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Transceptor NRT1.1 and receptor-kinase QSK1 complex controls PM H⁺-ATPase activity under low nitrate

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Running title: NRT1.1-RK complex controls proton pump activity in low nitrate

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1 **Summary**

2 NRT1.1, a nitrate transceptor, plays an important role in nitrate binding, sensing and
3 nitrate dependent lateral root (LR) morphology. However, little is known about NRT1.1-
4 mediated nitrate signaling transduction through plasma membrane (PM)-localized
5 proteins. Through in-depth phosphoproteome profiling using membranes of
6 *Arabidopsis* roots, we identified receptor kinase QSK1 and plasma membrane H⁺-
7 ATPase AHA2 as potential downstream components of NRT1.1 signaling in a mild low
8 nitrate (LN)-dependent manner. QSK1, as a functional kinase and molecular link,
9 physically interacts with NRT1.1 and AHA2 at LN, and specifically phosphorylates
10 AHA2 at S899. Importantly, we found that LN, not HN, induces formation of NRT1.1-
11 QSK1-AHA2 complex in order to repress the proton efflux into the apoplast by
12 increased phosphorylation of AHA2 at S899. Loss of either NRT1.1 or QSK1 thus
13 results in a higher T947/S899 phosphorylation ratio on AHA2, leading to enhanced
14 pump activity and longer LRs under LN. Our results uncover a regulatory mechanism
15 in which NRT1.1, under LN conditions, recruits coreceptor QSK1 into a complex to
16 transduce LN sensing to the PM H⁺-ATPase AHA2, controlling the phosphorylation
17 ratio of activating and inhibitory phosphorylation sites on AHA2. This then results in
18 altered proton pump activity, apoplast acidification, and regulation of NRT1.1-mediated
19 LR growth.

20

21 **Introduction**

22 Nitrogen is an essential macronutrient and nitrate serves as an important signaling
23 molecule required for growth, gene expression, and metabolism in plants.¹ The nitrate
24 concentration in soil can influence plant root growth, shoot elongation, as well as crop
25 yield. Improving nitrate use efficiency in plants is critical for increasing agricultural
26 production. The molecular mechanism of how nitrate is sensed and transported into
27 plants is well known since the characterization of the nitrate transporter and sensor
28 (transceptor) NRT1.1² (CHL1/NPF6.3) and the high-affinity nitrate transporter NRT2.1³
29 in *Arabidopsis thaliana*.

30 NRT1.1 in *Arabidopsis* is essential for nitrate transport, sensing, binding and
31 signaling.⁴ Phosphorylation of NRT1.1 at T101 by the CIPK23-CBL9 calcium sensor-
32 kinase complex switches NRT1.1 from a low-affinity to a high-affinity transporter and
33 increases NRT1.1-dependent nitrate uptake.⁵ Dephosphorylation of NRT1.1 is fine-
34 tuned by protein phosphatase ABI2 (abscisic acid insensitive 2).⁶ NRT1.1 activation
35 also induces a rapid increase in cytoplasmic Ca²⁺ levels.⁷ Recently, the NRT1.1-
36 CNGC15 module was found to be a molecular switch that controls calcium influx in a
37 nitrate-dependent manner.⁸ Calcium subsequently activates CPK10/30/32 to
38 phosphorylate transcription factor NLP7 at S205, promoting NLP7 retention in the
39 nucleus.⁹ Very recently, NLP7 was shown to be an intracellular nitrate sensor, as
40 nitrate can bind NLP7 directly.¹⁰ In rice, OsNRT1.1B-SPX4-NBIP-OsNLP3 was the first
41 module that demonstrated nitrate signaling from the plasma membrane (PM) to the
42 nucleus.¹¹ However, little is known about whether more regulatory components in
43 NRT1.1-mediated nitrate signal transduction exist in the PM.

44 NRT1.1 also plays an important role in root morphology by directly influencing lateral
45 root (LR) growth. Under low nitrate (LN) availability, NRT1.1 as well as phosphorylation
46 of NRT1.1 at T101 plays a key role in repression of LR growth, and NRT1.1 is
47 considered to repress LR growth through its auxin transport capacity.^{12,13} Other studies
48 also revealed that there is a crosstalk between nitrate and auxin in modulating of
49 NRT1.1-dependent LR growth.¹⁴ Plasma membrane-localized H⁺-ATPases pump H⁺
50 into the apoplast between the plasma membrane and cell wall, resulting in acidification
51 of the apoplast and increased cell elongation.^{15,16} However, it remains unknown

52 whether NRT1.1-mediated LR growth inhibition under LN relies on altered PM H⁺-
53 ATPase activity and apoplastic pH.

54 Many studies have suggested that genes involved in nitrate signaling are regulated at
55 the transcriptional level.¹⁷⁻¹⁹ However, there are indications of posttranslational
56 regulation of nitrogen uptake, sensing, and metabolism through phosphorylation.²⁰
57 Numerous nitrogen-dependent phosphoproteomic studies²¹⁻²⁴ identified PM-localized
58 transporters and receptor kinases, which showed nitrate-dependent phosphorylation
59 changes. However, whether, and by which molecular mechanisms these PM proteins
60 are involved in nitrate signaling and transport regulation still needs to be explored.

61 Here we carried out a systematic functional study of roots of WT and *nrt1.1-1* to
62 generate mild low and high nitrate-induced membrane protein phosphorylation profiles.
63 Using these profiles, we aimed to discover new regulatory components for NRT1.1-
64 mediated nitrate signal transduction. Ultimately, we uncovered a novel function of
65 NRT1.1, which interacts with the receptor kinase QSK1 to constitute a molecular switch
66 that controls PM H⁺-ATPase activity through phosphorylation balance on different
67 phosphorylation sites (T947/S899) of AHA2 in a mild low nitrate dependent manner.

68 **Results**

69 **Functional membrane phosphorylation profiling identifies downstream** 70 **components of NRT1.1**

71 We performed a comparative phosphoproteomic analysis of *Arabidopsis* root
72 membrane protein comparing WT and *nrt1.1-1* under mild low nitrate (LN) and high
73 nitrate (HN)-induced conditions after nitrogen starvation (NS) (Figure.1A) to identify
74 downstream components that may be involved in NRT1.1-dependent LR growth and
75 signal transduction. We identified 5,950 phosphosites corresponding to 1,945 proteins
76 (Figure.S1A). Among these, there were 2,390 previously uncharacterized
77 phosphosites and 3,246 new nitrate-induced phosphosites compared to the
78 PhosPhAt4.0 database²⁵ and published nitrate-induced phosphosites in *Arabidopsis*,
79 respectively²¹⁻²³ (Figure S1B and S1C). Among these were 2,295 phosphosites
80 corresponding to 1,280 proteins with localization probability >0.75 (class I sites), which
81 were used for quantitative statistical analysis (Supplementary Table 1). The
82 phosphorylation sites distributed to peptides with 93.4% single, 6% double, and 0.6%
83 triple or more phosphorylation (Figure S1D). Principal component analysis (PCA)

84 revealed a strong separation of WT under LN and HN along the first principal
85 component, and data points for WT at LN and *nrt1.1-1* at LN separated along the
86 second component. Data points for WT and *nrt1.1-1* at HN clustered tightly and
87 showed no separation (Figure 1B).

88 We performed stringent pairwise comparison analysis for genotypes and treatments to
89 separate LN and HN specific responses. In the WT, 642 phosphosites exhibited
90 significantly different abundance between LN vs NS or HN vs NS. Of these, 579 and
91 36 phosphosites were specifically changed under LN and HN, respectively. 515 of 579
92 phosphosites exhibited a higher abundance under LN (Figure 1C). Overall, LN induced
93 larger changes in the membrane phosphoproteome compared to HN. Subsequently,
94 we compared the phosphoproteome of the WT and *nrt1.1-1*. Under LN, 661
95 phosphosites were found with altered abundance in *nrt1.1-1*, out of which 608
96 phosphosites showed significantly decreased phosphorylation in *nrt1.1-1* (Figure 1D).
97 The asymmetry of the plots suggested that the absence of NRT1.1 had a strong impact
98 on the membrane phosphoproteome under LN. Overlap of phosphosites with
99 significantly changed phosphorylation in WT under LN vs. HN, and under LN in
100 *nrt1.1-1* compared to WT identified 307 phosphosites corresponding to 231 proteins
101 as candidates for being downstream regulators of NRT1.1 (Figure 1E), indicating LN-
102 induced phosphorylation of these proteins is dependent on the presence of NRT1.1.
103 These 231 proteins were enriched in functions of Signaling (bin30), Transport (bin34),
104 RNA (bin27), and Cell (bin31), and were overrepresented with subcellular locations at
105 the PM and in the N (nucleus) (Figure 1F).

106 We then ranked the phosphosites with significantly changed phosphorylation in *nrt1.1-1*
107 under LN (based on *p* values) and highlighted phosphosites on 19 transport-related
108 proteins (Figure S1E). Among the highly-ranked phosphorylation sites, T947 and S899
109 from the plasma membrane H⁺-adenosine triphosphatase 2 (PM H⁺-ATPase isoform,
110 AHA2) were also identified (Figure S2A and S2B). AHA2 is the major proton pump in
111 roots. These two residues were reported as resulting in activation (T947) and
112 inactivation (S899) upon phosphorylation^{26,27}, and are located in the C-terminal
113 autoinhibitory domain.²⁸ AHA2 is known to promote both lateral root and primary root
114 growth under LN.²⁹ T947 and S899 in AHA2 exhibited significantly higher
115 phosphorylation in WT in LN than in HN, and phosphorylation at both sites, but
116 especially S899 was drastically decreased in *nrt1.1-1* under LN, but not HN (Figure 2A

117 and 2B). AHA2 protein abundances showed no change (Figure 2C). These results
118 suggested that the presence of NRT1.1 is required for phosphorylation of AHA2 under
119 LN. Since phosphorylation at T947 and S899 have contrasting effects on AHA2 activity,
120 we used the ratio of T947 and S899 phosphorylation as an indication of the balance of
121 AHA2 activity. Interestingly, the phosphorylation ratio of T947/S899 was significantly
122 increased in WT at LN compared to HN, and an even more increased ratio was found
123 in *nrt1.1-1* compared to WT under LN, while no difference was observed under HN
124 (Figure 2D). This suggests that loss of NRT1.1 disrupted the balance of
125 phosphorylation between T947 and S899 at LN due to a stronger reduction of
126 phosphorylation at S899.

