NRT1.1-Related NH_4^+ Toxicity Is Associated with a Disturbed Balance between NH_4^+ Uptake and Assimilation[1][OPEN]

Shaofen Jian,[a] Qiong Liao,[a] Haixing Song,[a] Qiang Liu,[a] Joe Eugene Lepo,[b] Chunyun Guan,[c] Jianhua Zhang,[d] Abdelbagi M. Ismail,[e] and Zhenhua Zhang[a,2,3]

[a]Southern Regional Collaborative Innovation Center for Grain and Oil Crops in China, College of Resources and Environmental Sciences, Hunan Agricultural University, Changsha, China 410128
[b]Center for Environmental Diagnostics and Bioremediation, University of West Florida, Pensacola, Florida 32514
[c]National Center of Oilseed Crops Improvement, Hunan Branch, Changsha, China 410128
[d]Department of Biology, Hong Kong Baptist University, Hong Kong, China SCT704
[e]International Rice Research Institute, Metro Manila, Philippines DAPO 7777

ORCID IDs: 0000-0002-7553-642X (S.J.); 0000-0002-3819-2437 (J.Z.); 0000-0002-1961-3072 (A.M.I.); 0000-0002-8877-1090 (Z.Z.).

A high concentration of ammonium (NH_4^+) as the sole source of nitrogen in the growth medium often is toxic to plants. The nitrate transporter NRT1.1 is involved in mediating the effects of NH_4^+ toxicity; however, the mechanism remains undefined. In this study, wild-type Arabidopsis (*Arabidopsis thaliana* Columbia-0 [Col-0]) and *NRT1.1* mutants (*chl1-1* and *chl1-5*) were grown hydroponically in NH_4NO_3 and $(NH_4)_2SO_4$ media to assess the function of NRT1.1 in NH_4^+ stress responses. All the plants grew normally in medium containing mixed nitrogen sources, but Col-0 displayed more chlorosis and lower biomass and photosynthesis than the *NRT1.1* mutants in $(NH_4)_2SO_4$ medium. Grafting experiments between Col-0 and *chl1-5* further confirmed that NH_4^+ toxicity is influenced by NRT1.1. In $(NH_4)_2SO_4$ medium, NRT1.1 induced the expression of NH_4^+ transporters, increasing NH_4^+ uptake. Additionally, the activities of glutamine synthetase and glutamate synthetase in roots of Col-0 plants decreased and soluble sugar accumulated significantly, whereas pyruvate kinase-mediated glycolysis was not affected, all of which contributed to NH_4^+ accumulation. By contrast, the *NRT1.1* mutants showed reduced NH_4^+ accumulation and enhanced NH_4^+ assimilation through glutamine synthetase, glutamate synthetase, and glutamate dehydrogenase. Moreover, the up-regulation of genes involved in ethylene synthesis and senescence in Col-0 plants treated with $(NH_4)_2SO_4$ suggests that ethylene is involved in NH_4^+ toxicity responses. This study showed that NH_4^+ toxicity is related to a nitrate-independent signaling function of *NRT1.1* in Arabidopsis, characterized by enhanced NH_4^+ accumulation and altered NH_4^+ metabolism, which stimulates ethylene synthesis, leading to plant senescence.

Ammonium (NH_4^+) is a paradoxical inorganic nitrogen (N) source in the soil that is available to plants during growth and development. It is the preferred N source for some species such as rice (*Oryza sativa*) and tea (*Camellia sinensis*; Gao et al., 2010; Ruan et al., 2016). However, NH_4^+ toxicity occasionally occurs in terrestrial plants due to excessive N fertilizer application (Chen et al., 2013; Li et al., 2013). NH_4^+ toxicity typically takes place when plants are exposed to a high concentration, or alternatively, when plant cells themselves overproduce NH_4^+ under environmental stresses resulting from alterations in NH_4^+ metabolism (Barker and Corey, 1991; Feng and Barker, 1992; Bittsánszky et al., 2015). NH_4^+ toxicity often is accompanied by a depletion of organic acids and inorganic cations and by an accumulation of NH_4^+ (Hachiya et al., 2012). Several hypotheses have been proposed to explain the toxic effect of NH_4^+ on plants (Britto et al., 2001; Bittsánszky et al., 2015); however, the primary physiological mechanisms underlying NH_4^+ toxicity in plants remain elusive (Li et al., 2013).

NH_4^+ accumulation is widely observed when plants grow under high NH_4^+ concentration conditions. In rice, the application of high levels of NH_4^+ results in NH_4^+ accumulation and futile transmembrane NH_4^+ cycling (Chen et al., 2013). NH_4^+ accumulation occurs partially due to over uptake of NH_4^+. In Arabidopsis (*Arabidopsis thaliana*), four ammonium transporters (AMTs) function in NH_4^+ root acquisition, three of which, AtAMT1;1, AtAMT1;2, and AtAMT1;3, are

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[2]Author for contact: [zhzh1468@163.com].
[3]Senior author.
The author responsible for distribution of materials integral to the findings presented in this article in accordance with the policy described in the Instructions for Authors (www.plantphysiol.org) is: Zhenhua Zhang (zhzh1468@163.com).

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up-regulated by NH_4^+ and collectively are responsible for up to 90% of NH_4^+ import (Yuan et al., 2007a). Knockout of the *AMT1* gene significantly decreases NH_4^+ uptake, whereas overexpression of *AMT1* results in higher NH_4^+ permeability in roots (Ranathunge et al., 2014; Li et al., 2016). Wang et al. (2013) verified that, under high-NH_4^+ stress (30 mM NH_4^+), AtAMT1;3 is endocytosed from the plasma membrane to reduce NH_4^+ uptake and mitigate NH_4^+ toxicity.

NH_4^+ serves as a ubiquitous intermediate metabolite in N metabolism in plants (Joy, 1988; Glass et al., 1997). Its high concentration in plant cells is toxic to plant tissues. Therefore, the capacity of plant cells to detoxify excessive NH_4^+ is viewed as an important metabolic response to alleviate the consequences of this stress (Hoai et al., 2003). The incorporation of NH_4^+ into Gln and the synthesis of Glu are critical pathways of NH_4^+ assimilation and NH_4^+ detoxification (Li et al., 2014). Glutamine synthase (GS), glutamate synthase (GOGAT), and glutamate dehydrogenase (GDH) are important enzymes in these processes (Miflin and Habash, 2002; Skopelitis et al., 2006). NH_4^+-tolerant plants have higher GS activity and less NH_4^+ accumulation in tissues (Cruz et al., 2006; Omari et al., 2010). Konishi et al. (2017) reported that *GLN1* plays a dominant role in regulating NH_4^+ uptake in Arabidopsis. Indeed, pharmacological studies suggest that Gln, rather than NH_4^+, could be the real signaling molecule that regulates the expression of NH_4^+ transport and assimilation genes (Tabuchi et al., 2007). It is likely that NH_4^+ uptake and assimilation are integrated. Nevertheless, the coordination between NH_4^+ uptake and assimilation during NH_4^+ toxicity remains unclear.

NRT1.1 is the primary member of the nitrate transporter (*NRT*) gene family in higher plants (Tsay et al., 1993). It functions in multiple physiological processes in plants, such as NO_3^- and auxin transport, NO_3^- signal sensing, and stomatal movement (Tsay et al., 1993; Guo et al., 2003; Krouk et al., 2010; Bouguyon et al., 2015). Several studies have revealed that NRT1.1 is essential for plants to adapt to unfavorable environments, including cadmium stress, salt stress, and proton toxicity (Mao et al., 2014; Fang et al., 2016; Álvarez-Aragón and Rodríguez-Navarro, 2017). Interestingly, the Arabidopsis *NRT1.1* mutants *chl1-1* and *chl1-5* display higher resistance to a high concentration (10 mM) of NH_4^+ as the sole source of N than wild-type plants (Hachiya et al., 2011; Hachiya and Noguchi, 2011). However, the mechanisms by which NRT1.1 mediates NH_4^+ uptake and the subsequent symptoms of NH_4^+ toxicity have to be elucidated at the physiological and molecular levels.

Here, we analyzed gene expression, metabolites, and the physiological processes and chemical activities in roots and shoots of wild-type Arabidopsis plants and *NRT1.1* mutants grown in nutrient growth medium containing high concentrations of NH_4^+ as the sole N source. We aimed to clarify (1) whether NH_4^+ toxicity symptoms are NRT1.1 related and (2) the physiological roles of NH_4^+ uptake and assimilation during NH_4^+ toxicity. Our results provide further insights into NH_4^+ toxicity in plants.

RESULTS

NH_4^+ Toxicity Is Related to NRT1.1 Signaling Function

A previous study showed that 5 mM $(NH_4)_2SO_4$ (10 mM NH_4^+) as the sole source of N causes chlorosis in the leaves of Columbia-0 (Col-0), but not in the leaves of *chl1-1* and *chl1-5* mutant lines, in 11-d-old seedlings (Hachiya and Noguchi, 2011). In this study, with the equivalent stoichiometric sole NH_4^+ source supplied in different forms [$(NH_4)_2C_4H_6O_4$ (ammonium succinate), $(NH_4)_2SO_4$, or NH_4Cl] for 10 d to 4-week-old seedlings, we obtained the same results and observed severe chlorosis in plants treated with $(NH_4)_2SO_4$ (Fig. 1A). Accordingly, we used $(NH_4)_2SO_4$ treatment for all the subsequent experiments in this study.

High-concentration $(NH_4)_2SO_4$ treatment resulted in high amounts of sulfur (S) in the growth medium. Considering that S is an essential element for the synthesis of some amino acids, three different conditions were set to assess whether S was involved in $(NH_4)_2SO_4$-induced plant chlorosis: 5 mM $(NH_4)_2SO_4$ treatment as described above, 5 mM K_2SO_4 was added to control medium, or 5 mM NH_4NO_3 treatment was replaced with 10 mM KNO_3. We found that only the Col-0 plants treated with $(NH_4)_2SO_4$ displayed chlorosis (Supplemental Fig. S1). Therefore, the effect of S on $(NH_4)_2SO_4$-induced chlorosis in Col-0 plants was dismissed.

The application of sole NH_4^+ sources significantly reduced the plant size (Fig. 1A). The fresh weight of *chl1-1* and *chl1-5* was reduced by 44% and 39%, and the biomass was lowered by 10% and 16%, respectively, compared with the 58% and 36% reduction in fresh weight and biomass, respectively, observed in Col-0 (Fig. 1, B and C). There was no difference in photosynthetic rates or chlorophyll concentration between Col-0 and *NRT1.1* mutants under NH_4NO_3 treatment, whereas $(NH_4)_2SO_4$ reduced chlorophyll and photosynthetic rates in all genotypes, with greater reduction in Col-0 than in *chl1-1* and *chl1-5* (Fig. 1, D and E). Nevertheless, there was no significant difference in stomatal conductance between these genotypes with $(NH_4)_2SO_4$ treatment (Supplemental Fig. S2). These results indicate that NRT1.1 is involved in NH_4^+ toxicity in Arabidopsis.

