasticity, thus more stable growth, between limiting and optimal N comes at a cost of decreasing the maximum attainable size. Therefore, our findings point that PROTON1 mediates the trade-off between stable and maximal growth under different N conditions.

Gene expression analysis of rosette leaves of plants grown under limiting and optimal N revealed that *proton1* is a strong knockdown mutant, showing more than 1000 times reduced expression of*PROTON1* in comparison to WT (Figure 4a). Furthermore, the expression of *PROTON1* was increased in WT under limiting N (Figure 4b). Additionally, we observed that the *proton1* flowered earlier than the WT under limiting N, but that its FT was not different from that of the WT under optimal N (Figure 4c and d). To investigate if PROTON1 controlled plasticity of ERD in response to N availability is due to plasticity of N uptake, transport or assimilation, we evaluated expression of 18 genes involved in these processes in WT and*proton1* mutant grown under either limiting or optimal N. From the 18 analyzed genes, seven showed a significant expression difference between WT and the mutant line in both optimal and limiting conditions including the ammonium transporters *AMT1;1* , *AMT1;4* ,*AMT2;1* ; *NIA2* , involved in N assimilation; *NLP7*transcription factor; and *NRT1;5* and *NRT3;1* , which are nitrate transporters (Figure 4e and 4f). Furthermore, the levels of N assimilation *NIA1* gene and *NLP1* transcription factor under limiting N, and the ammonium transporter *AMT1;2* and *NLP6*transcription factor were also significantly different between*proton1* and the WT under optimal N (Figure 4f). All of the transcripts that showed significantly perturbed expression had higher expression in the mutant line in comparison to the WT, indicating that PROTON1 is involved in processes that lead to repression of expression of genes involved in the aforementioned N-related processes. In addition, it also suggests that the increased plasticity is associated with increased expression of genes involved in N responses. The mechanism by which *PROTON1* modulates the expression of these genes remains unknown.

To further confirm the role of *PROTON1* in controlling the plasticity of ERD to N availability, we searched the published genome sequences of Arabidopsis accessions for polymorphisms in *PROTON 1*(*At1g19880*) gene. We identified that Xan-5 has a missense substitution that results in a disruption in the start codon of*PROTON1* . The presence of this SNP in Xan-5 was further confirmed by sequencing. When we grew Xan-5, Col-0 and *proton1* under optimal and limiting N, we observed reduction in the plasticity of ERD in Xan-5 as well, thus further supporting the proposed role of*PROTON1* (Figure S3).

Metabolic changes associated with plasticity of studied phenotypes to N availability

Primary metabolism is associated with growth and levels of several primary metabolites are altered in response to changes in N availability (Sulpice et al., 2013). However, it is not clear if the plasticity of metabolites is associated with plasticity of the complex phenotypes studied here. To address this question, we first measured the levels of 66 primary metabolites in a subset of 43 accessions grown in the three N conditions (Table S6). To quantify plasticity, we calculated the CV of every metabolite across the three mean values for each of the 43 accessions (Table S7). The majority of primary metabolites are essential for plant growth and showed low plasticity across N conditions as expected (Figure 5a). The organic acids citrate and fumarate, and the amino acids ornithine, glutamine, lysine and arginine showed the highest median CV across the accessions (Figures 5a; Table S7). Of these, glutamine and glutamate are directly linked to N assimilation, whilst citrate is a precursor for 2-oxoglutarate needed in this process. Furthermore, malate and citrate valves are directly linked to carbon fixation and the tricarboxylic acid (TCA) cycle, providing the substrates for amino acids synthesis, and their conversion can favor the accumulation of other metabolites, such as fumarate (Eprintsev, Fedorin, Sazonova, & Igamberdiev, 2016).

