Identification of genomic regions regulating ammonium-dependent inhibition of primary root length in *Arabidopsis thaliana*

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Plant roots grow into the soil for efficient acquisition of various nutrients, such as inorganic nitrogen, ammonium, and nitrate. A previous study has revealed the genetic diversity of foliar functions of Arabidopsis thaliana ecotypes in an environment containing ammonium; however, the function of roots remains unclear. This work focuses on the root system architecture (RSA) of Arabidopsis ecotypes to investigate the genetic factors regulating ammonium-dependent RSA changes. Arabidopsis ecotypes were grown on vertical agar medium containing ammonium as a major nitrogen source, and root growth and RSA were determined. Arabidopsis ecotypes showed differential sensitivity to ammonium. The shoot dry weight of some ecotypes decreased, whereas that of other ecotypes increased in ammonium medium. The RSA changes also varied among the different ecotypes in response to ammonium. The total root length, measured as the sum of primary root and lateral root length, of some ecotypes was reduced, whereas that of other ecotypes showed no significant difference in ammonium medium. Compared with lateral roots, the primary roots showed a sharp response to ammonium supply. Notably, the RSA showed a partial correlation with shoot dry weight in ammonium medium. Because Col-4 and Ler-0 showed opposite RSA responses to ammonium supply, these two ecotypes were selected for further genetic analysis. Quantitative trait locus (QTL) analysis of recombinant inbred lines of Col and Ler showed the involvement of several genetic factors in ammonium-dependent RSA changes. Moreover, QTL analysis revealed that the primary structure of nitrogen-related enzymes do not account for changes in RSA in response to ammonium supply.

Keywords
Arabidopsis, ammonium, root, QTL, ecotypes

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Introduction

Nitrogen acquisition is one of the most important factors affecting plant growth (Marschner 1995). When the supply of inorganic nutrients in the soil is non-uniform, plant roots grow into regions of soil that contain higher concentrations of nutrients (Drew 1975). Plants uptake ammonium and nitrate from soil as the major nitrogen sources for growth (von Wirén 2000). Several lines of evidence suggest a strong relationship between nitrogen use and root development. Plant root plasticity depends on the integration of local and systemic signaling (Ruffel et al. 2011). Systemic signaling regulates root growth in response to the internal nutrient status of plants (Desnos 2008). A decrease in the shoot:root ratio due to a reduced nitrogen availability is typical for all plant species and cultivation practices (Ericsson 1995, Yasuda et al. 2017). Local signaling modulates root growth and branching in response to the local nutrient concentrations (Desnos 2008). Both ammonium and nitrate stimulate root development (Drew 1975); however, plants seem to have different responses to both these nitrogen sources. Forde and colleagues studied the mechanism of lateral root elongation in Arabidopsis in response to a localized nitrate supply (Zhang and Forde 2000). They concluded that meristematic activity in lateral root tips is stimulated by direct contact with an enriched source of nitrate and that lateral root development is highly susceptible to inhibition by a systemic signal. Ammonium stimulates lateral root branching in ammonium transporter dependent manner (Lima et al. 2010) and reduces the size of root apical meristem of the primary root (Giehl et al. 2014, Liu et al. 2013). Plants show differential nitrogen preferences (Sarasketa et al. 2014). The tolerance to ammonium varies not only among species but also among individuals within a species (Sarasketa et al. 2014). Recent studies comparing the responses of Arabidopsis ecotypes to different nitrogen regimes (Chardon et al. 2010, Masclaux-Daubresse & Chardon 2011, Ikram et al. 2012, Sarasketa et al. 2014) have shown that the key factor for adaptation to an ammonium-rich environment is the accumulation, but not assimilation, of foliar ammonium (Sarasketa et al. 2014). The ammonium assimilatory enzymes are highly expressed in the roots and are up-regulated by ammonium supply (Ishiyama et al. 2004, Konishi et al. 2014, Konishi et al. 2017, Konishi et al. 2018). Therefore, the root is an important plant organ for ammonium assimilation (Tobin & Yamaya 2001). Root development is strongly affected by the ammonium availability. It has been shown that local ammonium supply increases lateral root initiation and higher-order lateral root branching in Arabidopsis (Lima et al. 2010). By contrast, most of the nitrate assimilated by plants is translocated via xylem and assimilated in the shoot (Krapp 2015). Because previous studies have focused primarily on shoots, the function of roots remains unclear (Sarasketa et al. 2014). We hypothesized that roots partly account for the adaptation of plants to an ammonium-rich environment, as ammonium assimilation occurs mainly in the roots. The purpose of this study is to describe and examine (1) the relationship between root system architecture (RSA) and ammonium use in various Arabidopsis ecotypes, and (2) the identification of quantitative trait loci (QTLs) determining ammonium-dependent root development.