To further confirm that NH_4^+ toxicity is NRT1.1 related, we developed grafted Col-0 and *chl1-5* plants and subjected them to different NH_4^+ conditions. As shown in Figure 1, F and G, the plants with Col-0 shoots and/or roots developed chlorosis under $(NH_4)_2SO_4$ conditions, and the grafted plants with the shoot of Col-0 displayed more serious chlorosis than those with the roots of Col-0, although chlorosis was much milder than that of Col-0. This implies that NRT1.1-related NH_4^+ toxicity is independent of

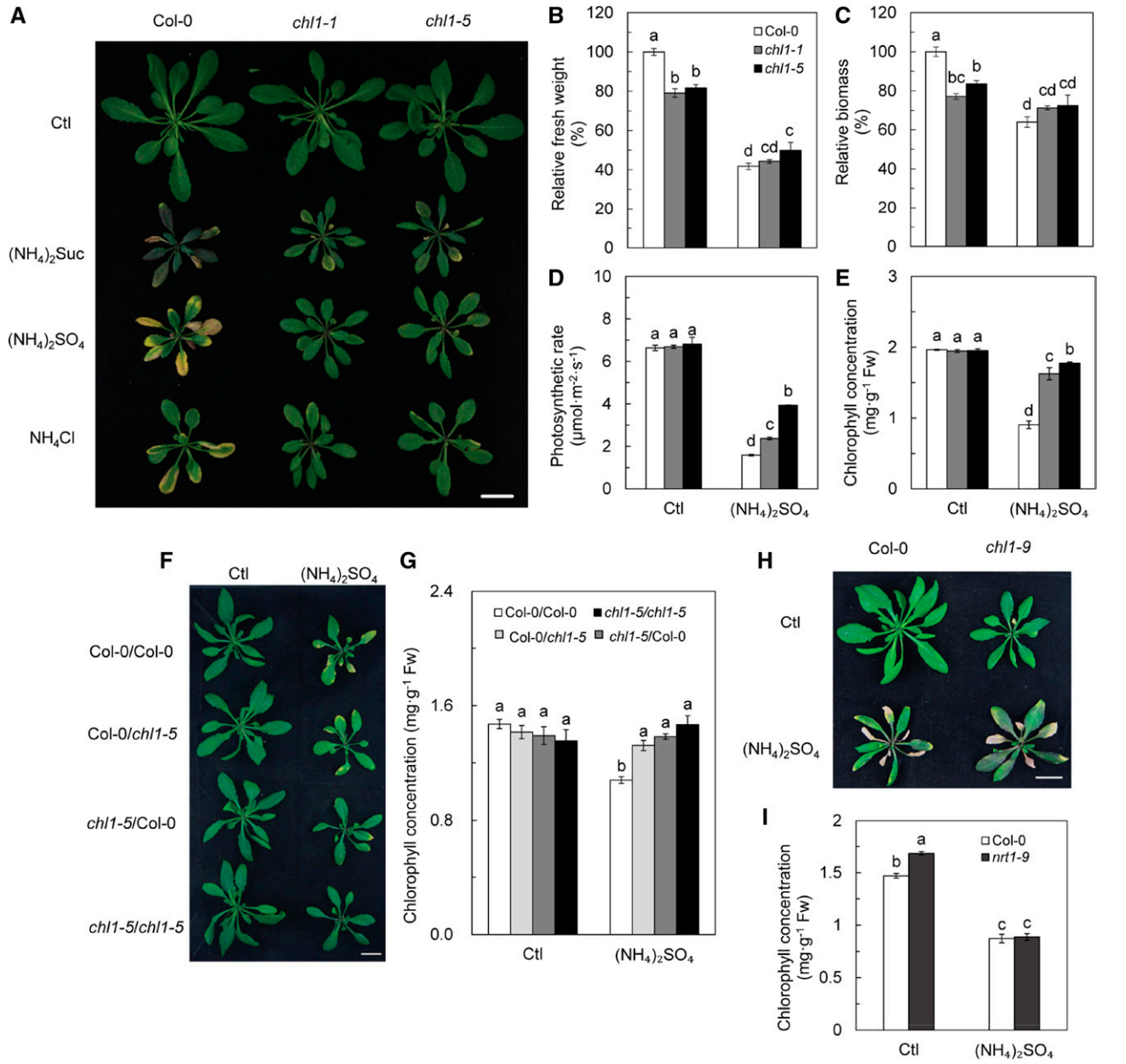

Figure 1. Effects of high concentration of a sole NH_4^+ source on Arabidopsis growth and photosynthesis. A, Plants were treated with 5 mM NH_4NO_3 (Ctl) and with different sources of NH_4^+ (10 mM) applied to rooting medium for 10 d. B to E, Relative fresh weight (B), relative biomass (C), photosynthetic rate (D), and chlorophyll concentration (E) of Col-0, *chl1-1*, and *chl1-5* grown in 5 mM NH_4NO_3 (Ctl) and $(NH_4)_2SO_4$ for 10 d. Fresh weight (Fw) and dry weight were obtained from pooled samples of seven plants from each replicate, and data were calculated relative to the average value of Col-0 under control conditions. F, Grafted plants grown in 5 mM NH_4NO_3 (Ctl) and $(NH_4)_2SO_4$ for 10 d. Plant material names on the left indicate the genotypes of the shoot/root combination. G, Chlorophyll concentration of the grafted plants grown in 5 mM NH_4NO_3 (Ctl) and $(NH_4)_2SO_4$ for 10 d. H and I, Phenotypes and chlorophyll concentrations of Col-0 and *chl1-9* grown in 5 mM NH_4NO_3 (Ctl) and $(NH_4)_2SO_4$ for 10 d. Data represent means ± SE (n = 4). Bars with the same letter indicate no significant difference at $P < 0.05$ using the LSD method. Bars = 1 cm in A, F, and H.

the nitrate transporter function of NRT1.1. The *chl1-9* mutant is defective in nitrate uptake but shows a normal primary nitrate response (Ho et al., 2009). In contrast to *chl1-1* and *chl1-5*, the phenotype of *chl1-9* was consistent with that of Col-0 (Fig. 1, H and I) when grown in $(NH_4)_2SO_4$ medium. These results indicate that the signaling function of *NRT1.1* is involved in NH_4^+ toxicity in Arabidopsis.

NRT1.1 Increases NH_4^+ Uptake and Accumulation

The total N content, NH_4^+ concentration, and expression of *AMT1* genes were assayed to identify the difference in NH_4^+ uptake between Col-0 and *NRT1.1* mutants in response to $(NH_4)_2SO_4$. There was no difference among Col-0, *chl1-1*, and *chl1-5* plants in total N content before $(NH_4)_2SO_4$ treatment (Fig. 2A). However, treatment with $(NH_4)_2SO_4$ for 10 d decreased total N content significantly in the three genotypes, but it remained significantly higher in Col-0 than in *chl1-1* and *chl1-5* (Fig. 2B). NH_4^+ concentration in the three genotypes showed no significant difference under control treatment (Fig. 2C); however, $(NH_4)_2SO_4$ treatment significantly reduced NH_4^+ concentrations in *chl1-1* and *chl1-5* roots and shoots, while the NH_4^+ concentration in Col-0 was stable in the root and increased significantly in the shoot (Fig. 2C). We found that the $^{15}NH_4^+$ uptake rate in Col-0 plants was significantly higher than that of *chl1-1* and *chl1-5* plants during both the first and second stages of $^{15}NH_4^+$ labeling (Fig. 2, E and F). This result was consistent with those of experiments in which $^{15}NH_4^+$ was not used and further verified that NRT1.1 enhanced NH_4^+ uptake under $(NH_4)_2SO_4$ treatment. Short-term $^{15}NH_4^+$ labeling for 5 and 10 min showed that the NH_4^+ uptake rate of Col-0 was significantly higher than that of *chl1-1* and *chl1-5* plants (Fig. 2D). The NH_4^+ uptake rate measured by short-term labeling was higher than that measured by long-term labeling. The decreasing concentration of NH_4^+ in growth medium over time could be an explanation for the lower NH_4^+ uptake rate observed by long-term labeling compared with short-term labeling. The circadian rhythm of nutrient uptake in plants also makes the average NH_4^+ uptake rate of long-term labeling lower than that of short-term labeling. NH_4^+ fluxes measured with noninvasive microtest technology (NMT) also showed that NH_4^+ influxed predominantly in Col-0 plants but effluxed in *chl1-1* and *chl1-5* (Supplemental Fig. S3, A and B).

The modifications in NH_4^+ uptake could be the result of the expression of *AMT1* genes. In Arabidopsis, *AMT1;1*, *AMT1;2*, *AMT1;3*, and *AMT1;5*, which encode AMT1 family transporters, are responsible for the majority of NH_4^+ acquisition in roots (Tegeder and Masclaux-Daubresse, 2018). The expression of these genes did not differ significantly between Col-0 and *nrt1.1* lines under control conditions. Generally, $(NH_4)_2SO_4$ resulted in the up-regulation of *AMT1;1*, *AMT1;3*, and *AMT1;5* in Col-0, particularly for *AMT1;5*, where the expression was 200-fold higher than that in the control (Fig. 3, C and D). However, the expression of *AMT1* genes in *chl1-1* and *chl1-5* decreased significantly (*AMT1;1*) or was unchanged (*AMT1;2* and *AMT1;3*) under $(NH_4)_2SO_4$ compared with that of the control and was significantly lower than that in Col-0 (Fig. 3). *NRT1.1* facilitates the expression of *AMT1s* in Arabidopsis in response to $(NH_4)_2SO_4$, which could lead to increased NH_4^+ uptake. The deficiency of *AMT1;1* causes a 30% decrease in NH_4^+ uptake by roots (Yuan et al., 2007a). The results in Supplemental Figure S4 show that *AMT1;1* deletion lowered the sensitivity of plants to high concentrations of NH_4^+ as the sole N source. This suggests that NH_4^+ uptake was responsible for NH_4^+ toxicity.

