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

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► **To cite this version:**

Takushi Hachiya, Nobue Makita, Liên Bach, Alain Gojon, Tsuyoshi Nakagawa, et al.. Genetic and transcriptomic dissection of nitrate-independent function of Arabidopsis NRT1.1/NPF6.3/CHL1 under high ammonium condition. 2024. hal-04493392

**HAL Id: hal-04493392**

**<https://hal.inrae.fr/hal-04493392>**

Preprint submitted on 7 Mar 2024

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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, NRT1.1

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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).

86 In addition to its nitrate-dependent functions, NRT1.1 exhibits significant roles in  
87 the absence of nitrate. It aids the basipetal transport of auxin out of the lateral root  
88 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.

104 The aforementioned observations clearly indicate a nitrate-independent function for  
105 NRT1.1, although the precise mechanism remains elusive. This study aimed to  
106 comprehensively uncover the primary nitrate-independent function of NRT1.1 in  
107 ammonium tolerance. Employing various NRT1.1-related lines, we conducted genetic  
108 analyses, tissue-specific expression studies using quantitative PCR (qPCR) and confocal  
109 microscopy, and transcriptome analyses. Our findings reveal that (i) plasma  
110 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 aliphatic  
113 glucosinolate (GSL) biosynthesis, ethylene signaling, and low pH stress.

114

## 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 *chll-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 Bioresource Center (BRC). The lines harboring the estradiol-inducible  
125 *NRT1.1-mCherry* and the *pNRT1.1:NRT1.1-GFPloop* in the *chll-5* background were  
126 used in previous studies (Bouguyon et al. 2015; Bouguyon et al. 2016). The *chll-9* in  
127 the Col background and the *T101D* and *T101A* lines in the *chll-5* background (Ho et al.  
128 2009) were provided by Dr. Yi-Fang Tsay (Academia Sinica).

129 The seeds underwent surface sterilization and were placed in plastic Petri dishes  
130 (diameter: 90 mm; depth: 20 mm; Iwaki, Tokyo, Japan) with approximately 30 mL of  
131 N-modified Murashige and Skoog medium. The medium included 4.7 mM MES-KOH  
132 (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 KNO<sub>3</sub> (10 mM NO<sub>3</sub><sup>-</sup> condition) or 5 mM  
134 (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> with 10 mM KCl (10 mM NH<sub>4</sub><sup>+</sup> 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<sup>-2</sup> s<sup>-1</sup> (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  
142 photon flux density of 100–130 μmol m<sup>-2</sup> s<sup>-1</sup> (16 h light/8 h dark cycle) at 23 °C. The  
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<sub>2</sub>,  
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- $\mu$ L  
158 KAPA SYBR FAST qPCR Kit (Nippon Genetics Co. Ltd., Tokyo, Japan), 0.4  $\mu$ L  
159 specific primers (0.2  $\mu$ M final concentration), and 7.2  $\mu$ L sterile water, 2  $\mu$ L 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  
171 (Affymetrix). Transcripts labeled as "absent" or "marginal" were excluded from  
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  $\lambda$  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  $\mu\text{g mL}^{-1}$  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,  
188 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  $\beta$ -estradiol (Bouguyon et al. 2015), significantly reduced shoot FW under  
207 ammonium, whereas  $\beta$ -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 *chl1-9* mutant exhibited markedly  
230 improved ammonium tolerance compared to the wild-type, displaying larger FWs and  
231 higher chlorophyll concentrations (Figure 1e–g). Except for slightly enhanced shoot  
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, l). 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, l, 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.

### 251 ***3.2. A significant expression of NRT1.1 is detectable both in shoots and roots of*** 252 ***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  
257 expression of *NRT1.1* remained consistently higher under 10 mM ammonium compared  
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

285 conducted using Metascape (Zhou et al. 2019) identified a significant enrichment of the  
286 term “response to extracellular stimulus” (Figure S3a). Additionally, the SUBA  
287 localization predictor (Hooper et al. 2017) indicated that the translational products  
288 corresponding to 9 out of the 26 DEGs were likely localized in the extracellular space  
289 (Figure S3b). These findings suggest that, under ammonium conditions, NRT1.1 could  
290 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* (*MAMI*), 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  
319 findings indicate an enhancement of photoautotrophic growth with reduced stress  
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, H<sub>2</sub>O<sub>2</sub>, IAA, and low pH under***  
323 ***ammonium condition***