Results

Correlation of RSA with shoot dry weight

Previously, Sarasketa and colleagues have shown a negative correlation between the shoot weight of Arabidopsis ecotypes and concentration of free ammonium (Sarasketa et al. 2014). In the present study, we sought to identify a response of Arabidopsis ecotypes to ammonium in roots because exogenously supplied ammonium is mainly assimilated by roots and to identify the relation between RSA and plant growth in the presence of ammonium. We evaluated 13 Arabidop-

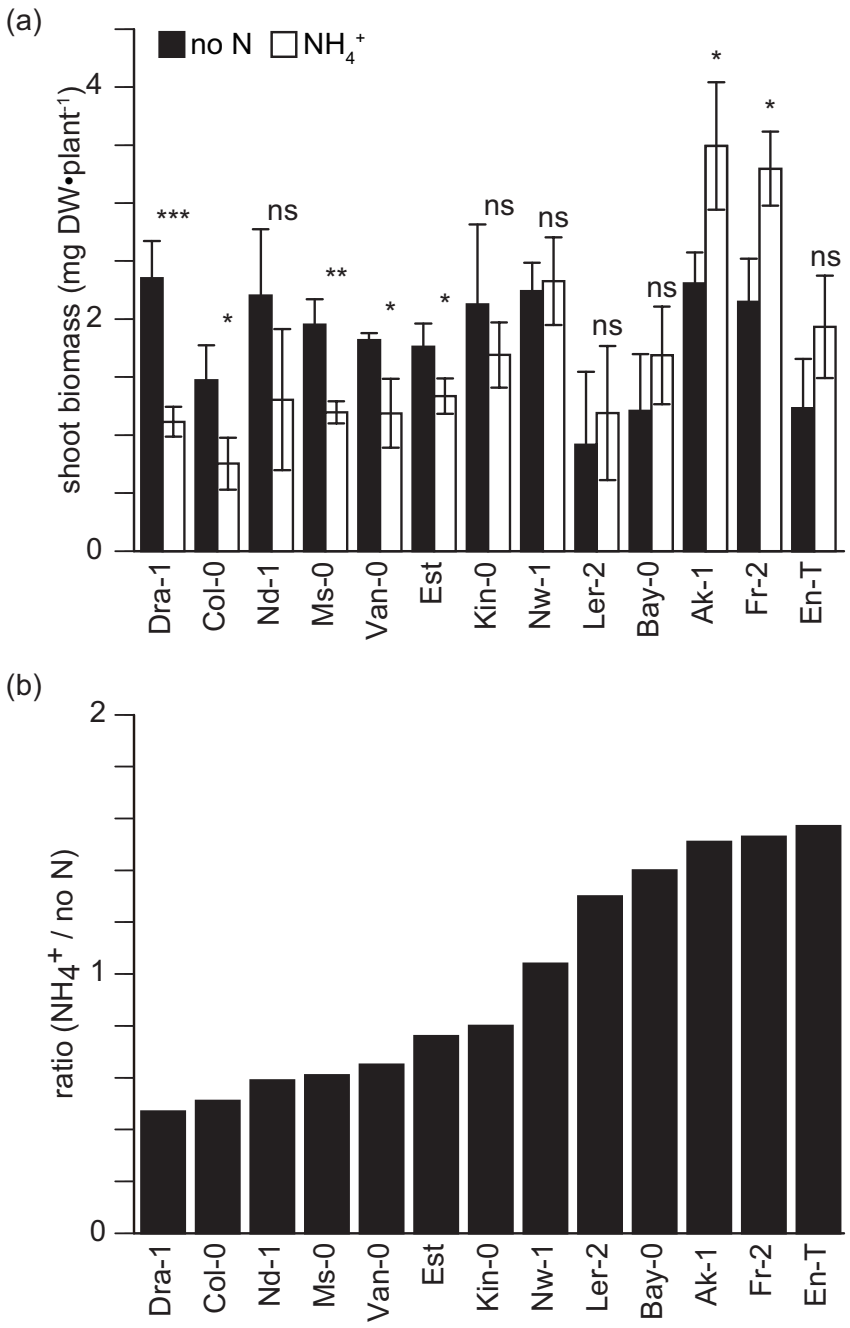


Figure 1. Growth of Arabidopsis seedlings on ammonium agar medium.

(a) Shoot dry weight of Arabidopsis ecotypes grown on a vertical agar plate containing either 10 μM KNO3 (–N) or 10 μM KNO3 supplemented with 10 mM NH4Cl (+NH4+) as a nitrogen source for 10 d. Significant differences, identified by the Student's t-test, are marked with asterisks: n. s. indicates not significant. (b) Shoot dry weight ratio of seedlings grown on medium with NH4+ to those grown on medium without NH4+. Significant differences were determined using one-way analysis of variance followed by Bonferroni. Significant differences ($p < 0.05$) within each line are indicated with different letters.

