NRT1.1s and NRT2.1 affect rhizosphere and apoplastic pH during nitrate uptake in *Medicago truncatula*

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Summary

- Nitrate uptake in plants is mediated by Nitrate Transporter 2 (NRT2) and NRT1.1 proteins via proton symport. The apoplast of root epidermal cells serves as an intermediate

compartment for plant nutrient uptake from the rhizosphere. We tested the effects of nitrate uptake on external media pH (pH_{med}) and apoplastic pH (pH_{apo}) in *nrt1.1* and *nrt2.1* mutants of *M. truncatula*.

- 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.
- Use of the pH sensitive dye HPTS showed that pH_{apo} of root cells decreased during nitrate uptake, and this affect was partly attenuated in the nitrate transporter mutants.
- To explain the discrepancies between the observed changes in pH_{med} and pH_{apo} during nitrate uptake, we propose the following model. We suggest that the depletion of protons in the media that occurs 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 is the primary source of nitrogen for most plants. Nitrate uptake depends on proton symport, which requires a supply of protons in the aploplast. This requirement is reflected in the apoplastic pH (pH_{apo}), which typically ranges from 4.5-5.5 (Tsai and Schmidt, 2021). The maintenance of pH_{apo} is thought to be partly dependent the high cation exchange capacity of the cell wall (Sentenac and Grignon, 1981; 1987). This property is mainly conferred by the carboxyl groups of pectins (Meychik et al., 2021), and is understood to provide a buffering effect on apoplastic pH. In addition to this buffering, pH_{apo} is thought to be largely determined by balancing the activity of the transporters that mediate the uptake of nutrients using proton symport mechanisms, with the action of plasma membrane (PM) H^+-ATPases. Nitrate is the major source of soil nitrogen for plants

under aerobic conditions. Its uptake 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 (McClure et al., 1990; Glass et al., 1992; Meharg and Blatt, 1995; Miller and Smith, 1996). In *Arabidopsis thaliana*, this role is fulfilled by the high affinity nitrate uptake transporter AtNRT2.1 and the low affinity transporter AtNRT1.1, also called Nitrate Transporter 1/Peptide Transporter 6.3 (Tsay et al., 1993; Cerezo et al., 2001). AtNRT1.1 also plays an important role in nitrate sensing (Bouguyon et al., 2015; Maghiaoui, Gojon and Bach, 2020). Loss of AtNRT1.1 greatly diminishes nitrate-induced rhizosphere alkalization (Fang et al., 2016), but NRT2.1's role in this process, in Arabidopsis or other plant species, has not been studied. Despite its playing a critical role in nitrate uptake, the dynamics of pH_{apo} in response to nitrate remains uninvestigated.

We addressed these questions in *Medicago truncatula* (Gaertn). Medicago contains three NRT1.1 paralogs, MtNRT1.1A, B, C (*syn*. NPF6.5, 6.7 and 6.6, respectively), and MtNRT2.1, the ortholog of AtNRT2.1, for which mutants are available (Xiao et al., 2021; Luo et al., 2023). Our previous work in *Xenopus laevis* oocytes showed that NRT1.1A and NRT1.1B selectively transport chloride and nitrate, respectively, while the substrate for NRT1.1C remains unknown (Xiao et al., 2021). Here we show that the dynamics of medium alkalization and apoplast acidification during nitrate uptake depend on NRT1.1A/B and NRT2.1.

Materials and Methods

Plant materials and growth conditions

M. truncatula (R108) was used as the wild-type (WT). The *nrt1.1a*, *nrt1.1b*, *nrt1.1c* and the

nrt1.1a/b double mutant are *Tnt1* insertion mutants isolated from the R108 background (Xiao et al., 2021). For seed germination, seeds were first 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. The sterilized seeds were then placed on 0.8 % agar plates (Beijing Vokai Biotechnology Co., China) 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

Five one week old 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. The pH was 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.

Aeration experiments

To provide gentle aeration of the medium a perforated plastic tube (0.5 mm outer diameter) was placed in each 15 ml tube and connected to an SB-748 silent air pump (Zhongshan SOBO electric appliance co., China) on its lowest setting.

