NRT1.1-dependent NH_4^+ toxicity in *Arabidopsis* is associated with disturbed balance between NH_4^+ uptake and assimilation

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Running title: NRT1.1-dependent NH_4^+ toxicity under sole NH_4^+ condition

Highlight: Nitrate transporter NRT1.1 enhances NH_4^+ accumulation, disturbs the NH_4^+ metabolism, and aggravates NH_4^+ toxicity in *Arabidopsis* when grown under sole NH_4^+ condition.

25 Abstract

26 A high concentration of a sole ammonium (NH_4^+) source in growth media is often
 27 toxic to plants. The nitrate transporter NRT1.1 is involved in plant NH_4^+ toxicity;
 28 however, its mechanism remains undefined. In this study, wild-type *Arabidopsis*
 29 (Col-0) and *NRT1.1* mutants (*chl1-1* and *chl1-5*) were grown hydroponically in
 30 NH_4NO_3 and $(\text{NH}_4)_2\text{SO}_4$ media to evaluate NRT1.1 function in NH_4^+ stress
 31 responses. All plants grew normally in mixed N sources, but Col-0 displayed more
 32 chlorosis, and lower biomass and photosynthesis than the *NRT1.1* mutants in the
 33 $(\text{NH}_4)_2\text{SO}_4$ condition. Grafting experiments between Col-0 and *chl1-5* further
 34 confirmed that NH_4^+ toxicity is NRT1.1-dependent. In $(\text{NH}_4)_2\text{SO}_4$ medium, NRT1.1
 35 facilitated the higher expression of NH_4^+ transporters, increasing NH_4^+ uptake.
 36 Additionally, glutamine synthetase (GS) and glutamate synthetase (GOGAT) in
 37 roots of Col-0 plants decreased and soluble sugar accumulated significantly, whereas
 38 pyruvate kinase (PK)-mediated glycolysis was not affected, all of which contributed
 39 to NH_4^+ accumulation. In contrast, the *NRT1.1* mutants reduced NH_4^+ accumulation
 40 and enhanced NH_4^+ assimilation through glutamate dehydrogenase (GDH) and
 41 glutamate-oxaloacetate transamination (GOT) activity. In addition, the upregulation
 42 of genes involved in senescence in Col-0 plants treated with $(\text{NH}_4)_2\text{SO}_4$ suggests that
 43 ethylene could be involved in NH_4^+ toxicity responses. Our results indicate that
 44 NH_4^+ toxicity is dependent on NRT1.1 in *Arabidopsis*, characterized by enhanced
 45 NH_4^+ accumulation and by perturbed NH_4^+ metabolism, which stimulated
 46 ethylene-induced plant senescence.

47 **Key words:** ammonium toxicity; ammonium uptake; ammonium assimilation;
 48 *Arabidopsis*; *CHL1/NRT1.1/NPF6.3*; ethylene

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53 **Abbreviations:**

54 *AMT*, ammonium transporter family; *CHL1/NRT1.1/NPF6.3*, nitrate transporter 1/peptide
55 transporter family; GS, glutamine synthetase; GOGAT, glutamate synthetase; GDH, glutamate
56 dehydrogenase; GOT, glutamate-oxaloacetate transaminase; GPT, glutamate-pyruvic
57 transaminase; PPFD, photosynthetic photon flux intensity; PK, pyruvate kinase.

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Introduction

Ammonium (NH_4^+) is a paradoxical inorganic nitrogen source in the soil that is available to plants during growth and development. It is the preferable N source for some species such as rice (*Oryza sativa* L.) and tea (*Camellia sinensis*) (Gao et al., 2010, Ruan et al., 2016). However, the high levels of NH_4^+ in the soil due to increased N fertilizer application, for example, is detrimental to higher terrestrial plants and can promote chlorosis, necrosis, and decreased biomass and yield (Chen et al., 2013; Li et al., 2013). To date, the primary underlying mechanisms of these forms of NH_4^+ toxicity in plants remain unclear (Li et al., 2013).

Excess NH_4^+ uptake and accumulation have been proposed to contribute to NH_4^+ toxicity in plants. Acidification of the rhizosphere from proton excretion during NH_4^+ uptake is considered to be the principal contributor to NH_4^+ toxicity (Chaillou et al., 1991); however, some studies suggest that the initial step of NH_4^+ toxicity is pH-independent (Britto and Kronzucker, 2002; van den Berg et al., 2005).

NH_4^+ serves as the ubiquitous intermediate metabolite in N metabolism in plants (Joy, 1988; Glass et al., 1997). Its high concentration in plant cells is damaging to plant tissues. NH_4^+ accumulation is commonly observed under high NH_4^+ conditions. In rice (*Oryza sativa* L.), the application of high levels of NH_4^+ resulted in NH_4^+ accumulation and futile NH_4^+ cycling (Chen et al., 2013). NH_4^+ accumulation occurs in part from NH_4^+ uptake. In *Arabidopsis thaliana*, the ammonium transporter (AMT) family consists of six protein isoforms, three of which are AtAMT1;1, AtAMT1;2, and AtAMT1;3 and are located in the root where they are responsible for about 90% of NH_4^+ uptake (Yuan et al., 2007a). 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. In addition, the capacity to detoxify excessive NH_4^+ accumulation in plant cells is thought to be an important metabolic factor for alleviating the consequence of this stress (Hoai et al., 2003). Glutamine synthetase (GS) acts in the center of NH_4^+ flow in ammonium metabolism (Mifflin and Habash, 2002), and glutamate dehydrogenase (GDH) is an

111 anti-stress enzyme involved in ammonia detoxification (Skopelitis et al., 2006). It
 112 has been reported that NH_4^+ -tolerant plants have greater GS activity and less NH_4^+
 113 accumulation in plant tissues (Cruz et al., 2006; Omari et al., 2010). Konishi et al.
 114 (2017) reported that *GLN1* plays a dominant role in regulating NH_4^+ uptake in
 115 *Arabidopsis*. Indeed, pharmacological studies suggest that Gln rather than NH_4^+
 116 could be the real signaling molecule that regulates the expression of NH_4^+ transport
 117 and assimilation genes (Tabuchi et al., 2007). It is suggested that NH_4^+ uptake and
 118 assimilation are integrated; nevertheless, the coordination between NH_4^+ uptake and
 119 assimilation during NH_4^+ toxicity remains unclear.

120 Ethylene serves as an intermediate for signal transduction and translation, and
 121 manipulates the physiological responses under stress (Ismail and Horie, 2017; Arraes
 122 et al., 2015; Zhang et al., 2014; Li et al., 2014; Cao et al., 2007). Conversely, severe
 123 stress causes disequilibrium in ethylene production and accelerates plant senescence
 124 (Djanaguiraman and Vara, 2010; Jing et al., 2005). The occurrence of stress
 125 symptoms frequently accompanies ethylene evolution and NH_4^+ accumulation under
 126 drought, salt stress, and NH_4^+ stress (Barker and Corey, 1991; Feng and Barker,
 127 1992). By exogenous application of NH_4^+ to leaves, Li et al. (2013) observed a
 128 significant increase in ethylene production and the inhibition of lateral root initiation
 129 in *Arabidopsis*. However, the relationship between NH_4^+ accumulation and ethylene
 130 evolution/production, as well as their contribution to stress induced symptoms differs
 131 between plant species. Lin et al. (2002) suggest that NH_4^+ accumulation in detached
 132 rice leaves does not change tissue sensitivity to ethylene, and ethylene is not
 133 involved in NH_4^+ -promoted senescence; however, the specifics of how ethylene
 134 could be involved in the development of NH_4^+ toxicity symptoms remain to be
 135 elucidated.