127 **NRT1.1 represses proton efflux at LN**

128 PM-localized ATPases fine-tunes proton efflux into the apoplast, resulting in apoplastic
129 acidification. To assess whether NRT1.1 is required for H⁺-ATPase activation under
130 LN, we applied membrane-impermeable 8-hydroxypyrene-1,3,6-trisulfonic acid
131 trisodium salt (HPTS) as a ratiometric fluorescent pH indicator for assessing changes
132 in the apoplastic pH at cellular resolution in the LR elongation zone.³⁰ Two different
133 forms of HPTS (protonated and deprotonated) were observed in two channels using a
134 confocal microscope with excitation wavelengths of 405 nm and 488 nm, respectively.
135 The ratiometric value (458/405) represents the apoplastic pH, and a high ratio
136 represents a relatively high pH. The WT exhibited a lower pH in LN compared to HN.
137 Moreover, an even lower pH was observed in *nrt1.1-1* under LN (Figure 2E and 2F),
138 uncovering that the higher phosphorylation ratio of T947/S899 (Figure 2D) resulted in
139 activation of H⁺-ATPase in WT under LN compared to HN, and an even more increased
140 activation of the H⁺-ATPase in *nrt1.1-1* than in WT under LN. These results suggested
141 that PM H⁺-ATPases function downstream of NRT1.1, mediating the LN-induced
142 proton efflux into the apoplast space. In turn, the phosphorylation ratio of T947/S899
143 controls the AHA2 activity.

144 As a result of enhanced proton pump activity, the average cortical cell length of LR
145 in the WT was longer under LN than HN, and an even longer cortical cell length was
146 observed in *nrt1.1-1* under LN, but not under HN (Figure 2G and 2H). Consequently,
147 WT displayed longer LR length under LN, and *nrt1.1-1* exhibited more enhanced LR
148 growth (Figure S2C and S2D). Taken together, our results suggested that the lower

149 apoplastic pH in the LR elongation zone led to longer cortical cell length and LR in
150 WT in LN, and that the even lower apoplastic pH in *nrt1.1-1* produced even longer
151 cortical cell length and LR. The longer LR in *nrt1.1-1* under HN was not correlated
152 with the apoplastic pH. Under LN, the reduced inhibition of AHA2 at S899 in *nrt1.1-1*
153 seems to override the co-occurring slightly decreased phosphorylation at T947, the
154 activating site. Direct interaction of NRT1.1 and AHA2 was not observed by ratiometric
155 bimolecular fluorescence complementation (rBiFC) assays (Figure 2I and S3A), the
156 known interaction of CBL9-CIPK23 and the known absent interaction of CBL9-CIPK14
157 were used as positive control and negative control, respectively. As NRT1.1 itself
158 cannot phosphorylate AHA2 at different sites, we postulate a kinase exists as a
159 molecular link between NRT1.1 and AHA2 under LN.

160 **Coreceptor QSK1 functions downstream of NRT1.1 to repress LR growth in LN**

161 To assess the potential kinases involved in this NRT1.1-mediated regulation, we
162 ranked phosphorylation sites (based on *p* value) on receptor kinases (Figure 3A and
163 Figure S3B), and found that phosphorylation of the coreceptor QSK1 at S621/S626
164 was detected in the WT under LN, however, no phosphorylation of QSK1S621/S626
165 was detectable either in the WT under HN or in *nrt1.1-1* (Figure 3B), while the protein
166 abundance of QSK1 was unchanged (Figure 3C). Therefore, we conclude that NRT1.1
167 is required for the phosphorylation of QSK1 at S621/S626 under LN. QSK1
168 phosphorylation at S621/S626 was also found in previous experiments under LN
169 condition.^{22,23}

170 QSK1 is strongly expressed in the root elongation zone and LR primordia³¹, which
171 overlaps with the expression pattern of NRT1.1.¹² To investigate whether QSK1 as well
172 as its phosphorylation sites regulates LN-induced LR growth in similar way as NRT1.1,
173 we generated transgenic plants overexpressing QSK1, phospho-dead (AA) and
174 phosphorylation-mimic QSK1 (DD) at S621S626 into *qsk1*. Compared to WT, the LR
175 length was significantly longer in *qsk1* under LN, and QSK1-OE/*qsk1* can complement
176 *qsk1* phenotype. Consistent with the observation of *qsk1* under LN, LR of QSK1-AA-
177 OE/*qsk1* were significantly longer when compared with the WT, while QSK1-DD-
178 OE/*qsk1* displayed the similar phenotype as WT under LN (Figure 3D and 3E), implying
179 that phosphorylation-mimic QSK1 can rescue the phenotype of *qsk1*, repressing LR
180 growth under LN.

181 Published protein-protein interaction networks from *Arabidopsis* roots suggested that
182 QSK1 may form a complex with NRT1.1 and AHA2 in a LN-dependent manner.³² rBiFC
183 assays confirmed that NRT1.1 directly interacts with QSK1 in the plasma membrane
184 (Figure 4A and Figure S4A). H356 of NRT1.1 is critical for nitrate binding and
185 uptake.^{33,34} To test whether nitrate binding is required for interaction of NRT1.1 with
186 QSK1, we mutated H356 to H356A. Indeed, interaction between NRT1.1H356A and
187 QSK1 was weaker compared to NRT1.1-QSK1 (Figure 4A and Figure S4A). To further
188 test whether LN-induced phosphorylation of NRT1.1 at T101² and QSK1 at S621S626
189 (Figure 3B) are essential for NRT1.1-QSK1 interaction, we analyzed the interaction of
190 different combinations of NRT1.1 and QSK1 mutants. We found that the NRT1.1-QSK1
191 interaction was not affected by either the phosphorylation-dead (NRT1.1-A) or
192 phosphorylation-mimic version (NRT1.1-D) of NRT1.1T101. The phospho-dead
193 QSK1S621/S626 (QSK1-AA) still interacted with NRT1.1-A, but the interaction of
194 NRT1.1 and QSK1 was significantly enhanced by phosphorylation mimic QSK1
195 (QSK1-DD) and NRT1.1 (NRT1.1-D) (Figure 4A and Figure S4A). Importantly, co-
196 immunoprecipitation (Co-IP) assays using membrane proteins isolated from tobacco
197 leaves expressing NRT1.1-eGFP and QSK1-FLAG showed that association between
198 NRT1.1-eGFP and QSK1-FLAG was enhanced when LN is present, not HN,
199 suggesting that LN promotes the NRT1.1-QSK1 interaction (Figure 4B). Altogether
200 these results demonstrated that NRT1.1 directly interacts with QSK1, and this
201 interaction is facilitated by the perception of LN by NRT1.1 and subsequent
202 phosphorylation events.

203 We next created a *nrt1.1-qsk1* double mutant to analyze the effect on LR growth.
204 Compared to WT, the LRs length was significantly longer in *qsk1*, *nrt1.1-1*, and the
205 *nrt1.1-qsk1* double mutant in LN, while the LR length in the double mutant resembled
206 that of the two single mutants. At HN, only the *nrt1.1-1* and the *nrt1.1-qsk1* double
207 mutant showed longer LRs length, with no effect observed in *qsk1* (Figure 4C and 4D).
208 These results suggested that QSK1 and NRT1.1 function in the same signaling
209 pathway to repress LR growth in LN. To elucidate whether QSK1 contributes to nitrate
210 uptake under LN, we performed nitrate influx assays on N starved plant or after
211 induction with 1 mM nitrate for 1 or 4 h (Figure 4E). Nitrate influx was measured after
212 supplying a nutrient solution containing 200 μ M K¹⁵NO₃ during 5 minutes. In N starved
213 plants, higher uptake rates were observed in *qsk1* compared to WT. This difference

214 was not observed when plants were treated with 1 mM NO₃⁻. These results indicated
215 that QSK1 inhibits LR growth and nitrate uptake in LN-dependent manner.

216 **QSK1 interacts with and phosphorylates AHA2S899 and represses proton pump** 217 **activity in LN**

218 To investigate association of QSK1 and AHA2, we then performed rBiFC and Co-IP
219 assays. rBiFC assays confirmed the interaction of QSK1-DD and AHA2, while QSK1-
220 AA and AHA2 interaction was not obvious (Figure 5A and Figure S4B). Using Co-IP
221 assays, a much stronger association was observed between QSK1-DD-FLAG and
222 AHA2-MYC compared to QSK1 or QSK1-AA with AHA2 (Figure 5B).

223 We next examined the phosphorylation status of AHA2 at T947 and S899 in WT and
224 *qsk1* roots. LN-induced phosphorylation of AHA2 at T947 and S899 was significantly
225 decreased in *qsk1*, while under HN no changes were observed for either phospho-site
226 (Figure 5C and 5D), suggesting that under LN, QSK1 is required for phosphorylation
227 of both sites on AHA2. The protein abundances of AHA2 displayed no change (Figure
228 5E). The phosphorylation ratio of T947/S899 was also disrupted in *qsk1*, being
229 significantly higher in *qsk1* compared to WT under LN, but not under HN (Figure 5F).
230 The AHA2 phosphorylation patterns found in *qsk1* resemble T947/S899 ratio found in
231 *nrt1.1-1* under LN, implying an increased activity of H⁺-ATPase in also *qsk1*.
232 Furthermore, an *in vitro* peptide phosphorylation assay using the recombinant
233 intracellular domain of QSK1 (QSK1C) and synthesized peptides covering S899 or
234 T947 site as substrates, confirmed that QSK1C could phosphorylate S899, but not
235 T947 on the synthesized peptide (Figure 5G).

236 To verify the role of QSK1 in impeding PM H⁺-ATPase activity under LN, we again
237 used HPTS as a pH indicator on the LR elongation zone of WT and *qsk1*. *qsk1* showed
238 lower apoplastic pH in the LR elongation zone under LN compared to WT (Figure 5H
239 and 5I), which correlates with longer cortical cell (Figure 5J and 5K) and LR length
240 (Figure 3E). These results suggested that QSK1 could serve as a downstream
241 component of NRT1.1 and that QSK1 has a key role in repressing apoplast acidification
242 through direct phosphorylation of AHA2 at S899 under LN, not HN condition.

