1 **Title**

2 STOP1 dominates Arabidopsis tolerance to ammonium over NRT1.1/NPF6.3/CHL1

3 **Running title**

4 STOP1 dominates NH_4^+ tolerance over NRT1.1

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Abstract

The Arabidopsis nitrate transporter/sensor NRT1.1/NPF6.3/CHL1 plays significant roles even in the absence of nitrate. The loss-of-function of NRT1.1 alleviates growth suppression and chlorosis under toxic levels of ammonium as the sole nitrogen source. Recently, we reported that acidic stress-inducible genes are downregulated by *NRT1.1* deficiency under ammonium toxicity conditions, implying that NRT1.1 may exacerbate ammonium-dependent acidic stress. The transcription factor SENSITIVE TO PROTON RHIZOTOXICITY (STOP1) enhances Arabidopsis tolerance to ammonium and acidic stresses. Furthermore, STOP1 directly activates *NRT1.1* transcription. These previous findings prompted us to analyze the association between NRT1.1 and STOP1 on ammonium tolerance. Here, we show that ammonium-inducible STOP1 plays a dominant role over NRT1.1 in ammonium tolerance and expression of direct target genes of STOP1. We present a novel scheme in which NRT1.1 and STOP1 jointly regulate common target(s) associated with ammonium tolerance.

Keywords: ammonium toxicity, CHL1, NPF6.3, NRT1.1, STOP1

45 **1. Introduction**

46 Nitrate serves not only as a substrate for nitrogen (N) assimilation but also as a
47 signaling molecule. The Arabidopsis nitrate transporter NRT1.1/NPF6.3/CHL1 acts as a
48 nitrate sensor and orchestrates various plant responses to the nitrate signal, including
49 gene expression and root system development (Bouguyon et al. 2015; Ho et al. 2009).
50 Apart from its nitrate-dependent functions, NRT1.1 exhibits significant roles in the
51 absence of nitrate (Hachiya et al. 2024). Previous studies have shown that the
52 loss-of-function of NRT1.1 alleviates growth suppression and chlorosis under toxic
53 levels of ammonium as the sole N source, i.e., ammonium toxicity (Hachiya et al. 2011;
54 Jian et al. 2018; Liu et al. 2020). Recently, we reported that in early seedlings grown
55 under 10 mM ammonium, the genes induced by acidic stress are downregulated in
56 *NRT1.1*-deficient mutants relative to the wild-type, whereas those repressed by acidic
57 stress are upregulated in the mutants (Hachiya et al. 2024). Moreover, an increase in
58 medium pH promotes shoot growth of the wild-type more intensely than that of the
59 *NRT1.1*-deficient mutant under 10 mM ammonium. Given that acidic stress is one of
60 the primary causes of ammonium toxicity (Hachiya et al. 2021), NRT1.1 may
61 exacerbate the ammonium-dependent acidic stress.

62 In *Arabidopsis*, the zinc finger transcription factor SENSITIVE TO PROTON
63 RHIZOTOXICITY (STOP1) positively regulates genes involved in the tolerance to
64 ammonium and acidic stresses, as well as abiotic stresses, including aluminum, drought,
65 salt, low phosphate, and low oxygen (Balzergue et al. 2017; Enomoto et al. 2019;
66 Hachiya et al. 2021; Sadhukhan et al. 2019; Sawaki et al. 2009; Tian et al. 2021).

67 Furthermore, STOP1 directly activates *NRT1.1* expression under an acidic rhizosphere
68 containing nitrate (Ye et al. 2021). These findings prompted us to analyze the
69 association between NRT1.1 and STOP1 under toxic levels of ammonium as the sole N
70 source. Here, we show that STOP1 dominates ammonium tolerance and expression of
71 direct target genes of STOP1 over NRT1.1.
72

73 **2. Materials and Methods**

74 ***2.1. Plant materials and growth conditions***

75 *Arabidopsis thaliana* accession Columbia (Col-0) was used as a control. T-DNA
76 insertion mutants *nrt1.1* (SALK_097431, Hachiya et al. 2011), $stop1^{KO}$ (SALK_114108,
77 Sawaki et al. 2007), and gamma ray-mutagenized mutant c*hl1-5* (Tsay et al. 1993) were
78 obtained from the European Arabidopsis Stock Center. $stop1^{KO}$ was backcrossed twice
79 with Col-0 before use. The line harboring *pSTOP1:GFP-STOP1* in the $stop1^{KO}$
80 background (Balzergue et al. 2017) was provided by Dr. Thierry Desnos (Aix-Marseille
81 University, France).

