

Genetic and transcriptomic dissection of nitrate-independent function of Arabidopsis NRT1.1/NPF6.3/CHL1 under high ammonium condition

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1 Title

- 2 Genetic and transcriptomic dissection of nitrate-independent function of Arabidopsis
- 3 NRT1.1/NPF6.3/CHL1 under high ammonium condition

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23 Abstract

24 The Arabidopsis nitrate transceptor NRT1.1/NPF6.3/CHL1 regulates physiological 25 responses to nitrate. Several studies have reported that Arabidopsis plants lacking 26 NRT1.1 show enhanced shoot growth under toxic levels of ammonium without nitrate, 27 suggesting a nitrate-independent function for NRT1.1. To further investigate this 28 nitrate-independent function and its impact on ammonium tolerance, we conducted 29 genetic analysis, tissue-specific expression analysis, and transcriptome analysis using 30 various NRT1.1-related lines. Transgenic plants expressing either nonphosphomimic or 31 phosphomimic mutants of NRT1.1 exhibited similar ammonium tolerance to the 32 wild-type. The chl1-9 mutant, in which NRT1.1 with the P492L substitution is localized 33 intracellularly rather than at the plasma membrane and fails to transport nitrate, showed 34 significantly improved ammonium tolerance. Confocal imaging revealed that the 35 NRT1.1-GFP signal was detected in the plasma membrane of various tissues, including 36 cotyledon pavement cells, hypocotyl epidermal cells, mesophyll cells, root cap cells, 37 and epidermal cells near root tips. In early seedlings, the absence of functional NRT1.1 38 altered the expression of genes associated with aliphatic glucosinolate biosynthesis, 39 ethylene signaling, and low pH stress. Genes predicted to encode products localized to 40 the extracellular space were enriched among those differentially expressed due to 41 NRT1.1 deficiency. Our data suggest that in the absence of nitrate, plasma 42 membrane-targeted NRT1.1 reduces ammonium tolerance irrespective of its 43 phosphorylation state with alterations of gene expression associated with stress and 44 senescence.

45	Key words: ammonium toxicity, CHL1, NPF6.3, NR11.1
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67 **1. Introduction**

68 Most land plants primarily rely on soil nitrate as their main nitrogen (N) source. Nitrate 69 serves not just as a substrate for N assimilation but also as a vital signaling molecule, 70 regulating genes related to nitrogen acquisition and root system development (Wang et 71 al. 2004; Okamoto et al. 2019). These nitrate responses require molecular components 72 to perceive nitrate and to drive nitrate-dependent signaling. The Arabidopsis nitrate 73 transporter NRT1.1/NPF6.3/CHL1 plays a crucial role as a nitrate sensor, orchestrating 74 various plant responses to nitrate supply (Remans et al. 2006; Ho et al. 2009; Bouguyon 75 et al. 2015). Downstream of NRT1.1, calcium ions serve as secondary messengers, 76 stimulating the expression of nitrate-responsive genes, including the major high-affinity 77 nitrate transport gene NRT2.1 (Riveras et al. 2015). Recent findings indicate that the 78 subgroup III protein kinase CPK perceives calcium signals and phosphorylates the 79 transcription factor NLP7 (Liu et al. 2017), which directly binds intracellular nitrate, 80 regulating nitrate-dependent gene expression (Liu et al. 2022). Furthermore, NRT1.1 81 enhances the expression of the transcription factor ANR1 and the AFB3 auxin receptor 82 gene in roots, promoting lateral root development and elongation in response to nitrate 83 supply (Remans et al. 2006; Vidal et al. 2010). The magnitude of these 84 NRT1.1-dependent responses is finely tuned by the phosphorylation status of NRT1.1 at 85 the T101 residue (Ho et al. 2009; Bouguyon et al. 2015).

In addition to its nitrate-dependent functions, NRT1.1 exhibits significant roles in the absence of nitrate. It aids the basipetal transport of auxin out of the lateral root primordia when nitrate is absent (Krouk et al. 2010). Exogenous nitrate inhibits auxin

89 transport, leading to auxin accumulation in primordia and the stimulation of lateral root 90 emergence in nitrate-rich patches (Mounier et al. 2014). Walch-Liu and Forde (2008) 91 observed that exogenous glutamate inhibits primary root elongation at the root tip, a 92 process antagonized by nitrate presence, depending on phosphorylated NRT1.1. 93 Intriguingly, overexpression of nonphosphomimetic NRT1.1 (T101A) heightens 94 glutamate sensitivity in primary roots even without nitrate. Furthermore, studies 95 including ours have demonstrated that the absence of functional NRT1.1 significantly 96 alleviates growth suppression and chlorosis under toxic ammonium levels as the sole N 97 source (Hachiya et al. 2011; Jian et al. 2018; Liu et al. 2020). NRT1.1 promotes 98 ammonium accumulation, altering ammonium metabolism and subsequently 99 upregulating ethylene signaling, resulting in ammonium toxicity (Jian et al. 2018). 100 Moreover, under high ammonium and NaCl concentrations without nitrate, NRT1.1 101 transports chloride, leading to chloride accumulation, especially in roots (Liu et al. 102 2020). This chloride accumulation contributes to ammonium toxicity under high salt 103 conditions.

