NRT1.1s and NRT2.1 affect rhizosphere and apoplastic pH during plant nitrate uptake

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Main Text

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Abstract

Nitrate is the primary source of nitrogen for plants in most environments. Nitrate uptake from the rhizosphere into root cells is mediated by Nitrate Transporter 2 (NRT2) and NRT1.1 proteins via proton symport, with the apoplast serving as an intermediate compartment. Using a simple root immersion system, we investigated the effects of NRT transporters on external media pH (pH_{med}) and apoplastic pH (pH_{apo}) in *M. truncatula* nitrate transporter mutants. Monitoring of pH_{med} showed that loss of NRT2.1 largely prevented nitrate induced alkalization of the media, while loss of NRT1.1A/B reduced media alkalization only during the initial stages of nitrate uptake, suggesting they have fundamentally different physiological roles. Use of the pH sensitive dye HPTS showed that the pH_{apo} of root cells initially decreased during nitrate uptake, and this required NRT1.1A/B. Unexpectedly, the observed changes in pH_{med} during nitrate uptake in WT and the mutants were not reflected in pH_{apo}. To explain these discrepancies, we propose a model in which the depletion of protons in the media occurring during nitrate symport is offset by the buffering of protons in the cell wall and the activity of the H^+-ATPase, allowing a lower pH_{apo} to be maintained. Such a mechanism would enable efficient nitrate uptake across a range of external pH values.

Introduction

Nitrate uptake in plants depends on proton symport, which requires a supply of protons from the apoplast. This requirement is reflected in the apoplastic pH (pH_{apo}), which typically ranges from 4.5-5.5 (1). Nitrate symport results in alkalization of the rhizosphere which occurs through the consumption of protons by nitrate proton symporters with a stoichiometry of at least 2 H^+ per nitrate absorbed (2). In *Arabidopsis thaliana*, this occurs through the activities of the high affinity nitrate uptake transporter AtNRT2.1 and the low or dual affinity transporter AtNRT1.1, also called Nitrate Transporter 1/Peptide Transporter 6.3 (3,4). AtNRT1.1 also plays an important role in nitrate sensing (5). Loss of AtNRT1.1 greatly diminishes nitrate-induced rhizosphere alkalization (6), but NRT2.1's role in this phenomenon has not been studied. Despite it playing a critical role in nitrate uptake, the dynamics of pH_{apo} in response to nitrate remains uninvestigated. Here we addressed these questions in *Medicago truncatula* (Gaertn) using NRT2.1 and NRT1.1A/B mutants, the respective orthologs of Arabidopsis NRT2.1 and NRT1.1 (7).

Results

To observe the effect of nitrate uptake on medium pH (pH_{med}), *M. truncatula* seedlings were grown on agar plates containing nitrate-free Fåhraeus nutrient solution (FP) for one week. The seedlings

36 were then transplanted into non-aerated liquid FP medium supplemented with different
37 concentrations of KNO_3 (initial pH_{med} of 6.0) with their roots fully submerged. In the absence of
38 nitrate, the seedlings acidified the FP medium to at ~4.0-4.2 by 24h (Fig. 1*A*). Treatment with high
39 concentrations of nitrate (≥5mM) significantly lowered pH_{med} at 3 and 6h compared to the FP control
40 by 0.2-0.4 pH units (Dunnett's test, p-value <0.05; inset Fig. 1*A*), but this trend reversed at 24h with
41 the nitrate containing samples becoming alkalized, with a pH_{med} ~1 pH unit higher than the nitrate-
42 free control, with even larger differences (~1.5-2.2 pH units) occurring at later time points. In
43 comparison, treatment with low nitrate (0.5 mM KNO_3) had only a slight effect on pH_{med}. These
44 results are consistent with media alkalization by the seedlings being due to nitrate/H^+ symport
45 mediated depletion of protons.