82 The seeds were surface sterilized, placed in plastic Petri dishes (diameter: 90 mm;
83 depth: 20 mm; Iwaki, Tokyo, Japan), and grown on a solid medium containing 30 mL
84 of Murashige and Skoog (MS) salts without N sources supplemented with 4.7 mM
85 MES-KOH (pH 5.7), 2% (w/v) sucrose, and 0.25% (w/v) gellan gum (Wako, Osaka,
86 Japan) with or without 100 nM indole-3-acetic acid (IAA). Two different N and K
87 sources were used: 10 mM KNO_3 (10 mM NO_3^- condition) or 5 mM $(NH_4)_2SO_4$ with 10
88 mM KCl (10 mM NH_4^+ condition). The plants were kept in the dark at 4°C for 3 days,

89 and then grown horizontally under a photosynthetic photon flux density of 100–130
90 μmol m^{-2} s^{-1} (16-h light/8-h dark cycle) at 23°C.
91
92 ***2.2. Extraction of RNA***
93 Frozen samples were ground using TissueLyser II (Qiagen, Tokyo, Japan) using
94 zirconia beads. Total RNA was extracted using an RNeasy Plant Mini Kit (Qiagen),
95 following the manufacturer's instructions.
96
97 ***2.3. Quantitative reverse transcription polymerase chain reaction***
98 Reverse transcription (RT) was conducted using ReverTra Ace qPCR RT Master Mix
99 with gDNA Remover (Toyobo, Tokyo, Japan), following the manufacturer's
100 instructions. The obtained cDNA was diluted 10-fold with distilled water for
101 quantitative PCR (qPCR). Transcript levels were evaluated using StepOnePlus
102 Real-Time PCR System (Thermo Fisher Scientific, Waltham, MA, USA). The diluted
103 cDNA (2 μL) was amplified in the presence of 10 μL KAPA SYBR FAST qPCR Kit
104 (Nippon Genetics, Tokyo, Japan), 0.4 μL specific primers (0.2 μM final concentration),
105 and 7.2 μL of sterile water. Relative transcript levels were calculated using the
106 comparative cycle threshold method with *ACTIN3* as an internal standard. Primer
107 sequences are shown in Supplementary Table 1.
108
109 ***2.4. Observation of fluorescent signal***

110 Confocal imaging was performed using a Leica SP5 Confocal Microscope (Leica
111 Microsystems, Wetzlar, Germany). GFP-STOP1 was excited with a 488-nm Ar laser,
112 and emission was detected from 500–530 nm using a Leica 25× objective (HCX IRAPO
113 L 25×/0.95 WATER).
114