sis ecotypes on agar medium containing 10 μM KNO3 and supplemented with either 0 or 1 mM NH4Cl (Fig. 1). A large variability in plant growth was observed in response to ammonium. Among the 13 ecotypes, Dra-1 and Col-0 were the most sensitive (Fig. 1a), showing a 50% reduction in fresh shoot weight in the presence of ammonium. By contrast, Ak-1 and Fr-2 were remarkably tolerant to ammonium (Fig. 1a); the shoot dry weight of these ecotypes was 50% higher in 1 mM NH4Cl than in 0 mM NH4Cl (Fig. 1b). Additionally, the RSA showed remarkable variability in response to ammonium. Figure 2 shows the RSA of 15 Arabidopsis ecotypes on vertical agar plates containing ammonium as a major nitrogen source. Of the 15 ecotypes, the TRL of 7 ecotypes was shorter in medium with ammonium than in medium without ammonium (Fig. 2a); Is-0 was the only ecotype with a significant increase in TRL (Fig. 2a). The PRL was the most sensitive component of the RSA (Fig. 2b). Neither LRL nor LRN showed remarkable changes in response to ammonium (Fig. 2c and 2d). Table 1 summarizes the correlation efficient and partial correlation efficient between RSA and shoot dry weight (Fig. 1). Shoot dry weight showed partial correlation with TRL ratio and LRL ratio, negatively correlated with PRL and no correlation with LRN ratio (Table 1).