NRT1.1 Inhibits the Activity of NH_4^+ Metabolism Enzymes under High Concentration of NH_4^+ as the Sole N Source

Under control conditions, GS activity was significantly lower in *chl1-1* and *chl1-5* than in Col-0 in roots (Fig. 4A). The GS activity decreased in Col-0 plants treated with $(NH_4)_2SO_4$ but was not affected in *chl1-1* and *chl1-5* under the same growth conditions (Fig. 4A). In shoots, $(NH_4)_2SO_4$ increased GS activity significantly in Col-0 and *chl1-1* but not in *chl1-5* (Supplemental Fig. S5A).

The GOGAT activity in the roots did not differ between genotypes under control conditions (Fig. 4B). $(NH_4)_2SO_4$ reduced GOGAT activity by 85.6% in Col-0 but to lesser extents in *chl1-1* and *chl1-5*, where it decreased by 48.1% and 21.5%, respectively (Fig. 4B). In shoots, $(NH_4)_2SO_4$ increased GOGAT activity significantly in *chl1-1* and *chl1-5* but not in Col-0, and no difference was observed among genotypes under control conditions (Supplemental Fig. S5B).

The activity of GDH was significantly higher in Col-0 than in *chl1-1* and *chl1-5* in roots under control conditions (Fig. 4C), and $(NH_4)_2SO_4$ treatment had no effect on GDH activity in Col-0 but increased GDH activity significantly in *chl1-1* and *chl1-5* by 23.4% and 70.6%, respectively, compared with the control (Fig. 4C). In shoots, $(NH_4)_2SO_4$ increased GDH activity in all genotypes, with no differences among genotypes under control or $(NH_4)_2SO_4$ treatments (Supplemental Fig. S5C).

No differences in glutamic-oxaloacetic transaminase (GOT) or glutamic-pyruvic transaminase (GPT) activities were observed in roots of the three genotypes under control conditions (Fig. 4, D and E); however, $(NH_4)_2SO_4$ increased GOT activity in *chl1-1* and *chl1-5* but not in Col-0 (Fig. 4D). GPT activity in the three genotypes was higher under $(NH_4)_2SO_4$ treatment than in the control, but with no significant difference between genotypes (Fig. 4E). In shoots, GOT and GPT activities were not affected by $(NH_4)_2SO_4$ treatment, and no difference in their activities could be measured between the wild-type and mutant genotypes (Supplemental Fig. S5, D and E). Taken together, these data suggest that *NRT1.1* had a negative effect on the activities of NH_4^+ assimilation enzymes and transaminases in response to a high concentration of the sole NH_4^+ source.

NRT1.1 Disturbs the Balance between Carbon and NH_4^+ Metabolism under NH_4^+ as the Sole N Source

The soluble protein concentration in shoots of *chl1-1* and *chl1-5* was significantly higher than that in Col-0

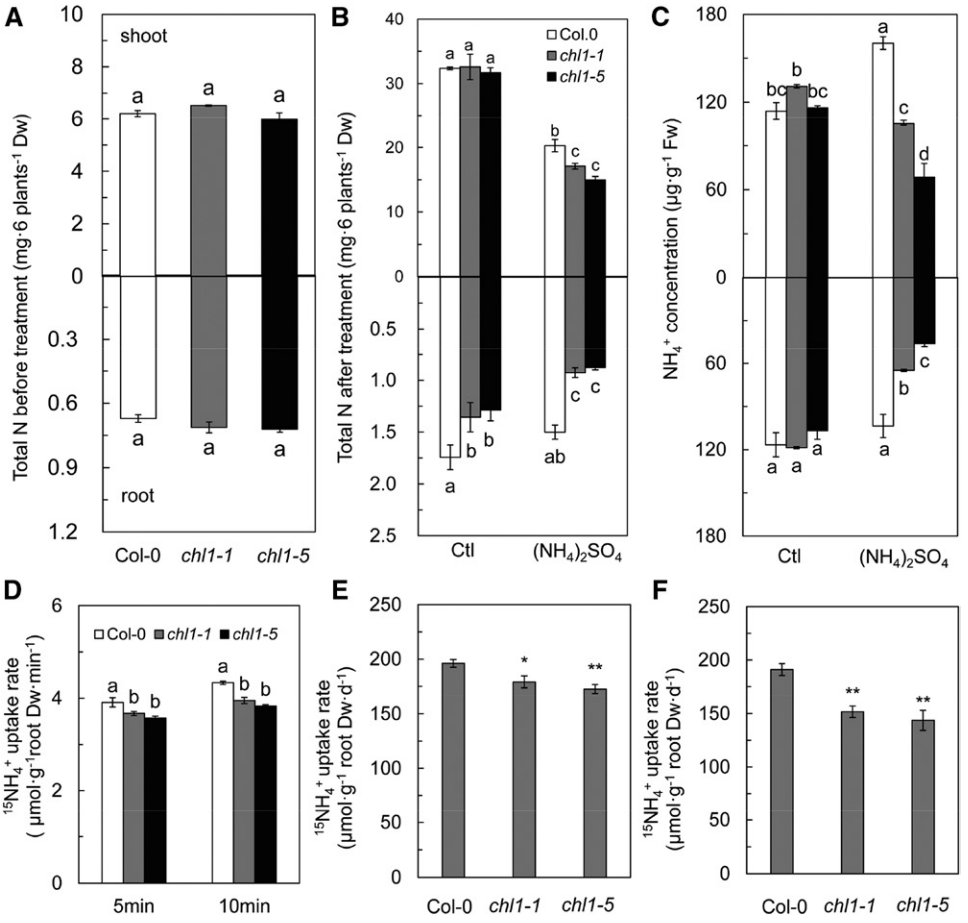

Figure 2. Effects of high concentration of a sole NH_4^+ source on N accumulation and NH_4^+ uptake in Arabidopsis. A, Total N content of Col-0, *chl1-1*, and *chl1-5* plants grown in 1.125 mM NH_4NO_3 medium before $(NH_4)_2SO_4$ treatment. B and C, Total N content (B) and NH_4^+ concentration (C) of Col-0, *chl1-1*, and *chl1-5* plants grown in 5 mM NH_4NO_3 (Ctl) and $(NH_4)_2SO_4$ for 10 d. D, $^{15}NH_4^+$ uptake rates in Col-0, *chl1-1*, and *chl1-5* plants labeled for 5 and 10 min. E and F, $^{15}NH_4^+$ uptake rates in Col-0, *chl1-1*, and *chl1-5* plants during the first (E) and second (F) stages of $(^{15}NH_4)_2SO_4$ treatment. Total N amount was obtained from pooled samples of six plants from each replicate. Data represent means ± SE ($n = 4$). Bars with the same letter are not significantly different at $P < 0.05$ using the LSD method. Bars with two and three asterisks indicate significant differences from the control at $P < 0.01$ and $P < 0.001$, respectively, using a two-tailed Student's *t* test. Dw, Dry weight; Fw, fresh weight.

under the control condition; however, *chl1-5* roots showed higher soluble protein concentrations than those of Col-0 (Fig. 5A). Irrespective of shoot or root, $(NH_4)_2SO_4$ increased soluble protein concentration in Col-0 but not in *chl1-1* and *chl1-5*. The mutants showed lower concentrations in roots than did Col-0 (Fig. 5A).

Soluble sugar concentrations in roots and shoots varied slightly between the wild type and mutants in the control treatment (Fig. 5B) and were 4.14 and 2.33 times higher in roots and shoots of Col-0, respectively, under $(NH_4)_2SO_4$ conditions than under control conditions (Fig. 5B). However, soluble sugar concentrations in *chl1-1* and *chl1-5* were not affected by $(NH_4)_2SO_4$ and were significantly lower than that in Col-0 (Fig. 5B). Pyruvate kinase (PK) activity was consistent among genotypes regardless of $(NH_4)_2SO_4$ treatment (Fig. 5C).

SNF1-RELATED PROTEIN KINASE1 (*SnRK1*) regulates the coordination between carbon (C) and N metabolism (Wang et al., 2012). $(NH_4)_2SO_4$ treatment led to inconsistent responses of *SnRK1* genes. As shown in Figure 5, D to G, the expression of *SnRK1;1* and *SnRK1;2* was unchanged in Col-0 with $(NH_4)_2SO_4$ treatment but was up-regulated in *chl1-1* and *chl1-5* when the plants were treated with $(NH_4)_2SO_4$ for 3 d (Fig. 5, D and F). Their expression on day 5 increased even further (Fig. 5, E and G).

To further investigate the effect of $(NH_4)_2SO_4$ on N metabolism, we compared the amino acid composition of Col-0 and *chl1-1* grown under different N sources. As shown in Figure 6, A to D, the major amino acids such as Asp and Asn were less affected by $(NH_4)_2SO_4$ in roots than in shoots of the wild-type and mutant lines (Fig. 6, A and B). Glu and Gln in the roots of *chl1-1* were significantly lower than in roots of Col-0 under $(NH_4)_2SO_4$ treatment (Fig. 6, C and D). In shoots, there were no significant differences in amino acid content

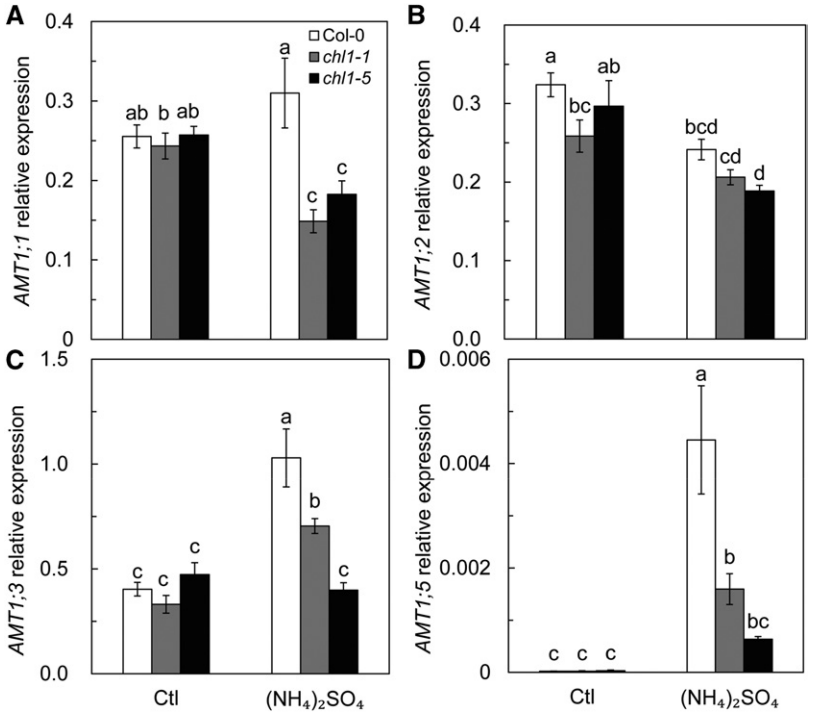

Figure 3. *AMT1* gene expression in roots of Col-0, *chl1-1*, and *chl1-5* plants grown in 5 mM NH_4NO_3 (Ctl) and $(NH_4)_2SO_4$ for 3 d. Roots were sampled for the expression of *AMT1;1* (A), *AMT1;2* (B), *AMT1;3* (C), and *AMT1;5* (D). Data represent means ± SE (n = 4). Bars with the same letter are not significantly different at $P < 0.05$ using LSD.

between Col-0 and *chl1-1* under control conditions (Fig. 6, A–D); however, $(NH_4)_2SO_4$ treatment increased the concentration of all measured amino acids, with the exception of Asn in Col-0, which was lower than that in the control (Fig. 6, A–D). In shoots, Asp, Asn, and Gln, but not Glu, were significantly higher in *chl1-1* than in Col-0 under $(NH_4)_2SO_4$ treatment (Fig. 6, A–D).