115 **3. Results and Discussion**

116 ***3.1. STOP1 dominates ammonium tolerance over NRT1.1***

117 To analyze the genetic relationship between *NRT1.1* and *STOP1*, *chl1-5* and *nrt1.1* were
118 crossed with *stop1* ^{KO}, and their double homozygous mutants were obtained (Figure 1a).
119 The growth of Col-0, *chl1-5*, *nrt1.1*, *stop1*^{KO}, *chl1-5 stop1*^{KO}, and *nrt1.1 stop1*^{KO} under
120 10 mM ammonium supplemented with 100 nM IAA was compared. It should be noted
121 that appropriate IAA concentrations stabilize the ammonium tolerance of
122 *NRT1.1*-deficient mutants (Hachiya and Noguchi 2011). The shoots of *chl1-5* and *nrt1.1*
123 were larger and greener than those of Col-0 (Figure 1b). The growth enhancement in
124 *NRT1.1*-deficient mutants was almost completely suppressed by *STOP1* deficiency.
125 Shoot fresh weights of *chl1-5* and *nrt1.1* were much larger than those of Col-0, whereas
126 those of *stop1*^{KO} were slightly smaller (Figure 1c). Fresh weights of the shoots of *chl1-5*
127 *stop1*^{KO} and *nrt1.1 stop1*^{KO} were comparable to those of *stop1*^{KO}. Also, under 10 mM
128 ammonium without IAA, the shoot fresh weights were increased by *NRT1.1* deficiency,
129 but they were reduced by *STOP1* deficiency to a level comparable to those of *stop1*^{KO}
130 (Figure 1d). Under 10 mM nitrate without IAA, the shoot fresh weights of
131 *NRT1.1*-deficient mutants were significantly smaller than those of other lines with or

132 without *STOP1*. Under both N conditions, the fresh weights of the root showed a similar
133 tendency to those of the shoot. Thus, our genetic analysis indicates that STOP1
134 dominates ammonium tolerance over NRT1.1.
135
136 ***3.2 STOP1 dominates expression of direct target genes of STOP1 over NRT1.1***
137 The above growth analysis suggests that NRT1.1 could act upstream of STOP1 and
138 downregulate *STOP1*/STOP1 activities under ammonium. To verify this, we checked
139 the transcript levels of known direct target genes of STOP1, i.e., *ALMT1*, *CIPK23*,
140 *GDH2*, and *STOP2* (Balzergue et al. 2017; Enomoto et al. 2019; Sadhukhan et al. 2019,
141 Tokizawa et al. 2021) in the absence of IAA. In five-day-old Col-0 shoots and roots, the
142 expression of *ALMT1*, *CIPK23*, *GDH2*, and *STOP2* was significantly higher under 10
143 mM ammonium than under 10 mM nitrate (Figure 2a). This ammonium-dependent
144 transcriptional induction was consistently decreased by *NRT1.1* deficiency (Figure 2a).
145 Also, *STOP1* expression in shoots and roots was not significantly increased by *NRT1.1*
146 deficiency under ammonium (Figure 2a). Moreover, in five-day-old seedlings grown
147 under 10 mM ammonium, the transcript levels of *ALMT1*, *CIPK23*, and *GDH2* were
148 lowest in *STOP1*-deficient mutants with or without *NRT1.1* (Figure 2b). Microarray
149 analysis using three- and five-day-old seedlings under 10 mM ammonium without IAA
150 revealed that *NRT1.1* deficiency did not generally induce genes whose expression is
151 upregulated via STOP1 under acidic pH (Sawaki et al. 2009) (Figure 2c and
152 Supplementary Table S2). The F-box protein RAE1 interacts with STOP1 and promotes
153 its degradation via the ubiquitin-26S proteasome pathway (Zhang et al. 2019),

154 suggesting the post-translational regulation of STOP1 activity. To analyze the effect of
155 NRT1.1 on STOP1 expression, *chl1-5* was crossed with *pSTOP1:GFP-STOP1* line in
156 the *stop1*^{KO} background (Balzergue et al. 2017) and produced the *pSTOP1:GFP-STOP1*
157 line in the *chl1-5 stop1*^{KO} background. The GFP-STOP1-derived fluorescence signal
158 was promoted in the roots under 10 mM ammonium, similar to a previous result (Tian
159 et al. 2021). Meanwhile, the GFP signal was little changed by *NRT1.1* deficiency under
160 ammonium (Figure 2d). These observations suggest that NRT1.1 does not downregulate
161 *STOP1*/STOP1 activities and that STOP1 dominates expression of direct target genes of
162 STOP1 over NRT1.1.
163
164 ***3.3 Possible scheme for regulation of ammonium tolerance by NRT1.1 and STOP1***
165 The present study shows that ammonium-inducible STOP1 dominates ammonium
166 tolerance and target gene expression of STOP1 over NRT1.1. A possible scheme for
167 regulation of ammonium tolerance by NRT1.1 and STOP1 is presented (Figure 2e),
168 noting that STOP1 directly upregulates *NRT1.1* expression (Ye et al. 2021). The STOP1
169 upregulated by ammonium induces the expression of *NRT1.1* and other target gene(s).
170 The function(s) of some target gene product(s) (i.e., Target X in Figure 2e) could
171 enhance ammonium tolerance but be suppressed by NRT1.1, possibly via protein–
172 protein interaction(s).
173 In *A. thaliana*, STOP1 directly induces *CIPK23* expression (Sadhukhan et al.
174 2019), and CIPK23 phosphorylates NRT1.1 to alter its affinity with nitrate (Ho et al.
175 2009). Since CIPK23 also inhibits ammonium transport via phosphorylation of