136 *NRT1.1* is the primary member of the nitrate transporter (*NRT*) gene family in
 137 higher plants (Tsay et al., 1993). It has multiple functions in physiological processes
 138 in plants such as NO_3^- and auxin transport, NO_3^- signal sensing, and stomatal
 139 movement (Tsay et al., 1993; Guo et al., 2003; Krouk G. et al., 2010; Bouguyon et
 140 al., 2015). Many studies have revealed that *NRT1.1* is essential for plants to defend

141 themselves against unfavorable environments, including cadmium stress, salt stress,
142 and proton toxicity (Mao et al., 2014; Aragón and Navarro, 2017; Fang et al., 2016).
143 Interestingly, the *Arabidopsis NRT1.1* mutants *chl1-1* and *chl1-5* display higher
144 resistance to high concentrations (10 mM) of sole NH_4^+ sources than that of its
145 wild-type plants (Hachiy et al., 2011; Hachiy and Noguch, 2011). However, the
146 mechanism for how *NRT1.1* mediates NH_4^+ uptake and the subsequent emergence of
147 NH_4^+ toxicity still need to be identified at physiological and molecular levels.

148 Here, we analyzed the gene expression, metabolites, and the physiological and
149 chemical activities in roots and shoots of wild type and *NRT1.1* mutant *Arabidopsis*
150 plants grown in nutrient growth media that contained high concentrations of NH_4^+ .
151 We aimed to clarify: 1) whether NH_4^+ toxicity symptoms are *NRT1.1*-dependent; 2)
152 the physiological role of NH_4^+ uptake and assimilation during NH_4^+ toxicity; and 3)
153 the function of ethylene in *NRT1.1*-mediated NH_4^+ toxicity. Our results provide
154 further insight into the understanding of NH_4^+ toxicity in plants.

155 **Materials and methods**

156 **Experimental materials and growth conditions**

157 Seeds of wild-type *Arabidopsis* (Col-0), *NRT1.1* knockout mutants (*chl1-1*,
158 *chl1-5*), an *NRT1.1* point mutation (*chl1-9*) line, and an ethylene signal mutant (*ein2*)
159 were sown in nutritional soil media in a growth chamber ($300 \mu\text{mol photons}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$,
160 16 h photoperiod, 22 °C) and allowed to germinate and grow for 10 d; then,
161 seedlings with one pair of true leaves were transplanted to 600-mL pots and
162 cultivated hydroponically with nutritional media. The growth media contained 1.125
163 mM NH_4NO_3 , 1.25 mM KCl, 0.625 mM KH_2PO_4 , 0.5 mM MgSO_4 , 0.5 mM CaCl_2 ,
164 1.25 μM Fe-EDTA, 17.5 μM H_3BO_3 , 3.5 μM MnCl_2 , 0.25 μM ZnSO_4 , 0.05 μM
165 NaMoO_4 , and 0.125 μM CuSO_4 . The pH of the nutrient media was adjusted to 6.0
166 and MES (2.5 mM) was added to the nutrient media to buffer any changes in the pH.
167 The media was refreshed every four days. There were 9 plants in each pot and the
168 growth condition was the same as mentioned above. After 4 weeks, half of the plants

169 were transferred to 5 mM (NH₄)₂SO₄ medium for an additional 10 d. The control
170 group was supplied with 5 mM NH₄NO₃. The experiment was arranged in a
171 completely randomized design. The position of the pots was interchanged when
172 refreshing the solution to eliminate any edge effects.

173 **¹⁵N pulse assay**

174 To investigate the uptake of NH₄⁺ during (NH₄)₂SO₄ treatment, Col-0, *chl1-1*, and
175 *chl1-5* seedlings were hydroponically cultivated for 4 weeks and then divided into
176 two groups for 5% atom abundance labeling (¹⁵NH₄)₂SO₄ (pH 6.0) at the start of the
177 sole NH₄⁺ treatment and 5 d after the treatment, respectively. Before ¹⁵N labeling,
178 roots were washed with 0.1 mM CaSO₄ for 1 min and then placed in 5 mM
179 (¹⁵NH₄)₂SO₄ medium for 5 days. When sampling, the roots were washed again with
180 0.1 mM CaSO₄ for 1 min and then with deionized water. Root and shoot tissues were
181 harvested separately and oven-dried at 70 °C for 48 h. The samples were ground into
182 powder with TissueLyser-48 (Jingxin Co. Ltd., China) and ¹⁵N abundance in samples
183 was determined using a continuous-flow isotope ratio mass spectrometer coupled
184 with a carbon-nitrogen elemental analyzer (ANCA-MS; PDZ Europa).

185 **Grafting experiment**

186 A grafting experiment was performed as described in [Rus et al. \(2006\)](#) using an
187 SZX-ILLB200 microscope (OLYMPUS OPTICAL CO., LTD, Japan). Col-0 and
188 *chl1-5* seeds were sterilized in 75% ethanol and germinated on ½ MS plates
189 containing 3 mg·L⁻¹ benomyl, 0.04 mg·L⁻¹ benzyladenine, 0.02 mg·L⁻¹
190 indole-3-acetic acid, and 17 g·L⁻¹ agar. All plates with stratified seeds were placed
191 vertically in a growth chamber as described above. Five-day-old seedlings were
192 grafted on the plate with a MANI Ophthalmic Knife (Straight 15, SWISSMED,
193 Japan). The grafted seedlings were placed on plates for another 5 d until the graft
194 union formed. Grafted seedlings without adventitious roots were cultivated
195 hydroponically and treated with (NH₄)₂SO₄ as described.

196 **Ethylene regulation experiment**

Four-week-old Col-0, *chl1-1*, and *chl1-5* seedlings were divided into three groups for (NH₄)₂SO₄ treatment. The leaves of the first and second groups were treated with 5 μM aminoethoxyvinylglycine (AVG) and 50 μM 1-aminocyclopropanecarboxylic acid (ACC), respectively, at 08:00 every day throughout the (NH₄)₂SO₄ treatment course. The third group was sprayed with deionized water and served as a control. Each plant was sprayed until the leaves were thoroughly wetted. After 10 d of treatment, chlorophyll content was determined and pictures were taken to determine the phenotype.

Photosynthesis measurements

Photosynthesis of the youngest fully expanded leaves in rosettes was measured 10 d after (NH₄)₂SO₄ treatment using an LI-6400 portable photosynthesis system (Li-Cor Inc., Lincoln, NE, USA). Air temperature in the cuvette during measurement was maintained at 22 °C and the photosynthetic photon flux intensity (PPFD) was 200 μmol·m⁻²·s⁻¹. The CO₂ concentration in the cuvette was adjusted to 500 μmol·mol⁻¹ with a CO₂ mixer and the VPD was at 1.0-1.5 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 Inc, USA). The data for gas exchange parameters were adjusted to the accurate leaf areas.

Measurement of chlorophyll concentration

Chlorophyll in fresh rosette leaves of NH₄NO₃ (control) and (NH₄)₂SO₄-treated plants was extracted according to the method reported by [Wellburn and Lichtenthaler \(1984\)](#). The absorbance of the extract was measured at 663 nm and 645 nm using a UV-VIS spectrophotometer (UV-2600, Shimadzu, Japan) to estimate total chlorophyll concentrations.