Under control conditions, there was no noticeable change in the root-to-shoot (R:S) ratios of amino acids between the wild type and mutants (Fig. 6, E–H). Although $(NH_4)_2SO_4$ promoted a higher accumulation of Asn in Col-0 roots than in shoots, the R:S ratio of Asn in *chl1-1* was not affected significantly (Fig. 6F). The R:S ratio of Gln similarly declined under $(NH_4)_2SO_4$ treatment in both Col-0 and *chl1-1* (Fig. 6H).

$(NH_4)_2SO_4$ treatment increased Thr, Ala, Ile, and Arg (Supplemental Fig. S6, A–C) in the shoots of *chl1-1* but not in those of Col-0. While Lys, Phe, and Ala were reduced or unchanged in *chl1-1*, their concentrations were lower than in Col-0 under $(NH_4)_2SO_4$ treatment (Supplemental Fig. S6, D–F).

NRT1.1-Related NH_4^+ Toxicity Is Associated with Ethylene and Plant Senescence

The expression of the senescence-related genes *WRKY45*, *WRKY53*, *SAG13*, *SAG29*, *SAG113*, and *GEN4* were assayed after treating Col-0, *chl1-1*, and *chl1-5* with $(NH_4)_2SO_4$. All genes were significantly up-regulated by $(NH_4)_2SO_4$ treatment in Col-0; but, together with *GEN4*, these genes were either slightly up-regulated or even down-regulated in *chl1-1* and *chl1-5* (Fig. 7, A–F). $(NH_4)_2SO_4$ treatment up-regulated the ethylene synthesis gene *ACS4* in Col-0 by 25.6% but inhibited *ACS4* expression by 84.3% and 90.7% in *chl1-1* and *chl1-5*, respectively. $(NH_4)_2SO_4$ treatment up-regulated *ACS11* expression by 606.3%, while *ACS11* expression in *chl1-1* was up-regulated by only 69.9% but inhibited in *chl1-5* by 36.8% (Fig. 7, G and H). Thus, it is apparent that ethylene synthesis and plant senescence are associated with NRT1.1-related NH_4^+ toxicity.

By using the ethylene signal mutant *ein2*, along with foliar application of the ethylene precursor ACC and the ethylene antagonist aminoethoxyvinylglycine (AVG) to the wild type and *NRT1.1* mutant lines, we found that the disruption of ethylene signaling did not induce plant chlorosis, although the NH_4^+ concentration in *ein2* was as high as that in Col-0 (Supplemental Fig. S7, A–C) and the inhibition of ethylene synthesis suppressed the effect of high NH_4^+ (Supplemental Fig. S7, D and E).

DISCUSSION

Nitrate-Independent Function of NRT1.1 in NH_4^+ Toxicity

Chlorosis and decline in biomass are the typical phenotypic markers of NH_4^+ toxicity in plants grown

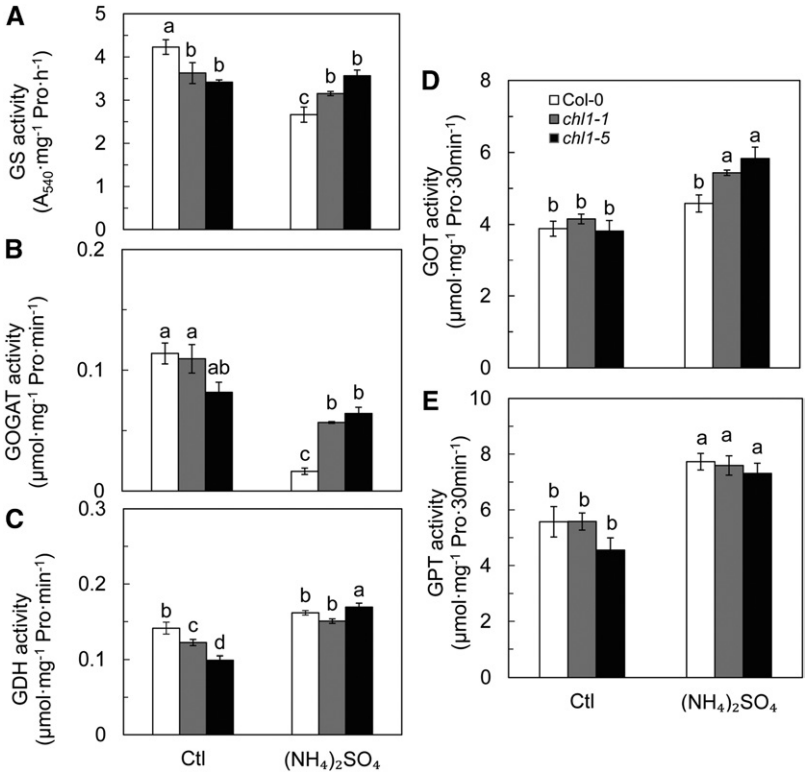

Figure 4. Activities of NH_4^+ assimilation enzymes and transaminases in roots of Col-0, *chl1-1*, and *chl1-5* plants. GS (A), GOGAT (B), GDH (C), GOT (D), and GPT (E) activities in roots of Col-0, *chl1-1*, and *chl1-5* plants grown in 5 mM NH_4NO_3 (Ctl) and $(NH_4)_2SO_4$ for 10 d are shown. Data represent means ± SE (n = 4). Bars with the same letter are not significantly different at $P < 0.05$ using LSD.

under high concentrations of sole NH_4^+ sources (Chen et al., 2013; Bittsánszky et al., 2015). These phenotypes are consistent with the reduced photosynthetic rate and chlorophyll concentration that were observed in Arabidopsis wild-type plants in this study (Fig. 1, A–E). Our results agree with those reported by Hachiya and coauthors (Hachiya and Noguchi, 2011; Hachiya et al., 2011) that the *NRT1.1* knockout mutants *chl1-1* and *chl1-5* exhibited stronger resistance to NH_4^+ toxicity relative to Col-0, showed weaker chlorosis, and experienced less reduction in biomass, photosynthetic rate, and chlorophyll concentration (Fig. 1, A–E).

These results suggest that NRT1.1 is responsible for NH_4^+ toxicity in Arabidopsis grown with a high concentration of NH_4^+ as the sole source of N. Our grafting experiment showed that the chlorosis induced by NH_4^+ occurred only in the presence of normal NRT1.1. This result further demonstrated that NH_4^+ toxicity in Arabidopsis is NRT1.1 related regardless of its expression in roots or shoots (Fig. 1, F and G).

NRT1.1 is a multifunctional gene that participates in plant development and in the interaction between plants and their environment (Bouguyon et al., 2015). In addition to being an NO_3^- transporter, NRT1.1 serves as an NO_3^- sensor in roots and also is a stomatal regulator in guard cells (Guo et al., 2003). *NRT1.1* expression in guard cells mediates the accumulation of NO_3^- in guard cells during stomatal opening (Guo et al., 2003). Stomatal closure increases the resistance of CO_2 diffusion from the ambient air into the intercellular space of leaves and, consequently, limits the rate of photosynthesis (Lawlor and Tezara, 2009). In this study, stomatal conductance in Col-0, *chl1-1*, and *chl1-5* showed no significant response to $(NH_4)_2SO_4$ treatment (Supplemental Fig. S2), suggesting that the NO_3^- transport and stomatal regulation functions of NRT1.1 are independent from NH_4^+ toxicity in Arabidopsis.

NRT1.1 can function in the absence of nitrate. The existence of a discrete nitrate-independent signaling pathway from NRT1.1 has been suggested by Hachiya et al. (2011). Since the *chl1-9* mutant shows normal function in primary nitrate signaling, even though it is defective in nitrate transport (Ho et al., 2009), the similar symptoms of *chl1-9* and Col-0 in response to $(NH_4)_2SO_4$ (Fig. 1, H and I) suggest that a specific deficiency in NO_3^- transport capacity is not sufficient to induce NH_4^+ toxicity in Arabidopsis in the absence of NO_3^-; the signaling function of