176 high-affinity ammonium transporter AMT1, the loss-of-function of *CIPK23* confers
177 ammonium-hypersensitivity (Hachiya et al. 2024; Straub et al. 2017). Meanwhile, a
178 recent study reported that STOP1 indirectly activates the plasma membrane H^+-ATPase
179 AHA2 by upregulating *SAUR55* whose translational product prevents PP2C.D2/5,
180 negative regulators of AHA2 (Agrahari et al. 2023). The plant Cistrome database
181 implies that STOP1 could directly bind to the promoter of *AHA2*. Moreover, NRT1.1
182 and receptor-kinase QSK1 inactivates the AHA2 via phosphorylation on S899 under
183 low nitrate (Zhu et al. 2024). Hence, CIPK23 and AHA2 could be possible candidates
184 for Target X in Figure 2e, although inhibition of CIPK23 function by NRT1.1 remains
185 unknown. It would be valuable to explore the common target(s) of NRT1.1 and STOP1
186 for better understanding ammonium toxicity.
187

188 **Acknowledgments**

189 We would like to thank Dr. Thierry Desnos (Aix-Marseille University) for providing the
190 *Arabidopsis* seeds, Dr. Alain Gojon for valuable discussion, and Mr. Shun Ohno for
191 supporting experiments. The authors would like to thank Enago (www.enago.jp) for the
192 English language review.
193

194 **Disclosure statement**

195 The authors report there are no competing interests to declare.
196

197 **Funding**

198 This study was supported by the Grants-in-Aid from the Ministry of Education, Culture,
199 Sports, Science and Technology, Japan under Grant
200 [number17K15237/20K05771/23K04978, the Inamori Foundation, the Agropolis
201 Foundation Grant [number1502-405], the Nagase Science and Technology Foundation,
202 and the Yanmar Joint Research to TH.

