Title

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

Running title

STOP1 overrides NRT1.1 in NH_4^+ tolerance

Authors and addresses

Takushi Hachiya[a,b†], Mako Sakai[a], Tsuyoshi Nakagawa[a], and Hitoshi Sakakibara[b,c]

[a]Department of Molecular and Functional Genomics, Interdisciplinary Center for Science Research, Shimane University, Matsue 690-8504, Japan

[b]RIKEN Center for Sustainable Resource Science, Yokohama 230-0045, Japan

[c]Graduate School of Bioagricultural Sciences, Nagoya University, Nagoya 464-8601, Japan

Corresponding Author

Takushi Hachiya

Department of Molecular and Functional Genomics, Interdisciplinary Center for Science Research, Shimane University, Matsue 690-8504, Japan

Email: takushi.hachiya@life.shimane-u.ac.jp, Tel: +81-852-32-6288

Type of contribution

Short paper

Division

Plant Nutrition

Abstract

The Arabidopsis nitrate transceptor NITRATE TRANSPORTER 1.1 (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 1 (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 1.1
48 (NRT1.1/NPF6.3/CHL1) acts as a nitrate sensor and orchestrates various plant
49 responses to the nitrate signal, including gene expression and root system development
50 (Bouguyon et al. 2015; Ho et al. 2009). Apart from its nitrate-dependent functions,
51 NRT1.1 exhibits significant roles in the absence of nitrate (Hachiya et al. 2024).
52 Previous studies have shown that the loss-of-function of NRT1.1 alleviates growth
53 suppression and chlorosis under toxic levels of ammonium as the sole N source, i.e.,
54 ammonium toxicity (Hachiya et al. 2011; Jian et al. 2018; Liu et al. 2020). Recently, we
55 reported that in early seedlings grown under 10 mM ammonium, the genes induced by
56 acidic stress are downregulated in *NRT1.1*-deficient mutants relative to the wild-type,
57 whereas those repressed by acidic stress are upregulated in the mutants (Hachiya et al.
58 2024). Moreover, an increase in medium pH promotes shoot growth of the wild-type
59 more intensely than that of the *NRT1.1*-deficient mutant under 10 mM ammonium.
60 Given that acidic stress is one of the primary causes of ammonium toxicity (Hachiya et
61 al. 2021), NRT1.1 may exacerbate the ammonium-dependent acidic stress.

62 In *Arabidopsis*, the zinc finger transcription factor SENSITIVE TO PROTON
63 RHIZOTOXICITY 1 (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 as the primary N source (Ye et al. 2021). These findings prompted us
69 to analyze the association between NRT1.1 and STOP1 under toxic levels of
70 ammonium as the sole N source. Here, we show that STOP1 dominates ammonium
71 tolerance and expression of 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 of KAPA SYBR FAST qPCR Kit
104 (Nippon Genetics, Tokyo, Japan), 0.4 μL of specific primers (0.2 μM final
105 concentration), and 7.2 μL of sterile water. Relative transcript levels were calculated
106 using the comparative cycle threshold method with *ACTIN3* as an internal standard.
107 Primer 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 shoot growth enhancement
124 in *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 is
134 epistatic to NRT1.1 regarding ammonium tolerance.
135
136 ***3.2 NRT1.1 does not downregulate* STOP1*/STOP1 and STOP1 dominates expression***
137 ***of direct target genes of STOP1 over NRT1.1***
138 The above growth analysis suggests that NRT1.1 could act upstream of STOP1 and
139 downregulate *STOP1*/STOP1 activities under ammonium. To verify this, we checked
140 the transcript levels of known direct target genes of STOP1, i.e.,
141 *ALUMINUM-ACTIVATED MALATE TRANSPORTER 1* (*ALMT1*), *CALCINEURIN*
142 *B-LIKE INTERACTING PROTEIN KINASE 23* (*CIPK23*), *GLUTAMATE*
143 *DEHYDROGENASE 2* (*GDH2*), and *SENSITIVE TO PROTON RHIZOTOXICITY 2*
144 (*STOP2*) (Balzergue et al. 2017; Enomoto et al. 2019; Sadhukhan et al. 2019, Tokizawa
145 et al. 2021) in the absence of IAA. In five-day-old Col-0 shoots and roots, the
146 expression of *ALMT1*, *CIPK23*, *GDH2*, and *STOP2* was significantly higher under 10
147 mM ammonium than under 10 mM nitrate (Figure 2a). However, this
148 ammonium-dependent transcriptional induction was consistently decreased by *NRT1.1*
149 deficiency (Figure 2a). Also, *STOP1* expression in shoots and roots was not
150 significantly increased by *NRT1.1* deficiency under ammonium (Figure 2a). Moreover,
151 our microarray analysis using three- and five-day-old seedlings under 10 mM
152 ammonium without IAA (Hachiya et al. 2024) revealed that *NRT1.1* deficiency did not
153 generally induce genes whose expression is upregulated via STOP1 under acidic pH