324 Initially, we concentrated on the genes involved in the biosynthesis of aliphatic GSL  
325 and indole GSL, based on the gene list from Harun et al. (2020). This focus was due to  
326 the enrichment of terms such as “glucosinolate biosynthesis from methionine,”  
327 “methionine-derived,” and “glucosinolate biosynthesis” among the upregulated genes in  
328 3- and 5-day-old *NRT1.1*-deficient mutants (Figures 3b, c, and S5b). The deficiency of



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

333 Our enrichment analysis revealed that the GO term “cellular response to ethylene  
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.

344 The GO term “response to salt stress” was found to be overrepresented in the  
345 downregulated genes of 3- and 5-day-old *NRT1.1*-deficient mutants (Figures 3d and  
346 S4c). A recent study found that in the presence of a 25 mM chloride ion with  
347 ammonium as the sole N source, *NRT1.1* supports excessive chloride absorption,  
348 resulting in severe root growth inhibition (Liu et al. 2020). *NRT1.1* deficiency may  
349 affect the expression of NaCl-responsive genes because our ammonium solution  
350 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 H<sub>2</sub>O<sub>2</sub> 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 H<sub>2</sub>O<sub>2</sub> were observed between Col and the  
363 *NRT1.1*-deficient mutants.

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

368 Interestingly, previous studies have shown that 43% of ammonium-inducible genes  
369 correspond to low pH (4.5)-inducible genes in Arabidopsis plants (Lager et al. 2010;  
370 Patterson et al. 2010). We have previously observed that the enhanced ammonium  
371 tolerance of *NRT1.1*-deficient mutants at pH 5.7 was mimicked in Col plants grown at  
372 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**

384 The Arabidopsis nitrate transceptor NRT1.1 and its orthologs are required for plant  
385 adaptation to nitrate-rich conditions (Wang et al. 2020). NRT1.1, on the other hand, has  
386 been shown to reduce ammonium tolerance in the absence of nitrate (Hachiya et al.  
387 2011; Jian et al. 2018). Thus, NRT1.1 and its orthologs are likely to determine adaption  
388 features for the two major N sources, nitrate and ammonium.

389 Excessive ammonium assimilation by plastidic glutamine synthetase produces  
390 proton accumulation and acidic stress in Arabidopsis shoots when exposed to high  
391 amounts of ammonium (Hachiya et al. 2021). Proton excretion from plants to external  
392 media is common in plant cultivation with high ammonium feeding (Britto and  
393 Kronzucker 2002). Moreover, in Arabidopsis plants, ammonium-inducible genes and  
394 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  $\text{NH}_3$  solution promoted shoot development of Col more intensely  
399 than that of *chl1-5* under ammonium conditions, virtually completely suppressing the  
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). Coletto 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

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

443

#### 444 **Acknowledgments**

445 We are grateful for Dr. Carine Alcon and Montpellier RIO Imaging on technical support  
446 with confocal microscopy and for Ms. Mako Sakai on technical support with expression  
447 analysis and on manuscript review. We would like to thank Dr. Yi-Fang Tsay  
448 (Academia Sinica) for giving the Arabidopsis seeds. We would also like to thank Enago  
449 ([www.enago.jp](http://www.enago.jp)) for the English language review.

450

#### 451 **Disclosure statement**

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

453

#### 454 **Funding**

455 This study was supported by the Grants-in-Aid from the Ministry of Education, Culture,  
456 Sports, Science and Technology, Japan (No. 17K15237, 20K05771, 23K04978 to TH),  
457 by the Inamori Foundation (to TH), by the Agropolis Foundation (No. 1502-405 to TH),  
458 and by the Nagase Science and Technology Foundation (to TH).

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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  $\beta$ -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  $\mu$ M  $\beta$ -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). (l) 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  $\mu$ m (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  
684 of each line of wild-type and mutant were placed and grown. \* $P < 0.05$ ; \*\* $P < 0.01$ ;  
685 \*\*\* $P < 0.001$  (unpaired two-tailed Welch's *t*-test). NS denotes not significant. Different  
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

702 for mesophyll protoplasts from 12-day-old plants (e). Mesophyll protoplasts were  
703 prepared from the plants which were grown under 10 mM nitrate for 9 days, then  
704 transferred to 10 mM ammonium, and further grown for 3 days. The scale bars  
705 represent 10  $\mu\text{m}$  (c, e–j) and 30  $\mu\text{m}$  (d). The purple signals represent autofluorescence  
706 (e) and PI fluorescence (f). The images were processed by Image J version 1.53c. Figure  
707 2h was generated by the maximum intensity projection.