To evaluate if metabolic plasticity relates to plasticity of the four phenotypes, we performed correlation

analysis between the CVs of metabolites and CVs of phenotypes ERD, FRD, FT and YIE over the 43 accessions (Figure 5b). We found a significant positive correlation between the plasticity of ERD and plasticities of benzoate, dehydroascorbate (dimer), glutamine, and phosphorate levels, and a significant negative correlation with plasticity of glucose levels. Therefore, our findings suggest that stable levels of glucose over different N conditions, in contrast to dehydroascorbate, are associated with the higher plasticity of ERD. Further, plasticity of FRD showed significant negative correlation with plasticity of fumarate and glycerate levels, and positive correlation with plasticities in lactate and fucose levels (Figure 5b). The plasticity of FT showed positive correlation with homoserine levels, while that of YIE positively correlated with plasticity of fructose, glucose, rhamnose and shikimate levels (Figure 5b). The latter indicates that stable sugar contents stabilizes yield in respond to N availability.

Next, we asked if the significant correlation between the plasticity of a complex phenotype and the plasticity of metabolite levels was observed between the mean metabolite levels and the mean levels of ERD, FRD, FT and YIE in each N condition separately. Only two metabolites, benzoate and lactate, showed significant correlation both between the mean and plasticity values of the same trait; benzoate to ERD and lactate to FRD respectively (Figure 5, Figure S4). This analysis supports the hypothesis that the plasticities and the mean values of the studied phenotypes are independently regulated.

Accessions showed different degrees of ERD and FT plasticity in response to different environments

Our results showed that plasticity of different phenotypes to N availability are not under the control of the same loci, and that plasticity of specific phenotypes varied between the studied accessions. Next, we asked whether plasticities of ERD and FT are specific to certain environmental cues, or whether different accessions show similar plasticities in ERD and FT independent of the environmental changes. To investigate this question, we selected 19 accessions, covering the range from lowest to highest CVs for ERD and FT (Table S3). These accessions were grown at 20°C/16°C (day/night) in four additional environments known to impact plant growth, including long days (16h/8h) with low light (20 µE) and high light (750 µE), short day (8h/16h, 120 µE) and in the polytunnel with natural light and temperature conditions. Plants grown at 20°C/16°C (day/night) under long days with light intensity of 120 µE were used as control. In the polytunnel, the average temperature during the experiment was 26.2°C, with minimum of 11.5°C and maximum of 59.5°C. Each accession was scored for ERD and FT. To quantify the plasticity of ERD and FT in response to the different conditions, we calculated the FC between each condition to the control condition (Figure 6a, b; Table S7). To investigate whether the same accessions showed high plasticity in the phenotypes in response to all growth limiting conditions, we conducted a correlation analysis of the plasticities of ERD and FT in response to the different conditions for each accession (Figure 6c and 6d). We did not find any significant correlations between the FCs of the different environments, suggesting that the mechanisms underlying plasticities of ERD and FT to different environmental cues may be uncoupled from each other.

Discussion

Plants cope with changing environments by adjusting their phenotype through phenotypic plasticity. The four adaptive phenotypes studied here, namely early and final rosette size, flowering time and seed yield, are known to respond to varying N availability (de Jong et al., 2019; Ikram et al., 2012; Masclaux-Daubresse & Chardon, 2011; Meyer et al., 2019; North et al., 2009). However, the genetic basis of their phenotypic plasticity in response to N availability is unknown. In this study, we quantified plasticity of the four phenotypes in response to N by using both CV and FC and used GWA analysis to identify candidate genes. None of the significantly associated loci contained genes known to be involved in N response, indicating that the plasticity to N availability has independent genetic basis in comparison to the focal N-related traits. This was further supported with our observation that the loci associated to the mean trait value of the accessions in different N condition were different from the loci associated with the trait plasticities to N. By using T-DNA mutant lines for the candidate genes, we verified that a regulator of chromosome condensation (RCC1) family protein, named here as PROTON1, influenced the plasticity of ERD in response to N availability.

The T-DNA mutant line for *PROTON1* (SALK_117261) showed significantly larger size under limiting N and was significantly smaller under optimal N (Figure 3c), resulting in reduced plasticity of ERD across the conditions (Figure 3b). This suggests that RCC1-mediated stability in growth is attained by improved performance in the limiting conditions, but with reduced performance in optimal conditions.