Measurements of biomass, total nitrogen content, and NH₄⁺ and soluble sugar concentrations

224 Plants in $(\text{NH}_4)_2\text{SO}_4$ treatment and control groups were harvested and the roots
225 and shoots were separated. The samples were oven-dried at 70 °C to a constant
226 weight. Dry samples were ground into a fine powder and about 100 mg of each
227 sample were digested with $\text{H}_2\text{SO}_4\text{-H}_2\text{O}_2$ at 350 °C for N quantification. Total N was
228 determined with an AA3 Autoanalyzer (SEAL, Germany).

229 NH_4^+ and soluble sugar concentrations in fresh samples were extracted with
230 deionized water. NH_4^+ concentrations were measured using the method of
231 indophenol blue colorimetry at 630 nm (Zanini, 2001) and $(\text{NH}_4)_2\text{SO}_4$ was used as a
232 standard. Soluble sugar concentrations were determined by the anthrone-sulfuric
233 acid method (Wang et al., 2002).

234 **Amino acid quantification**

235 Amino acid components in root and shoot of NH_4NO_3 and $(\text{NH}_4)_2\text{SO}_4$ -treated
236 plants were quantified with HPLC. The samples were frozen immediately in liquid
237 N_2 and stored at -80 °C. Frozen leaf samples (200 mg) were powdered with liquid N_2 ,
238 and homogenized with 1.5 mL 0.1% phenol in 6 M HCl. The homogenates were
239 hydrolyzed for 22 h at 100 °C. After cooling, 1 mL hydrolysate was dried with
240 organomation (NDK200-2, Hangzhou Mi'ou Instrument Co. Ltd. China) and
241 re-dissolved with 1 mL 0.1 M HCl. For derivation of amino acids, 200 μL
242 re-dissolved hydrolysate was mixed with 20 μL norleucine internal standard solution,
243 200 μL triethylamine acetonitrile ($\text{pH} > 7$), and 100 μL isothiocyanate acetonitrile
244 and placed at 25 °C for 1 h. Then 400 μL hexane was added and samples were
245 incubated for 10 min with shaking. The underlayer solution was filtered with 0.45
246 μm syringe filter. The analyses were performed on the Rigol L3000 (Rigol L3000,
247 Beijing RIGOL Technology Co., Ltd. China). Chromatographic separation was
248 operated in RP-HPLC ACE column (5C18-HL), particle size 5 μm (250 mm $\times$ 4.6
249 mm), at circumstances temperature through the binary gradient. Mobile phase A
250 contain 25 mM acetate buffer (pH 6.5) and 70 mL acetonitrile. Mobile phase B was
251 80% acetonitrile aqueous solution. The flow rate was 1.0 $\text{mL}\cdot\text{min}^{-1}$ and column
252 temperature was 40 °C. The sample was maintained for 45 min.

253 **Enzyme activity assays**

254 Frozen samples (100 mg) were ground into powder in liquid N₂, and homogenized
255 with 3 mL 50 mM Tris-HCl buffer (pH 8.0) containing 2 mM Mg²⁺, 2 mM DTT, and
256 0.4 M sucrose. The homogenate was centrifuged at 12×g for 10 min at 4 °C. The
257 supernatant was used to determine the activities of the N metabolic enzymes
258 glutamine synthase (GS), NADH-dependent glutamate synthase (NADH-GOGAT),
259 NADH-dependent glutamate dehydrogenase (NADH-GDH), glutamic-oxaloacetic
260 transaminase (GOT), and glutamic-pyruvic transaminase (GPT).

261 GS and GOGAT activities were measured according to the method described
262 previously by [Zhang et al. \(1997\)](#) and [Singh and Srivastava \(1986\)](#), respectively.
263 Activity of NADH-GDH was determined according to the method described by
264 [Loulakakis and Roubelakis-Angelakis \(1990\)](#). GOT and GPT activities were assayed
265 using the method described by [Wu et al. \(1998\)](#).

266 Frozen samples were pulverized in liquid N₂, and about 100 mg of each sample
267 were used to measure pyruvate kinase (PK) activity according to [Lepper et al. \(2010\)](#)
268 with some modification. The samples were extracted with 3 mL 100 mM Tris-HCl
269 (pH 7.5) containing 10 mM β-mercaptoethanol, 12.5% (v/v) glycerine, 1 mM
270 EDTA-Na₂, 10 mM MgCl₂, and 1% (m/v) PVP-40. These solutions were centrifuged
271 at 12×g and 4 °C for 10 min. The reaction mixture contained 100 mM Tris-HCl (pH
272 7.5), 10 mM MgCl₂, 0.16 mM NADH, 75 mM KCl, 5.0 mM ADP, 7.0 units L-lactate
273 dehydrogenase (LDH), and 1.0 mM phosphoenolpyruvate (PEP). The reaction was
274 started by adding 0.1 mL enzyme extract to 0.9 mL of the reaction mixture, and was
275 carried out at 25 °C to monitor the changes of absorbance at 340 nm for 180 s.

276 Enzyme activity was expressed as moles of metabolite generated/consumed per
277 mg protein per unit of time. Soluble protein content was measured using the
278 Bradford method with BSA as the standard ([Bradford, 1976](#)).

279 **RNA extraction and RT-PCR assay**

280 Seedlings were treated with 5 mM NH₄NO₃ and (NH₄)₂SO₄ for 3 d before the
281 roots and shoots were harvested separately for RNA analysis. Total RNA was

282 extracted with TRIzol (Invitrogen, USA), precipitated with an equal volume of
283 isopropanol, washed with 75% ethanol, and dissolved with RNase-free water.
284 cDNAs were synthesized using the PrimeScript™ RT Kit with gDNA Eraser
285 (Perfect Real Time) (TAKARA, Japan) following the manufacturer's protocol. The
286 relative expression of genes in roots and shoots was determined by quantitative
287 RT-PCR performed on an Applied Biosystems StepOne™ Real-Time PCR System
288 using SYBR Premix Ex-Taq (TAKARA) according to the manufacturer's protocol.
289 Primers used in the assays are listed in **Supplementary Table 1**, and the expression
290 data were normalized to *Actin2*.

291 **Statistics**

292 The experiment was conducted according to a completely random design. Four
293 biological replicates and two technical replicates were provided for each treatment.
294 Two-way analysis of variance (ANOVA) was conducted to analyze the effects of N
295 source and plant material. Multiple comparisons were performed using the least
296 significant difference (*LSD*) multiple range test. The differences between control and
297 (NH₄)₂SO₄ treatments in the same plant material were evaluated with the Student's
298 *t*-test. Differences were considered statistically significant at a *P* < 0.05 level.

299 **Results**

300 **NH₄⁺ toxicity is NRT1.1-dependent**

301 Compared to control, a high concentration of a sole NH₄⁺ source supplied in the
302 form of (NH₄)₂ succinate, (NH₄)₂SO₄, or NH₄Cl, respectively, for 10 days caused
303 chlorosis in the leaves in Col-0, with the most serious chlorosis observed in plants
304 treated with (NH₄)₂SO₄ (**Fig. 1A**). However, the leaf color of *chl1-1* and *chl1-5*
305 mutant plants was relatively less affected by sole NH₄⁺ source treatment. In this study,
306 we used (NH₄)₂SO₄ treatment for all subsequent experimentation and analysis.