The aforementioned observations clearly indicate a nitrate-independent function for NRT1.1, although the precise mechanism remains elusive. This study aimed to comprehensively uncover the primary nitrate-independent function of NRT1.1 in ammonium tolerance. Employing various NRT1.1-related lines, we conducted genetic analyses, tissue-specific expression studies using quantitative PCR (qPCR) and confocal microscopy, and transcriptome analyses. Our findings reveal that (i) plasma membrane-targeted NRT1.1 diminishes ammonium tolerance regardless of its

111	phosphorylation state, (ii) NRT1.1 is expressed across most seedling tissues, and (iii
112	NRT1.1 primarily modulates the expression of genes associated with aliphati
113	glucosinolate (GSL) biosynthesis, ethylene signaling, and low pH stress.

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115 **2. Materials and Methods**

116 2.1. Plant materials and growth conditions

117 In this study, Arabidopsis plants were used. The T-DNA insertion mutants nrt1.1 118 (Hachiya et al. 2011), cipk23-4 (Ho et al. 2009), and the gamma ray-mutagenized 119 mutant chl1-5 (Tsay et al. 1993a) in the Col background were obtained from the 120 European Arabidopsis Stock Center (NASC). The gamma-ray-mutagenized mutant 121 chll-6 in the Ler background (Tsay et al. 1993b) was sourced from the Arabidopsis 122 Biological Resource Center. The transposon tag line pst16286 in the Nossen 123 background (Ito et al. 2002; Kuromori et al. 2004) was purchased from the RIKEN 124 lines Bioresource Center (BRC). The harboring the estradiol-inducible 125 NRT1.1-mCherry and the pNRT1.1:NRT1.1-GFPloop in the chl1-5 background were 126 used in previous studies (Bouguyon et al. 2015; Bouguyon et al. 2016). The chl1-9 in the Col background and the T101D and T101A lines in the chl1-5 background (Ho et al. 127 128 2009) were provided by Dr. Yi-Fang Tsay (Academia Sinica).

The seeds underwent surface sterilization and were placed in plastic Petri dishes (diameter: 90 mm; depth: 20 mm; Iwaki, Tokyo, Japan) with approximately 30 mL of N-modified Murashige and Skoog medium. The medium included 4.7 mM MES-KOH (pH 5.7), 2% (w/v) sucrose, and 0.25% (w/v) gellan gum (Wako, Osaka, Japan). Two

133 different N and K sources were used: 10 mM KNO₃ (10 mM NO₃⁻ condition) or 5 mM 134 (NH₄)₂SO₄ with 10 mM KCl (10 mM NH₄⁺ condition). After being kept in the dark at 135 4 °C for 3 d, the plants were grown horizontally under a photosynthetic photon flux 136 density of 100-130 µmol m⁻² s⁻¹ (16 h light/8 h dark cycle) at 23 °C. For transfer 137 experiments, surface-sterilized seeds were sown in larger plastic Petri dishes (length: 138 140 mm; width: 100 mm; depth: 20 mm; Eiken Chemical Co. Ltd., Taito-ku, Tokyo, 139 Japan) containing 50 mL of half-strength modified Murashige and Skoog medium with 140 2.5 mM ammonium as the sole N source at pH 6.7 (Okamoto et al. 2019). After 3 days 141 in the dark at 4 °C, the plants were grown vertically for 5 d under a photosynthetic photon flux density of 100–130 μ mol m⁻² s⁻¹ (16 h light/8 h dark cycle) at 23 °C. The 142 143 plants were then transferred to different N conditions for subsequent experiments. 144 Further details regarding plantlet cultivation are provided in the Results section and 145 figure legends.

146 2.2. Extraction of RNA

147 The whole seedlings, shoots, and roots were harvested and promptly frozen in liquid N_2 , 148 then stored at -80 °C until needed. The frozen samples were ground using TissueLyser 149 II (QIAGEN) with zirconia beads (5 mm diameter). Total RNA was extracted using the 150 RNeasy Plant Mini Kit (Qiagen) and treated with on-column DNase digestion following 151 the manufacturer's instructions.

152 2.3. RT-qPCR

153 Reverse transcription (RT) was conducted using ReverTra Ace qPCR RT Master Mix

154 with gDNA Remover (Toyobo Co. Ltd., Tokyo, Japan) following the manufacturer's

155 guidelines. The resulting cDNA was diluted tenfold with distilled water for quantitative 156 PCR (qPCR). Transcript levels were assessed using a StepOnePlus Real-Time PCR 157 System (Thermo Fisher Scientific, Waltham, MA, USA). In the presence of 10-µL 158 KAPA SYBR FAST qPCR Kit (Nippon Genetics Co. Ltd., Tokyo, Japan), 0.4 µL 159 specific primers (0.2 µM final concentration), and 7.2 µL sterile water, 2 µL of obtained 160 cDNA was amplified. ACTIN3 (Hachiya et al. 2021) served as internal standards. 161 Standard curves were generated using plasmid DNA containing target cDNAs or total 162 cDNAs. Refer to Supplementary Table 1 for primer sequences.