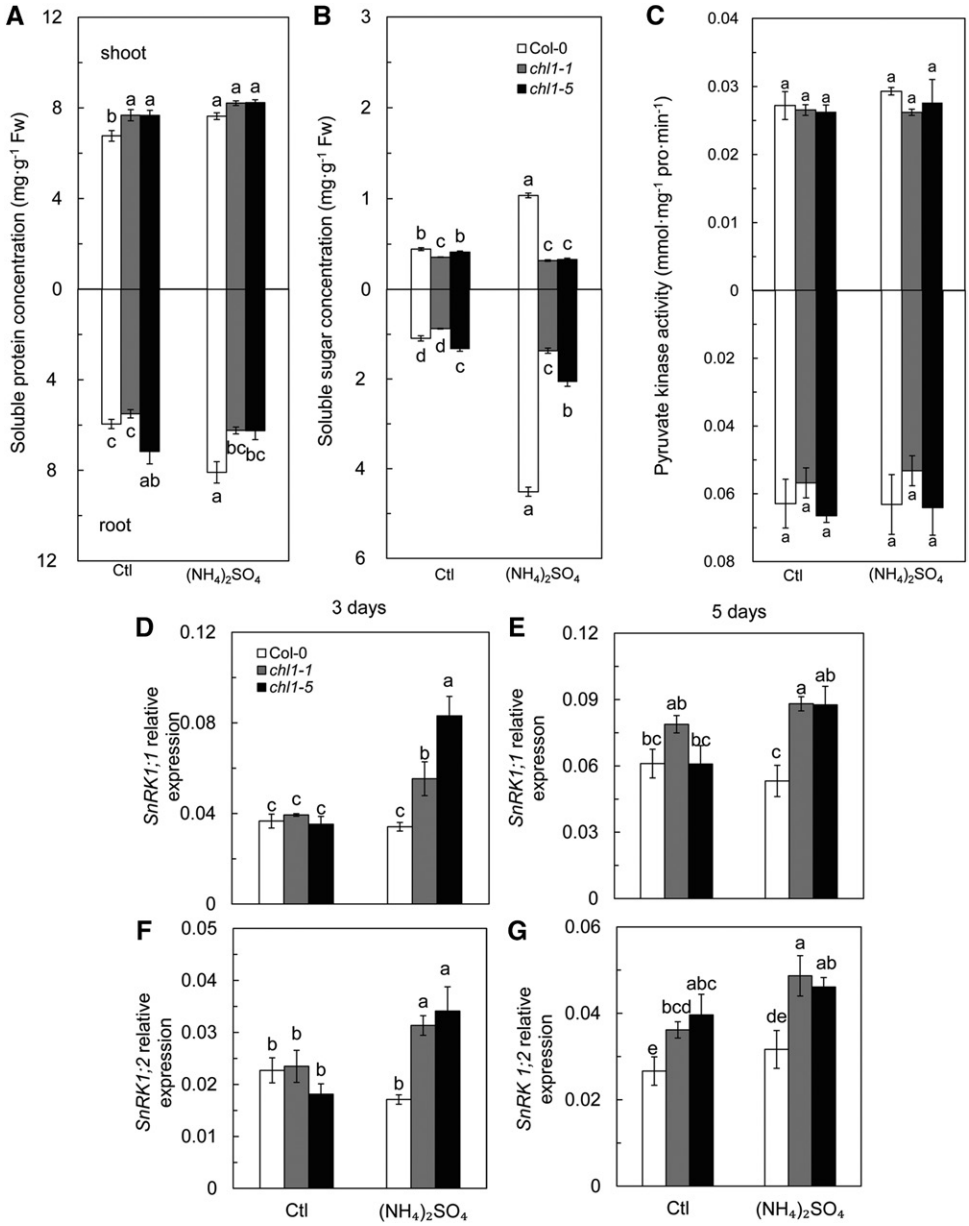

Figure 5. Effects of high concentration of NH_4^+ as the sole source of N on C and N metabolism. Soluble protein (A), soluble sugar content (B), activity of pyruvate kinase (C), and the expression of *SnRK1* genes (D–G) in Col-0, *chl1-1*, and *chl1-5* plants are shown. D and F represent *SnRK1;1* and *SnRK1;2* expression after 3 d, and E and G represent *SnRK1;1* and *SnRK1;2* expression after 5 d of $(NH_4)_2SO_4$ treatment. Plants measured in A to C were grown in 5 mM NH_4NO_3 (Ctl) and $(NH_4)_2SO_4$ media for 10 d. Data represent means ± SE ($n = 4$). Bars with the same letter are not significantly different at $P < 0.05$ using LSD. Fw, Fresh weight.

NRT1.1 could play an important role in mediating the effects of NH_4^+.

This notion was verified indirectly by the finding that the grafted plants with a Col-0 shoot showed more serious chlorosis than those with Col-0 roots when grown under $(NH_4)_2SO_4$. As the phosphorylation state and NRT1.1 level are similar in both Col-0 and *chl1-9* (Hachiya and Noguchi, 2011), we speculate that the altered phosphorylation state of NRT1.1 and the interaction between NRT1.1 and other proteins in metabolic pathways could be related to the occurrence of NH_4^+ toxicity. The difference in the degree of chlorosis in grafted plants with the shoot or roots of Col-0 also suggests that there could be different mechanisms in the shoot and roots through which NRT1.1 regulates the occurrence of NH_4^+ toxicity. Further studies are needed to elucidate the detailed mechanisms underlying the nitrate-independent signaling pathway of NRT1.1 in the regulation of NH_4^+ toxicity.

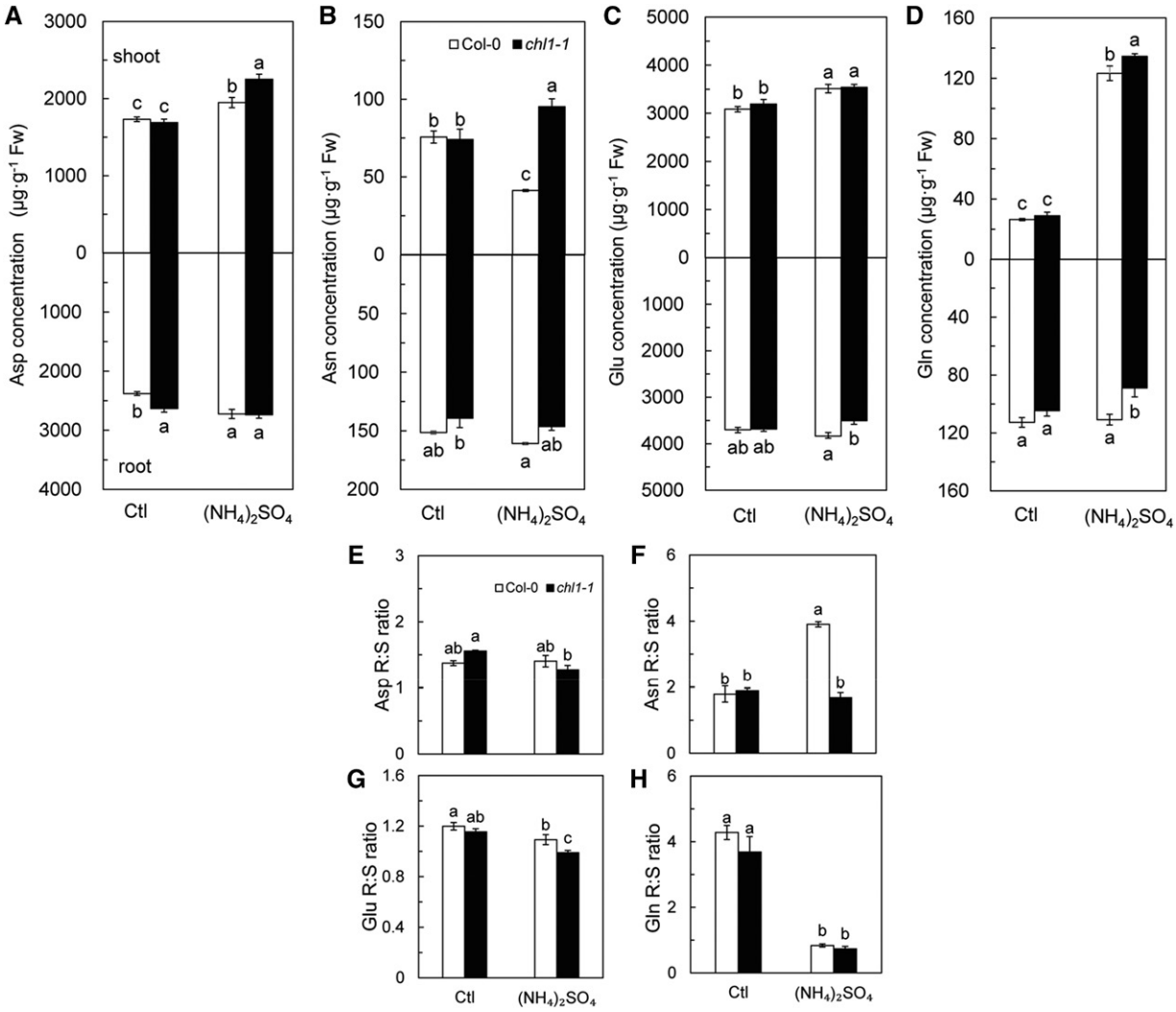

Figure 6. Effects of high concentration of NH_4^+ as the sole source of N on free amino acid concentration and their R:S ratios in Col-0 and *chl1-1*. Plants were grown in 5 mM NH_4NO_3 (Ctl) and $(NH_4)_2SO_4$ media for 10 d. Asp (A) Asn (B), Glu (C), and Gln (D) were measured in the shoots and roots, and then the R:S ratios of Asp (E), Asn (F), Glu (G), and Gln (H) were calculated. Data represent means ± SE ($n = 3$). Bars with the same letter are not significantly different at $P < 0.05$ using LSD. Fw, Fresh weight.

NRT1.1 Induces NH_4^+ Accumulation in Tissues under High NH_4^+ as the Sole Source of N

Excessive accumulation of NH_4^+ in plant tissues is believed to be one of the major causes of injury in plants under unfavorable conditions (Barker, 1999; Hoai et al., 2003; Nguyen et al., 2005). The accumulation of NH_4^+ is the first factor that is associated with NH_4^+ toxicity (Hachiya et al., 2012). In this study, NH_4^+ accumulated significantly in Col-0 but not in *chl1-1* and *chl1-5* under high $(NH_4)_2SO_4$ conditions (Fig. 2C). NH_4^+ equilibrium in plant cells is determined by NH_4^+ uptake, transport, and assimilation (Nguyen et al., 2005). Hachiya et al. (2011) proposed that increased NH_4^+ assimilation due to improved growth, rather than a decrease in NH_4^+ uptake, lowered the accumulation of NH_4^+ in *NRT1.1* mutants. In contrast, we found that the biomass and plant size of the wide type and *NRT1.1* mutant lines decreased under $(NH_4)_2SO_4$ treatment and that the increased accumulation of NH_4^+ in Col-0 (or the reduced accumulation of NH_4^+ in *NRT1.1* mutants) was caused largely by increased (or reduced) NH_4^+ uptake, according to $^{15}NH_4^+$ tracer and NH_4^+ flux experiments (Fig. 2, D–F; Supplemental Fig. S3). A rice variety sensitive to NH_4^+ toxicity displayed higher NH_4^+ accumulation and futile NH_4^+ cycling across the root-cell plasma membrane (Chen et al., 2013). However, in Arabidopsis, the efflux of NH_4^+ in *chl1-1* and *chl1-5* under high-NH_4^+ conditions could be beneficial by reducing NH_4^+ accumulation and alleviating its toxicity (Supplemental Fig. S3). Furthermore, the up-regulation of *AMT1* genes, which mediate the majority of NH_4^+ uptake in Arabidopsis roots (Yuan et al., 2007a), is associated