243 **NRT1.1-QSK1 complex coordinately transduce LN sensing to the proton pump**

244 Our results show that LN enhanced NRT1.1-QSK1 interaction (Figure 4B), induced
245 phosphorylation of QSK1 (Figure 3B), and phosphorylated QSK1 displayed stronger
246 association with AHA2 (Figure 5B). Thus, we propose that NRT1.1-mediated LN
247 sensing may modulate the QSK1-AHA2 interaction. To test this hypothesis, we
248 performed Co-IP assays using membrane protein mixture extracted from *Nicotiana*
249 *benthamiana* leaves harboring NRT1.1-eGFP, QSK1-FLAG and AHA2-MYC in
250 presence of LN, HN, or KCl. A strong interaction between QSK1-FLAG and AHA2-
251 MYC was observed in the presence of LN-NRT1.1, with the interaction becoming
252 weaker in the presence of HN-NRT1.1 or KCl- NRT1.1 (Figure 6A). We also analyzed
253 the effect of LN on apoplast pH and cortical cell length of LRs in double mutant *nrt1.1-*
254 *1qsk1*. As expected, *nrt1.1-1qsk1* double mutant showed lower apoplastic pH in LR
255 elongation zone and longer cells when compared to WT, but no differences when
256 compared to respective single mutants under LN (Figure 6B and 6C). Together, these
257 results suggested that LN promotes NRT1.1-QSK1-AHA2 complex in order to
258 transduce the LN signal from NRT1.1 to AHA2 through QSK1. Formation of this
259 complex then ultimately results in repression of the proton pump activity through
260 phosphorylation at inhibitory S899, which ultimately then affects cell elongation and
261 nitrate uptake.

262 Discussion

263 NRT1.1 is a plasma membrane-localized nitrate transceptor and critical for nitrate
264 binding and sensing. However, it is still poorly understood how NRT1.1 mediates
265 transduction of nitrate signals within plasma membrane localized proteins. Recent
266 findings that NRT1.1 can form a complex with Ca²⁺channel CNGC15 suggest that
267 protein-protein interactions are the molecular mechanism coupling nitrate sensing and
268 signaling to downstream processes such as Ca²⁺ signaling.⁸ Here, based on in-depth
269 membrane phosphoproteomics analysis, our data provide a first unbiased view of
270 NRT1.1-dependent phosphorylation events under LN (Figure 1). A noteworthy finding
271 is that phosphorylation of several receptor kinases and transporters under LN depends
272 on the presence of NRT1.1 (Figure 3A and Figure S1E), for example, three of the
273 known ammonium and nitrate-regulated phosphosites, T460 and S480/S489, in the
274 cytosolic C-terminal region of AtAMT1;1 were identified.³⁵ This opens the possibility
275 that some of these proteins could be involved in further complexes with NRT1.1
276 integrating, for example, nitrate and ammonium signaling.

277 Our work describes a new role of NRT1.1 in modulation of AHA2 activity by formation
278 of a protein-protein interaction complex. This is unlike the ability of NRT1.1 to directly
279 transport auxin.^{12,13} Since NRT1.1 is not directly associated with AHA2 (Figure 2I) we
280 postulated that kinases exist between NRT1.1 and AHA2, and at the plasma membrane,
281 receptor kinases are likely candidates linking NRT1.1 and proton pump AHA2 (Figure
282 3A). Receptor kinases play critical roles in perception and transduction of extracellular
283 signals, as well as in plant growth and defense.³⁶ Several studies have uncovered that
284 RKs could directly interact with PM-localized transport proteins and regulate their
285 activity. For example, TMK1, PSY1R, and BAK1 are well characterized in activation of
286 AHA2 through phosphorylation modification at different sites on AHA2^{27,37-40}, while
287 FERONIA can induce phosphorylation of S899 on AHA2, inhibiting proton efflux.²⁶
288 FLS2 interacts with Ca²⁺-ATPase ACA8 to regulate Ca²⁺ bursts⁴¹, and SIRK1 can
289 phosphorylate and activate aquaporins.^{42,43} These short, posttranscriptional and direct
290 regulatory circuits between RKs and transport proteins indicates a rapid and economic
291 mechanism, allowing plants to efficiently respond to local signals and rapidly adjust to
292 changes in the environment. Our findings provide a first evidence for a transceptor
293 being linked to another transmembrane transport proteins (here: AHA2) by a receptor
294 kinase, QSK1.

295 QSK1 (“thousand-hand” qiān shǒu kinase 1) is a highly expressed co-receptor kinase
296 in *Arabidopsis thaliana*. Depending of the physiological state of the plant, it is able to
297 directly phosphorylate specific substrate proteins, such as an ABC camalexin
298 transporter upon fungal infection⁴⁴ and potassium channel TPK1 during stomatal
299 closure.⁴⁵ It can also recruit substrates such as the aquaporin PIP2;4 to other receptor
300 kinases, such as SIRK1, forming a complex ultimately resulting in substrate
301 phosphorylation.^{42,43} Therefore, QSK1 is proposed to be a coreceptor and responsible
302 for recruiting different substrates to the respective signaling complexes. QSK1 is
303 known to be able to change localization, being depleted from ordered low-density
304 membranes (DRM) upon treatment with cytochalasin D or oryzalin.⁴⁶ Here, we show
305 that phosphorylated QSK1 recruits and phosphorylates AHA2 under LN (Figure 5).

306 Posttranslational regulation of PM H⁺-ATPases by phosphorylation and
307 dephosphorylation plays a crucial role in the activation or inactivation in response to
308 stress.⁴⁷⁻⁴⁹ Several phosphorylation sites within the autoinhibitory C-terminal domain
309 of PM H⁺-ATPases, which differentially affect pump activity, have been identified.

310 Among them, phosphorylation at T947 and T881 of AHA2 increases pump
311 activity^{27,37,38}, while phosphorylation at S899 and S931 inhibits pump activity.^{26,50} flg22,
312 a peptide derived from pathogenic bacterial flagella, elicits an increase in
313 phosphorylation at S899 and a decrease at T881 and T947, and leads to a rapid
314 apoplastic alkalinization.⁵¹ PM-localized ATPases regulates H⁺ efflux into the apoplast,
315 resulting in apoplastic acidification, which in turn affects LR growth. The proton
316 gradient is also used for ion uptake as well as cell elongation. Also, nitrate uptake has
317 been found to be coupled to protons.³³ We found that the elevated phosphorylation
318 ratio T947/S899 on AHA2 in *nrt1.1-1* and *qsk1* at LN results in higher AHA2 activity
319 and subsequently longer LRs (Figure 2 and Figure 5). Our results show that the PM
320 H⁺-ATPase activity is controlled coordinately by the balance of phosphorylation at
321 different sites. Since QSK1 specifically phosphorylates the S899 on AHA2 (Figure 5G),
322 thus, we propose there exist other proteins (e.g., RKs or other Kinases) downstream
323 of NRT1.1 which phosphorylate T947 on AHA2.

324 It is interesting to note that QSK1 is a coreceptor, not a ligand-binding receptor kinase.
325 Further research will be necessary to identify possible receptor kinase, as well as their
326 ligands, may function in a NRT1.1 dependent or independent manner. As NRT1.1
327 plays a critical role in the assembly of the here described signaling module, one may
328 speculate that NRT1.1, as a transceptor, functions as a main receptor to transduce the
329 LN signal to co-receptor QSK1. Mild nitrate deficiency activates BR signaling and
330 induces LR elongation⁵², while the role of auxin in LN-mediated LR elongation has
331 been well studied.^{12,13,53} Additional research will be directed in exploring whether BR
332 and auxin are involved in regulating the NRT1.1-QSK1-AHA2 module.

333 In summary, we demonstrate that at LN, nitrate binding to NRT1.1 is able to enhance
334 the NRT1.1-QSK1 interaction and phosphorylation of S621S626 on coreceptor QSK1.
335 This is the first example for a nutrient sensing transporter to recruit a co-receptor for
336 signal transduction. Phosphorylated QSK1 then recruits AHA2 into the complex in
337 order to phosphorylate S899 on AHA2. Since also phosphorylation of AHA2 at T947
338 can be affected by NRT1.1 or QSK1, we suspect further yet unknown kinases to be
339 involved in regulation of AHA2 activity directly or indirectly. (Figure 6D). Our results
340 have not only identified the key role of RKs and proton pump AHA2 under LN signaling
341 but has also coupled LN sensing from NRT1.1 to the activity of the proton pump.

342 **Methods**

343 **Plant Material and growth conditions**

344 For liquid growth cultures, *Arabidopsis* seeds of WT (Col-0), *qsk1* mutant
345 (SALK_019840), and *nrt1.1-1 mutant* (SALK_097431) were sterilized and grown in
346 basal medium composed of micro- and macronutrients with a total of 1 mM KNO₃. After
347 19 days, seedlings were starved for 2 days by changing the growth medium to a
348 nitrogen-free medium. Nitrate was then resupplied to a final concentration of 0.45 mM
349 (mild low nitrate, LN) and 9.4 mM (high nitrate, HN) for 15 minutes before harvesting
350 roots for microsomal protein preparation. All experiments consisted of at least three
351 biological replicates.

352 For LR growth analysis, WT (Col-0), *qsk1*, *35S::QSK1-GFP/qsk1* (QSK1-OE/ *qsk1*),
353 *35S::QSK1S621AS626A-GFP/qsk1* (QSK1-AA-OE/*qsk1*), *35S::QSK1S621DS626D-*
354 *GFP/qsk1* (QSK1-DD-OE/*qsk1*), and *nrt1.1-1* seeds were grown on medium
355 mentioned above with 1% agar and 0.45 mM or 9.4 mM KNO₃ for 10 days in a growth
356 chamber and positioned vertically (16/8 hours light/night). Seedlings were scanned as
357 pictures on the 10th day and LR length was measured using Fiji software.

358 **Constructs**

359 For ratiometric bimolecular fluorescence complementation (rBiFC) of *Arabidopsis*
360 proteins, cDNAs of the following genes were cloned into rBiFC plasmids⁵⁴: NRT1.1,
361 NRT1.1H356A, NRT1.1T101A, NRT1.1T101D and AHA2 were cloned as fusions with
362 the C-terminal half of YFP, whereas QSK1, QSK1 QSK1S621A/S626A,
363 QSK1S621D/S626D were cloned as fusions with the N-terminal half of YFP. All
364 constructs were transformed into *Agrobacterium tumefaciens* strain GV3101 by
365 electroporation. Positive colonies were confirmed by spectinomycin and rifampicin
366 resistance and colony PCR.

367 For purification of the cytoplasmic domain of QSK1 (QSK1C, amino acid 276–627)
368 were cloned into *Escherichia coli* BL21(DE3) expressing plasmid pETGST1a and
369 fused with His and GST tags, resulting in the plasmid His-GST-SIRK1C and His-GST-
370 QSK1C.

371 **Protein expression and purification**

372 Plasmids His-GST-SIRK1C and His-GST-QSK1C were transformed into *Escherichia*
373 *coli* BL21 (DE3). After 5 hours induction by IPTG (isopropyl- β -thiogalactopyranoside),
374 cells were harvested and lysed using lysis buffer (20mM Tris-HCl pH 7.4, 1mM EDTA,
375 200mM NaCl, 1mM PMSF, 1mM DTT), soluble fractions were used over gravity flow
376 Ni²⁺-NTA Sepharose columns for His-GST-SIRK1C and His-GST-QSK1C protein
377 purification.