307 Application of a sole NH₄⁺ source reduced the plant size of all plant materials
308 dramatically (**Fig. 1A**). The fresh weight of *chl1-1* and *chl1-5* was reduced by 44%
309 and 39%, and biomass was lowered by 10% and 16%, respectively, compared to the

58% and 36% reduction in fresh weight and biomass, respectively, observed for Col-0 (**Fig. 1B and C**). In contrast to *chl1-1* and *chl1-5*, the phenotype of *chl1-9* was consistent with Col-0 (**Fig. S1A and B**) when grown in $(\text{NH}_4)_2\text{SO}_4$ medium. There was no difference in photosynthetic rates and chlorophyll between Col-0 and *NRT1.1* mutants in NH_4NO_3 growth conditions, whereas $(\text{NH}_4)_2\text{SO}_4$ reduced chlorophyll and photosynthetic rates in all genotypes with a larger reduction measured in Col-0 than in *chl1-1* and *chl1-5* (**Fig. 1D and E**). Nevertheless, there was no significant difference in stomatal conductance between these genotypes under $(\text{NH}_4)_2\text{SO}_4$ treatment (**Fig. S1C**). These results indicate that NRT1.1 could be involved in NH_4^+ toxicity in *Arabidopsis*.

To further confirm that NH_4^+ toxicity is NRT1.1-dependent, we established grafted Col-0 and *chl1-5* plants that could be grown under NH_4^+ conditions. As shown in **Fig. 1F and G**, the plants with Col-0 roots and/or shoots developed chlorosis under $(\text{NH}_4)_2\text{SO}_4$ conditions. Plants with Col-0 shoots displayed worse chlorosis than those with Col-0 roots. Taken together, the results indicate that NH_4^+ toxicity is NRT1.1-dependent, but independent of the functions of *NRT1.1* in signaling and stomatal regulation (**Fig. S1**).

NRT1.1 increases NH_4^+ uptake and accumulation in roots and shoots

Total N content, NH_4^+ concentration, and the expression of *AMT1* genes in response to NH_4^+ transport were assayed to identify the difference in NH_4^+ uptake between Col-0 and *NRT1.1* mutants. As shown in **Fig. 2A**, there was no difference among Col-0, *chl1-1*, and *chl1-5* plants in total N before $(\text{NH}_4)_2\text{SO}_4$ treatment. In contrast, treatment with $(\text{NH}_4)_2\text{SO}_4$ for 10 d decreased total N significantly in the three genotypes, with the exception of Col-0 roots; the total N in Col-0 was significantly higher than that measured in *chl1-1* and *chl1-5* (**Fig. 2B**). There was no significant difference in NH_4^+ concentration in the three genotypes under control conditions (**Fig. 2C**). $(\text{NH}_4)_2\text{SO}_4$ 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 significantly increased in the shoot (**Fig. 2C**).

339 Similarly, the relative ^{15}N atom abundance dramatically decreased with $(\text{NH}_4)_2\text{SO}_4$
340 in the roots of *chl1-1* and *chl1-5*, but increased in shoots of Col-0 plants (**Fig. 2D**).
341 Although the relative ^{15}N atom abundance in *chl1-1* and *chl1-5* shoots significantly
342 increased under $(\text{NH}_4)_2\text{SO}_4$ treatment, the increase was much smaller than that
343 measured in Col-0 (**Fig. 2D**).

344 The expression of *AMT1;1*, *AMT1;2*, *AMT1;3*, and *AMT1;5* in *chl1-1* and *chl1-5*
345 was not significantly different from that in Col-0 under control condition, whereas
346 *AMT1;3*, and *AMT1;5* in Col-0 were up-regulated when treated with $(\text{NH}_4)_2\text{SO}_4$,
347 especially *AMT1;5*, which was expressed 200-fold higher than that in the control
348 (**Fig. 3C-D**). The expression level of *AMT1* genes in *chl1-1* and *chl1-5* was
349 significantly lower (*AMT1;1*) or unchanged (*AMT1;2* and *AMT1;3*) under $(\text{NH}_4)_2\text{SO}_4$
350 with respect to that of control (**Fig. 3A-D**). The expression of *AMT1;5* was
351 significantly higher in all genotypes in response to $(\text{NH}_4)_2\text{SO}_4$; however, the
352 expression in Col-0 was higher than that in *NRT1.1* mutants (**Fig. 3D**). With
353 $(\text{NH}_4)_2\text{SO}_4$ treatment, the expression of all *AMT1* genes in Col-0 was significantly
354 higher than that in *chl1-1* and *chl1-5* (**Fig. 3A-D**). These results suggest that *NRT1.1*
355 facilitates the expression of *AMT1*s in *Arabidopsis* in response to $(\text{NH}_4)_2\text{SO}_4$, and
356 ultimately results in increased NH_4^+ uptake.

357 ***NRT1.1* inhibits the activity of NH_4^+ metabolism enzymes under high** 358 **concentration of sole NH_4^+ condition**

359 Under control conditions, GS activity was significantly lower in *chl1-1* and *chl1-5*
360 than in Col-0 in roots (**Fig. 4A**). The GS activity was decreased in Col-0 plants
361 treated with $(\text{NH}_4)_2\text{SO}_4$, but was not affected in *chl1-1* and *chl1-5* under the same
362 growth conditions (**Fig. 4A**). In shoots, $(\text{NH}_4)_2\text{SO}_4$ significantly increased GS
363 activity in Col-0 and *chl1-1* but not in *chl1-5* (**Fig. S3A**).

364 GOGAT activity in roots did not differ between genotypes in control (**Fig. 4B**).
365 $(\text{NH}_4)_2\text{SO}_4$ significantly reduced GOGAT activity by 85.64% in Col-0, whereas
366 activity in *chl1-1* and *chl1-5* decreased by 48.13%, and 21.54%, respectively (**Fig.**
367 **4B**). In shoots, $(\text{NH}_4)_2\text{SO}_4$ significantly increased GOGAT activity in *chl1-1* and

368 *chl1-5* but not in Col-0, whereas no difference was observed in control conditions
369 among the genotypes (**Fig. S3B**).

370 GDH activity was significantly higher in Col-0 than in *chl1-1* and *chl1-5* in root
371 under control conditions (**Fig. 4C**). (NH₄)₂SO₄ had no significant influence on GDH
372 activity in Col-0, but increased GDH activity in *chl1-1* and *chl1-5* (**Fig. 4C**). In
373 shoots, (NH₄)₂SO₄ increased GDH activity in all genotypes, with no differences
374 among genotypes under control or (NH₄)₂SO₄ treatments (**Fig. S3C**).

375 There were no differences in GOT and GPT activities in roots of the three
376 genotypes under control conditions (**Fig. 4D-E**); however, (NH₄)₂SO₄ increased
377 GOT activity in *chl1-1* and *chl1-5* but not in Col-0 (**Fig. 4D**). Although GPT activity
378 in the three genotypes was higher under (NH₄)₂SO₄ treatment than in the control,
379 there was no significant difference between the genotypes (**Fig. 4E**). In shoots, GOT
380 and GPT activities were not affected by (NH₄)₂SO₄ treatment, and no difference in
381 their activities could be measured among the wild type and mutant genotypes (**Fig.**
382 **S3D-E**). Taken together, these results indicate that *NRT1.1* has a negative effect on
383 the activities of NH₄⁺ assimilation enzymes and transaminases in response to a high
384 concentration of a sole NH₄⁺ source.