154 (Sawaki et al. 2009) (Figure 2b and Supplementary Table S2). It is widely accepted that
155 the F-box protein REGULATION OF ATALMT1 EXPRESSION 1 (RAE1) interacts
156 with STOP1 and promotes its degradation via the ubiquitin-26S proteasome pathway
157 (Zhang et al. 2019), i.e., the post-translational regulation of STOP1 activity. Recent
158 studies have shown that aluminum stress induces phosphorylation of STOP1 by MAP
159 KINASE 4 and oxidative modification of RAE1 by reactive oxygen species (Zhou et al.
160 2023; Ding et al. 2024). These modifications inhibit RAE1-dependent proteolysis of
161 STOP1, thereby enhancing aluminum tolerance. Thus, to analyze the effect of NRT1.1
162 on STOP1 expression, *chl1-5* was crossed with *pSTOP1:GFP-STOP1* line in the
163 *stop1*^{KO} background (Balzergue et al. 2017) and produced the *pSTOP1:GFP-STOP1*
164 line in the *chl1-5 stop1*^{KO} background. The GFP-STOP1-derived fluorescence signal
165 was promoted in the roots under 10 mM ammonium compared to 10 mM nitrate (Figure
166 2c), similar to a previous result (Tian et al. 2021). However, the GFP signal was little
167 changed by *NRT1.1* deficiency. Meanwhile, in five-day-old seedlings grown under 10
168 mM ammonium, the transcript levels of *ALMT1*, *CIPK23*, and *GDH2* were similar
169 among *stop1*^{KO}, *chl1-5 stop1*^{KO}, and *nrt1.1 stop1*^{KO}, all of which were lower than those
170 observed in *chl1-5* and *nrt1.1* (Figure 2d). These observations suggest that NRT1.1 does
171 not downregulate *STOP1*/STOP1 activities and that STOP1 dominates expression of
172 direct target genes of STOP1 over NRT1.1.
173
174 ***3.3 Possible scheme for regulation of ammonium tolerance by NRT1.1 and STOP1***

175 Ye et al. (2021) demonstrated that, under acidic conditions with nitrate as the primary N
176 source, STOP1 directly upregulates *NRT1.1* expression in the root, which facilitates
177 co-uptake of proton and nitrate, elevating rhizosphere pH to a favorable level for
178 *Arabidopsis*: therefore, NRT1.1 was epistatic to STOP1 on root nitrate uptake and root
179 elongation. Meanwhile, the present study showed that, under toxic levels of ammonium
180 without nitrate, ammonium-inducible STOP1 dominates ammonium tolerance and
181 target gene expression of STOP1 over NRT1.1 (Figure 1 and 2). Moreover, we found
182 that ammonium-dependent transcriptional induction of shoot *NRT1.1* was completely
183 suppressed by *STOP1* deficiency (Supplementary Figure 1), supporting that *NRT1.1* is
184 under the control of STOP1. To reconcile these observations, a possible scheme for
185 regulation of ammonium tolerance by NRT1.1 and STOP1 is presented (Figure 2e). The
186 STOP1 upregulated by ammonium induces the expression of *NRT1.1* and other target
187 gene(s). The function(s) of some target gene product(s) (i.e., Target X in Figure 2e)
188 could enhance ammonium tolerance but be suppressed by NRT1.1, possibly via
189 protein–protein interaction(s).