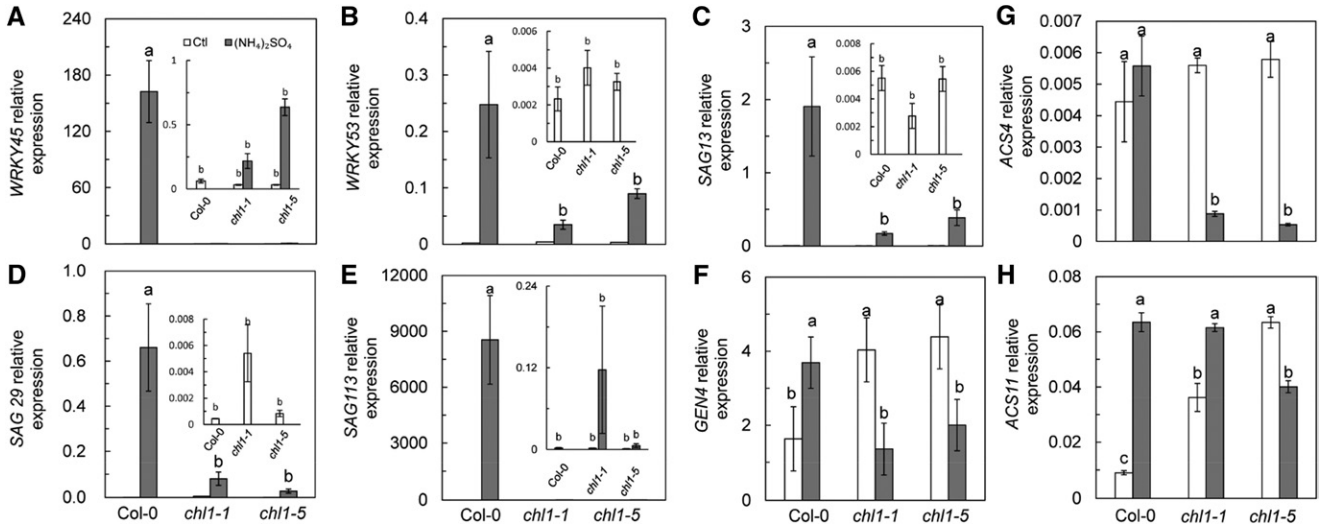

Figure 7. Gene expression related to senescence and 1-aminocyclopropane carboxylic acid (ACC) synthesis in leaves of Col-0, *chl1-1*, and *chl1-5*. Expression of *WRKY45* (A), *WRKY53* (B), *SAG13* (C), *SAG29* (D), *SAG113* (E), *GEN4* (F), *ACS4* (G), and *ACS11* (H) in Col-0, *chl1-1*, and *chl1-5* grown in 5 mM NH_4NO_3 (Ctl) and $(NH_4)_2SO_4$ media is shown. A to F show genes related to plant senescence and were measured 10 d after treatment, and G and H show genes related to ACC synthesis and were measured 3 d after treatment. Data represent means ± SE (n = 4). Bars with the same letter are not significantly different at $P < 0.05$ using LSD.

with the increased NH_4^+ accumulation in Col-0 (Fig. 3, A–D).

NRT1.1 can generate an unknown signal to regulate *NRT2.1* expression, hence, ensuring significant NO_3^- uptake in the presence of NH_4^+ in the external environment (Muños et al., 2004). Here, the distinct expression of *AMT1s* between Col-0 and *NRT1.1* mutants under NH_4^+ stress suggests that *NRT1.1* could be related to the response of *AMT1* genes to external NH_4^+ modification (Fig. 3, A, C, and D).

Proton secretion is accompanied by NH_4^+ uptake, leading to acidification of the growth medium. The acidification of medium has severe negative effects on Arabidopsis growth (Fang et al., 2016). Chaillou et al. (1991) suggested that NH_4^+ uptake induces a reduction in pH in the rhizosphere, which is one of the major factors of NH_4^+ toxicity. Our data excluded the effect of medium acidification on NH_4^+ toxicity, because the pH of the medium was similar among genotypes (Supplemental Fig. S8). Furthermore, although the *chl1* mutant has a pH-dependent phenotype (Fang et al., 2016), it was unharmed under a high concentration of NH_4^+. Contrarily, exogenously applied NH_4^+ acidifies the apoplast in plant tissues. However, NH_4^+ is not always related to cytosolic acidification in plant shoots (Husted and Schjoerring, 1995; Hachiya et al., 2012).

The capacity to ameliorate NH_4^+ toxicity has been viewed as an important factor in alleviating the consequences of this stress (Hoai et al., 2003; Nguyen et al., 2005). The GS-GOGAT cycle continuously provides the substrate Glu for the incorporation of NH_4^+ into Gln, a process that removes excess NH_4^+, thereby reducing toxicity symptoms (Bittsánszky et al., 2015). The reduced activities of GS and GOGAT in Col-0 under high-$(NH_4)_2SO_4$ conditions observed in this study indicated that attenuated NH_4^+ assimilation capacity contributed to the accumulation of NH_4^+ (Figs. 2C and 4, A and B). NADH-GDH plays a unique role independent of GS-GOGAT in N recycling (Masclaux-Daubresse et al., 2006). It has been widely reported that GDH responds positively to abiotic stress and is important for the detoxification of NH_4^+ under stress conditions (Boussama et al., 1999; Bittsánszky et al., 2015; Zhong et al., 2017). Compared with Col-0, the significantly higher activities of GDH, GS, and GOGAT in *chl1-1* and *chl1-5* under high-$(NH_4)_2SO_4$ conditions could decrease the accumulation of NH_4^+ (Fig. 4, A–C). Our results reveal that the higher N assimilation capacity of *chl1-1* and *chl1-5* mutants plays a vital role in mitigating NH_4^+ toxicity.

Taken together, this study revealed that enhanced NH_4^+ uptake accompanied by reduced NH_4^+ assimilation causes higher NH_4^+ accumulation in Col-0 than in the *NRT1.1* mutants when grown with high concentrations of $(NH_4)_2SO_4$. NH_4^+ uptake and assimilation are tightly linked. Gln is the actual signal regulating NH_4^+ uptake and assimilation (Tabuchi et al., 2007). The expression of the cytosolic isoform of GS, *AtGLN1;2*, is induced strongly in the roots by NH_4^+, contributing to NH_4^+ assimilation in the root (Konishi et al., 2017). Furthermore, differences in NH_4^+ uptake and assimilation between Col-0 and *NRT1.1* mutants suggest that NRT1.1 plays a significant role in regulating these processes. Bouguyon et al. (2015) hypothesized that NRT1.1 affects AMT1s by regulating N assimilation enzymes, as NRT1.1 regulates the expression level of

several genes involved in the N assimilation pathway. The substantial reductions in the activities of GS and GOGAT in Col-0 plants grown in $(NH_4)_2SO_4$ medium (Fig. 4, A and B) suggest that the pathway for N assimilation regulated by NRT1.1 is perturbed by high concentrations of NH_4^+.

Numerous studies have demonstrated the combined effects of ethylene production and NH_4^+ accumulation in the development of NH_4^+ toxicity symptoms in plants (You and Barker, 2005; Li et al., 2011, 2013). Ethylene production could be the link between NH_4^+ accumulation and the symptoms of NH_4^+ toxicity, as the inhibition of ethylene production ameliorated the symptoms of NH_4^+ toxicity even when NH_4^+ concentration in leaves was high (Li et al., 2013). In our study, the accumulation of NH_4^+ was consistent with the up-regulation of ACC synthesis genes and senescence-related genes (Fig. 7). In addition, the ethylene mutant *ein2* grown in $(NH_4)_2SO_4$ medium was not affected, although the concentration of NH_4^+ was comparable to that in Col-0. NH_4^+ toxicity symptoms in Col-0 were alleviated by the application of AVG, whereas NH_4^+ toxicity symptoms in *chl1-1* and *chl1-5* were accelerated by the application of ACC (Supplemental Fig. S8, A–E). These results suggest that NH_4^+ accumulation-induced ethylene synthesis and plant senescence are the two major reasons for the development of NH_4^+ toxicity symptoms.

NRT1.1-Related Perturbation of C and N Metabolism under High Concentration of NH_4^+ as the Sole Source of N

The interaction between C and N metabolism is of paramount importance to improve plant stress tolerance (Reguera et al., 2013). The assimilation of NH_4^+ requires carbohydrates to provide the C skeletons of 2-oxoglutarate (2-OG) generated from the tricarboxylic acid cycle (Yuan et al., 2007b). Nguyen et al. (2005) proposed that a reduction in photosynthetic capacity and a decrease in 2-OG production might be responsible for the excessive accumulation of NH_4^+. The availability of sugar in plant tissues may regulate N (including NH_4^+) uptake and assimilation but depends on a series of C metabolic steps downstream of hexose phosphorylation (Lejay et al., 2003). PK is at a crucial position to control glycolysis and the assimilation of C to provide energy and C skeletons (2-OG) for N metabolism (Stitt, 1999). In this study, the activity of PK was statistically similar in Col-0 and *NRT1.1* mutants and between experimental treatments, suggesting that the availability of C skeletons is not a limiting factor in N assimilation (Fig. 5C). The significant increase in the accumulation of sugars in Col-0 plants under NH_4^+ stress suggested that C and N metabolism in Col-0 were uncoupled when plants were exposed to high concentrations of NH_4^+ in the medium (Fig. 5B).

Complex regulatory mechanisms are involved in the coordination between N and C metabolism, including sensing of sugars, nitrate, and amino acids, as well as the effects of phytohormones (Nunes-Nesi et al., 2010). SnRK1 plays a vital role in plant energy homeostasis, growth and development, and stress tolerance (Baena-González et al., 2007; Jossier et al., 2009) by regulating the key enzymes involved in C and N metabolism. The up-regulated expression of *SnRK1;1* and *SnRK1;2* in *NRT1.1* mutants was consistent with the increased N metabolism and resistance to NH_4^+ toxicity, suggesting that *SnRK1* plays an essential role in the homeostasis of C and N metabolism under NH_4^+ stress (Fig. 5, D–G).

The accumulation of NH_4^+ often is accompanied by the accumulation of free amino acids (Hoai et al., 2003), probably due to the diminished demand for NH_4^+ during amino acid biosynthesis. In this study, however, *chl1-1* presented a higher amino acid content and a lower NH_4^+ content under $(NH_4)_2SO_4$ conditions; this could have resulted from greater NH_4^+ assimilation capacity and higher amino acid biosynthesis. Gln and Asn are important amides for organic N storage and transport in plant tissues. They function as important signals that reflect the N status in plants and mediate NH_4^+ uptake and assimilation (Tabuchi et al., 2007; Konishi et al., 2017). The accumulation of Gln and Asn was less in the roots of *chl1-1* than in the roots of Col-0 during $(NH_4)_2SO_4$ treatment (Fig. 6, E and F), which potentially benefits *chl1-1* plants. This is because less root Gln and Asn accumulation might increase the requirement for NH_4^+ incorporation into de novo amino acids and, thus, mitigate NH_4^+ accumulation. Additionally, Asn plays an important role in NH_4^+ detoxification (Masclaux-Daubresse et al., 2006). The significantly higher Asn concentration in $(NH_4)_2SO_4$-treated *chl1-1* plants compared with that in Col-0 indicates that Asn could have contributed to the lower accumulation of NH_4^+ in *chl1-1* (Fig. 6B).