385 **NRT1.1 disturbs the balance between carbon and NH₄⁺ metabolism in sole NH₄⁺** 386 **source conditions**

387 Soluble protein concentrations in *chl1-1* and *chl1-5* were significantly higher than
388 those in Col-0 under control conditions with the exception of *chl1-1* roots, which
389 showed lower soluble protein concentrations than the wild type in the control
390 treatment (**Fig. 5A**). Irrespective of shoot or root, (NH₄)₂SO₄ increased soluble
391 protein concentrations in Col-0, but not in *chl1-1* and *chl1-5*; while the
392 concentrations in shoots did not differ between the genotypes; mutants showed lower
393 concentrations in roots than did Col-0 (**Fig. 5A**).

394 Soluble sugar concentrations in roots and shoots varied slightly in the wild type
395 and mutants in the control treatment (**Fig. 5B**); whereas the soluble sugar
396 concentrations were 4.14 and 2.33 times higher in roots and shoots of Col-0,

397 respectively, under $(\text{NH}_4)_2\text{SO}_4$ conditions than under control conditions (**Fig. 5B**).
 398 However, soluble sugar concentrations in *chl1-1* and *chl1-5* were not as affected by
 399 $(\text{NH}_4)_2\text{SO}_4$, and were significantly lower than that in Col-0 (**Fig. 5B**). Pyruvate
 400 kinase (PK) activity was consistent among genotypes regardless of $(\text{NH}_4)_2\text{SO}_4$
 401 treatment (**Fig. 5C**).

402 The gene expression of the carbon and nitrogen metabolic regulator *SnRK1* was
 403 studied to evaluate the coordination of carbon and nitrogen metabolism. As shown in
 404 **Fig. 5D-G**, after 3 d in control conditions, the expression of *SnRK1;1* and *SnRK1;2*
 405 was unchanged in wild type and mutant lines; however, *SnRK1;2* expression
 406 increased after 5 d in the mutants compared to wild type. With $(\text{NH}_4)_2\text{SO}_4$ treatment
 407 for 3 d, we observed an up-regulation of these two genes in the mutants, but their
 408 expression in Col-0 was unchanged (**Fig. 5D and F**). After 5 d of treatment, *NRT1.1*
 409 mutants expressed higher levels of the *SnRK1* genes than Col-0 with $(\text{NH}_4)_2\text{SO}_4$
 410 treatment (**Fig. 5E and G**).

411 To further investigate the effect of $(\text{NH}_4)_2\text{SO}_4$ on nitrogen metabolism, we
 412 compared the difference in amino acid composition between Col-0 and *chl1-1* grown
 413 with different N sources. As shown in **Fig. 6A-D**, major amino acids such as
 414 aspartate (Asp) and asparagine (Asn) were less affected by $(\text{NH}_4)_2\text{SO}_4$ in roots than
 415 in shoots in wild type and mutant lines (**Fig. 6A and B**). Glutamate (Glu) and
 416 glutamine (Gln) in roots of *chl1-1* were significantly lower than that in Col-0 with
 417 $(\text{NH}_4)_2\text{SO}_4$ treatment (**Fig. 6C and D**). In shoots, there were no significant
 418 differences in amino acid content between Col-0 and *chl1-1* under control conditions
 419 (**Fig. 6A-D**); however, $(\text{NH}_4)_2\text{SO}_4$ treatment increased the amino acid content with
 420 the exception of Asn in Col-0, which was lower than that measured in control
 421 conditions (**Fig. 6A-D**). In shoots, Asp, Asn, and Gln, but not Glu, were significantly
 422 higher in *chl1-1* than in Col-0 under $(\text{NH}_4)_2\text{SO}_4$ treatment (**Fig. 6A-D**). The ratio of
 423 amino acids in root and shoot were calculated and **Fig. 6E-H** indicates that in control
 424 conditions, there was no noticeable change in amino acid ratios between the wild
 425 type and mutants. $(\text{NH}_4)_2\text{SO}_4$ promoted a higher accumulation of Asn in Col-0 roots
 426 than in shoots, whereas the R:S ratio of Asn in *chl1-1* was not significantly affected

(**Fig. 6F**). The R:S ratio of Gln dramatically declined with $(\text{NH}_4)_2\text{SO}_4$ treatment in both Col-0 and *chl1-1*; however, no difference was observed between these lines (**Fig. 6H**).

***NRT1.1*-dependent NH_4^+ toxicity is associated with ethylene-induced plant senescence**

Using ethylene mutant *ein2*, and foliar application of the ethylene precursor 1-aminocyclopropanecarboxylic acid (ACC) and ethylene antagonist aminoethoxyvinylglycine (AVG) in wild type and *NRT1.1* mutants, we tested whether *NRT1.1*-dependent NH_4^+ toxicity is associated with ethylene evolution. As shown in **Fig. 7A**, the growth of *ein2* plants under $(\text{NH}_4)_2\text{SO}_4$ treatment did not differ from that of the Col-0; furthermore, it maintained a higher chlorophyll concentration under these conditions as well (**Fig. 7B**). While $(\text{NH}_4)_2\text{SO}_4$ treatment significantly increased NH_4^+ concentration in *ein2* and Col-0, there was no significant difference between the two lines (**Fig. 7C**).

Applying AVG alleviated the chlorosis in Col-0 after $(\text{NH}_4)_2\text{SO}_4$ treatment, whereas *chl1-1* and *chl1-5* were unchanged (**Fig. 7D and E**). ACC application caused similar levels of chlorosis with $(\text{NH}_4)_2\text{SO}_4$ treatment in Col-0, and the chlorophyll content in *chl1-1* and *chl1-5* significantly decreased with ACC treatment in the presence of $(\text{NH}_4)_2\text{SO}_4$ (**Fig. 7D and E**).

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 $(\text{NH}_4)_2\text{SO}_4$ (**Fig. S5A-F**). All genes were significantly up-regulated by $(\text{NH}_4)_2\text{SO}_4$ treatment in Col-0; in contrast, the expression of these genes in *chl1-1* and *chl1-5* was less up-regulated or even down-regulated, as was the case for *GEN4* (**Fig. S5A-F**). These results suggest that *NRT1.1*-dependent NH_4^+ toxicity is associated with ethylene and its induction of plant senescence.

Discussion

***NRT1.1*-dependent symptoms of NH_4^+ toxicity**

Chlorosis and a decline in biomass are the most typical phenotypes in plants grown in high concentration of sole NH_4^+ sources (Chen et al., 2013). These phenotypes are consistent with the reduced photosynthetic rates and chlorophyll concentrations that were observed in *Arabidopsis* wild-type plants in this study (Fig. 1 A-E). Similar to the results reported by Hachiya and Noguchi (2011), the *NRT1.1* knockout mutants *chl1-1* and *chl1-5* had a stronger resistance to NH_4^+ toxicity relative to Col-0, had weaker chlorosis, and less reduction in biomass, photosynthetic rate, and chlorophyll content (Fig. 1 A-E). These results indicated that *NRT1.1* is responsible for the NH_4^+ toxicity in *Arabidopsis* when grown under high concentrations of a sole NH_4^+ source. Our grafting experiment further demonstrated that NH_4^+ toxicity in *Arabidopsis* is *NRT1.1*-dependent regardless of its expressing in roots or shoots (Fig. 1 F and G).