190 In *A. thaliana*, STOP1 directly induces *CIPK23* expression under toxic levels
191 of ammonium as the sole N source (Tian et al. 2021). CIPK23 inhibits ammonium
192 transport via phosphorylation of high-affinity AMMONIUM TRANSPORTER 1;1
193 (AMT1;1) and 1;2 (AMT1;2) (Straub et al. 2017). Since the loss-of-function of *CIPK23*
194 causes excessive ammonium uptake and thus ammonium hypersensitivity (Hachiya et al.
195 2024; Straub et al. 2017), STOP1 improves ammonium tolerance by upregulating
196 *CIPK23* (Tian et al. 2021). Notably, under low nitrate conditions, CIPK23 physically

interacts with NRT1.1 and phosphorylates it, increasing the affinity of NRT1.1 for nitrate (Ho et al. 2009). It remains unclear whether NRT1.1 reduces ammonium tolerance through its interaction with CIPK23 as a candidate of Target X (Figure 2e). Meanwhile, a recent study reported that STOP1 indirectly activates the ARABIDOPSIS PLASMA-MEMBRANE H^+-ATPase 2 (AHA2) by upregulating *SMALL AUXIN-UP RNA 55* (*SAUR55*) whose translational product prevents PP2C.D2/5, negative regulators of AHA2 (Agrahari et al. 2023). The plant Cistrome database implies that STOP1 could directly bind to the promoter of *AHA2*. By contrast, NRT1.1 and receptor-kinase QIAN SHOU KINASE 1 (QSK1) inactivate the AHA2 via phosphorylation on S899 under low nitrate (Zhu et al. 2024). Hence, STOP1 and NRT1.1 may affect ammonium-derived acidic stress via AHA2 in opposite directions and therefore, AHA2 could also be a possible candidate for Target X in Figure 2e. It would be valuable to explore the common target(s) of NRT1.1 and STOP1 for better understanding ammonium toxicity.

Acknowledgments

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

Disclosure statement

The authors report there are no competing interests to declare.

Funding

This study was supported by the Grants-in-Aid from the Ministry of Education, Culture, Sports, Science and Technology, Japan under Grant [17K15237/20K05771/23K04978], the Inamori Foundation, the Agropolis Foundation Grant [1502-405], and the Nagase Science and Technology Foundation, and the Yanmar Joint Research to TH.

References

Agrahari, R. K., Y. Kobayashi, T. Enomoto, T. Miyachi, M. Sakuma, M. Fujita, T. Ogata, et al. 2023. "STOP1-regulated *SMALL AUXIN UP RNA55* (*SAUR55*) is involved in proton/malate co-secretion for Al tolerance in Arabidopsis." *Plant Direct* 8(1): e557. doi: 10.1002/pld3.557.

Balzergue, C., T. Dartevelle, C. Godon, E. Laugier, C. Meisrimler, J. M. Teulon, A. Creff, et al. 2017. "Low phosphate activates STOP1-ALMT1 to rapidly inhibit root cell elongation." *Nature Communications* 8 (1): 15300. doi: 10.1038/ncomms15300.

Bouguyon, E., F. Brun, D. Meynard, M. Kubeš, M. Pervent, S. Leran, B. Lacombe, et al. 2015. "Multiple mechanisms of nitrate sensing by *Arabidopsis* nitrate transceptor NRT1.1." *Nature Plants* 1 (3): 15015. doi:10.1038/nplants.2015.15.