HPTS staining and imaging

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 as previously described (Barbez et al., 2017). 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. HPTS fluorescence was detected using dual excitation at 405 nm and 458 nm, with emission collected at 514 nm. The overall 458/405 ratio was calculated for each image using Fiji software.

PS-1 treatment

PS-1 (TargetMol Chemicals Inc., USA) was stored as a 25 mM stock solution in DMSO. One-week-old seedlings were prepared as described above were transferred to 15 mL tubes containing aerated FP solution supplemented with PS-1 or mock treatment (equivalent amount of 25 µM DMSO) for 6 hours. The pH of the liquid medium was then measured.

Measurement of oxygen levels

Plants were grown on FP medium for one week as described above and then transferred to aerated or non-aerated FP or FP supplemented with 5 mM KNO_3 and left for approximately 30 hours.

Oxygen concentrations were then measured using an optical oxygen microsensor (PreSens; Regensburg, Germany).

Statistical analyses

All means were compared using Student's *t*-test, or compared to controls using Dunett's Test to correct for multiple comparisons.

Results

To observe the effect of nitrate uptake on medium pH (pH_{med}), *M. truncatula* seedlings were grown agar plates containing Fåhraeus nutrient solution (FP), which is nitrate-free, for one week. Seedlings were then transplanted into liquid FP medium adjusted to an initial pH_{med} of 6.0 with their roots fully submerged. The experimental groups were supplemented with 0.5, 5, 10, or 15 mM KNO_3, while the control group contained FP medium only. In the absence of nitrate the seedlings acidified the FP medium to ~4.2 by 24h, which stablized at ~4.0 at later time points (Fig. 1a). Treatment with high concentrations of nitrate (≥5mM) significantly lowered pH_{med} at 3 and 6h compared to the FP control by 0.2-0.4 pH units (Dunnett's test, p-value <0.05; inset Fig. 1a), but this trend reversed at 24h with the nitrate containing samples becoming alkalized, with a pH_{med} ~1 pH unit higher than the nitrate-free control. This trend continued at the 48 and 80h time points, with the nitrate treated samples reaching pH_{med} ~1.5 and ~2.2 pH units higher than the control,

respectively. In comparison, treatment with low nitrate (0.5 mM KNO_3) had only a slight effect on pH_{med} that was only statistically significant at the 24h time point (Dunnett's test, p-value <0.05). These results are are consistent with media acidification being due to nitrate/H^+ symport mediated consumption of protons by the seedlings.