378 **Co-immunoprecipitation assays**

379 Membrane protein was extracted from infiltrated Tobacco (*Nicotiana Benthamian*)
380 leaves with IP buffer (50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1% Triton
381 X-100, 10% glycerol, 1 mM PMSF, 1× protease inhibitor cocktail), 0.45 mM potassium
382 nitrate or potassium chloride was then added and incubated with anti-GFP magnetic
383 agarose beads (Chromotek, gta-20) for 3 h at 4 °C. The beads were washed five times
384 with wash buffer (50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1× protease
385 inhibitor cocktail). The precipitated proteins were eluted with 2×SDS buffer loading
386 buffer at 95 °C for 10 min for immunoblot analysis using the indicated antibodies
387 (Abcam).

388 **Microsomal membrane preparation, tryptic digestion, and phosphopeptide** 389 **enrichment**

390 Microsomal membrane (MF) preparation and phosphopeptide enrichment were
391 performed as described in the “ShortPhos” workflow⁵⁵. A total of 1 g of roots (fresh
392 weight) was homogenized in 10 ml extraction buffer (330mM sucrose, 100 mM KCl, 1
393 mM EDTA, 50 mM Tris-MES, fresh 5 mM DTT, and 1 mM phenylmethylsulfonylfluoride,
394 pH 7.5) in the presence of 0.5% v/v proteinase inhibitor mixture (MedChemExpress)
395 and phosphatase inhibitors (25 mM NaF, 1 mM Na₃VO₄, 1 mM benzamidin, 3 μ M
396 leupeptin) in Dounce homogenizers. The homogenate was centrifuged for 15 minutes
397 at 7500 × g at 4 °C. The pellet was discarded, and the supernatant was centrifuged for
398 75 minutes at 48,000 × g at 4 °C. The microsomal pellet was re-suspended in 100 μ l
399 UTU (6 M urea, 2 M thiourea, pH 8). Protein concentrations were determined using a
400 Bradford (Sigma–Aldrich) assay with BSA (Bovine serum albumin) as protein standard.
401 Samples were stored at -80°C.

402 150 μ g MF were aliquoted for tryptic digestion and phosphopeptides enrichment. MF
403 were predigested for 3 h with endoproteinase Lys-C (0.5 μ g/ μ l; Wako Chemicals) at

404 room temperature. After a 4-fold dilution with 10 mM Tris-HCl (pH 8), samples were
405 digested with 3 μ l sequencing-grade modified trypsin (0.5 μ g/ μ l; Promega) overnight
406 at 37 °C. After overnight digestion, 10% v/v trifluoroacetic acid (TFA) was added (until
407 the pH was 3 or less) to stop digestion. Digested peptides were dried in a vacuum
408 concentrator.

409 Dry peptides were dissolved in 200 μ l of 1 M glycolic acid in 80% v/v acetonitrile (ACN)
410 and 5% v/v trifluoroacetic acid (TFA). Phosphopeptides were enriched over titanium
411 dioxide (TiO₂) (GL Sciences). 1.5 mg TiO₂ beads per sample were washed once with
412 100 μ l of 1% v/v ammonia solution and equilibrated three times with 50 μ l of 1 M
413 glycolic acid in 80% v/v ACN and 6% v/v TFA. 200 μ l digested peptides were mixed
414 with equilibrated TiO₂ for 30 min incubation. Peptides and TiO₂ beads mixture were
415 washed once with 100 μ l of 1 M glycolic acid in 80% v/v ACN and 6% v/v TFA, and
416 three times with 100 μ l of 80% v/v ACN and 1% v/v TFA. Phosphopeptides were eluted
417 from TiO₂ beads three times with 1% v/v ammonia solution. Eluates were immediately
418 acidified with 70 μ l of 10% v/v formic acid. Acidified phosphopeptides were desalted
419 over a C18 stage tip prior to mass spectrometric analysis.

420 **LC-MS/MS of Peptides and Phosphopeptides**

421 Enriched phosphopeptides were resuspended in 5 μ l resuspension buffer (0.2% v/v
422 TFA, 5% v/v ACN) and analyzed via LC-MS/MS using standard setting as described³³
423 with nanoflow Easy-nLC 1200 (Thermo Scientific) as an HPLC system and an Orbitrap
424 hybrid mass spectrometer (Orbitrap exploris 480, Thermo Scientific) as a mass
425 analyzer. Peptides were eluted from a 75 μ m homemade 25 cm analytical column on
426 a linear gradient running from 4 to 64% acetonitrile over 130 min and sprayed directly
427 into the mass spectrometer. Peptides were identified via MS/MS based on the
428 information-dependent acquisition of fragmentation spectra of multiple charged
429 peptides. A data-dependent acquisition MS method was used, and for full-scan
430 spectrum and MS/MS scan acquired at resolution of 60,000 and 15,000 at m/z 200.

431 **Protein Identification and Ion Intensity Quantitation**

432 Raw data acquired by the mass spectrometer were processed using MaxQuant⁵⁶,
433 version 1.6.5.0 using settings as described for protein identification and label-free
434 intensity quantitation (LFQ). Spectra were matched against the *Arabidopsis* proteome
435 UP000006548 (UniProt database, 39,324 entries). Common contaminants (trypsin,

436 keratin, etc.) were included during database searches. Carbamidomethylation of
437 cysteine was set as a fixed modification, and the oxidized methionine (M), acetylation
438 (protein N-term) and phosphorylation (STY) were set as variable modifications. Trypsin
439 was specified as the digesting protease, and up to two missed cleavages were allowed.
440 The mass tolerance for the database search was set to 20 ppm for full scans and 0.5
441 Da for ions fragment. The multiplicity was set to 1. For label-free quantitation, retention
442 time matching between runs was chosen within a time window of 0.7 min. False
443 discovery rate cutoffs were set to 0.01 for peptide and protein identification, and to 0.05
444 for phosphorylation site assignment. Hits to contaminants (e.g., keratins) and reverse
445 hits identified by MaxQuant were excluded from further analysis.

446 **Bioinformatics and statistical Analysis**

447 Bioinformatics analysis was performed with Perseus software (version 1.6.4.0).⁵⁷ For
448 the analysis of phosphoproteomic results, reported label free intensity (LFQ) values
449 (Phospho (STY)Sites. txt) were used for data analysis. Missing values were imputed
450 from a normal distribution around the detection limit of the mass spectrometer. For
451 each phosphosite, imputed values from five or six biological replicates in different
452 genotypes or treatment were used for student *t* tests (FDR<0.05, S0=0.1).

453 Annotations were extracted from MapMan⁵⁸, subcellular locations were obtained from
454 SUBA3.⁵⁹ Functional enrichment analysis was done via Fisher's exact test, *p* values
455 were adjusted using Bonferroni correction. *p* value indicates a degree of significance
456 and enrichment factor represent the level of enrichment with respect to the background.

457 **Ratiometric bimolecular fluorescence assay**

458 *Agrobacterium tumefaciens* harboring the relevant constructs described above were
459 injected into 5 to 6 weeks old *Nicotiana benthamiana* leaves for 2 days before
460 observation. Fluorescence was observed using a Zeiss LSM800 confocal microscope
461 (20× water immersion objective). In all cases, excitation intensities, filter settings,
462 photomultiplier gains and other parameters were standardized. The YFP and RFP
463 fluorochromes were excited with 488 nm and 561 nm, respectively. Emitted light was
464 collected at a range of 500–560 nm for YFP and 575–625 nm for RFP. All images
465 throughout all experiments were collected using the same settings. The collected
466 images were processed and both YFP and RFP intensity was measured using the FIJI
467 software and the YFP/RFP ratio was calculated. RFP is internal control. At least 20

468 different cells from leaves of the 2 plants were analyzed. Statistical significance was
469 determined using one-way ANOVA test with a Tukey test. To calibrate YFP/RFP ratios,
470 known interaction of CBL9 with CIPK23 was used as positive control, whereas the
471 interaction of CBL9 with CIPK14 was used as negative control.⁶⁰ YFP/RFP ratio
472 greater than 1 indicate interaction of two proteins, while YFP/RFP ratio smaller than 1
473 suggest weak or no interaction.

474 **HPTS assay**

475 HPTS staining was performed as described³⁰ with minor modifications. 10 days old
476 seedlings grown on LN and HN medium were transferred into liquid LN and HN
477 medium supplemented with 1mM HPTS for 30 min. The seedlings were subsequently
478 mounted in the same growth medium supplemented with HPTS on a microcopy slide.
479 HPTS stained images of lateral roots elongation zone was performed using an inverted
480 Zeiss LSM800 confocal microscope equipped with a highly sensitive GaAsP detector.
481 Fluorescent signals for the protonated HPTS form (Excitation 405 nm, emission peak
482 514 nm), as well as the deprotonated HPTS form (excitation 488 nm, emission peak
483 514 nm) were detected with a 20× (water immersion) objective. The image analysis
484 was performed using the Fiji macro. The ratiometric value (488/405) correlates with
485 the apoplastic pH. The higher value represents higher pH.

486 **¹⁵NO₃ influx experiment**

487 Root NO₃-influx was assayed as described by Laugier et al.⁶¹ Five weeks old
488 hydroponically-grown plants were nitrate-starved for 5 days, then subjected to 1 mM
489 NO₃ for 0h, 1h or 4h. After washing with 0.1 mM CaSO₄, roots were subsequently
490 transferred into a nutrient solution containing 200μM ¹⁵NO₃ (99 atom% excess 15N)
491 for 5 minutes. Roots were harvested and dried at 70°C for 48 h, and samples were
492 analyzed for total N and atom% 15N using a continuous flow isotope ratio mass
493 spectrometer coupled with a C/N elemental analyzer (model Euroflash; Eurovector,
494 Pavia, Italy). 6-12 replicates were performed in each genotype and condition.

495 **An *in vitro* peptide phosphorylation assay**

496 *In vitro* peptide phosphorylation assays were performed as described in published
497 paper⁴³ with minor modification. In brief, kinase activity assays were performed with 10
498 pmol of the peptides GLDIETPSHYTV (covering T947) or EAVNIFPEKGSYR

499 (covering S899) as a substrate and His-GST-QSK1-Cterminal Domain (QSK1C) as
500 kinases in 30 μ l kinase reaction buffer (20 mM Tris/HCl pH 7.5, 10 mM $MgCl_2$, 1 mM
501 $MnCl_2$, 0.1% BSA, 2 mM DTT, 100 μ M ATP). After incubation for one hour, the reaction
502 mixture was acidified with TFA. Acidified mixture was then desalted over C18 column.
503 The phosphorylation of peptides was analyzed vis mass spectrometry and quantified
504 based on the intensity of signature fragment ion.