203

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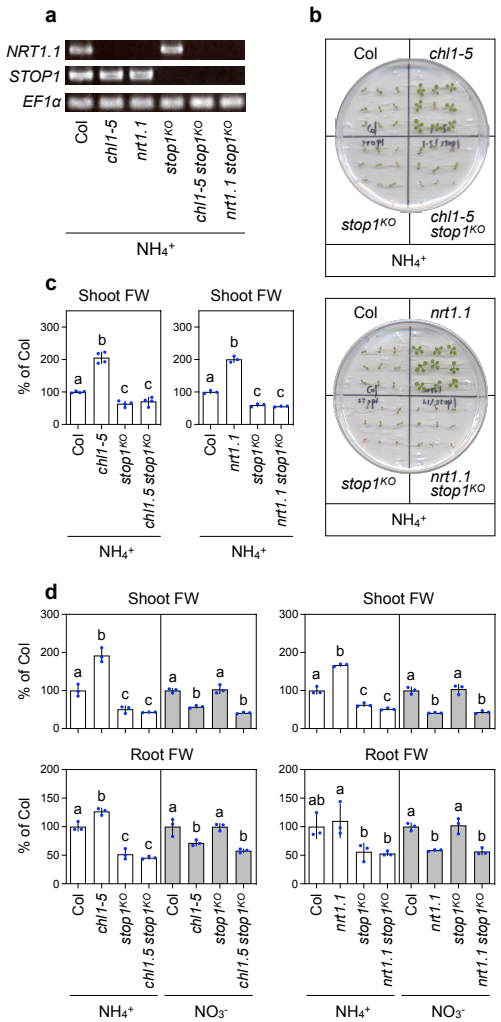
296
297 **Figure legends**
298 **Figure 1.** *STOP1* deficiency suppresses enhanced ammonium tolerance in
299 *NRT1.1*-deficient mutants. (a) RT-PCR and agarose gel electrophoresis for *NRT1.1*,
300 *STOP1*, and *EF1α* using five-day-old seedlings of Col-0, *chl1-5*, *nrt1.1*, *stop1*^{KO}, *chl1-5*
301 *stop1*^{KO}, and *nrt1.1 stop1*^{KO} grown under 10 mM ammonium. (b,c) Representative
302 photographs (b) and fresh weights (c) of shoots from 11-day-old Col-0, *chl1-5*, *nrt1.1*,
303 *stop1*^{KO}, *chl1-5 stop1*^{KO}, and *nrt1.1 stop1*^{KO} seedlings grown under 10 mM ammonium
304 with 100 nM IAA (using 100 μM IAA (in dimethyl sulfoxide) stock solution). (d) Fresh
305 weights of shoots and roots from 11-day-old Col-0, *chl1-5*, *nrt1.1*, *stop1*^{KO}, *chl1-5*
306 *stop1*^{KO}, and *nrt1.1 stop1*^{KO} seedlings grown under 10 mM ammonium without IAA.

307 (b-d) In one dish, nine seedlings of each line were regarded as a single biological
308 replicate (Mean ± SD; n = 3–4). Different lowercase letters indicate significant
309 differences evaluated by the Tukey–Kramer multiple comparison test conducted at a
310 significance level of $P < 0.05$.
311
312 **Figure 2.** NRT1.1 does not downregulate STOP1. (a) Relative transcript levels of
313 *ALMT1*, *CIPK23*, *GDH2*, *STOP1*, and *STOP2* in shoots and roots of five-day-old
314 seedlings from Col-0 and *chl1-5* grown under 10 mM ammonium or 10 mM nitrate
315 conditions without IAA. (b) Relative transcript levels of *ALMT1*, *CIPK23*, and *GDH2*
316 in five-day-old seedlings from Col-0, *chl1-5*, *nrt1.1*, *stop1*^{KO}, *chl1-5 stop1*^{KO}, and *nrt1.1*
317 *stop1*^{KO} grown under 10 mM ammonium without IAA. (a, b) Thirty-seven seedlings
318 from one plate were regarded as a single biological replicate (Mean ± SD; n = 3–5). (c)
319 Comparisons of the expression of STOP1-upregulated genes under acidic pH (Sawaki et
320 al. 2009) between Col-0 and *NRT1.1*-deficient mutants (*chl1-5*, *nrt1.1*) grown under 10
321 mM ammonium without IAA based on the microarray results (Hachiya et al. 2024).
322 Changes in gene expression levels were shown as logarithms to base 2 of fold change
323 (Mean ± SD). (d) GFP-STOP1 signals from the primary root tips of five-day-old
324 seedlings grown under 10 mM ammonium or 10 mM nitrate without IAA. The left four
325 images and lower right image were generated by the maximum intensity projection of
326 multiple slices of the range where fluorescence is clearly observed. The single cell
327 image in the upper right is a composite of bright and fluorescent images. Scale bars: 50

328 μm (left), 5 μm (upper right), and 20 μm (lower right). (e) A possible scheme for

329 ammonium tolerance regulation by NRT1.1 and STOP1.