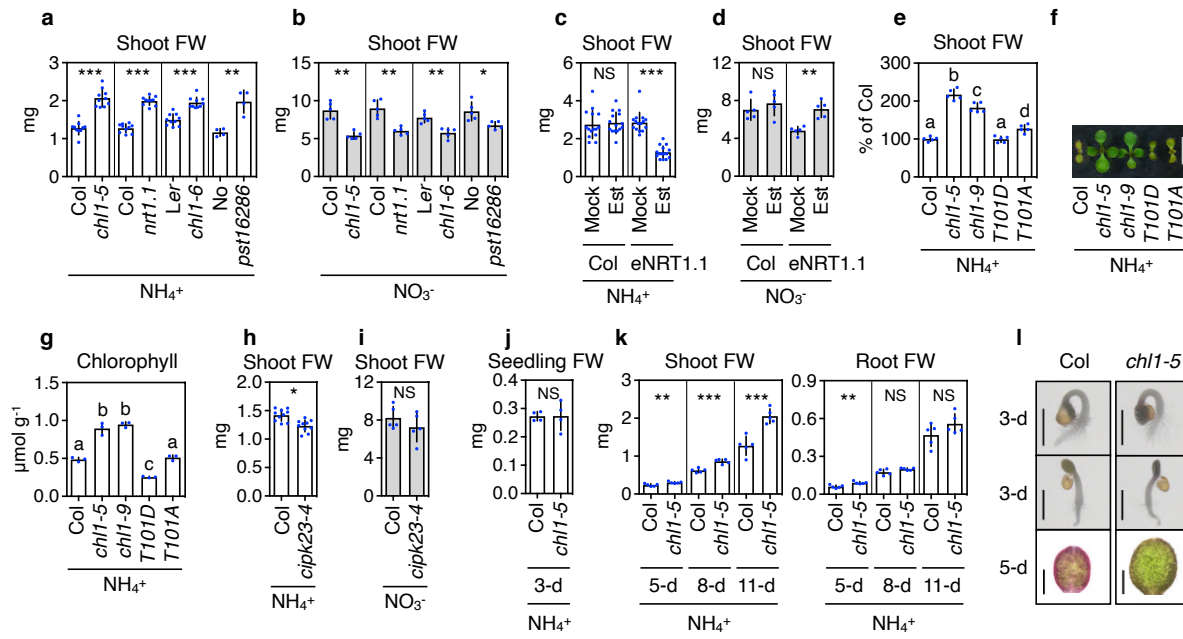
708

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)  
722 and 5-day-old (120 h) whole seedlings of Col, *chl1-5*, and *nrt1.1*.

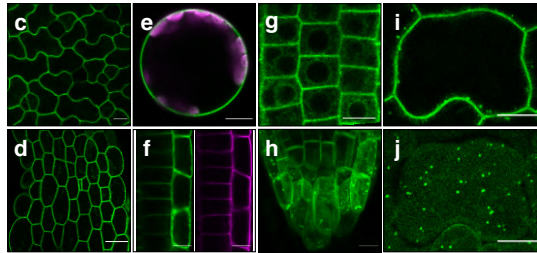
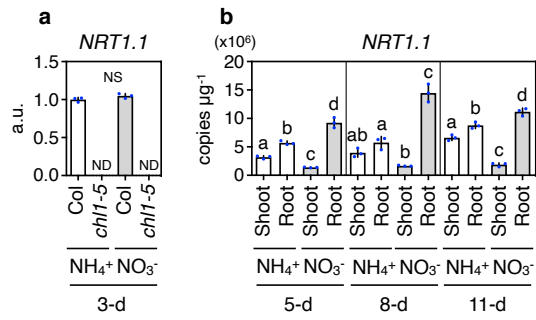
723

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 H<sub>2</sub>O<sub>2</sub> 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 (*chll-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 *chll-5* and Col  
737 and *nrt1.1* were represented as logarithms to base 2 of ratios in signal intensities. *l-5*  
738 and *l.1* denote *chll-5* and *nrt1.1*, respectively. \**P* < 0.05; \*\**P* < 0.01; \*\*\**P* < 0.001  
739 (two-tailed ratio paired *t*-test).