Several genetic regions determine the RSA under high ammonium concentration

We conducted QTL analysis using Col-4 X Ler-0 RIL population

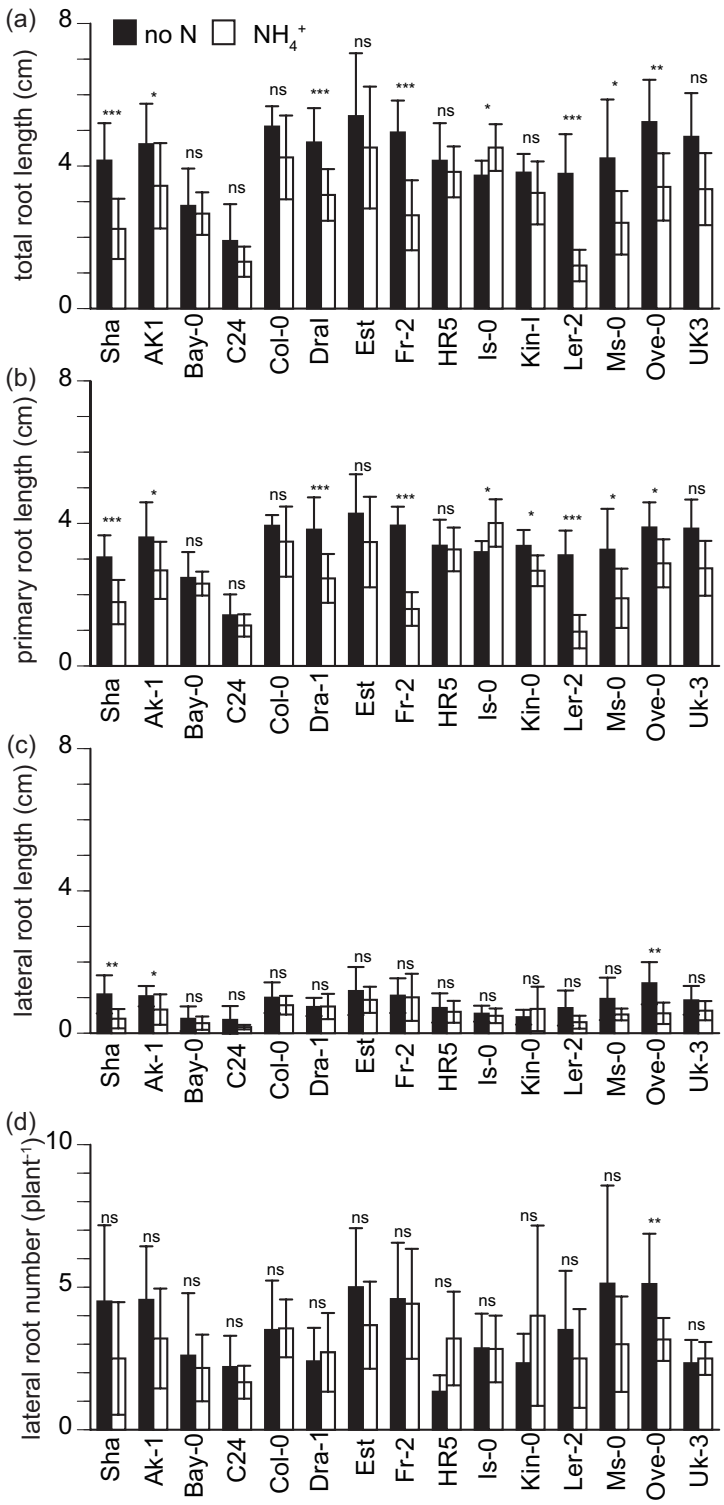


Figure 2. . Root system architecture of 15 Arabidopsis ecotypes grown on vertical agar plates containing 1 mM ammonium

Data showing total root length (TRL) (a), primary root length (PRL) (b), lateral root length (LRL) (c), and lateral root number (LRN) (d) per plant. Arabidopsis ecotypes were grown on a vertical agar plate for 14 d. The MGRL medium (Fujiwara et al. 1992) containing no nitrogen was supplemented with 10 μM KNO3 was used as a basic medium and further supplemented with 1 mM NH4Cl. Data represent mean ± standard deviation (SD) (n = 3–12). Significant differences, identified by the Student's t-test, are marked with asterisks (*p < 0.05, *** p < 0.005): n. s. indicates not significant.

grown on 0.5 mM ammonium and 10 μM nitrate to investigate the genetic factors underlying different RSA responses to ammonium. Several QTLs affecting RSA response to ammonium were mapped. Figure 3 shows TRL and PRL of parental lines, Col-4 and Ler-0, for QTL analysis. Values of TRL (Fig. 3a) and PRL (Fig. 3b) were lower in Ler-0 than in Col-4, and that of TRL and PRL were significantly lower in Ler-2 than in Col-0 (Fig 2a and 2b), indicating that the different RSA responses of Col-0/Ler-2 to ammonium are well conserved in Col-4/Ler-0. The TRL and PRL of the 98 RILs derived from Col-4 X Ler-0 showed normal frequency distribution (Fig. 4a and 4b). A total of 6 putative QTLs associated with the response to ammonium were identified (Fig. 5 and Table 2). Two QTLs for TRL were mapped near the markers C1_164 and C2_086 on chromosomes 1 and 2, respectively, and percentages of phenotypic variation explained by these QTLs were 17.6% and 18.6%, respectively. Ler-0 alleles increased the LOD score of TRL in the case of chromosome 2 QTL, whereas Col-4 alleles increased the TRL score in the case of chromosome 1 QTL. Four QTLs for PRL were mapped near C1_165 on chromosome 1, C2_051 and C2_087 on chromosome 2, and C5_148 on chromosome 5; each of these QTLs explained 22.2%, 12.0%, 16.9%, and 12.7%,