Figure 1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NRT1.1
STOP1
EF1α
Col
chl1-5
nrt1.1
stop1KO
chl1-5 stop1KO
nrt1.1 stop1KO
NH4+
b
Col
chl1-5
stop1KO
chl1-5 stop1KO
NH4+
Col
nrt1.1
stop1KO
nrt1.1 stop1KO
NH4+
c
Shoot FW
% of Col
Col
chl1-5
stop1KO
chl1.5 stop1KO
NH4+
Shoot FW
Col
nrt1.1
stop1KO
nrt1.1 stop1KO
NH4+
d
Shoot FW
Root FW
% of Col
Col
chl1-5
stop1KO
chl1.5 stop1KO
NH4+
NO3-
Shoot FW
Root FW
Col
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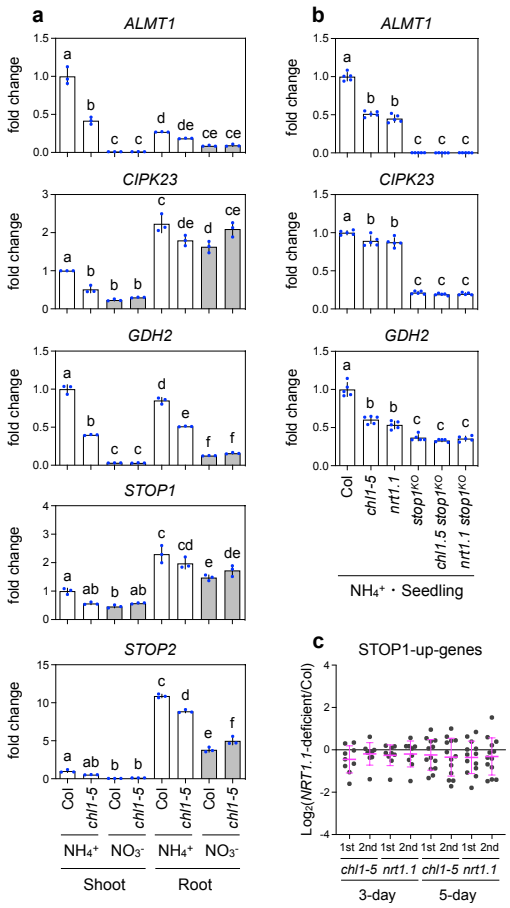
stop1KO
nrt1.1 stop1KO
NH4+
NO3-

Figure 2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ALMT1
CIPK23
GDH2
STOP1
STOP2
fold change
Col
chl1-5
NH4+
NO3-
Shoot
Root

b
ALMT1
CIPK23
GDH2
fold change
Col
chl1-5
nrt1.1
stop1KO
chl1.5 stop1KO
nrt1.1 stop1KO
NH4+ · Seedling

c
STOP1-up-genes
Log2(NRT1.1-deficient/Col)
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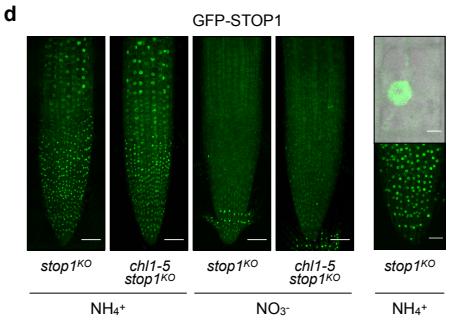
chl1-5 nrt1.1 chl1-5 nrt1.1
3-day
5-day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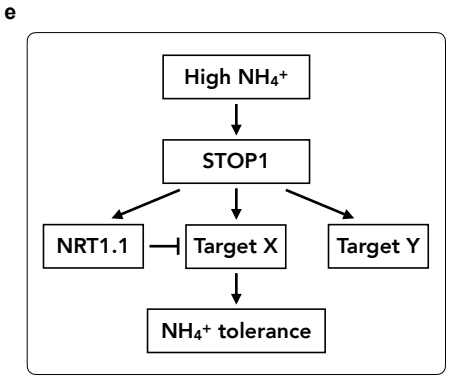
stop1KO
chl1-5 stop1KO
NH4+
NO3-


e
High NH4+
STOP1
NRT1.1
Target X
Target Y
NH4+ tolerance