505 **Author contributions**

506 Z.Z and L.K performed most experiments. X.N.W, L.K and W.X.S designed research;
507 S.L ran samples in mass spectrometry; X.L and T.W assisted with confocal microscope
508 for rBiFC; Z.L, Z.H, X.Y and S.G for phosphoproteomic sample preparation; H.L, M.G,
509 M.H and J.Z for partial data analysis. B.N, A.J and L.L for ^{15}N uptake assay. X.N.W,
510 W.X.S and L.K wrote paper.

511 **Data availability**

512 The raw MS data from this study was deposited at the ProteomeXchange Consortium
513 via the PRIDE partner repository with the identifier PXD045818 (User name:
514 reviewer_pxd045818@ebi.ac.uk Password: KiHE8l6e).

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528 **Declaration of interests**

529 The authors declare no competing interest.

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Figure legends

Figure 1. The global membrane phosphoproteome of Arabidopsis roots was changed in the *nrt1.1-1* mutant under LN condition

(A) The work flow of High-throughput phosphoproteomics to identify NRT1.1-mediated LN and HN-induced signaling components.

(B) Principal components analysis (PCA) of WT and *nrt1.1-1* under LN and HN conditions. The first and second components are shown.

(C) Student *t*-test difference between LN-induced and nitrogen starvation (NS) at membrane phosphoproteome (*x* axis) and the difference between HN-induced and nitrogen starvation (NS) in membrane phosphoproteome (*y* axis) in WT. Significant phosphosites were determined using a permutation-based false discovery rate calculation ($FDR \leq 0.05$, $S_0 = 0.1$). The phosphosites were colored for significant changed abundance in *x* axis (orange), *y* axis (red), and both (purple), respectively.

(D) Student *t*-test difference between *nrt1.1-1* and WT in LN-induced membrane phosphoproteome (*x* axis) and the difference between *nrt1.1-1* and WT in HN-induced membrane phosphoproteome (*y* axis). Significant phosphosites were determined using a permutation-based false discovery rate calculation ($FDR \leq 0.05$, $S_0 = 0.1$). The phosphosites were colored for significant changed abundance in *x* axis (green), *y* axis (blue) and, both (black), respectively.

(E) Venn diagram showing overlap for phosphosites with significantly changed phosphorylation under LN compared to HN (C) and *nrt1.1-1* compared to WT in LN (D).

(F) Fisher exact test (2% FDR) on the group of 307 phosphosites corresponding to 231 proteins that are indicated in (E). Enriched Mapman terms and SUBA are displayed.

791 **Figure 2. NRT1.1 represses T947/S899 phosphorylation ratio on AHA2 and**
792 **proton pump activity in LN**

793 (A and B) Difference of phosphorylation levels induced by LN and HN at T947 and
794 S899 sites of PM H⁺-ATPase AHA2 in WT and *nrt1.1-1*.

795 (C) Difference in protein levels of AHA2 induced by LN and HN in WT and *nrt1.1-1*.

796 (D) Box plots show T947/S899 phosphorylation ratio for WT and *nrt1.1-1* under LN and
797 HN. Center lines of boxes represent medians, vertical line indicate the
798 minimal/maximal value.

799 Data are mean + SD, at least 4 biological replicates (A-D), statistical analysis (A-D)
800 was performed using Student t-test (**** $P < 0.0001$; NS, not significant).

801 (E and F) Quantification (E) and confocal images (F) of apoplastic pH in the lateral root
802 elongation zone (EZ) of WT and *nrt1.1-1* grown in LN and HN medium. Ratiometric
803 value (488 nm/405 nm) of fluorescent HPST was used to monitor pH in the EZ of lateral
804 roots, $n \geq 38$ individual root for each. For F, scale bar, 20 μm .

805 (G and H) Quantification (G) and images (H) of cortical cell length of lateral roots
806 mature zone of WT and *nrt1.1-1* grown in LN and HN medium, $n \geq 63$ cells for each.

807 For H, scale bar, 50 μm . Statistical analysis (E and G) was performed using one-way
808 ANOVA with a Tukey test (**** $P < 0.0001$; NS, not significant).

809 (I) Quantification of the in vivo interaction of NRT1.1, with AHA2. Around 20 randomly
810 selected cells were quantified. The known interaction of CBL9-CIPK23 and the known
811 absent interaction of CBL9-CIPK14 were used as positive control and negative control,
812 respectively. Scale bar, 20 μm . Different letters represent significant differences at
813 $p < 0.05$ according to one-way ANOVA with a Tukey test.

814 **Figure 3. LN promotes NRT1.1-QSK1 interaction and QSK1 phosphorylation**

815 (A) Ranking of phosphosites significantly upregulated in WT under LN and
816 downregulated in *nrt1.1-1* in LN. 10 named receptor-like kinases are highlighted in red.

817 (B) Phosphorylation differences of S621/S626 on QSK1 in WT and *nrt1.1-1* induced
818 by LN and HN.

819 (C) Differences of protein levels in QSK1 induced by LN and HN in WT and *nrt1.1-1*.

820 Data are mean + SD, at least 4 biological replicates (B-C), statistical analysis (B-C)
821 was performed using Student *t*-test (different letter indicates $P < 0.05$).

822 (D) Representative images of WT, *qsk1*, transgenic line overexpressing QSK1, QSK1
823 phospho-dead and as well as phosphorylation-mimic version in *qsk1* mutant (QSK1-
824 OE/*qsk1*, QSK1-AA-OE/*qsk1* and QSK1-DD-OE/*qsk1*) in LN and HN medium, scale
825 bar 1cm.

826 (E) Total lateral root length of WT, *qsk1*, QSK1-OE/*qsk1*, QSK1-AA-OE/*qsk1* and
827 QSK1-DD-OE/*qsk1* in LN and HN medium. Bar plots indicate means \pm SEM (at least
828 17 independent seedlings). Lateral roots were measured after 8 days on LN and HN.

829 Statistical analysis was performed using one-way ANOVA (**** $P < 0.0001$; NS, not
830 significant).

831 **Figure 4. QSK1 functions downstream of NRT1.1 to modulate LRs growth in LN**

832 (A) Quantification of the *in vivo* rBiFC interaction of QSK1, QSK1SASA, and
833 QSK1SDSD with NRT1.1, NRT1.1H356A, NRT1.1T01A, and NRT1.1T01D. rBiFC
834 calibration is shown in Figure S3. 18-35 randomly selected cells were quantified.
835 (B) Co-IP assays using membrane protein mixture extracted from tobacco leaves
836 (*Nicotiana benthamiana*) expressing NRT1.1-eGFP and QSK1-FLAG in the presence
837 of LN (0.45 mM KNO₃), HN (9.4 mM KNO₃) or potassium chloride (0.45 mM).
838 (C) Representative images of WT, *nrt1.1-1*, *qsk1* and *nrt1.1-1qsk1* in LN and HN
839 medium, scale bar, 1cm. Different letters (A and D) represent significant differences at
840 $p < 0.05$ according to one-way ANOVA with a Tukey test.
841 (D) Total lateral root length of WT, *nrt1.1-1*, *qsk1*, and *nrt1.1-1qsk1* in LN and HN
842 medium. Bar plots indicate means \pm SEM (16-20 independent seedlings). Lateral roots
843 were measured after 10 days on LN and HN medium.
844 Different letters (A and D) represent significant differences at $p < 0.05$ according to one-
845 way ANOVA with a Tukey test.
846 (E) ¹⁵Nitrate uptake activity under LN. Bar plots indicate means \pm SEM (at least 10
847 biological replicates). Statistical analysis was performed using two-tailed Student *t*-test
848 ($P < 0.05$; NS, not significant).

849

850 **Figure 5. phosphorylated QSK1 forms a strong complex with AHA2 and**
851 **represses proton pump activity in LN**

852 (A) Quantification of the *in vivo* rBiFC interaction of QSK1, QSK1SASA and
853 QSK1SDSD with AHA2. rBiFC calibration is shown in Figure S3. 50 randomly selected
854 cells were quantified. Different letters represent significant differences at $p < 0.05$
855 according to one-way ANOVA with a Tukey test.
856 (B) Co-IP assays using membrane protein mixture extracted from tobacco leaves
857 (*Nicotiana benthamiana*) expressing QSK1-FLAG, QSK1-AA-FLAG and QSK1-DD-
858 FLAG with AHA2-MYC.
859 (C and D) Difference of phosphorylation levels of T947 (C) and S899 (D) on AHA2
860 induced by LN and HN in WT and *qsk1*.
861 (E) Difference of protein levels of AHA2 under LN and HN in WT and *qsk1*.
862 (F) Box plots show T947/S899 phosphorylation ratio for WT and *qsk1* under LN. Center
863 lines of boxes represent medians, vertical line indicates the minimal/maximal value.
864 Data (C, D and E) are mean+SD. Statistical analysis (C-F) was performed using two-
865 tailed Student *t*-test (**** $P < 0.0001$; NS, not significant).
866 (G) *In vitro* peptide phosphorylation assay using GLDIETPSHYTV (covering T947) and
867 EAVNIFPEKGSYR (covering S899) from AHA2 as substrates for the QSK1C terminus.
868 nd indicates not detected.
869 (H and I) Quantification (H) and confocal images (I) of apoplastic pH in the lateral root
870 EZ of WT and *qsk1* grown on LN and HN medium. Ratiometric value (488 nm/405 nm)

871 of fluorescent HPST was used to monitor pH in the EZ of lateral roots, $n \geq 39$ individual
872 root for each. For I, scale bar 20 μm .
873 (J and K) Quantification (J) and images (K) of cortical cell length of the lateral roots
874 mature zone of WT and *qsk1* grown on LN and HN medium. $n \geq 61$ cells for each. For
875 K, scale bar 50 μm .
876 Statistical analysis (H and J) was performed using one-way ANOVA with a Tukey test
877 (**** $P < 0.0001$; NS, not significant).