Table 1. Correlation coefficient and partial correlation coefficient of root system architecture and shoot dry weight in Arabidopsis plants

	Correlation coefficient	Partial correlation coefficient
total root length (NH_4^+ / no N)	-0.235	**0.859***
primary root length (NH_4^+ / no N)	-0.294	**-0.865***
lateral root length (NH_4^+ / no N)	-0.267	**-0.821***
lateral root number (NH_4^+ / no N)	-0.272	0.762

*, $P<0.05$

Correlation analysis was performed to identify the strength of relationship between root system architecture (total root length, primary root length, lateral root length and lateral root number) and shoot dry weight.

The ratio (grown in NH_4^+ medium and grown in no nitrogen medium) of root system architecture and the ratio of shoot dry weight were used for the analysis.

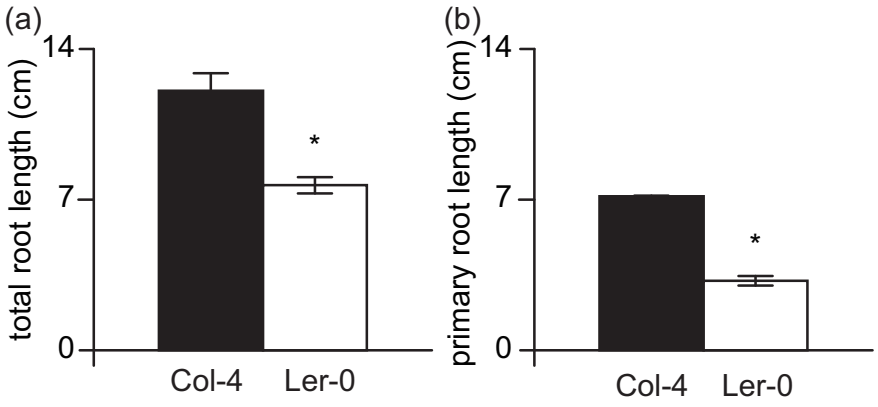


Figure 3. Ammonium-dependent change in root system architecture (RSA) of Col-4 and Ler-0

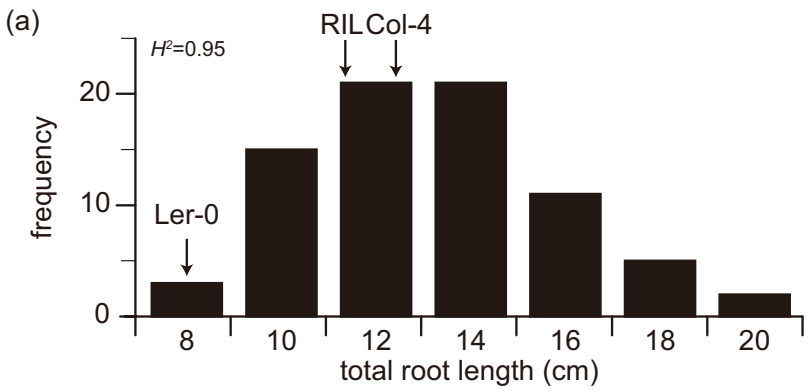
(a) Total root length (TRL) and (b) primary root length (PRL). Plants were grown for 21 d on a vertical agar plate. The MGRL medium (Fujiwara et al. 1992) containing no nitrogen was supplemented with 10 μM KNO3 was used as a basic medium and further supplemented with 0.5 mM NH4Cl. Data represent mean ± SD (n = 9–13), and significant differences are indicated with asterisks (*p < 0.05). Significant differences, identified by the Student's t-test.

respectively, of the phenotypic variation. Ler-0 alleles increased the score of PRL in the case of QTL near C2_087, whereas Col-4 alleles increased PRL score in the case of QTLs near C1_165, C2_051, and C5_148.