N in rooting medium affects the accumulation of amino acids in plants (Noguchi et al., 2015). Increased amino acid synthesis can be advantageous for the adaptation to stress and for the accumulation of precursors for biosynthetic pathways (Joshi et al., 2010). Glu can be converted into Asp and Ala by GOT and GPT, respectively (Brock et al., 1970) and used subsequently for the synthesis of branched-chain amino acids. Branched-chain amino acids such as Met, Thr, and Ile are derived from the Asp pathway and act as precursors for many plant secondary metabolites such as ethylene, which are involved in the responses of plants to stresses (Joshi et al., 2010). In this study, *chl1-1* and *chl1-5* showed enhanced GOT activity in the roots under $(NH_4)_2SO_4$ treatment (Fig. 4D), which was accompanied by significant increases in the intracellular pools of Ile and Thr (Supplemental Fig. S6). Thus, our results suggest that the Asp-derived pathway for amino acid synthesis is of great significance to improve the tolerance of plants to NH_4^+ toxicity.

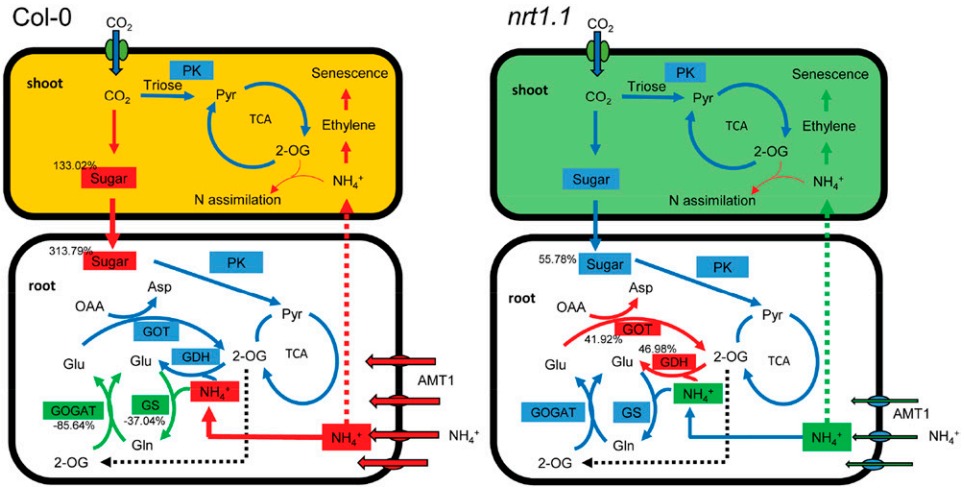

Figure 8. Proposed model for NH_4^+ metabolism in Col-0 and NRT1.1 mutants grown under high concentration of NH_4^+ (10 mM) as the sole source of N. Effects in Col-0 cells (A) and *nrt1.1* mutants cells (B) are shown. The colored rectangles are as follows: red, molecules or physiological processes that increased; blue, molecules or physiological processes that did not change significantly; and green, molecules or physiological processes that decreased. Red or green dotted arrows represent NH_4^+ flow from roots to shoots speculated as being higher or lower. Red or green solid arrows represent the physiological processes getting stronger or weaker; blue solid arrows represent physiological processes that were not changed significantly; and numbers nearest to a physiological process indicate the change in percentage of that process. Compared with plants grown under control conditions, *NRT1.1* up-regulates the expression of *AMT1* genes in roots and facilitates NH_4^+ uptake. Simultaneously, GS and GOGAT activities are inhibited by NH_4^+ as the sole source of N. Furthermore, sugars accumulate and are stored in roots, disturbing C and NH_4^+ metabolism. As a result, free NH_4^+ increases and can be transported to the shoot. High NH_4^+ concentrations in the shoot induce ethylene synthesis, which causes plant senescence. In *NRT1.1* mutants, NH_4^+ uptake, the GS-GOGAT cycle, and C and NH_4^+ metabolism are not affected by NH_4^+ as the sole source of N, and the activities of NADH-dependent GDH and GOT are enhanced under NH_4^+, which diminish NH_4^+ accumulation and improve amino acid metabolism. Thus, *NRT1.1* mutant plants can better survive environments with high concentrations of NH_4^+ as the main source of N than the wild type. TCA, Tricarboxylic acid; Pyr, pyruvate; OAA, oxaloacetic acid.

CONCLUSION

Our results suggest that NRT1.1 plays a signaling role in mediating NH_4^+ uptake and downstream NH_4^+ assimilation into amino acid metabolism, thus playing a role in NH_4^+ toxicity. In wild-type Arabidopsis, *NRT1.1* increased the expression of *AMT1s* and NH_4^+ uptake when plants were grown in the presence of high concentrations of a sole NH_4^+ source. However, the ability to assimilate NH_4^+ decreased when plants were grown under such conditions. Furthermore, with a sole NH_4^+ source, the metabolism of C and N was uncoupled, causing an accumulation of soluble sugars and reduced amino acid metabolism. As a result, NH_4^+ accumulated, increasing the production of ethylene and the expression of genes related to plant senescence and, finally, NH_4^+ toxicity (Fig. 8A).

In the *NRT1.1* mutants *chl1-1* and *chl1-5*, the expression of *AMT1s* was lower than that in Col-0. In addition, the metabolism of C and N in the mutants was less affected by the sole NH_4^+ source, and NH_4^+ assimilation by the GS-GOGAT and GDH pathways and amino acid synthesis by GOT led to reductions in the accumulation of NH_4^+. Ultimately, chlorosis did not occur in *NRT1.1* mutants (Fig. 8B). Clearly, the interaction between nitrate and AMTs is complex and is an attractive research area. To further explore the regulatory pathways that involve NRT1.1 during NH_4^+ uptake, transport, and assimilation is of great importance to further elucidate the mechanisms of NH_4^+ toxicity and to develop effective approaches for NH_4^+ nutrient management.

MATERIALS AND METHODS

Experimental Materials and Growth Conditions

Seeds of wild-type Arabidopsis (*Arabidopsis thaliana* Col-0), *NRT1.1* knockout mutants (*chl1-1* and *chl1-5*), an *NRT1.1* point mutation (*chl1-9*) line, the *amt1;1* mutant, and an ethylene signal mutant (*ein2*) were sown in nutrient-supplemented soil medium in a growth chamber (300 µmol photons m^{-2} s^{-1}, 16-h photoperiod, and 22°C) and allowed to germinate and grow for 10 d. Subsequently, the seedlings with one pair of true leaves were transplanted in 600-mL pots and grown hydroponically using nutrient medium containing 1.125 mM NH_4NO_3, 1.25 mM KCl, 0.625 mM KH_2PO_4, 0.5 mM $MgSO_4$, 0.5 mM $CaCl_2$, 1.25 µM Fe-EDTA, 17.5 µM H_3BO_3, 3.5 µM $MnCl_2$, 0.25 µM $ZnSO_4$, 0.05 µM $NaMoO_4$, and 0.125 µM $CuSO_4$. The pH of the nutrient medium was adjusted to 6, and MES (2.5 mM) was added to the nutrient medium to buffer changes in pH. The medium was refreshed every 4 d. Nine plants were grown in each pot, and the growth conditions were the same as described above. After 4 weeks, half of the plants were transferred to 5 mM $(NH_4)_2SO_4$, 5 mM $(NH_4)_2C_4H_6O_4$, or 10 mM NH_4Cl medium for an additional 10 d, with the control pots supplied with 5 mM NH_4NO_3. To assess the effect of SO_4^{2-} on NH_4^+ toxicity, 4-week-old

seedlings were treated with 5 mM $(NH_4)_2SO_4$ medium or 5 mM NH_4NO_3 medium containing 5 mM K_2SO_4 for 10 d, with the control supplied with 10 mM KNO_3. The experiment was arranged in a completely randomized design. The positions of the pots were interchanged when refreshing the medium to eliminate edge effects.

^{15}N Pulse Assay

To investigate the uptake of NH_4^+ during $(NH_4)_2SO_4$ treatment, Col-0, *chl1-1*, and *chl1-5* seedlings were hydroponically grown for 4 weeks, then divided into two groups for ^{15}N labeling with 5% atom abundance of $(^{15}NH_4)_2SO_4$, with one group at the start of $(NH_4)_2SO_4$ treatment and the other at 5 d after the start of the treatment. To perform a short-term NH_4^+ uptake analysis, 99.9% atom abundance of $(^{15}NH_4)_2SO_4$ was used for labeling for 5 and 10 min at 10 AM. Growth conditions were as described above. Before ^{15}N labeling, the roots were washed with 0.1 mM $CaSO_4$ for 1 min and then placed in growth medium containing 5 mM $(^{15}NH_4)_2SO_4$ for 5 d. While sampling, the roots were washed again with 0.1 mM $CaSO_4$ for 1 min and then with deionized water. The root and shoot tissues were harvested separately and oven dried at 70°C for 48 h. The samples were pulverized with TissueLyser-48 (Jingxin), and ^{15}N abundance in the samples was determined using a continuous-flow isotope ratio mass spectrometer coupled with a C-N elemental analyzer (ANCA-MS; PDZ Europa).

Grafting Experiment

A grafting experiment was performed as described by Rus et al. (2006) using an SZX-ILLB200 microscope (Olympus Optical). Col-0 and *chl1-5* seeds were sterilized with 75% ethanol and germinated on one-half-strength Murashige and Skoog plates containing 3 mg L^{-1} benomyl, 0.04 mg L^{-1} benzyladenine, 0.02 mg L^{-1} indole-3-acetic acid, and 17 g L^{-1} agar. All the plates with stratified seeds were placed vertically in a growth chamber as described above. Five-day-old seedlings were grafted on the plate with a MANI Ophthalmic Knife (Straight 15; SWISSMED). The grafted seedlings were placed on plates for another 5 d until the graft union formed. The grafted seedlings without adventitious roots were grown hydroponically and treated with $(NH_4)_2SO_4$ as described.

Determination of NH_4^+ Flux at the Root Surface with NMT

NMT is a method that measures the gradient of specific molecules or ions by means of a selective flux microsensor vibrating repeatedly between two predefined points. The molecular/ionic fluxes are calculated based on Fick's law of diffusion: $J = -D_0 \cdot (dc/dx)$, where J is the ion flux (pmol cm^{-2} s^{-1}), dc is the concentration gradient, dx is the distance between the two points, and D_0 is the diffusion constant. The direction of flux is derived from Fick's law of diffusion.