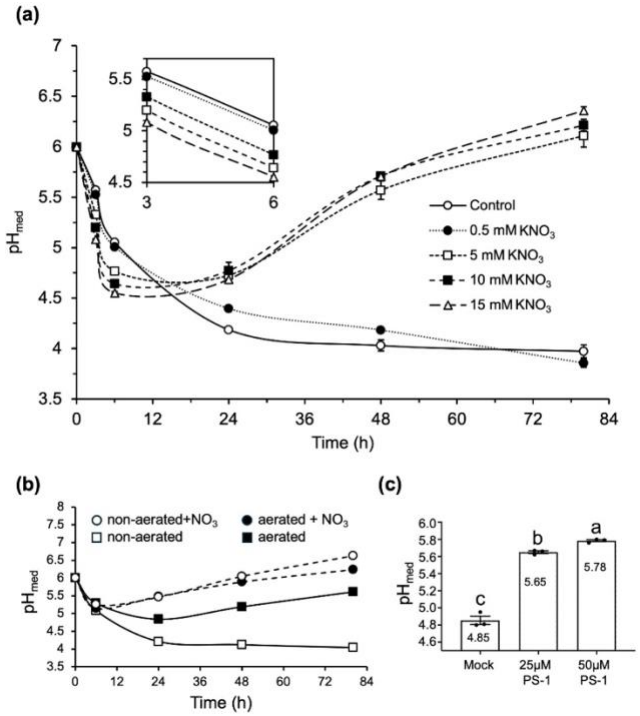

Fig 1. The effect of different concentrations of KNO_3 on hypoxia-induced media acidification by *M. truncatula* seedlings. (a) One week old WT seedlings grown on nitrate-free FP agar plates were transferred to either FP, or FP supplemented with varying concentrations of KNO_3 and pH_{med} was monitored. n=3, with 5 plants per biological replicate. Error bars indicate SEM. (b) One week old WT seedlings grown on nitrate-free FP agar plates were transferred to either FP or FP containing 5 mM KNO_3 with and without aeration. The treatments were carried out in 50 ml of FP nutrient solution adjusted to an initial pH of 6.0 with 15 plants in each tube. (c) Seedlings were germinated and then grown on FP (initial pH 6.0) agar plates. After 7 days they were removed from the plates and their roots were immersed in FP containing the indicated concentrations of PS-1 for 6h and then pH_{med} was determined.

To better characterize our experimental conditions we investigated the the cause of the medium acidification that was observed in the absence of nitrate. A similar acidification phenomenon was reported after root immersion in *A. thaliana*, which was shown to require H^+-ATPases 1 and 2 (Haruta et al., 2010). Furthermore, it was shown that hypoxia induces increased proton efflux in barley root tips, which was attributed to PM H^+-ATPase activity (Pang et al., 2006), suggesting this is a general hypoxia-related phenomenon. We tested this possibility by gently aerating the FP media, and found that the pH drop was largely attenuated (Fig. 1b, Fig. S1). We then investigated the involvement of the PM H^+-ATPase using the specific inhibitor protonstatin-1 (PS-1). Treatment with PS-1 essentially blocked root proton extrusion, with 91 and 95% inhibition by 25 and 50mM PS-1, respectively (Fig. 1c). Together, our results suggest that the observed media acidification is mediated by the PM ATPase and is induced by short-term hypoxia.

We then used this root immersion system for comparing the relative contributions of NRTs to the alkalization of pH_{med} during nitrate uptake. We first tested whether the acidification was affected by the absence of NRTs. Five mutants were tested, *nrt2.1*, *nrt1.1a*, *nrt1.1b*, *nrt1.1c*, and the *nrt1.1a/b* double mutant. All of these mutants were found to acidify pH_{med} to ~3.8-4.0 within 24h, similar to WT (Fig. S2a).

We then tested the effect of these mutations on pH_{med} in the presence of various concentrations of KNO_3. We found that the *nrt1.1a/b* double mutant and *nrt2.1* mutant affected pH_{med} in different ways (Fig. 2), while the single *nrt1.1* mutants had little effect (Fig. S2b). The *nrt1.1a/b* mutant showed a small but significant (~0.1 pH unit; Dunnett's test, p-value <0.05) decrease in pH_{med} at 3 and 6h after nitrate addition at all nitrate concentrations except under 15 mM KNO_3. This effect grew stronger at 24h (~0.2-0.4 pH units, depending on the nitrate concentration) but was greatly diminished or absent at the later time points. This sharply contrasted

with *nrt2.1*, which showed no phenotype at 3 and 6h (Fig. S3), and had a greatly diminished ability (~1.4-1.7 pH units) to alkalize the media at 80h. This *nrt1.1a/b* phenotype was stable across experiments, with sometimes showing larger differences in pH_{med} (Fig. S4). Similar results were also obtained when the initial pH of the FP medium was 4.5, but the phenotypes were less pronounced (Fig. S5).