Discussion

Growth in ammonium-containing medium was partially correlated with RSA

To investigate the genetic diversity among Arabidopsis ecotypes, we first focused on the relation between the growth in ammonium-containing medium and the RSA. Differ-



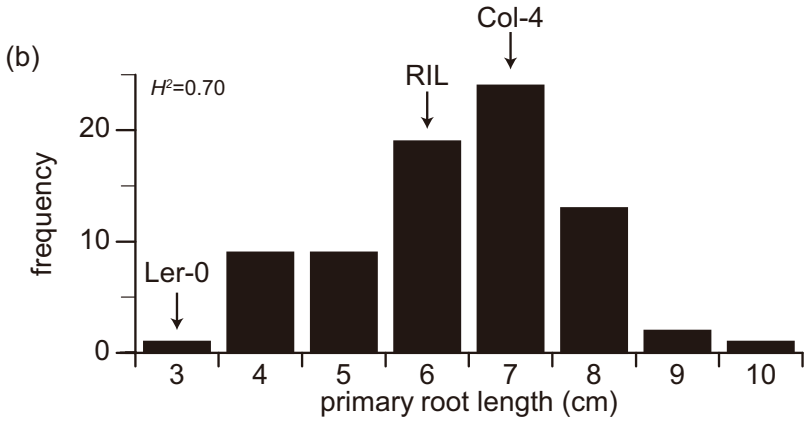


Figure 4. Frequency distribution and quantitative trait locus (QTL) map for RSA changes in response to ammonium levels
Frequency distribution of total root length (TRL) (a) and primary root length (PRL) (b) in 98 recombinant inbred lines (RILs) derived from a cross between Col-4 and Ler-0. Arrow indicates the mean value of the parental ecotypes.

ences were observed in the growth (Fig. 1) and RSA (Fig. 2) of Arabidopsis ecotypes on medium containing ammonium as a major nitrogen source. The growth of some ecotypes was enhanced, whereas that of other ecotypes was inhibited on ammonium-containing medium. Significant partial correlation was observed between growth and RSA on ammoni-

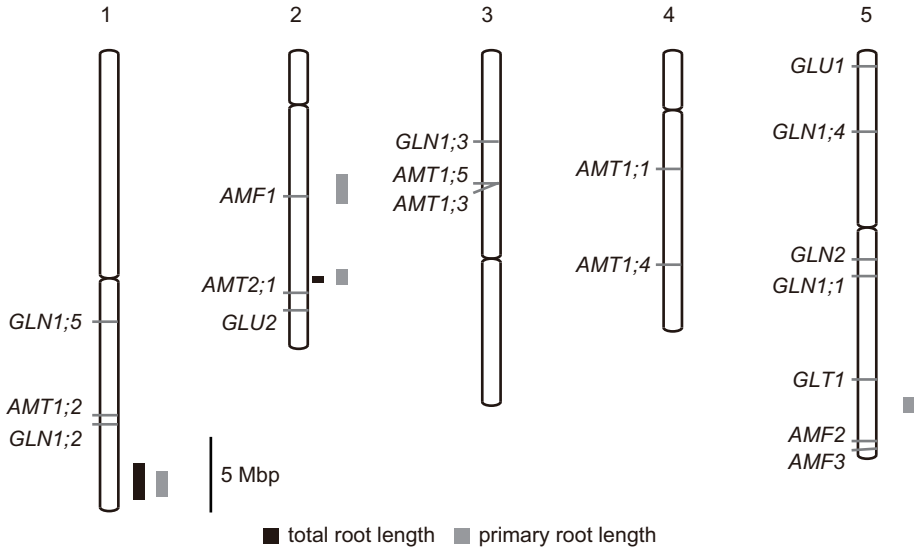


Figure 5. QTL map for RSA responding to ammonium nutrition and ammonium assimilatory genes on the linkage map
The location of QTLs responsible for total root length (TRL, dark gray column) and primary root length (PRL, light gray column) is shown. Cytosolic glutamine synthetase (GLN1), ammonium transporter (AMT), Fd-dependent glutamate synthase (GLU), ammonium facilitator (AMF), chloroplastic glutamine synthetase (GLN2), and NADH-dependent glutamate synthase (GLT) are indicated. (a) At low nitrogen level, the symplastic route of ammonium mainly consists of AMT1;1, AMT1;3, and AMT1;5 at the plasma membrane of the epidermis. The apoplastic route of ammonium mainly consists of AMT1;2 at the cortex and endodermis. GLN1;1 and GLN1;2 assimilate ammonium from both routes in the epidermis and endodermis.