Ding, Z. J., C. Xu, J. Y. Yan, Y. X. Wang, M. Q. Cui, J. J. Yuan, Y. N. Wang, et al. (2024). "The LRR receptor-like kinase ALR1 is a plant aluminum ion sensor." *Cell Research*, 34 (4): 281−294. doi: 10.1038/s41422-023-00915-y.

Enomoto, T., M. Tokizawa, H. Ito, S. Iuchi, M. Kobayashi, Y. Y. Yamamoto, Y. Kobayashi, et al. 2019. "STOP1 regulates the expression of *HsfA2* and *GDHs* that are critical for low-oxygen tolerance in Arabidopsis." *Journal of Experimental Botany* 70 (12): 3297−3311. doi:10.1093/jxb/erz124.

Hachiya, T., Y. Mizokami, K. Mizokami, D. Tholen, C. K. Watanabe, and K. Noguchi. 2011. "Evidence for a nitrate-independent function of the nitrate sensor NRT1.1 in *Arabidopsis thaliana*." *Journal of Plant Research* 124 (3): 425−430. doi:10.1007/s10265-010-0385-7.

Hachiya, T. and K. Noguchi. 2011. "Mutation of NRT1.1 enhances ammonium/low pH-tolerance in *Arabidopsis thaliana*." *Plant Signaling & Behavior* 6 (5): 706−708. doi:10.4161/psb.6.5.15068.

Hachiya, T., J. Inaba, M. Wakazaki, M. Sato, K. Toyooka, A. Miyagi, M. Kawai-Yamada et al. 2021. "Excessive ammonium assimilation by plastidic glutamine synthetase causes ammonium toxicity in *Arabidopsis thaliana*." *Nature Communications* 12 (1): 4944. doi:10.1038/s41467-021-25238-7.

Hachiya, T., N. Makita, L. Bach, A. Gojon, T. Nakagawa, and H. Sakakibara. 2024. " Genetic and transcriptomic dissection of nitrate- independent function of Arabidopsis NRT1.1/ NPF6.3/CHL1 under high ammonium condition." *Soil Science and Plant Nutrition* in press. doi: 10.1080/00380768.2024.2326086.

Ho, C. H., S. H. Lin, H. C. Hu, and Y. F. Tsay. 2009. “CHL1 functions as a nitrate sensor in plants.” *Cell* 138 (6): 1184−1194. doi:10.1016/j.cell.2009.07.004.

Jian, S., Q. Liao, H. Song, Q. Liu, J. E. Lepo, C. Guan, J. Zhang, et al. 2018. "NRT1.1-related NH_4^+ toxicity is associated with a disturbed balance between NH_4^+ uptake and assimilation." *Plant Physiology* 178 (4): 1473−1488. doi:10.1104/pp.18.00410.

Liu, X. X., Y. X. Zhu, X. Z. Fang, J. Y. Ye, W. X. Du, Q. Y. Zhu, X. Y. Lin, et al., 2020. "Ammonium aggravates salt stress in plants by entrapping them in a chloride over-accumulation state in an NRT1.1-dependent manner." *Science of The Total Environment* 746: 141244. doi:10.1016/j.scitotenv.2020.141244.

Sawaki, Y., S. Iuchi, Y. Kobayashi, Y. Kobayashi, T. Ikka, N. Sakurai, M. Fujita, K., et al. 2009. "STOP1 regulates multiple genes that protect Arabidopsis from proton and aluminum toxicities." *Plant Physiology* 150 (1): 281−294. doi:10.1104/pp.108.134700.

Sadhukhan, A., T. Enomoto, Y. Kobayashi, T. Watanabe, S. Iuchi, M. Kobayashi, L. Sahoo, et al. 2019. "Sensitive to proton rhizotoxicity1 regulates salt and drought tolerance of *Arabidopsis thaliana* through transcriptional regulation of *CIPK23*." *Plant and Cell Physiology* 60 (9): 2113−2126. doi:10.1093/pcp/pcz120.

Straub, T., U. Ludewig, and B. Neuhäuser. 2017. "The kinase CIPK23 inhibits ammonium transport in *Arabidopsis thaliana*." *The Plant Cell* 29 (2): 409−422. doi: 10.1105/tpc.16.00806.