NRT1.1 is a multifunctional gene that participates in plant development and the interaction between plants and their environment (Bouguyon et al., 2015). In addition to being an NO_3^- transporter, *NRT1.1* serves as NO_3^- sensor in roots and is also a stomatal regulator in guard cells (Guo et al., 2003), which are tightly correlated with plant growth and physiological responses to environment. The *chl1-9* mutant is defective in nitrate uptake but shows a normal primary nitrate response (Ho et al., 2009) and showed NH_4^+ toxicity symptoms similar to that in Col-0 in response to $(\text{NH}_4)_2\text{SO}_4$ (Fig. S1A and B), suggesting a specific deficiency in NO_3^- transport capacity is not enough to cause NH_4^+ toxicity in *Arabidopsis* in the absence of NO_3^- . As the phosphorylation state and amount of *NRT1.1* protein are similar in both wild-type 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 relate to the occurrence of NH_4^+ toxicity. The performance of *chl1-9* in response to $(\text{NH}_4)_2\text{SO}_4$ in this study was different from that reported by Hachiya and Noguchi (2011); this discrepancy could be due to the variation in sensitivity to NH_4^+ at different growth stages. *NRT1.1* expression in guard cells mediates the accumulation of NO_3^- 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 photosynthesis (Lawlor and Tezara, 2009). In this study, stomatal conductance (g_s) in Col-0, *chl1-1*, and *chl1-5* displayed no significant difference with $(\text{NH}_4)_2\text{SO}_4$ treatment (Fig. S1C). These results reveal that NRT1.1-dependent NH_4^+ toxicity in *Arabidopsis* is independent of the function of NRT1.1 in NO_3^- transport and stomatal regulation.

NRT1.1-related perturbation of carbon and nitrogen metabolism under high concentration of a sole NH_4^+ source

The interaction between carbon (C) and nitrogen (N) metabolism is of paramount importance to improve stress tolerance of plants (Reguera et al., 2013). The assimilation of NH_4^+ requires carbohydrates to provide the carbon skeletons of 2-oxoglutarate (2-OG) generated from the tricarboxylic acid (TCA) cycle (Yuan et al., 2007b). Nguyen et al. (2005) proposed that a reduction in photosynthetic capacity and decrease in 2-OG production might be responsible for the excess 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 carbon metabolic steps downstream of hexose phosphorylation (Lejay et al., 2003). Pyruvate kinase (PK) is at a crucial position to control the glycolysis of assimilated C for providing energy and carbon skeletons (2-OG) for N metabolism (Stitt, 1999). In this study, the activity of PK was not significantly different between Col-0 and *NRT1.1* mutants or between the control and experimental treatments, suggesting the availability of C skeletons is not a limiting factor in N assimilation, and that C and N metabolism in Col-0 were uncoupled when the plants were subjected to high concentrations of a sole NH_4^+ source. Complex regulatory machineries are involved in the coordination between N and C metabolism, including sugar, nitrate, and amino acid sensing, as well as phytohormones (Nunes-Nesi, et al., 2010). SnRK1 (SNF1-related protein kinase) plays a vital role in plant energy homeostasis, growth and development, and stress tolerance (Baenagonzález et al., 2007), where it regulates the key enzymes in C and N metabolism. The up-regulated expression of *SnRK1;1* and *SnRK1;2* in *NRT1.1* mutants were consistent with the increased N

514 metabolism and resistance to NH_4^+ toxicity, suggesting *SnRK1* is an essential factor
515 in C and N metabolism under NH_4^+ stress (**Fig. 5D-G**).

516 Accumulation of NH_4^+ was often accompanied by the accumulation of free amino
517 acids (Hoai et al., 2003), probably due to the lower requirement of NH_4^+ for amino
518 acid synthesis. In this study, however, *chl1-1* had a higher amino acid content but
519 lower NH_4^+ in $(\text{NH}_4)_2\text{SO}_4$ conditions. This could have resulted from a greater NH_4^+
520 assimilation capacity and amino acid metabolism (**Fig. 4, Fig. 6, and Fig. S4**).
521 Glutamine (Gln) and asparagine (Asn) are important for organic N storage and
522 transport in plant tissues. They function as important signals that can reflect the N
523 status in plants and mediate NH_4^+ uptake and assimilation (Tabuchi et al., 2007;
524 Konishi et al., 2017). There was less Gln and Asn accumulation in roots in *chl1-1*
525 than in Col-0 during $(\text{NH}_4)_2\text{SO}_4$ treatment (**Fig. 6E and F**). This potentially
526 benefited the *chl1-1* mutant because less root Gln and Asn accumulation might
527 increase the requirement for NH_4^+ incorporation into *de novo* amino acids, and help
528 to mitigate NH_4^+ accumulation.

529 Increased amino acid synthesis could be advantageous to physiological processes
530 such as stress defense and the accumulation of precursors for biosynthetic enzymes
531 (Joshi et al., 2010). Glu can be converted into Asp and Ala by glutamic-oxaloacetic
532 transaminase (GOT) and glutamic-pyruvic transaminase (GPT), respectively (Brock
533 et al., 1970), and subsequently used for the synthesis of branched-chain amino acids
534 (BCAAs). BCAAs such as Met, Thr, and Ile are derived from Asp pathway and
535 provide precursors for many plant secondary metabolites, which could improve the
536 resistance of plants to stresses (Joshi et al., 2010). In this study, *chl1-1* and *chl1-5*
537 showed enhanced GOT activity under $(\text{NH}_4)_2\text{SO}_4$ conditions (**Fig. 4D**). Furthermore,
538 this was consistent with the increase in Ile and Thr contents (**Fig. S4**). Our results
539 indicate that the Asp-derived pathway for amino acid synthesis is of great
540 significance for improving the NH_4^+ toxicity tolerance in plants.

541 **NRT1.1 induces NH_4^+ accumulation in tissues under high sole NH_4^+ source**
542 **medium and facilitates ethylene synthesis and plant senescence**

Excessive NH_4^+ accumulation is the major cause for injury of plants in many unfavorable conditions (Barker, 1999; Hoai et al., 2003; Nguyen et al., 2005). In this study, NH_4^+ significantly accumulated in Col-0 under $(\text{NH}_4)_2\text{SO}_4$ conditions (**Fig. 2C**). The NH_4^+ equilibrium in plant cells is determined by NH_4^+ uptake, transport, and assimilation (Nguyen et al., 2005). The results obtained from our measurement of an ^{15}N tracer revealed that NH_4^+ uptake was substantially higher in Col-0 than in mutants grown in sole NH_4^+ source conditions, and the absorbed NH_4^+ was mainly distributed in shoots (**Fig. 2D**). This result indicates that increased NH_4^+ uptake in Col-0 made a significant contribution to NH_4^+ accumulation under such NH_4^+ conditions. *AtAMT1;1*, *AtAMT1;2*, and *AtAMT1;3* are the dominant genes required for NH_4^+ uptake in *Arabidopsis* (Yuan et al., 2007a). The up-expression of *AMT1* genes further suggests that they are regulated by NRT1.1 and account for the higher NH_4^+ accumulation in Col-0 than that in the *NRT1.1* mutants (**Fig. 3 A, B, and D**).

Proton secretion is accompanied with NH_4^+ uptake and leads to acidification of the growth medium. Acidification of media has severely negative effects on *Arabidopsis* growth (Fang et al., 2016). It has been suggested that NH_4^+ uptake induces reduction in pH in the rhizosphere, which is one of the major effects of NH_4^+ toxicity (Chaillou et al., 1991). Present data excluded the effect of medium acidification on NH_4^+ toxicity, as the symptoms of NH_4^+ toxicity appeared only in Col-0, whereas the pH was not significantly different among the genotypes evaluated (**Fig. S2**).