163 2.4. Microarray analysis

164 Microarray analysis was conducted using 3-day-old (72 h) and 5-day-old (120 h) whole 165 seedlings of Col, chl1-5, and nrt1.1 following the protocol outlined in Hachiya et al. 166 (2021). RNA quality was evaluated using an Agilent 2100 bioanalyzer (Agilent 167 Technologies). RNA amplification, labeling, hybridization, and scanning were 168 performed using the 3' IVT Express Kit (Affymetrix) and GeneChip Arabidopsis 169 Genome ATH1 Array (Affymetrix) as per the manufacturer's instructions. Data from 170 the microarray chips were normalized using the Microarray Suite 5.0 (MAS5) method (Affymetrix). Transcripts labeled as "absent" or "marginal" were excluded from 171 172 subsequent quantitative analysis. The raw microarray data used in this study are 173 accessible in the ArrayExpress database at EMBL-EBI under accession number 174 E-MTAB-13395.

175 2.5. Observation of cotyledon

176 Bright-field imaging of Arabidopsis early seedling cotyledons was conducted using an 177 all-in-one fluorescence microscope (BZ-X710, KEYENCE, Japan) equipped with a 178 Nikon 40× objective (CFI Plan Apo λ 40×/0.95). To create a high-resolution and 179 wide-area continuous image of the cotyledon, individual images were seamlessly

180 merged using the image joint function of the BZ analyzer software (KEYENCE).

181 2.6. Observation of fluorescent signal

182 Confocal imaging of NRT1.1-GFPloop and propidium iodide (PI) was performed using

183 a Leica SP5 Confocal Microscope (Leica Microsystems). Roots were stained with a 10

184 $\mu g m L^{-1}$ PI solution to outline cell shapes. Mesophyll protoplasts were prepared from

185 cotyledons of 12-day-old plants (Plants grown under 10 mM nitrate for 9 days were

186 transferred to 10 mM ammonium and grown for 3 days.) following the method of Endo

187 et al. (2016). NRT1.1-GFPLoop and PI were excited with 488 nm and 543 nm lasers,

and emissions were detected from 500 to 530 nm and 590 to 660 nm, respectively,

189 using a Leica 25× objective (HCX IRAPO L 25×/0.95 WATER).

190 2.7. Statistical analysis

191 The unpaired two-tailed Welch's *t*-test and the Tukey–Kramer multiple comparison test 192 were conducted using R software v.2.15.3. Additionally, the two-tailed ratio paired 193 t-test was performed using GraphPad Prism software v.9.3.1, assuming a Gaussian 194 distribution.

195

196 **3. Results**

197 3.1. NRT1.1 targeted to the plasma membrane reduces ammonium tolerance almost

198 regardless of its phosphorylation status

199 In our previous study, NRT1.1-deficient mutants in the Col background exhibited 200 enhanced shoot growth under 10 mM ammonium conditions (Hachiya et al. 2011). In 201 this study, we consistently observed larger shoot fresh weights (FWs) in 202 NRT1.1-deficient mutants from three Arabidopsis accessions (Col; chl1-5, nrt1.1, Ler; 203 chl1-6, and Nossen; pst16286) compared to wild-type plants under 10 mM ammonium. 204 Conversely, under 10 mM nitrate conditions, the FWs were lower in these mutants 205 (Figure 1a, b). Intriguingly, the induction of NRT1.1-mCherry in the chl1-5 background, 206 controlled by β-estradiol (Bouguyon et al. 2015), significantly reduced shoot FW under 207 ammonium, whereas β-estradiol-treated Col plants did not show a change in FW 208 (Figure 1c). Under nitrate, shoot growth was restored by the induction of 209 NRT1.1-mCherry, confirming its functionality (Figure 1d). These findings underscore 210 the significant role of Arabidopsis NRT1.1 in ammonium tolerance.

211 NRT1.1 exhibits diverse molecular functions, including nitrate uptake, nitrate 212 sensing/signaling, and auxin transport (Ho et al. 2009; Krouk et al. 2010; Bouguyon et 213 al. 2015; Zhang et al. 2019). Modifications at specific residues in the protein alter these 214 functions. In the chl1-9 mutant with the P492L substitution, NRT1.1 primarily localizes 215 intracellularly rather than at the plasma membrane (Bouguyon et al. 2015). Surprisingly, 216 whilst P492L lacks the ability to transport nitrate and auxin, it can still drives some of 217 the nitrate-dependent signaling, including short-term induction of NRT2.1 by nitrate (Ho 218 et al. 2009; Bouguyon et al. 2015). This suggests that NRT1.1 functions as a nitrate

219 sensor, independent of its uptake activity. The phosphorylation status of NRT1.1 at the 220 T101 residue alters its affinity for nitrate; Non-phosphorylated NRT1.1 forms a 221 homodimer with the NRT1.1 located in close proximity to the dimer interface, allowing 222 low-affinity nitrate uptake, whereas phosphorylated NRT1.1 decouples the dimer 223 configuration to act as a high-affinity nitrate transporter (Sun and Zheng 2015). 224 Moreover, the phosphorylated form transports auxin better than the non-phosphorylated 225 form in the absence of nitrate (Bouguyon et al. 2015). To reveal how P492L substitution 226 and phosphorylation status of NRT1.1 affects ammonium tolerance, we analyzed 227 growth of chl1-9 and transgenic plants expressing nonphosphomimic (T101A) or 228 phosphomimic (T101D) mutants of NRT1.1.