Tian, W. H., J. Y. Ye, M. Q. Cui, J. B. Chang, Y. Liu, G. X. Li, Y. R. Wu, et al. 2021. "A transcription factor STOP1-centered pathway coordinates ammonium and phosphate acquisition in *Arabidopsis*." *Molecular Plant* 14 (9): 1554−1568. doi:10.1016/j.molp.2021.06.024.

Tokizawa, M., T. Enomoto, H. Ito, L. Wu, Y. Kobayashi, J. Mora-Macías, D. Armenta-Medina, et al. 2021. "High affinity promoter binding of STOP1 is essential for early expression of novel aluminum-induced resistance genes *GDH1* and *GDH2* in Arabidopsis." *Journal of Experimental Botany* 72 (7): 2769−2789. doi:10.1093/jxb/erab031.

305 Tsay, Y. F., J. I. Schroeder, K. A. Feldmann, and N. M. Crawford. 1993. "The herbicide
306 sensitivity gene *CHL1* of Arabidopsis encodes a nitrate-inducible nitrate transporter."
307 *Cell* 72 (5): 705−713. doi:10.1016/0092-8674(93)90399-B.
308
309 Ye, J. Y., W. H. Tian, M. Zhou, Q. Y. Zhu, W. X. Du, Y. X. Zhu, X. X. Liu, et al. 2021.
310 "STOP1 activates NRT1.1-mediated nitrate uptake to create a favorable rhizospheric pH
311 for plant adaptation to acidity." *The Plant Cell* 33 (12): 3658−3674.
312 doi:10.1093/plcell/koab226.
313
314 Zhang, Y., J. Zhang, J. Guo, F. Zhou, S. Singh, X. Xu, Q. Xie, et al. "F-box protein
315 RAE1 regulates the stability of the aluminum-resistance transcription factor STOP1 in
316 *Arabidopsis*." *Proceedings of the National Academy of Sciences USA* 116 (1): 319−327.
317 doi: 10.1073/pnas.181442611.
318
319 Zhou, F., S. Singh, J. Zhang, Q. Fang, C. Li, J. Wang, C. Zhao, et al. "The
320 MEKK1-MKK1/2-MPK4 cascade phosphorylates and stabilizes STOP1 to confer
321 aluminum resistance in Arabidopsis." *Molecular Plant* 16 (2): 337−353. doi:
322 10.1016/j.molp.2022.11.010.
323
324 Zhu, Z., L. Krall, Z. Li, L. Xi, H. Luo, S. Li, M. He, et al. "Transceptor NRT1.1 and
325 receptor-kinase OSK1 complex controls PM H^+-ATPase activity under low nitrate."
326 *Current Biology* 34(7): 1479−1491. doi: 10.1016/j.cub.2024.02.066.

327

328 **Figure legends**

329 **Figure 1.** *STOP1* deficiency suppresses enhanced ammonium tolerance in
330 *NRT1.1*-deficient mutants. (a) RT-PCR and agarose gel electrophoresis for *NRT1.1*,
331 *STOP1*, and *EF1α* using five-day-old seedlings of Col-0, *chl1-5*, *nrt1.1*, *stop1*^{KO}, *chl1-5*
332 *stop1*^{KO}, and *nrt1.1 stop1*^{KO} grown under 10 mM ammonium. (b,c) Representative
333 photographs (b) and fresh weights (c) of shoots from 11-day-old Col-0, *chl1-5*, *nrt1.1*,
334 *stop1*^{KO}, *chl1-5 stop1*^{KO}, and *nrt1.1 stop1*^{KO} seedlings grown under 10 mM ammonium
335 with 100 nM IAA (using 100 μM IAA (in dimethyl sulfoxide) stock solution). (d) Fresh
336 weights of shoots and roots from 11-day-old Col-0, *chl1-5*, *nrt1.1*, *stop1*^{KO}, *chl1-5*
337 *stop1*^{KO}, and *nrt1.1 stop1*^{KO} seedlings grown under 10 mM ammonium without IAA.
338 (b-d) In one dish, nine seedlings of each line were regarded as a single biological
339 replicate (Mean ± SD; n = 3–4). Different lowercase letters indicate significant
340 differences evaluated by the Tukey–Kramer multiple comparison test conducted at a
341 significance level of $P < 0.05$.