46 To determine if the medium acidification in our system was caused by flood-induced
47 hypoxia, we repeated the experiment with gentle aeration. This increased the O_2 concentration of
48 the media from 7.2 (±0.04 SEM) to 9.6 (±0.17) ppm and largely attenuated the acidification (Fig.
49 1*B*). We then investigated the potential involvement of the PM H^+-ATPase using the specific
50 inhibitor protonstatin-1 (PS-1). Treatment with PS-1 essentially blocked root proton extrusion, with
51 91 and 95% inhibition by 25 and 50mM PS-1, respectively (Fig. 1*C*). These results suggest that the
52 observed media acidification is mediated by the PM H^+-ATPase and is induced by short-term
53 hypoxia resulting from the immersion.

54 The root immersion system provides a means of evaluating the H^+ consumption by NRTs
55 during nitrate uptake. First, we tested whether the acidification of the media was affected by the
56 absence of NRTs. Five mutants were tested, *nrt2.1*, *nrt1.1a*, *nrt1.1b*, *nrt1.1c*, and the *nrt1.1a/b*
57 double mutant. Every mutant acidified pH_{med} to ~3.8-4.0 within 24h, similar to WT (Fig. 1*D*). We
58 then tested the effect of these mutations on pH_{med} in the presence of various concentrations of
59 KNO_3. We found that the *nrt1.1a/b* double mutant and *nrt2.1* mutant affected pH_{med} in different
60 ways (Fig. 1*E-G*). The *nrt1.1a/b* mutant showed a small but significant (~0.1 pH unit; Dunnett's test,
61 p-value <0.05) decrease in pH_{med} at 3 and 6h after nitrate addition at all nitrate concentrations. This
62 effect grew stronger at 24h (~0.2-0.4 pH units, depending on the nitrate concentration) but was
63 greatly diminished or absent at the later time points. This sharply contrasted with *nrt2.1*, which
64 showed no phenotype at 3 and 6h, but had a significantly diminished ability to alkalize the media
65 at 24 and 48 under all nitrate concentrations, which grew more pronounced at 80h (~1.4-1.7 pH
66 units) under 5 and 10 mM KNO_3. Under 0.5mM the pH_{med} phenotype of *nrt2.* at 24h and 48h was
67 weaker, and no difference was seen at 80h.

68 We then investigated whether changes occur in pH_{apo} occur during nitrate uptake. We used
69 HPTS, a widely used fluorescent membrane-impermeant pH sensitive dye, to stain immersed roots
70 and made observations on root tip cells using confocal microscopy (see *S1 Appendix*). After
71 transfer to 5 mM nitrate the pH_{apo} of WT seedlings significantly decreased, indicating a pH drop, at

72 4h (Fig. 2*A*). The *nrt1.1a/b* mutant failed to respond, indicating it has a reduced ability to acidify the
73 apoplast. Observations at the 24- and 48-hour marks indicate that pH_{apo} of WT recovered, while
74 that of *nrt1.1a/b* was higher at 48h.
75
76 **Discussion**
77
78 The seedling immersion approach provides a convenient means to observe the effect of root nitrate
79 uptake on external pH. Our results indicate that the initial drop in pH_{med} after seedling transfer was
80 caused by hypoxia. A similar drop in pH_{med} was reported in *A. thaliana* after root immersion, which
81 was shown to require H^+-ATPases 1 and 2 (8). Furthermore, it was shown that hypoxia induces
82 increased proton efflux in barley root tips, which was attributed to PM H^+-ATPase activity (9),
83 suggesting this is a general hypoxia-related phenomenon. Using this approach, we found that
84 nitrate initially induced a small drop in pH_{med} and pH_{apo}. It was shown in maize that following an
85 initial depolarization after exposure to nitrate, the PM becomes hyperpolarized through
86 overcompensation of the H^+-ATPase (10). Such hyperpolarisation could explain the observed drop
87 in pH_{med} and pH_{apo} at early time points. Consistent with this, reports in maize showed an increase
88 in root PM H^+-ATPase activity after nitrate supply (11,12).
89 The initial nitrate-induced drop in pH_{apo} was compromised in the *nrt1.1a/b* mutant. This
90 requires an explanation, since decreased nitrate/H^+ symport would be expected to lower, not raise,
91 the pH of this compartment. We suggest that this may occur through reduced activation of the PM
92 H^+-ATPase due to lower nitrate uptake in the mutants. While the trigger for nitrate induced
93 hyperpolarization is unknown, it is possible that the increased influx of protons into the cytoplasm
94 by nitrate/H^+ symport could trigger higher H^+-ATPase activity to maintain a stable cytoplasmic pH
95 (13). In this case, a decrease in nitrate/H^+ symport activity would lessen the stimulation of the PM
96 H^+-ATPase by alleviating symport-mediated cytosolic acidification, resulting in a higher pH_{apo} in the
97 mutants. Another, non-exclusive possibility, is the inhibitory effect of nitrate on vacuolar (V)-
98 ATPases (14). Such downregulation of the V-ATPase would serve a complementary role,
99 preventing competition with the PM-ATPase for cytosolic protons during nitrate uptake.
100 The strong alkalization observed in the medium at later time points did not occur in the
101 apoplast. Similarly, the much lower pH_{med} of *nrt2.1* compared to WT at 48h was not reflected in its
102 pH_{apo}, which was similar to WT. It is generally assumed that pH_{apo} and pH_{med} are the same since
103 these compartments are physically connected. These discrepancies can be explained by the role
104 the cell wall plays in maintaining a low pH_{apo}. The cell wall's apoplast has two zones: the Water
105 Free Space, which mirrors the ionic concentration of the external solution (pH_{med}), and the Donnan
106 Free Space, where electrostatic charges from the wall concentrate cations (including protons) and
107 depletes anions (15). We propose that the proton binding capacity of the cell wall results in pH_{apo}