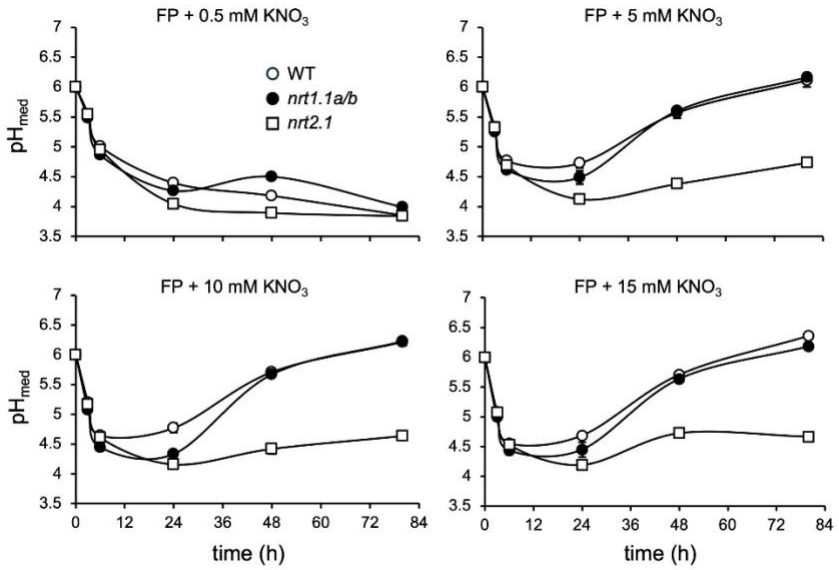

Figure 2. Loss of NRT1.1A/B or NRT2.1 affects pH_{med} during nitrate uptake. *M. truncatula* seedlings were grown for one week on nitrate-free FP agar plates, and then transferred to either FP, or FP with varying concentrations of KNO_3 and pH_{med} was monitored. n=3, with 5 plants per biological replicate. Error bars indicate SEM.

To investigate whether similar changes occur in pH_{apo} occur during nitrate uptake, we used HPTS, a widely used fluorescent membrane-impermeant pH sensitive dye (Wang et al., 2025; Lin et al., 2021; Barbez et al., 2017), to stain roots treated using the seedling immersion approach described above. After transfer to 5 mM nitrate the pH_{apo} of WT seedlings dropped slightly at 1.5

h, and further at 4h (Fig. 3a,b). The *nrt2.1* and *nrt1.1* mutants failed to respond, indicating they have a reduced ability to acidify the apoplast. At the 24- and 48-hour marks, no large differences were seen in the mutants, with *nrt1.1b* and *nrt1.1a/b* showing a slightly higher pH_{apo} at 48h (Fig. 3b,d).

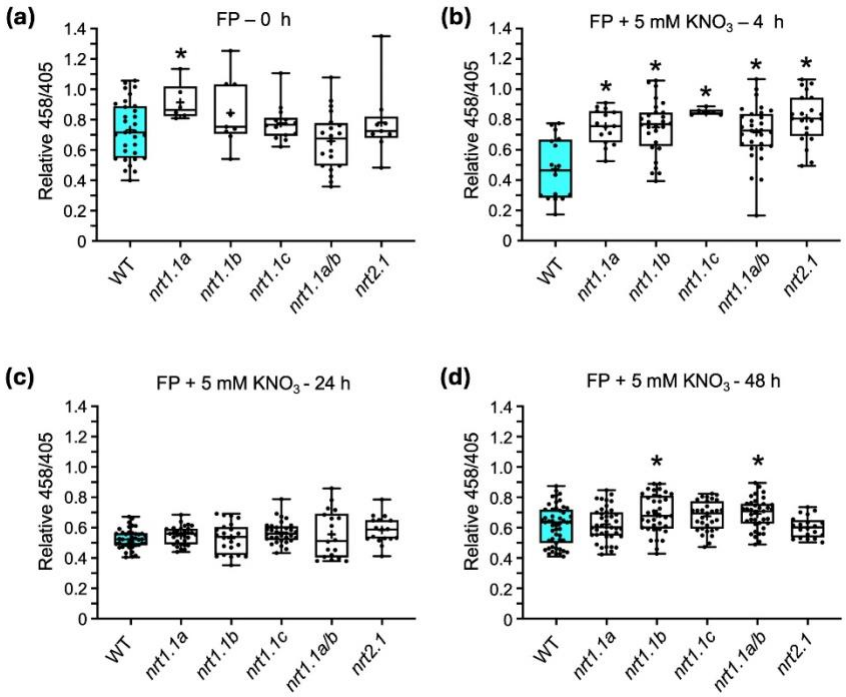

Figure 3. Loss of NRT2.1 and NRT1.1s affect affect pH_{apo} during nitrate uptake. *M. truncatula* seedlings were grown for 5-7 days on nitrate-free FP agar plates and the transferred to FP supplemented with 5 mM KNO_3: (a) 0h, (b) 4h, (c) 24h, and (d) 48h. The roots of the seedlings were then stained with HPTS and then imaged with a confocal microscope after 30 min. The media was adjusted to an initial pH of 6.0. *P-value<0.05, Dunnett's test for mutants vs WT at each time point, n=6-32. Boxplot shows individual measurements as dots with box and whiskers indicating 25th to 75th percentiles, and 10th and 90th percentiles, respectively. Line indicates median, + indicates mean.