Because stress generally causes NH_4^+ accumulation in plant cells, a high capacity to ameliorate NH_4^+ toxicity has been viewed as an important factor in alleviating the consequence of this stress (Hoai et al., 2003; Nguyen et al., 2005). The GS-GOGAT cycle continuously provides the substrates glutamate (Glu) for incorporation of NH_4^+ into glutamine (Gln) to detoxify excess NH_4^+ (Bittsánszky et al., 2015). The reduced activities of GS and GOGAT in Col-0 under the $(\text{NH}_4)_2\text{SO}_4$ conditions in this study indicated that attenuated NH_4^+ assimilation capacity contributed to the accumulation of NH_4^+ (**Fig. 2 C and D; Fig. 4 A-E**). NADH-GDH plays a unique role from GS-GOGAT in N recycling (Masclaux-Daubresse et al., 2006) and 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). The significant increase in GDH activity in *chl1-1* and *chl1-5* in $(\text{NH}_4)_2\text{SO}_4$ conditions could diminish the accumulation of NH_4^+ (Fig. 4C), and our results reveal that the higher N assimilation capacity in the *chl1-1* and *chl1-5* mutants plays a vital role in mitigating NH_4^+ toxicity.

Taken together, our results reveal that enhanced NH_4^+ uptake combined with reduced NH_4^+ assimilation causes higher NH_4^+ accumulation in Col-0 than in the *NRT1.1* mutant lines under high concentrations of $(\text{NH}_4)_2\text{SO}_4$, indicating that NH_4^+ uptake and assimilation are tightly linked. It has been well documented that Gln is the signal for the regulation of NH_4^+ uptake and assimilation (Tabuchi et al., 2007). A previous study revealed that the expression of the cytosolic isoform of GS, *AtGLN1;2*, was strongly induced in roots by NH_4^+ and contributed to NH_4^+ assimilated in the root (Konishi et al., 2017). Furthermore, the different NH_4^+ uptake and assimilation observed in Col-0 and *NRT1.1* mutants suggest that NRT1.1 plays a significant role in regulating these processes. The interaction between *NRT1.1* and *AMT1* genes has been implicated at a physiological level to facilitate N uptake in plants grown in mixed N sources that contain NO_3^- and NH_4^+ (Hachiya and Sakakibara, 2017). It is hypothesized that NRT1.1 could affect AMT1s by regulating N assimilation enzymes, as it has been reported that NRT1.1 regulates the expression levels of many N assimilation pathway genes (Bouguyon et al., 2015). The substantial reduction in GS and GOGAT activity in Col-0 plants grown in $(\text{NH}_4)_2\text{SO}_4$ (Fig. 4A and B) suggests that the pathway for N assimilation enzymes regulated by NRT1.1 is perturbed by high concentration of NH_4^+ .

Numerous studies have reported the combination of ethylene and NH_4^+ accumulation in the development of NH_4^+ toxicity symptoms in plants (You and Barker, 2005; Li et al., 2011; Li et al., 2013). Ethylene production could be the link between NH_4^+ accumulation and NH_4^+ toxic symptoms, as the inhibition of ethylene production ameliorated the symptoms of NH_4^+ toxicity even when NH_4^+ concentrations in leaves are still high (Li et al., 2013). In our study, when the

ethylene mutant *ein2* was grown in $(\text{NH}_4)_2\text{SO}_4$ medium, it had a comparable level of NH_4^+ concentration to Col-0; furthermore, the growth of Col-0 was unaffected when the plants were grown in $(\text{NH}_4)_2\text{SO}_4$ medium and treated with the ethylene inhibitor AVG, whereas ACC induced chlorosis even in *chl1-1* and *chl1-5* plants (**Fig 7A and D**). These results indicate that NH_4^+ accumulation induced ethylene synthesis, which is involved in the development of NH_4^+ toxicity in plants. In addition, $(\text{NH}_4)_2\text{SO}_4$ up-regulated the expression of genes related to plant senescence in Col-0 plants but not in *chl1-1* and *chl1-5* mutant lines (**Figure S5**), suggesting the symptoms of NH_4^+ toxicity resulted from ethylene induced plant senescence.

Conclusions

In conclusion, our results suggested that an increase in NH_4^+ uptake and reduction in capacity of NH_4^+ metabolism is NRT1.1-dependent and the main factor responsible for NH_4^+ toxicity (**Fig. 8A and B**). In wild-type, *NRT1.1* induced higher expression of *AMT1s* and increased NH_4^+ uptake in the presence of high concentrations of a sole NH_4^+ source. Nevertheless, the ability for NH_4^+ assimilation decreased when the plants were grown under high concentrations of sole NH_4^+ source medium. Furthermore, with sole NH_4^+ source, C and N metabolism was uncoupled, causing an accumulation of soluble sugars and diminished amino acid metabolism. NH_4^+ accumulation results in the increased production of ethylene and subsequently higher 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 less than that of Col-0. In addition, the C and N metabolism in *nrt1.1* plants were less affected with sole NH_4^+ source, and NH_4^+ assimilation by the GDH pathway and amino acid synthesis by GOT ensured reduction in the adverse effects of NH_4^+ . Ultimately, chlorosis did not occur in *NRT1.1* mutants (**Fig. 8B**). At present, the interaction between nitrate and ammonium transport is a complex and attractive research area. To further explore the regulatory pathways that involve NRT1.1 during NH_4^+ uptake, transport, and assimilation are of great importance to elucidate

632 the mechanism of NH_4^+ toxicity and to develop methods for managing NH_4^+ toxicity.

633

634 **Supplementary information**

635 **Supplementary Table S1** Primers for genes used in this study.

636 **Supplementary Fig. S1** Effects of $(\text{NH}_4)_2\text{SO}_4$ on the phenotype (**A**) and chlorophyll
637 concentration (**B**) of Col-0 and *chl1-9*, and the stomatal conductance (**C**) of Col-0,
638 *chl1-1*, and *chl1-5* plants.

639 **Supplementary Fig. S2** Comparison of pH values of growth media.

640 **Supplementary Fig. S3** Effects of $(\text{NH}_4)_2\text{SO}_4$ on activity of NH_4^+ assimilation
641 enzymes and transaminase in the shoots of Col-0, *chl1-1*, and *chl1-5* plants.

642 **Supplementary Fig. S4** Effects of $(\text{NH}_4)_2\text{SO}_4$ on Thr (**A**), Met (**B**), Ile (**C**), and Ala
643 (**D**) content in roots and shoots of Col-0 and *chl1-1* plants.

644 **Supplementary Fig. S5** Effects of $(\text{NH}_4)_2\text{SO}_4$ on the relative expression of genes
645 related to senescence in Col-0, *chl1-1*, and *chl1-5* plants.