RCC1-family proteins were first identified to function in regulating cell cycle, but since then different members of this family are known to be involved in diverse functions (Hadjebi et al., 2008). Arabidopsis contains 24 RCC1-family genes, but only four of them have been characterized so far. PROTON1 is the most highly expressed in roots and young leaves (BAR eFP Browser, Arabidopsis.org), and we found that it was induced by limiting N in young leaves (Figure 4b). Co-expression analysis of *PROTON1* showed that it is co-expressed with eight genes with function in the spliceosome machinery (Figure S5) that mediates alternative splicing plants. The four genes in primary co-expression network of *PROTON1* include two RNA-binding family protein members. One of these is SR34, a known splicing factors in Arabidopsis (Stankovic et al., 2016). The role of alternative splicing controlling PROTON1-mediated plasticity of ERD and FT in response to N availability remains to be investigated. Alternative splicing regulates gene expression and protein diversity by producing multiple mRNA isoforms from a single gene. In *Arabidopsis* , alternative splicing is known to regulate transitioning to flowering, but less is known how the alternative splicing is regulated by environmental cues. An intriguing question for future studies would be to investigate if variability in spliceosome, similar to the HSP90 system(Queitsch, Sangster, & Lindquist, 2002; Salathia & Queitsch, 2007; Sangster & Queitsch, 2005; Sangster et al., 2008; Zabinsky, Mason, Queitsch, & Jarosz, 2019), plays a central role in regulating plasticity in plants and animals.

In addition to the genetic basis of plasticity, we used correlation analysis to investigate whether plasticity of specific primary metabolites was associated with the plasticity of any of the four phenotypes. We identified that plasticities of the fumarate and glycerate levels showed significant negative correlation with the plasticity of FRD. The mean levels of fumarate and glycerate were previously found to negatively correlate with the biomass of Arabidopsis plants grown under low N conditions (Sulpice et al., 2013). In our experiments, the mean levels of fumarate showed negative correlation with the mean ERD only under optimal N conditions (Figure S4c). This suggests, similar to the four phenotypes, that the mean metabolites levels associated with N responses are different from the plasticity of metabolite levels in response to N.

To rapidly respond to changes in surroundings, plasticity in an important trait for survival of homozygous organisms, such as Arabidopsis. We found that plasticities of ERD and FT and the plasticity of FRD correlated in across the studied accessions (Figure 2c). In addition, *proton1* mutant line was associated with both altered ERD and FT plasticity in response to N availability. Further, in*proton1,* the ERD and FT plasticities were due to faster development and earlier FT in limiting N conditions (Figure 4c, d). These findings indicate that these traits respond simultaneously to the changing N. Furthermore, we found that the different environments had different impact on the focal traits. This enhances our understanding of the complexity of possible constraints and consequences of selection when acting on plasticity in response to climate change. Altogether, these results highlight the importance to investigate plasticity of different phenotypes in multiple environments alone and in combination, when understanding the past, present and future relationships between the plants and environment.

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Tables

Table 1. Significant associations identified for ERD and FT in response to different nitrogen conditions using 250 K SNPs and whole-genome SNPs. GWA analysis was done using easyGWAS web-application (https://easygwas.ethz.ch) with minimum allele frequency of 10% and Bonferroni correction α=10%.

Hosted file

`image1.emf` available at https://authorea.com/users/389902/articles/504419-plasticity-of-rosette-size-in-response-to-nitrogen-availability-is-controlled-by-an-rcc1-family-protein

Figure legends

Figure 1. Mean trait values in response to different N availability in Arabidopsis accessions. Screening of Arabidopsis accessions for early rosette diameter (ERD, n = 176), final rosette diameter (FRD, n = 155), flowering time (FT, n = 163), and yield (YIE, n = 134) at optimal (Opt), intermediate (Int), and limiting (Lim) N conditions. **A.** Boxplots showing the mean values for the complex traits across accessions in each N condition. The line indicates the median value. **B.** k -means clustering of the reaction norms representing groups of accessions with similar profiles in response to N availability for ERD (n = 142), FRD (n = 109), FT (n = 127), and YIE (n = 102) at optimal, intermediate or limiting N. For each cluster, the profile is represented by a different color. The accessions belonging to each cluster are given in Table S2. The optimal number, k, of clusters for each trait was determined by silhouette index (see Methods).