Table 2. Location and effect of QTL for response to ammonium ion evaluated from morphology of root under agar condition.

Trait	QTL	Chr.	Mbp	Marker [a]	LOD	R^2 [b] (%)	A [c]
TRL		1		C1_164	5.36	17.6	1.3
		2		C2_086	4.24	18.6	-1.4
PRL		1		C1_165	6.89	22.2	0.8
		2		C2_051	4.07	12.0	0.5
		2		C2_087	5.49	16.9	-0.6
		5		C5_148	4.47	12.7	0.5

[a] The nearest marker to the QTL

[b] Proportion of the phenotypic variation explained by the nearest marker of QTL.

[c] Additive effect of the allele from Col-4 compared with Ler-0.

[*] Putative QTLs with significant LOD scores on 1,000 permutations tested at the 5% level.

um-containing medium (Table 1). Several lines of evidence suggest nitrogen use and root development. First, a decrease in shoot:root ratio in response to reduced availability of nitrogen is typical for all plant species and culturing methods (Ericsson 1995). Second, localized ammonium supply stimulates the branching of roots (Drew 1975, Lima et al. 2010). Third, ammonium supply stimulates the formation of lateral roots and reduces the size of parental root meristem (Giehl et al. 2014, Liu et al. 2013). Our results are in general agreement with previous studies on Arabidopsis ecotypes. The current study suggests a link between growth and RSA of Arabidopsis ecotypes grown on ammonium-containing medium. Since we analyzed only 13 Arabidopsis ecotypes, it was not possible to identify the genetic factors regulating ammonium-dependent changes in RSA using genome-wide association study. We selected two Arabidopsis ecotypes, Col and Ler, because of their different responses to ammonium supply in RSA.

Primary structures of nitrogen-related enzymes do not account for ammonium-dependent RSA changes

Since there was a clear difference in PRL between Ler-0 and Col-4 under high ammonium conditions (Fig. 3), we mapped the QTLs associated with PRL. The supply of ammonium resulted in various changes in the RSA of Ler-0 and Col-4 (Fig. 4 and Fig. 5), which were associated with several QTLs. Initial symptoms of ammonium toxicity appear in the roots with a severe modification of the RSA (Esteban et al. 2016). Ammonium-induced growth response is characterized by a change in the root:shoot ratio (Britto & Kronzucker 2002). Stunted root growth is the main phenotypic marker of ammonium toxicity in plants (Bittsánszky et al. 2015). On the other hand, nitrogen supply generally increases the shoot:root ratio (Andrew et al. 1999). Reduced PRL in Ler-0 may not indicate growth inhibition but may indicate adaptation to an ammonium-rich environment. Previously, we have shown that Arabidopsis does not suffer from ammonium toxicity at 1 mM NH4+ and 10 μM NO3-; instead, Arabidopsis plants utilize ammonium as a beneficial nitrogen source (Konishi et al 2017). In this study, QTLs involved in the assimilation and transport of ammonium were mapped in Arabidopsis (Fig. 5). Ammonium-induced lateral root branching is dependent on ammonium transporter 1;3 (AMT1;3) located on chromosome 3 (Lima

et al. 2010). In this study, no QTL affecting RSA was detected on chromosome 3, suggesting that the difference in RSA between Col-4 and Ler-0 is not explained by AMT1;3. Additionally, none of the other ammonium-related genes overlapped the QTLs identified in this study, except for ammonium facilitator 1 (AMF1, Chiasson et al. 2014). The deduced amino acid sequence of AMF1 in Ler-0 has been compared with that in Col-4 (Lu et al. 2012); however, there is no association between AMF1 amino acid sequences and plant phenotype. This suggests that the primary structures of nitrogen-related enzymes, including AMT1;3, do not account for changes in RSA in response to ammonium supply. It is possible that a single nucleotide polymorphism in nitrogen-related genes rather than a structural change in these proteins is responsible for changes in RSA in response to ammonium. Alternatively, an unknown gene might be responsible for changes in ammonium-dependent RSA. The identification of genes responsible for QTLs regulating changes in RSA would further our understanding of the adaptation of plants to ammonium-rich environment.