342

343 **Figure 2.** NRT1.1 does not downregulate *STOP1*/STOP1 and STOP1 dominates
344 expression of direct target genes of STOP1 over NRT1.1. (a) Relative transcript levels
345 of *ALMT1*, *CIPK23*, *GDH2*, *STOP1*, and *STOP2* in shoots and roots of five-day-old
346 seedlings from Col-0 and *chl1-5* grown under 10 mM ammonium or 10 mM nitrate
347 conditions without IAA. (b) Comparisons of the expression of STOP1-upregulated
348 genes under acidic pH (Sawaki et al. 2009) between Col-0 and *NRT1.1*-deficient

349 mutants (*chl1-5*, *nrt1.1*) grown under 10 mM ammonium without IAA based on the
350 microarray results (Hachiya et al. 2024). Changes in gene expression levels were shown
351 as logarithms to base 2 of fold change (Mean ± SD). (c) GFP-STOP1 signals from the
352 primary root tips of five-day-old seedlings grown under 10 mM ammonium or 10 mM
353 nitrate without IAA. The left four images and lower right image were generated by the
354 maximum intensity projection of multiple slices of the range where fluorescence is
355 clearly observed. The single cell image in the upper right is a composite of bright and
356 fluorescent images. Scale bars: 50 µm (left), 5 µm (upper right), and 20 µm (lower
357 right). (d) Relative transcript levels of *ALMT1*, *CIPK23*, and *GDH2* in five-day-old
358 seedlings from Col-0, *chl1-5*, *nrt1.1*, *stop1*^{KO}, *chl1-5 stop1*^{KO}, and *nrt1.1 stop1*^{KO} grown
359 under 10 mM ammonium without IAA. (a, d) Thirty-seven seedlings from one plate
360 were regarded as a single biological replicate (Mean ± SD; n = 3–5). (e) A possible
361 scheme for ammonium tolerance regulation by NRT1.1 and STOP1.