878 **Figure 6. NRT1.1-QSK1 complex regulates AHA2 activity in LN**

879 (A) Co-IP assays using membrane protein mixture extracted from tobacco leaves
880 (*Nicotiana benthamiana*) expressing NRT1.1-eGFP, QSK1-FLAG and AHA2 in the
881 presence of LN (0.45 mM KNO_3), HN (9.4 mM KNO_3) or potassium chloride (0.45 mM).
882 (B) Quantification of apoplastic pH in the lateral root EZ of WT, *nrt1.1-1*, *qsk1* and
883 *nrt1.1-1qsk1* grown in LN and HN medium. Ratiometric value (488 nm/405 nm) of
884 fluorescent HPST was used to monitor pH in the EZ of lateral roots, $n \geq 16$ individual
885 root for each.
886 (C) Quantification of the cortical cell length of lateral roots mature zone of WT, *nrt1.1-1*,
887 *qsk1* and *nrt1.1-1qsk1* grown on LN and HN medium, $n \geq 152$ cells for each.
888 Statistical analysis (B and C) was performed using one-way ANOVA with a Tukey test
889 (**** $P < 0.0001$; NS, not significant).
890 (D) Proposed model for LN signal transduction through NRT1.1-QSK1 to AHA2.
891 Perception of LN leads to NRT1.1 interacting with and phosphorylation of QSK1.
892 Subsequently QSK1 recruits AHA2 and directly phosphorylates AHA2S899. NRT1.1
893 and QSK1 also induces AHA2T947 phosphorylation indirectly through unknown
894 proteins (e.g. Kinases). As a result, phosphorylation ratio of T947/S899 and thus H⁺-
895 ATPase activity is affected. In both the *nrt1.1-1* and *qsk1* mutants, phosphorylation
896 ratio of T947/S899 is dramatically increased due to a stronger reduction of
897 phosphorylation at S899, resulting in an even higher proton activity, longer cells and
898 longer LRs.

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900 **Supplementary Figures and Tables**

901 **Figure S1 The global phosphoproteome of *Arabidopsis* roots of WT and *nrt1.1-1*** 902 **under LN and HN-induced conditions.**

903 (A) Distribution of the assigned phosphorylated amino acid residues for class I
904 phosphosites.
905 (B) Venn diagram showing the overlap of the phosphosites identified in this study and
906 published in previous nitrate-induced experiments.
907 (C) Venn diagram showing the overlap of the phosphosites identified in this study and
908 published in previous nitrate-induced experiments.
909 (D) Summary of identified and quantified class I phosphosites (localization probability
910 of > 0.75) corresponding to number of proteins in this study.

911 (E) Ranking of phosphosites significantly upregulated in WT under LN and
912 downregulated in *nrt1.1-1* in LN. 19 named transport proteins are highlighted in blue.
913 **Figure S2** (A and B) Representative spectra of AHA2 C-terminal peptides
914 GLDIETPSHYTV (containing phosphorylated T947 (A) and EAVNIFPEKGSYR
915 (containing phosphorylated S899) (B) directly exported from MaxQuant version 1.6.4.0.
916 The phosphorylated amino acid is indicated by (ph).
917 (C) Representative images for total lateral root length of WT and *nrt1.1-1* in LN and
918 HN, scale bar 1cm.
919 (D) Total lateral root length of WT and *nrt1.1-1* in LN and HN. Bar plots indicate means
920 \pm SEM (at least 16 independent seedlings). Lateral roots were measured after 10 days
921 on LN and HN medium. Different letters represent significant differences at $P < 0.05$
922 according to one-way ANOVA.
923 **Figure S3** (A) Representative images of the in vivo interaction of NRT1.1, with AHA2.
924 The known interaction of CBL9-CIPK23 and the known absent interaction of CBL9-
925 CIPK14 were used as positive control and negative control, respectively. Cartoons
926 show the respective T-DNA of the rBiFC-2in1-CC vector, harboring different versions
927 of CIPK23-nYFP and CIPK14-nYFP with cYFP-CBL9, NRT1.1-cYFP and AHA2-nYFP.
928 Scale bar, 20 μ m.
929 (B) Representative spectra of QSK1 C-terminal peptides LIEEVSHSSGSPNPVSD
930 (containing phosphorylated S621S626) directly exported from MaxQuant version
931 1.6.4.0. The phosphorylated amino acid is indicated by (ph).
932 **Figure S4** (A) Representative images of the in vivo interaction of QSK1, QSK1SASA,
933 and QSK1SDSD with NRT1.1, NRT1.1H356A, NRT1.1T01A, and NRT1.1T01D.
934 (B) Representative images of the in vivo interaction of QSK1, QSK1SASA and
935 QSK1SDSD with AHA2 (C).
936 Cartoons show the respective T-DNA of the rBiFC-2in1-CC vector, harboring different
937 versions of QSK1s-nYFP, NRT1.1s-cYFP and AHA2-cYFP. Scale bar, 20 μ m.
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939 **Supplementary Table 1** Quantitative phospho-profiling in WT and *nrt1.1-1* mutant.
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Figures:

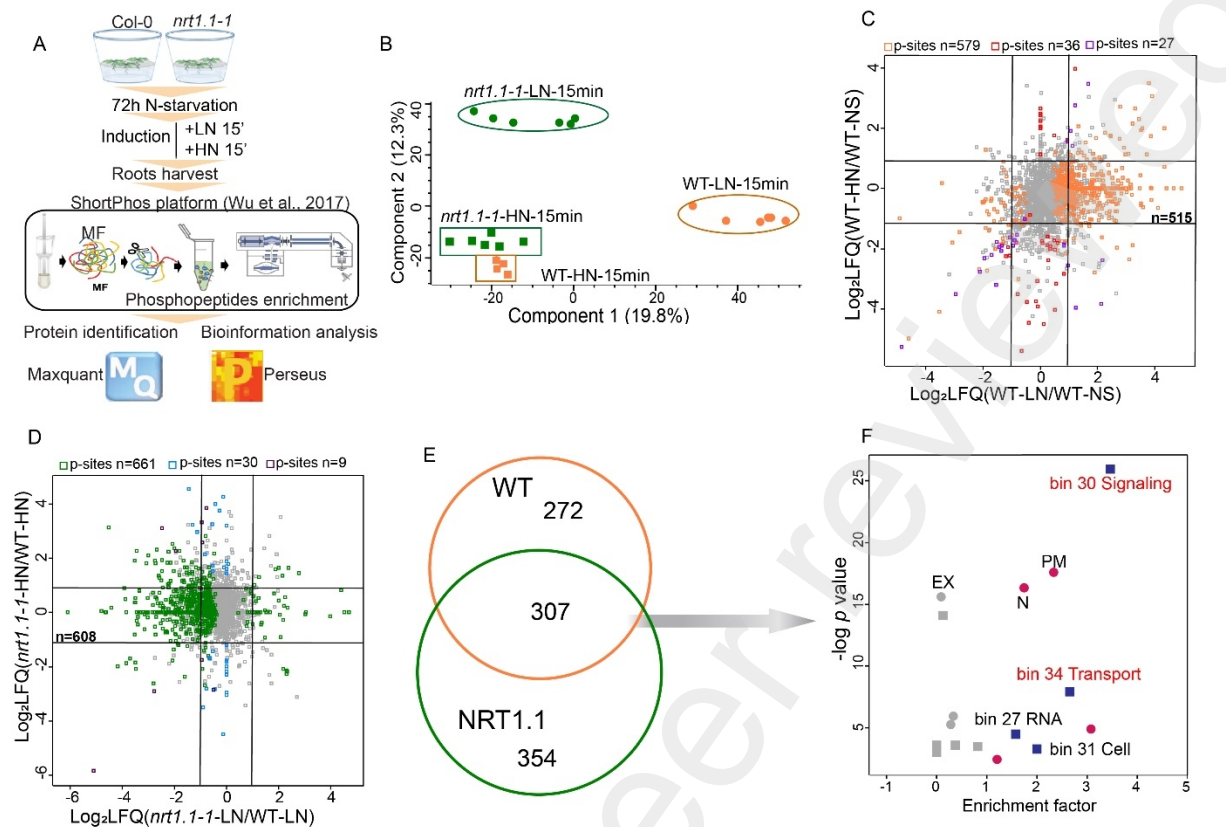


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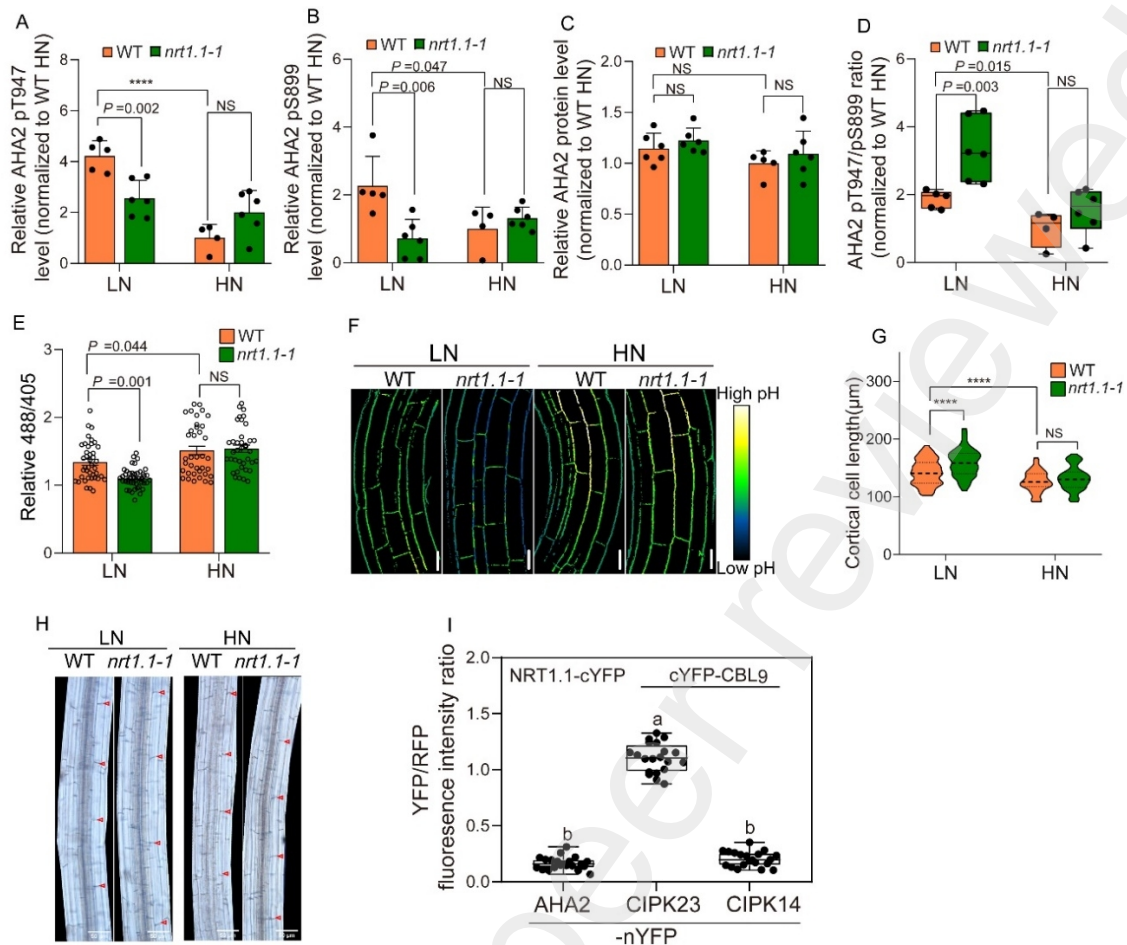


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(C) Difference in protein levels of AHA2 induced by LN and HN in WT and *nrt1.1-1*.