The experiment was carried out at the YoungerUSA-Xuyue BioFunction Institute using NMT Physiolyzer (YoungerUSA, Xuyue) and imFluxes V2.0 (YoungerUSA) software. Measurements were performed at room temperature (24°C–26°C). Before determination, the roots were equilibrated immediately in measuring solution [5 mM $(NH_4)_2SO_4$, 0.1 mM $CaCl_2$, and 0.3 mM MES, pH 6] for 10 min, then transferred to the measuring chamber, a small dish containing measuring solution. The ion flux microsensor was calibrated using different NH_4^+ concentrations [10 mM $(NH_4)_2SO_4$ and 1 mM $(NH_4)_2SO_4$, with other conditions consistent with the test solution] before NH_4^+ flux measurement. An NH_4^+-selective microsensor used for the net NH_4^+ flux measurement was vibrated in two measuring solutions between two positions, 1 and 30 μm at the meristem. Only the flux microsensor with a Nernstian slope > 50 mV $decade^{-1}$ was used in this study. The same flux microsensor was calibrated again according to the same procedure and standards after each test. The μV differences were exported as raw data before they were imported and converted into net NH_4^+ fluxes using the JCal V3.3 software (a free MS Excel spreadsheet; YoungerUSA, Xuyue). Four plants were used for measurements in each treatment with each plant measured once.

Ethylene Regulation Experiment

Four-week-old Col-0, *chl1-1*, and *chl1-5* seedlings were divided into three groups for $(NH_4)_2SO_4$ treatment. The leaves of the first and second groups were sprayed with 5 μM AVG and 50 μM ACC, respectively, at 8 AM every day throughout the $(NH_4)_2SO_4$ treatment period. The third group was sprayed with deionized water and served as the control. Each plant was sprayed until the leaves were thoroughly wetted. After 10 d of treatment, chlorophyll concentration was determined and photographs were taken to determine the phenotypes.

Photosynthesis Measurements

Photosynthesis of the youngest fully expanded leaves in rosettes was measured 10 d after $(NH_4)_2SO_4$ treatment using an LI-6400XT portable photosynthesis system (Li-Cor). Air temperature in the cuvette during measurement was maintained at 22°C, and the photosynthetic photon flux intensity was 200 μmol m^{-2} s^{-1}. The CO_2 concentration in the cuvette was adjusted to 500 μmol mol^{-1} with a CO_2 mixer, and the vapor pressure deficit (VPD) was at 1 to 1.5 kPa. Data were recorded after equilibration to a steady state. After photosynthetic determination, the leaf area was measured with a leaf area meter (CI-202 Portable Laser Area Meter; CID Bio Science). The data of gas-exchange parameters were adjusted to the actual leaf areas.

Measurement of Chlorophyll Concentration

Chlorophyll in fresh rosette leaves of NH_4NO_3 (control)- and $(NH_4)_2SO_4$-treated plants was extracted according to the method of Wellburn and Lichtenthaler (1984) with 80% (v/v) acetone. The absorbance of the extract was measured at 663 and 645 nm using a UV-VIS spectrophotometer (UV-2600; Shimadzu) to estimate total chlorophyll concentrations.

Measurements of Biomass, Total Nitrogen, and NH_4^+ and Soluble Sugar Concentrations

Control and $(NH_4)_2SO_4$-treated plants were harvested, and roots and aerial parts were separated, oven dried at 70°C to a constant weight, and then ground to a fine powder. About 100 mg of each sample was digested with H_2SO_4-H_2O_2 at 350°C for N quantification using a high-resolution digital colorimeter (Autoanalyzer 3; SEAL).

NH_4^+ and soluble sugar were extracted from each sample with deionized water. The NH_4^+ concentration was measured colorimetrically using the method of Indophenol Blue colorimetry at 630 nm (Zanini, 2001), with $(NH_4)_2SO_4$ used as a standard. Soluble sugar concentration was determined by the anthrone-sulfuric acid method (Wang et al., 2002).

Amino Acid Quantification

Amino acids in roots and shoots were quantified using HPLC (del Campo et al., 2009). Samples were harvested and immediately frozen in liquid N_2, then stored at −80°C until analyses. Frozen leaf samples (200 mg) were pulverized with liquid N_2 and homogenized in 1.5 mL of 0.1% phenol and 6 M HCl. The homogenates were hydrolyzed for 22 h at 100°C and cooled, and 1 mL of hydrolysate was dried with organomation (NDK200-2; Hangzhou Mi'ou Instrument) and redissolved in 1 mL of 0.1 M HCl. To determine the amino acids, 200 μL of redissolved hydrolysate was mixed with 20 μL of nor-Leu internal standard solution, 200 μL of triethylamine acetonitrile (pH > 7), and 100 μL of isothiocyanate acetonitrile, then incubated at 25°C for 1 h. Then, 400 μL of hexane was added and the samples were incubated for 10 min with shaking. The underlayer solution was filtered with a 0.45-μm syringe filter. The analyses were performed on a Rigol L3000 device (Beijing Rigol Technology). Chromatographic separation was accomplished using an RP-HPLC ACE column (5C18-HL) with particle size of 5 μm (250 mm × 4.6 mm) at room temperature through a binary gradient. Mobile phase A was 25 mM acetate buffer (pH 6.5) and 70 mL acetonitrile. Mobile phase B was 80% acetonitrile aqueous solution. The flow rate was 1 mL min^{-1} and the column temperature was 40°C.

Enzyme Activity Assays

Frozen samples (100 mg) were powdered with liquid N and homogenized with 3 mL of 50 mM Tris-HCl buffer (pH 8) containing 2 mM Mg^{2+}, 2 mM DTT, and 0.4 M Suc. The homogenate was centrifuged at 10,000*g* and 4°C for 10 min. The supernatant was used to determine the activities of the N metabolic enzymes GS, NADH-GOGAT, NADH-GDH, GOT, and GPT.

The activities of GS and GOGAT were measured according to the method described by Zhang et al. (1997) and Singh and Srivastava (1986), respectively. The activity of NADH-GDH was determined according to the method of Loulakakis and Roubelakis-Angelakis (1990). GOT and GPT activities were assayed using the method described by Wu et al. (1998).

Frozen samples were pulverized in liquid N, and about 100 mg of each sample was used to measure PK activity according to Lepper et al. (2010) with some modifications. The samples were extracted with 3 mL of 100 mM Tris-HCl (pH 7.5) containing 10 mM β-mercaptoethanol, 12.5% (v/v) glycerine, 1 mM EDTA-Na_2, 10 mM $MgCl_2$, and 1% (m/v) polyvinylpyrrolidone-40. These solutions were centrifuged at 10,000*g* and 4°C for 10 min. The reaction mixture contained 100 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 0.16 mM NADH, 75 mM KCl, 5 mM ADP, 7 units of L-lactate dehydrogenase, and 1 mM phospho*enol*pyruvate. The reaction was started by adding 0.1 mL of enzyme extract to 0.9 mL of the reaction mixture and was carried out at 25°C to monitor the absorbance changes at 340 nm for 180 s.

Enzyme activities were expressed as mol of metabolite generated/consumed per mg of protein per unit of time. Soluble protein content was measured by the Bradford method with BSA as the standard (Bradford, 1976).

RNA Extraction and Reverse Transcription Quantitative PCR Assay

The seedlings were treated with 5 mM NH_4NO_3 and $(NH_4)_2SO_4$ for 3 d before roots and shoots were harvested separately for RNA analysis. Total RNA was extracted with TRIzol (Invitrogen), precipitated with an equal volume of isopropanol, washed with 75% ethanol, and dissolved in RNase-free water. cDNAs were synthesized using the PrimeScript RT Kit with gDNA Eraser (Perfect Real Time; Takara) following the protocol of the manufacturer. The relative expression of genes in roots and shoots was determined by reverse transcription quantitative PCR performed on an Applied Biosystems StepOne Real-Time PCR System with SYBR Premix Ex-Taq (Takara) according to the protocol of the manufacturer. Primers used in the assays are listed in Supplemental Table S1, and the expression data were normalized to *ACTIN2* or *SAND*.

Statistical Analysis

All experiments were conducted using a completely randomized design. Four samples used as replicates and two technical replicates were used for each treatment. Two-way ANOVA was conducted to analyze the effects of N source and plant material. Multiple comparisons were performed using the LSD multiple range test. The differences between control and $(NH_4)_2SO_4$ treatments in the same plant material were evaluated with Student's *t* test. Differences were considered statistically significant at $P < 0.05$.

Accession Numbers

Sequence data from this article can be found in the GenBank/EMBL data libraries under accession numbers AT3G18780 (ACTIN2), AT3G01970 (WRKY4), AT4G23810 (WRKY53), AT2G29350 (SAG13), AT5G13170 (SAG13), AT5G59220 (SAG113), AT4G30270 (SEN4), AT4G13510 (AMT1;1), AT1G64780 (AMT1;2), AT3G24300 (AMT1;3), AT3G24290 (AMT1;5), AT3G01090 (SnRK1.1), AT3G29160 (SnRK1.1), AT2G22810 (ACS4), AT4G08040 (ACS11), and AT2G28390 (SAND).

Supplemental Data

The following supplemental materials are available.

Supplemental Figure S1. Phenotypes and chlorophyll concentrations of Col-0, *chl1-1*, and *chl1-5* plants treated with $(NH_4)_2SO_4$, K_2SO_4, and KNO_3.

Supplemental Figure S2. Effects of higher $(NH_4)_2SO_4$ on the stomatal conductance of Col-0, *chl1-1*, and *chl1-5* plants.

Supplemental Figure S3. Effects of high concentrations of NH_4^+ as the sole source of N on NH_4^+ uptake using NMT.

Supplemental Figure S4. Phenotypes of Col-0 and *amt1;1* plants treated with 5 mM $(NH_4)_2SO_4$ for 10 d.

Supplemental Figure S5. Effects of $(NH_4)_2SO_4$ on the activity of NH_4^+ assimilation enzymes and transaminase in the shoots of Col-0, *chl1-1*, and *chl1-5* plants.

Supplemental Figure S6. Effects of $(NH_4)_2SO_4$ on free amino acid content in Col-0 and the *chl1-1* mutant.

Supplemental Figure S7. Ethylene is involved in *NRT1.1*-related NH_4^+ toxicity.

Supplemental Figure S8. Comparison of pH values of growth media.

Supplemental Table S1. Primer sequences for gene expression used in this study.

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