229 When grown on 10 mM ammonium, the chll-9 mutant exhibited markedly 230 improved ammonium tolerance compared to the wild-type, displaying larger FWs and higher chlorophyll concentrations (Figure 1e-g). Except for slightly enhanced shoot 231 232 growth in T101A compared to Col, both T101 mutants displayed ammonium toxicity 233 similar to Col. Moreover, *cipk23-4*, lacking CIPK23 that phosphorylates NRT1.1 at the 234 T101 residue (Ho et al. 2009), did not exhibit enhanced shoot growth under ammonium 235 or nitrate conditions (Figure 1h, i). These results imply that NRT1.1-induced 236 ammonium toxicity is nearly independent of its phosphorylation at the T101 residue but 237 requires correct plasma membrane targeting dependent on the P492 residue. Given that 238 the phosphorylating status alters auxin transport in the absence of nitrate (Bouguyon et 239 al. 2015), changes in auxin distribution and action may have little effect on ammonium 240 toxicity.

241 To determine the developmental stages at which NRT1.1 affects ammonium 242 tolerance, we monitored the growth progression of Col and *chl1-5* plants under 10 mM 243 ammonium. In 3-day-old seedlings, there were no significant difference in FW, and 244 their appearances were similar (Figure 1j, 1). By day 5, *chl1-5* seedlings exhibited larger 245 shoot and root FWs, and their cotyledons were more expanded and greener compared to 246 Col (Figure 1k, 1, and S1a). Additionally, at this stage, *chl1-5* and *nrt1.1* seedlings had 247 significantly higher chlorophyll concentrations than Col (Figure S1b). As plants aged 248 between 8 and 11-days, the impact of NRT1.1 deficiency on shoot growth intensified 249 compared to root growth (Figure 1k). These findings highlight that NRT1.1's influence 250 on ammonium tolerance initiates at the early stages of seedling growth.

3.2. A significant expression of NRT1.1 is detectable both in shoots and roots of ammonium-grown seedlings

253 To decipher how NRT1.1 impacts ammonium tolerance, we examined NRT1.1 254 expression patterns in Col plants grown under 10 mM ammonium or 10 mM nitrate. In 255 3-day-old Col seedlings, NRT1.1 expression showed little disparity between ammonium 256 and nitrate conditions (Figure 2a). However, in 5-, 8-, and 11-day-old plants, shoot expression of NRT1.1 remained consistently higher under 10 mM ammonium compared 257 258 to 10 mM nitrate, whereas in the roots, this trend reversed (Figure 2b). Consequently, 259 the disparity in NRT1.1 transcript levels between shoot and root was smaller under 260 ammonium than nitrate. Furthermore, to investigate NRT1.1 protein expression under 261 ammonium conditions, we utilized the pNRT1.1:NRT1.1-GFPloop line for confocal 262 observation (Bouguyon et al. 2016). Clear GFP signals were localized in the plasma

263 membrane of various cells, including pavement cells of cotyledons (Figure 2c, i), 264 epidermal cells of hypocotyl (Figure 2d), mesophyll protoplasts (Figure 2e), root cap 265 cells, and epidermal cells near primary root tips (Figure 2f, g). Intriguingly, dotted GFP 266 signals were frequently detected in intracellular regions of pavement cells and root tips 267 (Figure 2h, j). These expression analyses indicate that NRT1.1 might function in diverse 268 cells and tissues under high ammonium conditions.

269 3.3. Overview of genome-wide transcriptional responses caused by NRT1.1 deficiency

270 To unravel the primary mechanisms by which NRT1.1 controls genome-wide gene 271 expression under ammonium conditions, we conducted independent microarray 272 experiments using 3-day-old (72 h) and 5-day-old (120 h) whole seedlings of Col, 273 chl1-5, and nrt1.1. In 3- and 5-day-old seedlings, we identified 20 and 176 transcripts, 274 respectively, in which expression was at least two-fold higher in both NRT1.1-deficient 275 mutants compared to Col (Table S2). Conversely, 13 and 80 transcripts in the mutants 276 exhibited levels less than or equal to half of those in Col (Table S2). To capture more 277 NRT1.1-regulated genes in the 3-day-old seedlings, we re-explored differentially 278 expressed genes (DEG) at a 1.5-fold threshold. This analysis identified 120 upregulated 279 genes and 71 downregulated genes in the mutants relative to Col (Table S3). RT-qPCR 280 analysis of 12 DEGs confirmed that the results were consistent with those of the 281 microarray (Figure S2 and Tables S2, 3). For subsequent analyses, we concentrated on 282 DEGs at a 1.5-fold threshold for 3-day-old seedlings and a 2-fold threshold for 283 5-day-old seedlings. A Venn diagram revealed that only 26 DEGs were common to both 284 3-day-old and 5-day-old seedlings (Figure 3a and Table S4). Enrichment analysis

conducted using Metascape (Zhou et al. 2019) identified a significant enrichment of the
term "response to extracellular stimulus" (Figure S3a). Additionally, the SUBA
localization predictor (Hooper et al. 2017) indicated that the translational products
corresponding to 9 out of the 26 DEGs were likely localized in the extracellular space
(Figure S3b). These findings suggest that, under ammonium conditions, NRT1.1 could
influence extracellular events.