being lower than pH_{med}, providing the PM ATPase is sufficently active. In line with this, the apoplast contained within the cell wall was found to be as much as 2 units more acidic than the remaining apoplastic liquid, which was attributed the cell wall's high cation exchange capacity (16). Moreover, Martinière et al. (17) found that the apoplast is considerably more acidic than the apoplastic liquid immediately adjacent to the PM. Hence, the cell wall may act as a proton reservoir, that charges itself through the action of the PM ATPase and by acquiring protons from the surrounding medium, thus helping to sustain apoplastic acidity. To explain how NRT1.1A/B enhances apoplast acidification, we posit that nitrate symport enhances the activation of H^+-ATPase, in order to maintain a stable cytosolic pH (Fig. 2*B*).

Materials and Methods

The detailed methods are described in SI Appendix. Briefly, *M. truncatula* seedlings were grown on agar plates containing nitrate-free FP nutrient solution for one week. The seedlings were then transplanted into non-aerated liquid FP medium supplemented with different concentrations of KNO_3 with their roots fully submerged and pH was monitored using a pH meter. For HPTS staining, the seedlings were prepared in the same manner and then transferred to FP medium containing 5 mM KNO_3 for the given time, and then stained with HPTS for 30 minutes, rinsed three times with FP, then root epidermal cells near the root tip were imaged using a confocal microscope. The *M. truncatula nrt1.1* and *nrt2.1* mutants were previously described (7, 18).

Acknowledgments

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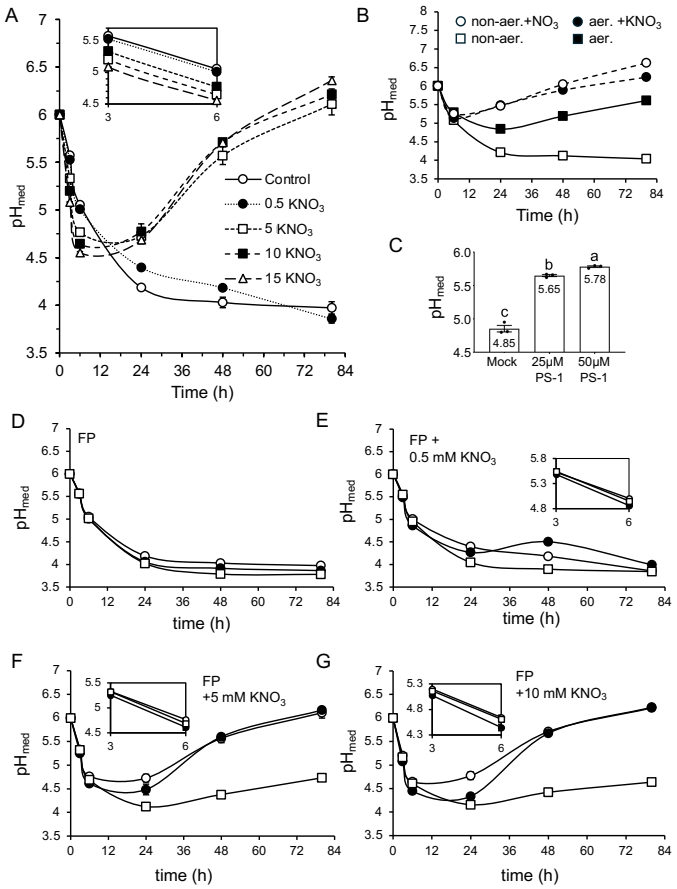
185 **Figures**