646

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Figures

Fig. 1 Effects of high concentrations of sole NH_4^+ sources on *Arabidopsis* growth and photosynthesis **(A)** Plants were grown in 5 mM NH_4NO_3 (control) and different sources of ammonium (10 mM) media for 10 d. **(B)** Relative fresh weight, **(C)** biomass, **(D)** photosynthetic rates, and **(E)** chlorophyll content of Col-0, *chl1-1*, and *chl1-5* grown in 5 mM NH_4NO_3 (control) or $(\text{NH}_4)_2\text{SO}_4$ for 10 d. **(F)** Grafted plants grown in 5 mM NH_4NO_3 (control) and $(\text{NH}_4)_2\text{SO}_4$ for 10 d. Plant material names on the left of indicate the genotypes of the shoot/root combination. **(G)** Chlorophyll content of the grafted plants grown in 5 mM NH_4NO_3 (control) and $(\text{NH}_4)_2\text{SO}_4$ for 10 d. The relative values were calculated with the average value of Col-0 in control conditions as a reference. Fresh weight and dry weight were obtained from pooled samples of seven plants from each replicate. Data represent means $\pm$ SE ($n = 4$). Bars with the same letter indicate no significant difference at $\alpha = 0.05$ using the *LSD* method. Scale bars in (A) and (F) represent 1 cm.

Fig. 2 Effects of a high 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 $(\text{NH}_4)_2\text{SO}_4$ treatment. **(B)** Total N content and **(C)** NH_4^+ concentration of Col-0, *chl1-1*, and *chl1-5* plants grown in 5 mM NH_4NO_3 (control) and $(\text{NH}_4)_2\text{SO}_4$ for 10 d. **(D)** Relative ^{15}N abundance (%) in Col-0, *chl1-1*, and *chl1-5* plants grown in 5 mM NH_4NO_3 (control) and $(\text{NH}_4)_2\text{SO}_4$ for 5 d. Total nitrogen content was obtained from pooled samples of seven plants from each replicate. Data represent means $\pm$ SE ($n = 4$). Bars with the same letter are not significantly different at $\alpha = 0.05$ by the *LSD* method. Bars with two (**) and three (***) asterisks indicate significant differences from the control at $\alpha = 0.01$ and $\alpha = 0.001$, respectively, using a two-tailed Student's *t*-test.

Fig. 3 *AMT1* gene expression under high sole NH_4^+ source treatment in roots of *Col-0*, *chl1-1*, and *chl1-5* plants grown in 5 mM NH_4NO_3 (control) and $(\text{NH}_4)_2\text{SO}_4$ for 3 d. Roots were sampled for the expression of (A) *AMT1;1* (B) *AMT1;2* (C) *AMT1;3* and (D) *AMT1;5*. Data represent means $\pm$ SE ($n = 4$). Bars with the same letter are not significantly different at $\alpha = 0.05$ using the *LSD* method.

Fig. 4 Activity of NH_4^+ assimilation enzymes and transaminases under high sole NH_4^+ source treatment in roots of *Col-0*, *chl1-1*, and *chl1-5* plants grown for 10 d in 5 mM NH_4NO_3 (control) and $(\text{NH}_4)_2\text{SO}_4$. **(A)** Glutamine synthetase (GS), **(B)** glutamate synthetase (GOGAT), **(C)** glutamate dehydrogenase (GDH), **(D)** glutamate-oxaloacetate transaminase (GOT), and **(E)** glutamate-pyruvic transaminase (GPT) activity in roots of *Col-0*, *chl1-1*, *chl1-5* plants. Data represent means $\pm$ SE ($n = 4$). Bars with the same letter are not significantly different at $\alpha = 0.05$ by the *LSD* method.

Fig. 5 Soluble protein concentration (A), soluble sugar content (B), activity of pyruvate kinase (C) and expression of *SnRK1* genes with high sole NH_4^+ source treatment in Col-0, *chl1-1*, and *chl1-5* plants (D-G). *SnRK1;1* and *SnRK1;2* expression after 3 d (D and F) and 5 d (E and G) of $(\text{NH}_4)_2\text{SO}_4$ treatment is shown. Plants measured in A-C were grown in 5 mM NH_4NO_3 (control) and $(\text{NH}_4)_2\text{SO}_4$ media for 10 d before the roots and shoots were harvested. Plants measured in D-G

was grown in 5 mM NH_4NO_3 (control) and $(\text{NH}_4)_2\text{SO}_4$ medium for 3 and 5 d, respectively, before mRNA was extracted from roots. Data represent means $\pm$ SE ($n = 4$). Bars with the same letter are not significantly different at $\alpha = 0.05$ by the *LSD* method.

Fig. 6 Free-amino acid content and their R:S ratios with high concentrations of a sole NH_4^+ source in Col-0 and *chl1-1*. Plants were grown in 5 mM NH_4NO_3 (control) and $(\text{NH}_4)_2\text{SO}_4$ media for 10 d. Asp (A), Asn (B), Glu (C), and Gln (D) was measured in the shoots and roots, and then the root/shoot ratios of Asp (E), Asn (F), Glu (G), and Gln (H) were calculated. Data represent means $\pm$ SE ($n = 3$). Bars with the same letter are not significantly different at $\alpha = 0.05$ according to the LSD method.

Fig. 7 Ethylene is involved in NRT1.1-dependent NH_4^+ toxicity (A) Col-0 and the ethylene mutant *ein2* were grown in 5 mM NH_4NO_3 (control) and $(\text{NH}_4)_2\text{SO}_4$ media for 10 d. (B) Chlorophyll content and (C) NH_4^+ concentration of Col-0 and *ein2* plants grown in 5 mM NH_4NO_3 (control) and $(\text{NH}_4)_2\text{SO}_4$ media for 10 d. (D) Col-0, *chl1-1*, and *chl1-5* were grown in 5 mM $(\text{NH}_4)_2\text{SO}_4$ media and then their leaves were sprayed with 50 μM ACC or 5 μM AVG, respectively, at 08:00 for 10 d throughout the treatment period. The control was sprayed with deionized water. (E) Chlorophyll content of Col-0, *chl1-1* and *chl1-5* plants grown in 5 mM $(\text{NH}_4)_2\text{SO}_4$ medium and treated with ACC and AVG, respectively, for 10 d. Data represent means $\pm$ SE ($n = 4$). Bars with the same letter are not significantly different at $\alpha = 0.05$ by the *LSD* method.

Fig. 8 A proposed model of NH_4^+ metabolism in *Col-0* (A) and *NRT1.1* mutants (B) grown under high concentrations of a sole NH_4^+ source (10 mM in this study). The colors of the rectangles indicate: red, the material or physiological progress was increased; blue, the material or physiological progress was not significantly changed; and green, the material or physiological progress was decreased. Red or green dotted arrows represent NH_4^+ flow from roots to shoots speculated to be higher or lower. Red or green solid arrows represent the physiological progress getting stronger or weaker; the numbers nearest to the physiological process indicates the percentage of change in this progress. Compared to plants grown under control conditions, *NRT1.1* induces up-regulated expression of *AMT1* genes in roots and facilitates NH_4^+ uptake. Simultaneously, glutamine synthetase (GS) and glutamate synthetase (GOGAT) activities are inhibited by sole NH_4^+ source. Furthermore, sugars accumulate and are stored in roots, leading to the perturbation of carbon and ammonium metabolism. As a result, free NH_4^+ is higher and can be transported to the shoot. High NH_4^+ concentrations in the shoot induce ethylene synthesis and subsequently cause plant senescence. In *NRT1.1* mutants, the NH_4^+ uptake, GS-GOGAT cycle, and carbon and ammonium metabolism are not affected by sole NH_4^+ source; in addition, the activity of NADH-dependent glutamate dehydrogenase (GDH) and glutamate-oxaloacetate transaminase (GOT) are enhanced in sole NH_4^+ condition, which diminished NH_4^+ accumulation and improved amino acid metabolism. Thus, *NRT1.1* mutant plants can better survive environments with high concentrations of a sole NH_4^+ source than does the wild type.