291 In 3-day-old seedlings, the absence of NRT1.1 led to significant gene expression 292 changes without altering seedling growth (Figure 1e, 3a). Thus, we focused on the 293 transcriptome profile of 3-day-old seedlings to uncover the primary functions of 294 NRT1.1 under ammonium conditions. Gene cloud analysis (Krouk et al. 2015) revealed 295 a 168-fold enrichment in "methionine-derived" and a 120-fold enrichment in 296 "4-reductase" genes in the upregulated genes of 3-day-old NRT1.1-deficient mutants 297 (Figure 3b). "Methionine-derived" genes included At3g19710 (BCAT4), At4g12030 298 (BAT5), and At5g23010 (MAM1), essential for aliphatic glucosinolate biosynthesis. The 299 "4-reductase" genes included At2g47460 (MYB12), At4g09820 (bHLH042), and 300 At5g42800 (DFR), contributing to flavonoid biosynthesis. Metascape analysis also 301 overrepresented terms like "glucosinolate biosynthesis from methionine" and 302 "flavonoid metabolic process" for upregulated genes in the mutants (Figure 3c). 303 Downregulated genes in the 3-day-old mutants enriched terms like "interpro-ipr008263 304 (glycoside hydrolase, family 16, active site)" from genes encoding cell wall-modifying 305 enzymes like xyloglucan endotransglucosylase/hydrolase (At4g25810 (XTH23), 306 At4g14130 (XTH15), and At5g57560 (XTH22)) (Figure 3b). Intriguingly, DEGs in the

307 3-day-old mutants often overrepresented terms related to the extracellular space, such as
308 "plant-type cell wall loosening," "secretory vesicle," "apoplast," "xyloglucan metabolic
309 process," "plant-type cell wall," and "xyloglucan:xyloglucosyl transferase activity"
310 (Figure 3c, d).

311 In 5-day-old seedlings, terms related to photosynthesis were predominant among 312 upregulated genes in response to NRT1.1 deficiency (Figure S4a, b). This aligns with 313 the observed greener and more expanded cotyledons and higher chlorophyll 314 concentrations in NRT1.1-deficient seedlings compared to Col (Figure 1m and S1a, b). 315 Conversely, downregulated genes were associated with terms related to abiotic and 316 biotic stresses (Figure S4c). MapMan analysis clearly illustrated the upregulation of 317 genes encoding components for light reactions, chlorophyll biosynthesis, and the 318 Calvin-Benson cycle in 5-day-old NRT1.1-deficient mutants (Figure S5b). These findings indicate an enhancement of photoautotrophic growth with reduced stress 319 320 response in the 5-day-old mutants.

321 3.4. Effects of NRT1.1 deficiency on expression of genes for glucosinolate 322 biosynthesis and genes responsive to ACC, NaCl, H₂O₂, IAA, and low pH under 323 ammonium condition

Initially, we concentrated on the genes involved in the biosynthesis of aliphatic GSL and indole GSL, based on the gene list from Harun et al. (2020). This focus was due to the enrichment of terms such as "glucosinolate biosynthesis from methionine," "methionine-derived," and "glucosinolate biosynthesis" among the upregulated genes in 3- and 5-day-old *NRT1.1*-deficient mutants (Figures 3b, c, and S5b). The deficiency of

NRT1.1 significantly upregulated the aliphatic GSL biosynthesis genes and their positive regulator genes, *AT5G61420 (MYB28)* and *AT5G07690 (MYB29)*, while it had little effect on the expression of indole GSL biosynthesis genes (Figure 4a and Tables S2, 3, and 5).

Our enrichment analysis revealed that the GO term "cellular response to ethylene 333 334 stimulus" was overrepresented among the downregulated genes in 3-day-old 335 NRT1.1-deficient mutants (Figure 3d). This aligns with a previous study that reported a 336 down-regulation of genes involved in ethylene biosynthesis and senescence under toxic 337 ammonium conditions in NRT1.1-deficient mutants (Jian et al. 2018). Consequently, we 338 examined the genes responsive to the application of 1-aminocyclopropane-1-carboxylic 339 acid (ACC), a precursor of ethylene whose biosynthesis is a rate-limiting step for 340 ethylene production, based on the gene list from Goda et al. (2008) (Figure 4b and 341 Table S6). The ACC-induced genes were significantly downregulated in both 3- and 342 5-day-old NRT1.1-deficient mutants grown under 10 mM ammonium, whereas the 343 ACC-repressed genes did not show a significant response.