Figure 2. Plasticity of complex traits in response to N availability in Arabidopsis accessions. **A.** Boxplots showing the CV of the accessions for each trait across the three nitrogen conditions (ERD, n = 142, FRD, n = 109, FT, n = 127, YIE, n = 102).**B.** Clustering of the accessions based on the CVs for the four phenotypes (n=77, with the CVs for all phenotypes available).**C.** Spearman correlation between the CV of complex traits across the three nitrogen conditions. Adjusted p-values were calculated using Benjamini-Hochberg correction. Significant $r_s$correlations (FDR < 0.05) are represented as red, for positive, and blue, for negative.

Figure 3. Genetic architecture underlying plasticity of ERD in response to N availability.

A. Manhattan plot representing the significant association for FC between optimal and limiting N of ERD on chromosome 1. The analysis was run with easyGWAS (https://easygwas.ethz.ch/), using the sequenced genomes dataset (n=58) with FaST-LMM and minimum allele frequency > 10 %. The green line represents the Bonferroni significance level ($\alpha < 0.1$), and the associations are shown as dots (-log10 p-value). The chromosomal position (bp) is represented under the graphs. **B.** FC of ERD for Col-0 WT and for the T-DNA lines of the candidate genes between the plants grown at limiting and at optimal N conditions (n=10). **C.** ERD of Col-0 WT and of the T-DNA lines grown under limiting (Lim) and optimal (Opt) N (n=10). Significant differences to the WT according to Mann–Whitney U test are represented by one (p-value $<$ 0.05), or two (p-values < 0.01) asterisks above each column. **D.** Boxplot representing the two haplogroups for the FC between optimal and limited N of ERD, including the accessions which were sequenced in this work (n = 148). The significance of the difference between the haplogroups based on their ERD FC was tested using Mann–Whitney U test.

Figure 4. The role of *PROTON1* in plasticity of ERD in response to N availability. **A.** Relative expression of *PROTON1*(*At1g19880*) in *proton1* T-DNA line, represented as the Log2 fold change (FC) in comparison to the WT in each N conditions.**B.** Relative expression of *PROTON1* (*At1g19880*) in response to limiting N (Lim), using the expression levels at optimal N (Opt) as reference. **C.** WT and *proton1* grown under limiting N. Photo was taken 18 days after pricking (DAT). **D.**Number of days to the first open flower in WT and *proton1* under both limiting and optimal N. **E.** Relative expression of N-related genes in Col WT and *proton1* under limiting N, represented as the fold change (FC) to the WT (n=5). **F.**Relative expression of N-related genes in Col WT and *proton1*under optimal N, represented as the FC to the WT (n=5). Significant differences to the WT according to Mann-Whitney U test are represented by one (p-value < 0.05), or two (p-value > 0.01) asterisks above each column.

Figure 5. Plasticity of primary metabolites in response to N availability of Arabidopsis accessions. **A.** Boxplots for CV of each metabolite across accessions grown under limiting, intermediate and optimal N. The metabolites are colored according to their classification. **B.** Metabolites whose CV levels

showed significant correlation with plasticity of the four complex traits. Significant Spearman correlation after Benjamini-Hochberg correction (FDR < 0.05) are represented as red for positive, or blue for negative. Abbreviations: adenosine-5-monophosphate (AMP), dehydroascorbate (DHA), fructose-6-phosphate (F6P), gamma aminobutyric acid (GABA), glucose-6-phosphate (G6P), glycerol-3-phosphate (G3P), n= 43.

Figure 6. Plasticity of ERD and FT in response to different environments in Arabidopsis accessions. A. Fold changes (FC) of ERD between indicated conditions. **B.** Fold change of flowering time (FT) between indicated conditions. The FCs were calculated for plants grown under low light (LL , n = 10), high light (HL, n=15), short days (SD, n=10), or in the polytunnel (PT, n=15) in comparison to those grown under long days and normal lights (Ctrl, n=10). The FC values are represented on a scale from ranging from 2 (red) to 0 (blue) in comparison to the control. They were calculated based on the means from plants grown under limiting (Lim) or intermediate (Int) N and those grown under optimal (Opt) N (n = 4 for each condition). Black denotes missing data. **C.** Pearson correlation analysis between the FCs of ERD. **D.** Pearson correlation analysis between the FCs of FT. Adjusted p-values were calculated using Benjamini-Hochberg correction. Significant Spearman r_s correlations (FDR < 0.05) are represented as red for positive, or blue for negative, n = 19.