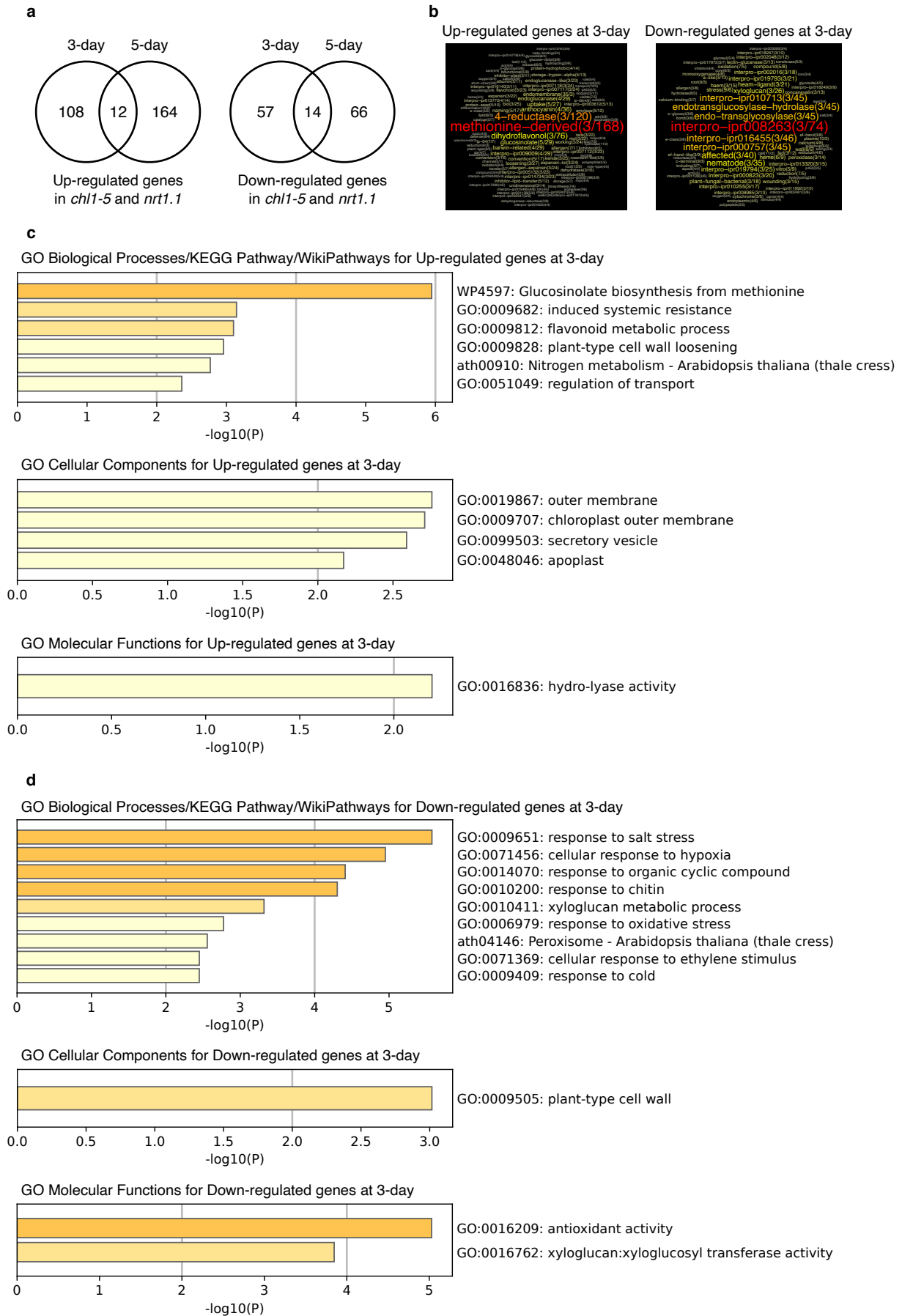
**Figure 1**



**Figure 2**



**Figure 3**



**Figure 4**