Methods

Plant material

Seeds of Arabidopsis ecotypes Ak-1 (CS6602), Bay-0 (CS954), C24 (CS906), Col-0 (CS1092), Dra-1 (CS6686), En-T (CS6176), Est (CS6173), Fr-2 (CS6708), HR5 (CS22205), Is-0 (CS6741), Kin-0 (CS6755), Ler-2 (CS8581), Ms-0 (CS6797), Nd-1 (CS1636), Nw-1 (CS6812), Ove-0 (CS6823), Shahdara (Sha, CS929), Uk-3 (CS6880), and Van-0 (CS6884) were obtained from the Arabidopsis Biological Resource Center, The Ohio State University, USA. The ecotypes were selected from the previous work (Lempe et al. 2005).

Plant growth conditions

For all experiments, the MGRL medium (Fujiwara et al. 1992), designed to eliminate nitrate and produce a nitrogen-free medium, was supplemented with 10 µM KNO3 and 1% agar for plant growth (Wako Pure Chemical Industries, Ltd., Osaka, Japan). Plants were cultured in a growth cabinet under controlled environment at 22°C temperature, 60% relative humidity, and 16 h light /8 h dark photoperiod (Ishiyama et al. 2004). The modified MGRL medium (described above) was supplemented with either 1 mM NH4Cl (+NH4+) or 1 mM KCl (–N), pH was adjusted to 5.8. Arabidopsis ecotypes were cultured under 40 µmol m–2 s–1 light intensity for 10 d. After 10 d of culture, shoots were cut using scissors and weighed with a UMX2Ultra-Microbalance (Mettler Toledo International Inc., Tokyo, Japan).

Root length measurement

Plants were cultured on the modified MGRL medium supplemented with 0, 0.5, 1, 5, or 10 mM NH4Cl for 14 d. Root systems were scanned with an ES-10000G scanner (Seiko Epson Corporation, Nagano, Japan) at a resolution of 300 dpi, and background noise was removed from the images using Adobe Photoshop software package (Adobe Systems Incorporated, CA, USA). Root length was measured by analyzing scanned images using WinRHIZO version Pro2007d (Regents Instruments Inc., Canada).

Quantitative trait locus (QTL) analysis

Plants were grown on the modified MGRL medium supplemented with 0.5 mM NH4Cl for 21 d, and the root systems were scanned as described above. A recombinant inbred line (RIL) population (TAIR accession number, CS1899) derived from a cross between Landsberg erecta (Ler-0; CS20) and Columbia (Col-4; CS933) (Lister & Dean 1993) was obtained from the Nottingham Arabidopsis Stock Center (NASC; http://arabidopsis.info/). A set of 98 RILs with 395 markers (Lu et al. 2012) covering all five chromosomes was used in this study. Linkage analyses were performed using Kosambi (Mapmaker/EXP 3.0; Lander et al. 1987), and QTL analyses were performed using QTL

Cartographer ver. 2.5 (Wang et al. 2007). After 1,000 permutations, log of the odds (LOD) score were chosen for composite interval mapping of root morphology. Permutation testing on six data sets indicated that the LOD scores of 2.6, 2.8, 2.6, and 2.7 for the total root length (TRL), primary root length (PRL), and lateral root length (LRL), and lateral root number (LRN), respectively, were suitable as genome-wide 5% significance thresholds for both measured traits. The percentage of total phenotypic variation explained by QTLs for each trait was estimated as R2. Positions of QTLs were assigned at regions under consideration. Two QTL positions on the same chromosome were regarded as different when the distance of the nearest marker of each QTL was > 5 cM.

Statistics

Data were analyzed using Microsoft Excel add-in software (Social Survey Research Information Co., Ltd, Tokyo, Japan). Correlation and partial correlation coefficients were determined between the ratio of the plants grown in NH4+ and in no nitrogen of both RSA and shoot dry weight. Test for the population partial correlation coefficient was performed. The data Correlation with a P-value < 0.05 was considered as statistically significant.

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Disclosures

Conflicts of interest: No conflicts of interest are declared.

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