Supporting information

Table S1. *Arabidopsis thaliana* accessions used for the plasticity screening in response to N availability.

Table S2. Average scores of early rosette diameter (ERD), final rosette diameter (FRD), flowering time (FT), and yield (YIE) of Arabidopsis global accessions in response to nitrogen.

Table S3. Plasticity scores for early rosette diameter (ERD), final rosette diameter (FRD), flowering time (FT), and yield (YIE) of Arabidopsis global accessions in response to nitrogen.

Table S4. Description of the associated loci identified by GWAS using ERD and FT plasticity to nitrogen.

Table S5. ERD FC of the haplogroups carrying the major or minor alleles for the GWA association identified at Chromosome 1.

Table S6. Primary metabolites levels from 43 Arabidopsis global accessions grown under limiting, intermediate, and optimal N conditions.

Table S7. Plasticity (CV) of 66 primary metabolites from 43 Arabidopsis global accessions grown under limiting, intermediate, and optimal N conditions.

Table S8. Plasticity of early rosette diameter and flowering time in response to different environmental cues for accessions selected based on their plasticity to N.

Figure S1. Number of rosette leaves, bolting time (B) and flowering time (F) for accessions grown under limiting (lim), intermediate (int) and optimal (opt) N conditions . The seeds were stratified for 5 days at 4°C/dark, following germination for 5 days on the respective soil (short days, 20°C/16°C day/night), and vernalization for 60 days (long days, 4°C). After vernalization, the plants were acclimated for one day at long days with 21°C/17°C day/night). Next, for each accession and condition, one plant was pricked to individual pots (n = 5) containing new soil with the same N concentration. The plants were grown under long days and 21°C/17°C (day/night). The number of days was counted after pricking. Bolting time was scored when the stem reached 1 cm height; flowering time was scored when the first flower opened.

Figure S2. Flowering time (FT) performance of T-DNA lines for candidate genes related to FT plasticity in response to N. Days to the first open flower (FT) of Col-0 WT and T-DNA lines under **A.**limiting (Lim) N, **B.** intermediate (Int) N, **C.** optimal (Opt) N. For each line and condition, n = 10. **D.**

FT plasticity measured as the fold change (FC) between limiting and optimal N.**E.** FT plasticity measured as the coefficient of variation (CV) across the three N conditions.

Figure S3 . Early rosette diameter (ERD) performance of Col-0, Gy-0, Xan-5, and *proton1* in response to N. Col-0 and Gy-0 belong to the haplogroup carrying the major allele at the promoter of*PROTON1* . Xan-5 has a missense substitution that causes the loss of *PROTON1* start codon, while *proton1* is a strong knockdown line (Figure 4a). **A.** ERD in response to limiting (Lim) and optimal (Opt) N (n = 10 for each condition and line).**B.** ERD fold change (FC) between limiting to optimal N for each of the lines, indicating that loss of *PROTON1* function reduces ERD plasticity, as observed for Xan-5 and *proton1* FC.

Figure S4. Correlation of the mean levels of metabolites and the mean trait values of ERD, FRD, FT and YIE in accessions grown at limiting, intermediate and optimal N conditions. Significant Spearman correlation after Benjamini-Hochberg correction (FDR < 0.05) are represented as red for positive, or blue for negative. Abbreviations: adenosine-5-monophosphate (AMP), dehydroascorbate (DHA), fructose-6-phosphate (F6P), gamma aminobutyric acid (GABA), glucose-6-phosphate (G6P), glycerol-3-phosphate (G3P).

Figure S5. Co-expression network of At1g19880 (*PROTON1*).Co-expression analysis adapted from ATTED-II v.10.1 (https://atted.jp/). KEGG (Kyoto Encyclopedia of Genes and Genomes) IDs are shown. Murual ranks for the edge strength of co-expressed gene network are represented as bold (MR < 5), normal (MR < 30), or thin (MR > 30) edges.