Figure 1

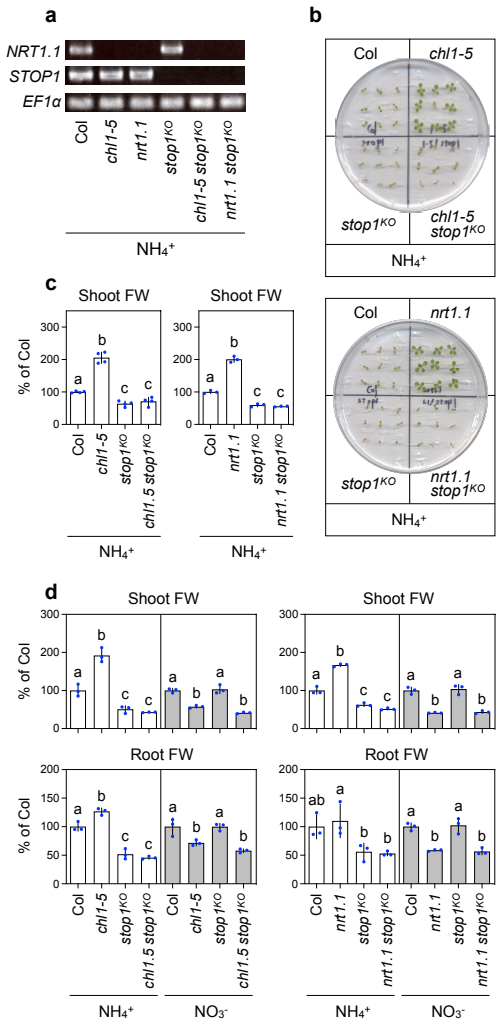
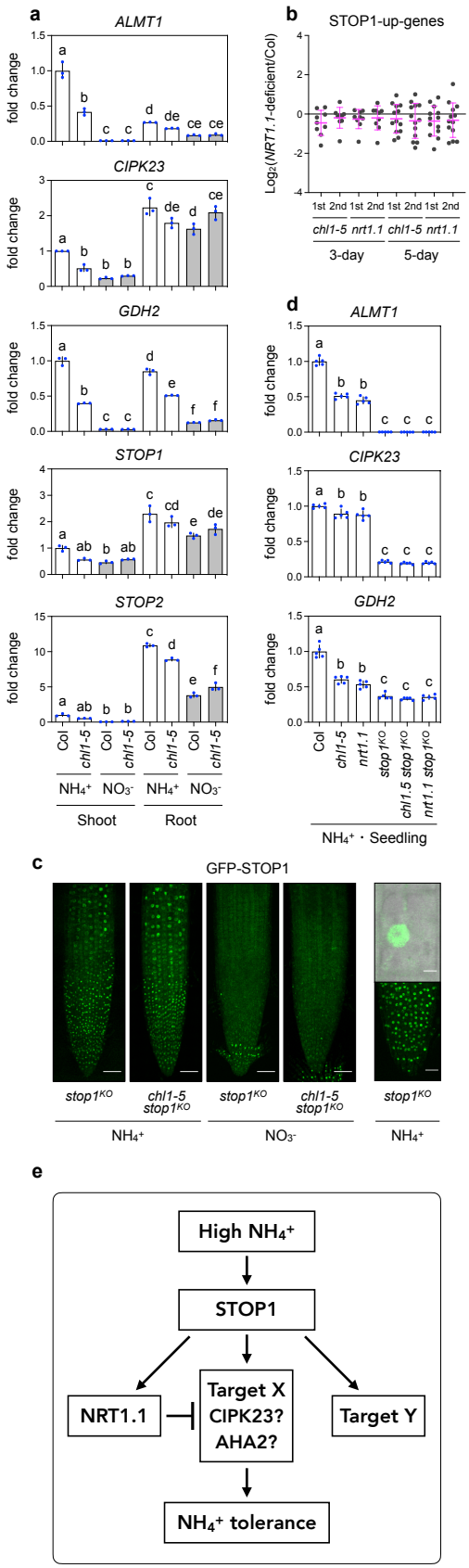

a
NRT1.1
STOP1
EF1α
Col
chl1-5
nrt1.1
stop1KO
chl1-5 stop1KO
nrt1.1 stop1KO
NH4+
b
Col
chl1-5
stop1KO
chl1-5 stop1KO
NH4+
Col
nrt1.1
stop1KO
nrt1.1 stop1KO
NH4+
c
Shoot FW
% of Col
Col
chl1-5
stop1KO
chl1.5 stop1KO
NH4+
Shoot FW
Col
nrt1.1
stop1KO
nrt1.1 stop1KO
NH4+
d
Shoot FW
Root FW
% of Col
Col
chl1-5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

Figure 2


a
ALMT1
CIPK23
GDH2
STOP1
STOP2
fold change
Col
chl1-5
NH4+
NO3-
Shoot
Root
b
STOP1-up-genes
Log2(NRT1.1-deficient/Col)
1st 2nd 1st 2nd 1st 2nd 1st 2nd
chl1-5 nrt1.1 chl1-5 nrt1.1
3-day
5-day
d
ALMT1
CIPK23
GDH2
Col
chl1-5
nrt1.1
stop1KO
chl1.5 stop1KO
nrt1.1 stop1KO
NH4+ · Seedling
c
GFP-STOP1
stop1KO
chl1-5 stop1KO
NH4+
NO3-
e
High NH4+
STOP1
NRT1.1
Target X
CIPK23?
AHA2?
Target Y
NH4+ tolerance