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188 **Figure 1. NRT2.1 and NRT1.1s affect nitrate-induced media alkalization.** WT *M. truncatula*
189 seedlings were transferred to either FP (control), or FP supplemented with varying concentrations
190 of KNO_3 and pH_{med} was monitored (*A*). *M. truncatula* seedlings were transferred to either FP or FP
191 containing 5 mM KNO_3 with and without aeration (aer.) and pH_{med} was monitored (*B*). Seedlings
192 grown on FP were transferred to FP containing the indicated concentrations of PS-1 for 6h and
193 then pH_{med} was determined (*C*). WT and mutant *M. truncatula* seedlings were transferred to either
194 FP (*D*), or FP with varying concentrations of KNO_3 (*E*-*G*) and pH_{med} was monitored. In (*A*), the inset
195 shows a detailed view of the 3 and 6 h timepoints. For all experiments *N*=3, with 5 plants per
196 biological replicate. Error bars indicate SEM.

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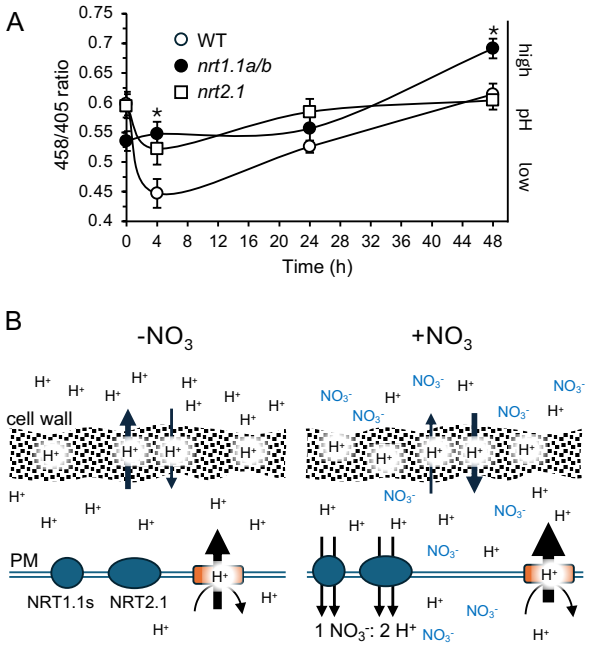

Figure 2. Nitrate induces a short-term decrease in pH_{apo} which requires NRT1.1A/B. Roots of *M. truncatula* seedlings were stained with HPTS and 458/405 ratios of the root tip cells were determined by confocal microscopy after immersion in FP (0 h) or at different times after addition of FP containing 5mM KNO_3 (*A*). *P-value<0.05, Dunnett's test, mutants vs WT at each time point. *N*=6-32. A model for nitrate uptake in plants (*B*). (left) Without aeration and in the absence of nitrate the rhizosphere and apoplast of immersed plants are acidified through the activity of H^+-ATPases that serve to maintain cytosolic pH during hypoxia. (right) When nitrate is added to the media, the activation of NRT1.1 and NRT2.1 symporters triggers an overcompensation of the PM H^+-ATPase through an unknown mechanism, which more than offsets H^+ uptake via nitrate symport, leading to a small decrease in pH_{apo}. The ongoing uptake of nitrate and protons via the apoplast by the symporters eventually leads to the alkalization of the rhizosphere.