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letters represent significant differences at $p < 0.05$ according to one-way ANOVA with a Tukey test.

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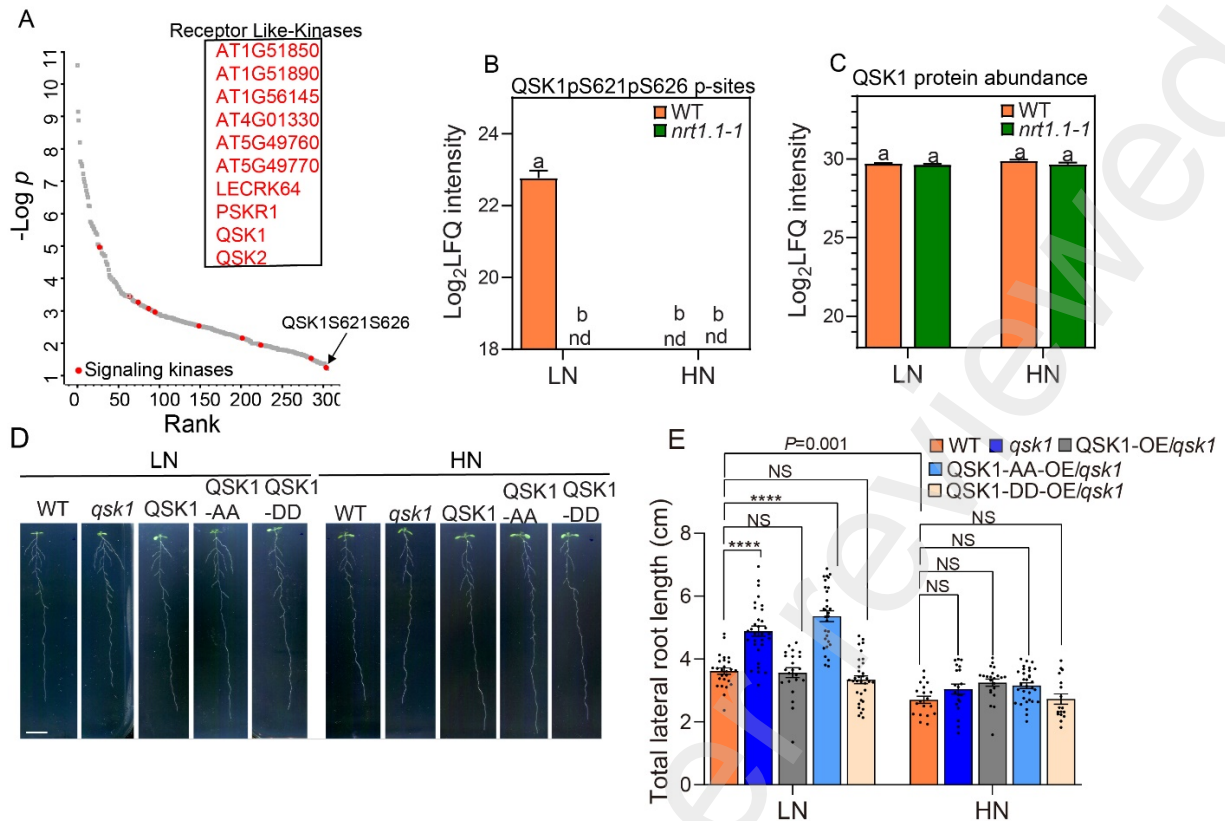


Figure 3. LN promotes NRT1.1-QSK1 interaction and QSK1 phosphorylation

(A) Ranking of phosphosites significantly upregulated in WT under LN and downregulated in *nrt1.1-1* in LN. 10 named receptor-like kinases are highlighted in red.

(B) Phosphorylation differences of S621/S626 on QSK1 in WT and *nrt1.1-1* induced by LN and HN.

(C) Differences of protein levels in QSK1 induced by LN and HN in WT and *nrt1.1-1*.

Data are mean + SD, at least 4 biological replicates (B-C), statistical analysis (B-C) was performed using Student *t*-test (different letter indicates $P < 0.05$).

(D) Representative images of WT, *qsk1*, transgenic line overexpressing QSK1, QSK1 phospho-dead and as well as phosphorylation-mimic version in *qsk1* mutant (QSK1-OE/*qsk1*, QSK1-AA-OE/*qsk1* and QSK1-DD-OE/*qsk1*) in LN and HN medium, scale bar 1cm.

(E) Total lateral root length of WT, *qsk1*, QSK1-OE/*qsk1*, QSK1-AA-OE/*qsk1* and QSK1-DD-OE/*qsk1* in LN and HN medium. Bar plots indicate means \pm SEM (at least 17 independent seedlings). Lateral roots were measured after 8 days on LN and HN. Statistical analysis was performed using one-way ANOVA (**** $P < 0.0001$; NS, not significant).

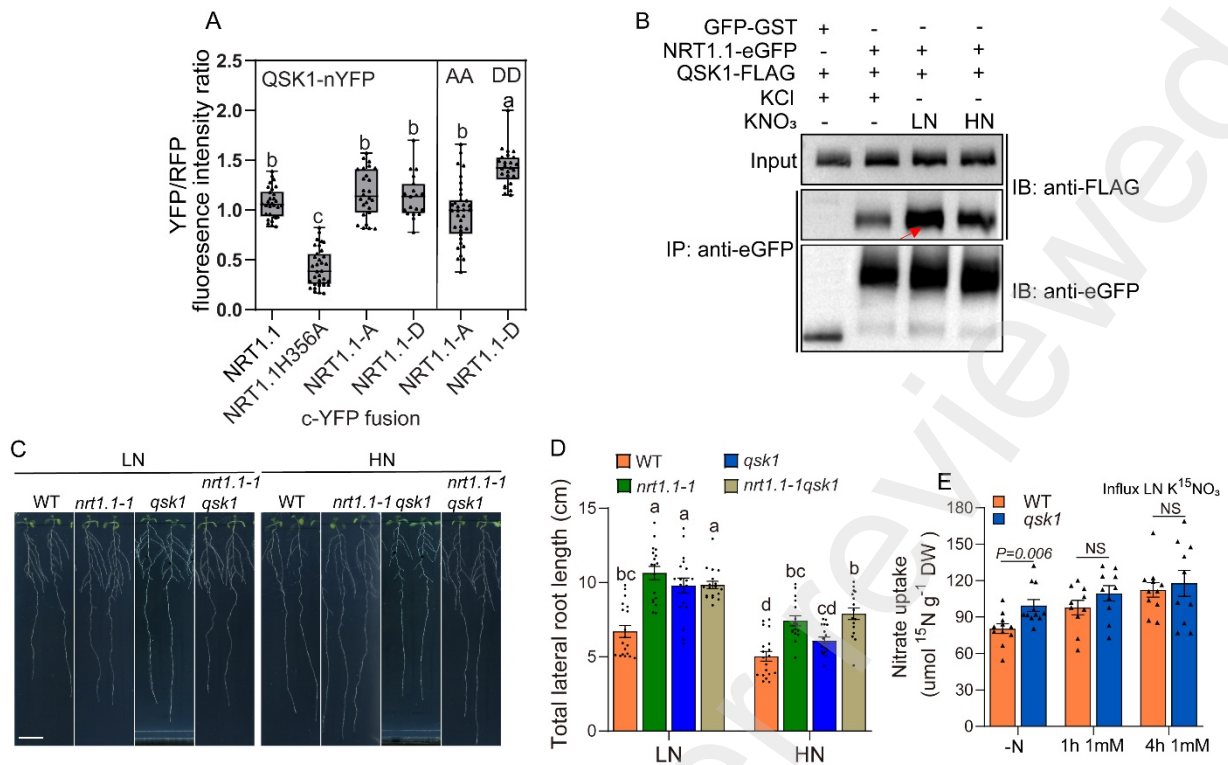


Figure 4. QSK1 functions downstream of NRT1.1 to modulate LRs growth in LN

(A) Quantification of the *in vivo* rBiFC interaction of QSK1, QSK1SASA, and QSK1SDSD with NRT1.1, NRT1.1H356A, NRT1.1T01A, and NRT1.1T01D. rBiFC calibration is shown in Figure S3. 18-35 randomly selected cells were quantified.

(B) Co-IP assays using membrane protein mixture extracted from tobacco leaves (*Nicotiana benthamiana*) expressing NRT1.1-eGFP and QSK1-FLAG in the presence of LN (0.45 mM KNO₃), HN (9.4 mM KNO₃) or potassium chloride (0.45 mM).

(C) Representative images of WT, *nrt1.1-1*, *qsk1* and *nrt1.1-1qsk1* in LN and HN medium, scale bar, 1cm. Different letters (A and D) represent significant differences at $p < 0.05$ according to one-way ANOVA with a Tukey test.

(D) Total lateral root length of WT, *nrt1.1-1*, *qsk1*, and *nrt1.1-1qsk1* in LN and HN medium. Bar plots indicate means \pm SEM (16-20 independent seedlings). Lateral roots were measured after 10 days on LN and HN medium.

Different letters (A and D) represent significant differences at $p < 0.05$ according to one-way ANOVA with a Tukey test.

(E) ¹⁵Nitrate uptake activity under LN. Bar plots indicate means \pm SEM (at least 10 biological replicates). Statistical analysis was performed using two-tailed Student *t*-test ($P < 0.05$; NS, not significant).