The GO term "response to salt stress" was found to be overrepresented in the downregulated genes of 3- and 5-day-old *NRT1.1*-deficient mutants (Figures 3d and S4c). A recent study found that in the presence of a 25 mM chloride ion with ammonium as the sole N source, NRT1.1 supports excessive chloride absorption, resulting in severe root growth inhibition (Liu et al. 2020). *NRT1.1* deficiency may affect the expression of NaCl-responsive genes because our ammonium solution contains 16 mM chloride ions. The NaCl-induced genes listed from Shen et al. (2014)

351 were significantly downregulated in the 5-day-old *NRT1.1*-deficient mutants, but not in 352 the 3-day-old mutants (Figures 4c and Table S7). The expression of NaCl-repressed 353 genes differed little between Col and *NRT1.1*-deficient mutants.

354 It has been reported that ammonium nutrition disrupts redox homeostasis, leading 355 to the apoplastic accumulation of reactive oxygen species and oxidative stress 356 (Podgórska et al. 2015). In line with this, our enrichment analysis revealed the presence 357 of the GO terms "response to oxidative stress" and "antioxidant activity" among the 358 downregulated genes in both 3-day-old and 5-day-old NRT1.1-deficient mutants (Figure 359 3d and S4c). Genes that are upregulated in response to H₂O₂ application, as documented 360 by Hieno et al. (2019), were downregulated in the 5-day-old NRT1.1-deficient mutants, 361 but not in the 3-day-old mutants (Figure 4d and Table S8). No significant alterations in 362 the expression of genes downregulated by H₂O₂ were observed between Col and the NRT1.1-deficient mutants. 363

Regarding auxin responses, although NRT1.1 facilitates auxin influx into the cell in the absence of nitrate (Krouk et al. 2010), its deficiency did not significantly alter the expression of typical genes responsive to indole-3-acetic acid (IAA) application (Goda et al. 2008) (Figure 4e and Table S9).

Interestingly, previous studies have shown that 43% of ammonium-inducible genes correspond to low pH (4.5)-inducible genes in Arabidopsis plants (Lager et al. 2010; Patterson et al. 2010). We have previously observed that the enhanced ammonium tolerance of *NRT1.1*-deficient mutants at pH 5.7 was mimicked in Col plants grown at pH 6.7 (Hachiya et al. 2011). These findings suggest that *NRT1.1*-deficient mutants may

373	exhibit enhanced tolerance to ammonium-derived acidic stress. Consequently, we
374	examined the microarray data with a focus on low pH stress-responsive genes listed in
375	Lager et al. (2010). Remarkably, the low pH stress-inducible genes were significantly
376	downregulated both in the 3- and 5-day-old NRT1.1-deficient mutants relative to Col,
377	whereas the low pH stress-repressive genes were generally upregulated in the mutants
378	(Figure 4f and Table S10).

379 Collectively, we conclude that NRT1.1 primarily alters the expression of genes 380 associated with aliphatic glucosinolate biosynthesis, ethylene signaling, and low pH 381 stress.

382

383 4. Discussion

The Arabidopsis nitrate transceptor NRT1.1 and its orthologs are required for plant adaptation to nitrate-rich conditions (Wang et al. 2020). NRT1.1, on the other hand, has been shown to reduce ammonium tolerance in the absence of nitrate (Hachiya et al. 2011; Jian et al. 2018). Thus, NRT1.1 and its orthologs are likely to determine adaption features for the two major N sources, nitrate and ammonium.

Excessive ammonium assimilation by plastidic glutamine synthetase produces proton accumulation and acidic stress in Arabidopsis shoots when exposed to high amounts of ammonium (Hachiya et al. 2021). Proton excretion from plants to external media is common in plant cultivation with high ammonium feeding (Britto and Kronzucker 2002). Moreover, in Arabidopsis plants, ammonium-inducible genes and low pH (4.5)-inducible genes overlap at a significant rate (Lager et al. 2010, Patterson et

395 al. 2010). These findings imply that acidic stress is a major source of ammonium 396 toxicity. Our transcriptome analysis revealed that NRT1.1 deficiency dampens low pH 397 responses (Figure 4f). We observed that increasing the pH of the medium from 5.7 to 398 6.7 by adding alkaline NH₃ solution promoted shoot development of Col more intensely than that of chl1-5 under ammonium conditions, virtually completely suppressing the 399 400 difference in growth (Figure S6a). Expression of ALMT1 was upregulated in Col shoots 401 and roots under 10 mM ammonium compared with under 10 mM nitrate, and this 402 ammonium induction was significantly suppressed in chl1-5 shoots and roots (Figure 403 S6b). Importantly, the magnitude of ammonium induction and suppression by NRT1.1 404 deficiency of ALMT1 was much larger in shoots than in roots (Figure S6b). This 405 corresponds to the observation that the impact of NRT1.1 deficiency on shoot growth 406 intensified compared to root growth (Figure 1k). These suggest that NRT1.1 407 exacerbates acidic stress in the presence of ammonium, mainly in shoots. Interestingly, 408 an acidic rhizosphere induces NRT1.1 expression in a STOP1 transcription 409 factor-dependent way, which enhances symport of proton and nitrate through NRT1.1 410 and adjusts rhizosphere pH to a more suitable range (Ye et al. 2021). Furthermore, 411 NRT1.1 and SLAH3 collaborate to drive a nitrate cycle across the plasma membrane, 412 reducing acidification of the rhizosphere (Xiao et al. 2022). It is uncertain how NRT1.1 413 affects intra- and extracellular pH under nitrate-free ammonium conditions.