Extended methods

Seed sterilization

Wild type *M. truncatula* (ecotype R108) and *nrt1.1a*, *nrt1.1b*, *nrt1.1c, nrt2.1* and the *nrt1.1a/b* double mutant seeds were scarified with sandpaper, then surface-sterilized with 70% ethanol for 1 minute, then 10% bleach for 3 minutes, and then rinsed five times with sterile distilled water.

Plant culture

Sterilized *M. truncatula* (ecotype R108) and *nrt1.1a*, *nrt1.1b*, *nrt1.1c*, *nrt2.1* and the *nrt1.1a/b* double mutant seeds were placed on 0.8 % agar plates and stratified in darkness at 4°C for 3 days. The plates containing the seedlings were then placed upside down in the dark at 22°C to allow the roots to extend overnight. The seedlings were then transferred to fresh 0.8 % agar plates containing Fahraeus nitrogen free nutrient medium (FP) for one week with the plates vertically positioned.

pH measurements

For the pH measurements five of the seedlings were transferred to graduated 15 ml plastic tubes filled to the 15 ml mark with FP medium (~13.2 ml of FP) supplemented with different concentrations of nitrate with the pH adjusted using H_2SO_4 or KOH. Plants were grown under controlled conditions with a 16-hour light period at 22°C and an 8-hour dark period at 20°C. The pH of the liquid medium (pH_{med}) was measured every few hours using a pH meter (Mettler Toledo FE28) with a reported accuracy of ± 0.01.

HPTS staining and imaging

HPTS is a membrane impermeant pH sensitive fluorescent dye[1,2,3]. For HPTS staining, plants were grown on FP medium for one week as described above and then transferred to FP medium containing 5 mM KNO_3 for 0, 1.5, 4, 24, or 48h. Seedlings were then transferred to the same medium supplemented with 1 mM 8-hydroxypyrene-1,3,6-trisulfonic acid (HPTS) (Abcam, USA) for 30 minutes. Roots were then rinsed three times with HPTS-free growth medium, placed on a microscope slide, and covered with a coverslip. Imaging of epidermal cells near the root tip was carried out using an inverted Leica SP8 confocal microscope with a 20X water immersion objective.

250 HPTS fluorescence was detected using dual excitation at 405 nm and 458 nm, with emission
251 collected at 514 nm. The overall 458/405 ratio was calculated for each image using Fiji software.
252
253 **Seed sterilization**
254
255 Wild type *M. truncatula* (ecotype R108) and *nrt1.1a*, *nrt1.1b*, *nrt1.1c, nrt2.1* and the *nrt1.1a/b*
256 double mutant seeds were scarified with sandpaper, then surface-sterilized with 70% ethanol for
257 1 minute, then 10% bleach for 3 minutes, and then rinsed five times with sterile distilled water.
258
259 **Aeration experiments**
260
261 To provide gentle aeration of the medium a perforated plastic tube (0.5 mm outer diameter) was
262 placed in each 15 ml tube and connected to an SB-748 silent air pump (Zhongshan SOBO
263 electric appliance co., China) on its lowest setting.
264
265 **Measurement of oxygen levels**
266
267 Plants were grown on FP medium for one week as described above and then transferred to
268 aerated or non-aerated FP or FP supplemented with 5 mM KNO_3 and left for approximately 30
269 hours. Oxygen concentrations were then measured using an optical oxygen microsensor
270 (PreSens; Regensburg, Germany).
271
272 **PS-1 treatment**
273
274 PS-1 (TargetMol Chemicals Inc., USA) was stored as a 25 mM stock solution in DMSO. One-
275 week-old seedlings were prepared as described above were transferred to 15 mL tubes
276 containing aerated FP solution supplemented with PS-1 or mock treatment (equivalent amount of
277 25 μM DMSO) for 6 hours. The pH of the liquid medium was then measured.
278
279
280 **Statistical analyses**
281
282 All means were compared using Student's *t*-test or, when more than two means were compared
283 to controls using Dunett's Test to correct for multiple comparisons.
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