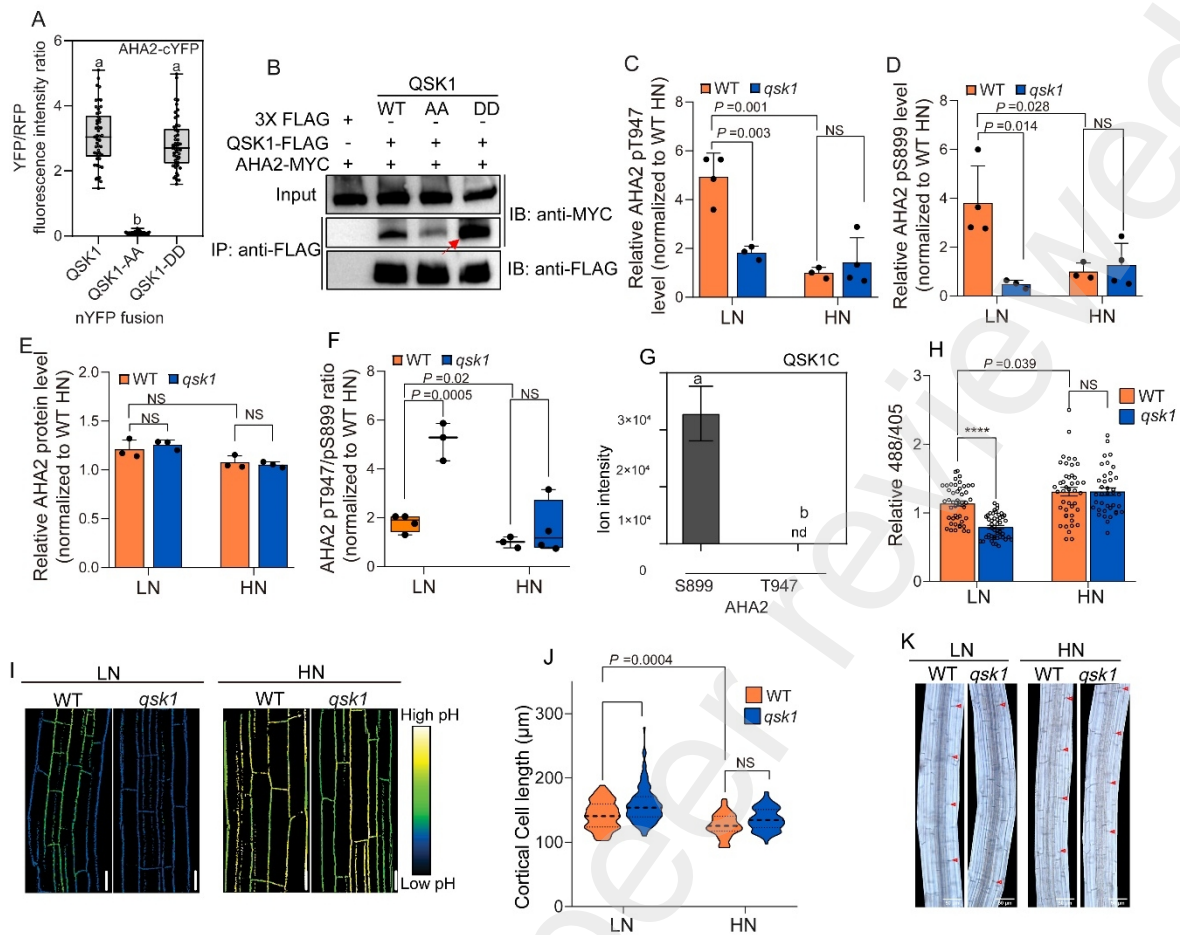


Figure 5. phosphorylated QSK1 forms a strong complex with AHA2 and represses proton pump activity in LN

(A) Quantification of the *in vivo* rBiFC interaction of QSK1, QSK1SASA and QSK1SDSD with AHA2. rBiFC calibration is shown in Figure S3. 50 randomly selected cells were quantified. Different letters represent significant differences at $p < 0.05$ according to one-way ANOVA with a Tukey test.

(B) Co-IP assays using membrane protein mixture extracted from tobacco leaves (*Nicotiana benthamiana*) expressing QSK1-FLAG, QSK1-AA-FLAG and QSK1-DD-FLAG with AHA2-MYC.

(C and D) Difference of phosphorylation levels of T947 (C) and S899 (D) on AHA2 induced by LN and HN in WT and *qsk1*.

(E) Difference of protein levels of AHA2 under LN and HN in WT and *qsk1*.

(F) Box plots show T947/S899 phosphorylation ratio for WT and *qsk1* under LN. Center lines of boxes represent medians, vertical line indicates the minimal/maximal value.

Data (C, D and E) are mean+SD. Statistical analysis (C-F) was performed using two-tailed Student *t*-test (**** $P < 0.0001$; NS, not significant).

(G) *In vitro* peptide phosphorylation assay using GLDIETPSHYTV (covering T947) and EAVNIFPEKGSYR (covering S899) from AHA2 as substrates for the QSK1C terminus. nd indicates not detected.

(H and I) Quantification (H) and confocal images (I) of apoplastic pH in the lateral root EZ of WT and *qsk1* grown on LN and HN medium. Ratiometric value (488 nm/405 nm) of fluorescent

HPST was used to monitor pH in the EZ of lateral roots, $n \geq 39$ individual root for each. For I, scale bar 20 μm .

(J and K) Quantification (J) and images (K) of cortical cell length of the lateral roots mature zone of WT and *qsk1* grown on LN and HN medium. $n \geq 61$ cells for each. For K, scale bar 50 μm .

Statistical analysis (H and J) was performed using one-way ANOVA with a Tukey test (**** $P < 0.0001$; NS, not significant).

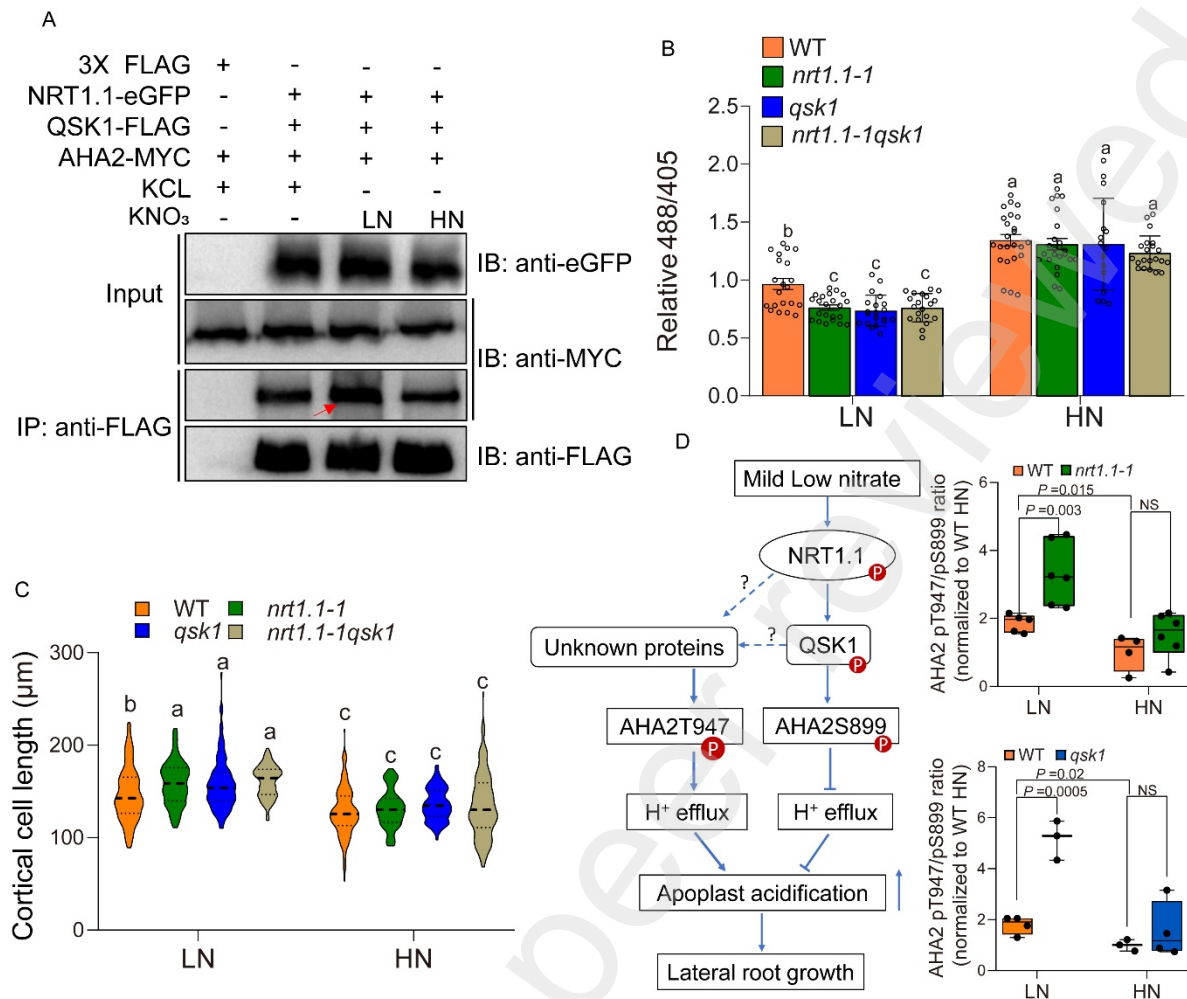


Figure 6. NRT1.1-QSK1 complex regulates AHA2 activity in LN

(A) Co-IP assays using membrane protein mixture extracted from tobacco leaves (*Nicotiana benthamiana*) expressing NRT1.1-eGFP, QSK1-FLAG and AHA2 in the presence of LN (0.45 mM KNO₃), HN (9.4 mM KNO₃) or potassium chloride (0.45 mM).

(B) Quantification of apoplastic pH in the lateral root EZ of WT, *nrt1.1-1*, *qsk1* and *nrt1.1-1qsk1* grown in LN and HN medium. Ratiometric value (488 nm/405 nm) of fluorescent HPST was used to monitor pH in the EZ of lateral roots, n≥16 individual root for each.

(C) Quantification of the cortical cell length of lateral roots mature zone of WT, *nrt1.1-1*, *qsk1* and *nrt1.1-1qsk1* grown on LN and HN medium, n≥152 cells for each.

Statistical analysis (B and C) was performed using one-way ANOVA with a Tukey test (****P<0.0001; NS, not significant).

(D) Proposed model for LN signal transduction through NRT1.1-QSK1 to AHA2. Perception of LN leads to NRT1.1 interacting with and phosphorylation of QSK1. Subsequently QSK1 recruits AHA2 and directly phosphorylates AHA2 at S899. NRT1.1 and QSK1 also induces AHA2T947 phosphorylation indirectly through unknown proteins (e.g. Kinases). As a result, phosphorylation ratio of T947/S899 and thus H⁺-ATPase activity is affected. In both the *nrt1.1-1* and *qsk1* mutants, phosphorylation ratio of T947/S899 is dramatically increased due to a stronger reduction of phosphorylation at S899, resulting in an even higher proton activity, longer cells and longer LRs.

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