414 Our transcriptome analysis highlighted the extracellular space as an early site of 415 NRT1.1 action (Figure 3c, d, and S3a, b, S4b, c). A study by Podgórska et al. (2017) in 416 Arabidopsis plants demonstrated that ammonium toxicity is associated with smaller

417 mesophyll cells characterized by a more rigid cell wall structure, containing increased 418 phenolic compounds and boron ions. In our study, the GO terms "plant-type cell wall 419 loosening" and "plant-type cell wall modification" were enriched in the genes 420 upregulated by NRT1.1 deficiency (Figure 3c and S4b). These terms originated from 421 genes encoding proteins involved in cell wall loosening and cell expansion, such as 422 AT1G69530 (EXPA1), AT1G74670 (GASA6), AT2G20750 (EXPB1), AT2G40610 423 (EXPA8), AT3G29030 (EXPA5), and AT4G28250 (EXPB3). This suggests that NRT1.1 424 might influence ammonium tolerance via alterations in cell wall modification processes. 425 We discovered that genes for aliphatic GSL biosynthesis and their positive 426 regulator genes, MYB28 and MYB29, were induced by NRT1.1 deficiency (Figure 4a, 427 S2b, Table S2, 4). Coleto et al. (2021) reported that ammonium toxicity was 428 exacerbated in the myb28myb29 mutant but not in the myc234 mutant. Given that both 429 mutants are almost devoid of aliphatic GSL, the ammonium hypersensitivity of 430 myb28myb29 is not linked with a lack of aliphatic GSL. The authors provided evidence 431 that MYB28 and MYB29 maintain intracellular iron homeostasis under ammonium 432 conditions, thereby attenuating ammonium toxicity. It remains to be seen whether the 433 absence of NRT1.1 enhances ammonium tolerance via upregulation of MYB28 and 434 MYB29. Meanwhile, our transcriptome data suggested that NRT1.1 enhances ethylene 435 signaling under ammonium conditions. Plants emit ethylene as a phytohormone in 436 response to various stresses, including ammonium toxicity (Britto and Kronzucker 437 2002). Inhibition of ethylene signaling by gene knockout and chemical treatment 438 alleviates ammonium toxicity (Jian et al. 2018, Li et al. 2019). These findings suggest

that NRT1.1 reduces ammonium tolerance through ethylene signaling. Although there is
little overlap among aliphatic GSL biosynthesis genes and ACC- and low
pH-responsive genes (Tables S5, 6, 10), it would be worthwhile to scrutinize these
associations.

443

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450

451 **Disclosure statement**

452 The authors report there are no competing interests to declare.

453

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658 Figure legends

659 Figure 1. Plasma membrane-targeted NRT1.1 reduces ammonium tolerance almost 660 regardless of its phosphorylating status. (a, b) Fresh weights (FWs) of shoots from 661 11-day-old Col, chl1-5, nrt1.1, Ler, chl1-6, Nossen, and pst16286 plants grown under 662 10 mM ammonium (a) or 10 mM nitrate (b) conditions (Mean \pm SD; n = 5-10). (c, d) 663 Shoot FWs in Col and NRT1.1-mCherry (eNRT1.1) lines under the control of the 664 β -estradiol-inducible promoter in the *chl1-5* background (Mean \pm SD; n = 15). In one 665 dish, five seedlings of each line grown under the 10 mM nitrate condition for 5 days 666 were transferred to the 10 mM ammonium (c) or 10 mM nitrate (d) condition in the 667 absence (ethanol as a mock) or presence of 1 μM β-estradiol and further grown for 6 668 days. (e, f) Shoot FWs (e) and a representative photograph (f) from 11-day-old Col, 669 chl1-5, chl1-9 (P492L), T101D, and T101A plants grown under 10 mM ammonium 670 conditions (Mean \pm SD; n = 5). The scale bar represents 5 mm. Twelve shoots from one 671 plate were regarded as a single biological replicate. (g) The chlorophyll (a + b)672 concentrations of shoots from 5-day-old Col, chl1-5, chl1-9, T101D, and T101A 673 seedlings grown under 10 mM ammonium (Mean \pm SD; n = 3). Thirty-seven shoots 674 from one plate were regarded as a single biological replicate. (h, i) FWs of shoots from 675 11-day-old Col and cipk23-4 plants grown under 10 mM ammonium (h) or 10 mM 676 nitrate (i) conditions (Mean \pm SD; n = 5-10). (j) FWs of 3-day-old Col and *chl1-5* 677 seedlings grown under 10 mM ammonium (Mean \pm SD; n = 4). (k) FWs of shoots and 678 roots from 5-, 8-, or 11-day-old Col and chl1-5 grown under 10 mM ammonium (Mean 679 \pm SD; n = 5). (1) Representative photographs of 3-day-old Col and *chl1-5* seedlings

680 grown under 10 mM ammonium and representative photographs of the adaxial side of 681 cotyledons from 5-day-old Col and *chl1-5* seedlings grown under 10 mM ammonium. 682 The scale bars represent 1 mm (3-day) and 500 µm (5-day). (a, b, h-k) Six shoots or 683 roots from one plate were regarded as a single biological replicate. In one dish, six seeds of each line of wild-type and mutant were placed and grown. *P < 0.05; **P < 0.01; 684 ***P < 0.001 (unpaired two-tailed Welch's *t*-test). NS denotes not significant. Different 685 686 lowercase letters indicate significant differences evaluated by the Tukey-Kramer 687 multiple comparison test conducted at a significance level of P < 0.05.

688

689 Figure 2. NRT1.1 is expressed in various tissues under ammonium conditions. (a) 690 Relative transcript levels of NRT1.1 in 3-day-old Col whole seedlings grown under 10 691 mM ammonium or 10 mM nitrate conditions (Mean \pm SD; n = 3). 45 seedlings from 692 one plate were regarded as a single biological replicate. (b) Relative transcript levels of 693 NRT1.1 in shoots and roots from 5-, 8-, or 11-day-old Col grown under 10 mM 694 ammonium or 10 mM nitrate conditions (Mean \pm SD; n = 3). 12 shoots and 12 roots 695 from one plate were regarded as a single biological replicate. (c-j) Signals of 696 NRT1.1-GFPloop from pavement cells of cotyledons from 3-day-old plants (c), 697 epidermal cells of hypocotyl from 3-day-old plants (d), mesophyll protoplasts from 698 12-day-old plants (e), root cap and epidermal cells near primary root tips from 699 5-day-old plants (f, g), primary root tip from 5-day-old plants (h), and pavement cells of 700 cotyledons from 8-day-old plants (i: horizontal central section of the cell, j: horizontal 701 section below the cell surface layer) grown under 10 mM ammonium condition except

for mesophyll protoplasts from 12-day-old plants (e). Mesophyll protoplasts were prepared from the plants which were grown under 10 mM nitrate for 9 days, then transferred to 10 mM ammonium, and further grown for 3 days. The scale bars represent 10 μ m (c, e–j) and 30 μ m (d). The purple signals represent autofluorescence (e) and PI fluorescence (f). The images were processed by Image J version 1.53c. Figure 2h was generated by the maximum intensity projection.

708

709 Figure 3. Genome-wide transcriptional responses caused by NRT1.1 deficiency. (a) 710 Venn diagram showing the number of genes upregulated and downregulated in the 3-711 and 5-day-old NRT1.1-deficient mutants (chl1-5, nrt1.1) compared with Col grown 712 under 10 mM ammonium. The transcripts whose expression was at least 1.5-fold 713 (3-day) or 2-fold (5-day) higher or at most 1/1.5 (3-day) or 1/2 lower (5-day) in both 714 mutants relative to that in Col were counted. The gene lists are shown in Supplementary 715 Tables S2, 3. (b) Outputs derived from GeneCloud analysis of genes upregulated and 716 downregulated in the 3-day-old NRT1.1-deficient mutants (chl1-5, nrt1.1) compared 717 with Col grown under 10 mM ammonium. The numbers next to the term denote the 718 number of genes containing the term and the fold enrichment. (c, d) Outputs derived 719 from Metascape analysis of genes upregulated (c) and downregulated (d) in the 720 3-day-old NRT1.1-deficient mutants (chl1-5, nrt1.1) compared with Col grown under 10 721 mM ammonium. Note that microarray analysis was conducted using 3-day-old (72 h) and 5-day-old (120 h) whole seedlings of Col, *chl1-5*, and *nrt1.1*. 722

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724 Figure 4. NRT1.1 primarily alters the expression of aliphatic glucosinolate biosynthesis 725 genes, ACC-responsive genes, and low pH-responsive genes under ammonium 726 conditions. (a-f) Comparisons of the expression of biosynthesis genes for aliphatic 727 glucosinolate (AGSL) and indole glucosinolate (IGSL) (a), genes upregulated and 728 downregulated by ACC application (b), genes upregulated and downregulated by NaCl 729 stress (c), genes upregulated and downregulated by H₂O₂ application (d), genes 730 upregulated and downregulated by IAA application (e), and genes upregulated and 731 downregulated by low pH treatment (f) between Col and NRT1.1-deficient mutants 732 (chl1-5, nrt1.1) grown under 10 mM ammonium. The samples harvested from two 733 independent experiments were subjected to microarray analysis. E1 and E2 denote 1st 734 experiment and 2nd experiment, respectively. One hundred thirty-five seedlings (3-day) 735 from three plates and 74 seedlings (5-day) from two plates were regarded as a single 736 biological replicate. Changes in gene expression levels between Col and *chl1-5* and Col 737 and *nrt1.1* were represented as logarithms to base 2 of ratios in signal intensities. 1-5 738 and 1.1 denote chll-5 and nrt1.1, respectively. *P < 0.05; **P < 0.01; ***P < 0.001739 (two-tailed ratio paired *t*-test).