In conclusion, addition of high concentrations of nitrate to *M. truncatula* seedlings resulted in a slightly lower pH_{med} initially (3-6h) followed by a strong alkalization at later time points (24-80h). Loss of NRT1.1A/B caused a stronger drop in pH_{med} during the early, but not later stages, of nitrate uptake. By contrast, loss of NRT2.1 had sustained effect on pH_{med} at later time points, but did no affect the early stages. Nitrate induced a slight drop in pH_{apo} 1.5-4h after nitrate addition that was attenuated in the nitrate transporter mutants.

Discussion

The seedling immersion approach provides a convenient means to assay the affect of root nitrate uptake on external pH. Using this approach, we found that nitrate initially induced a small drop in pH_{med} and pH_{apo}. It was shown in maize that following an initial depolarization after exposure to nitrate, the PM becomes hyperpolarized, which was proposed to occur through overcompensation of the H^+-ATPase (McClure et al., 1990). Such hyperpolarisation could explain the observed drop in pH_{med} and pH_{apo} at early time points. Consistent with this, a report in maize showed an increase in root PM H^+-ATPase activity after nitrate supply (Santi et al., 1995, 2003).

The initial drop in pH_{apo} was compromised by mutation of NRT1.1s or NRT2.1. This requires an explanation, since decreased nitrate/H^+ symport would be expected to lower, not raise, the pH of this compartment. We suggest that this may occur through reduced activation of the PM H^+ATPase due to lower nitrate uptake in the mutants. While the trigger for nitrate induced hyperpolarization is unknown, it is possible that the increased flux of protons into the cytoplasm by nitrate/H^+ symport could trigger higher H^+-ATPase activity in order to maintain a stable cytoplasmic pH (Espen et al., 2004). In this case a decrease in nitrate/H^+ symport activity would

lessen the stimulation of the PM H^+-ATPase by alleviating symport-mediated cytosolic acidification, resulting in a higher pH_{apo} in the mutants. Another, not-exclusive possibility, is the inhibitory affect of nitrate on vacuolar ATPases (Tu et al., 1987; Sze et al., 1992). Such regulation would serve a complementary role, preventing competition with the PM-ATPase for cytosolic protons during nitrate uptake.

The relative discrepancies in pH_{apo} and pH_{med} also requires an explanation. A clear example of this is the *nrt2.1*—by 80h the pH_{med} for *nrt2.1* was almost 2 pH units below that of the control, while no difference in pH_{apo} was observed. It is generally assumed that pH_{apo} and pH_{med} are correlated since these compartments are physically connected. This discrepancy may be partly explained by the role the cell wall plays in maintaining a low pH_{apo}. The cell wall's apoplast has two zones: the Water Free Space, which mirrors the ionic concentration of the external solution (pH_{med}), and the Donnan Free Space, where electrostatic charges from the wall concentrate cations (including protons) and depletes anions (Bush and McColl, 1987; Dainty and Hope, 1959; Briggs et al., 1958). We propose that the proton binding capacity of the cell wall enables pH_{apo} to be lower than pH_{med} providing that 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 the cell wall's high cation exchange capacity (Sentenac and Grignon, 1981; 1987). Moreover, Martinière et al. (2018) found that the apoplast is considerably more acidic than the apoplastic liquid immediately adjacent to the PM, leading the authors to propose a role for the cell wall in pH homeostasis. 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, helping to sustain apoplastic acidity. To explain how nitrate symporters enhance apoplast acidification, we posit that NRT1.1 and NRT2.1 transporters promote

proton influx during nitrate uptake, resulting in stronger H^+-mediated activation of H^+-ATPase activity, in order to maintain a stable cytosolic pH. In this manner the NRTs form part of a pH homeostat that underpins nitrate uptake (Fig. 4).

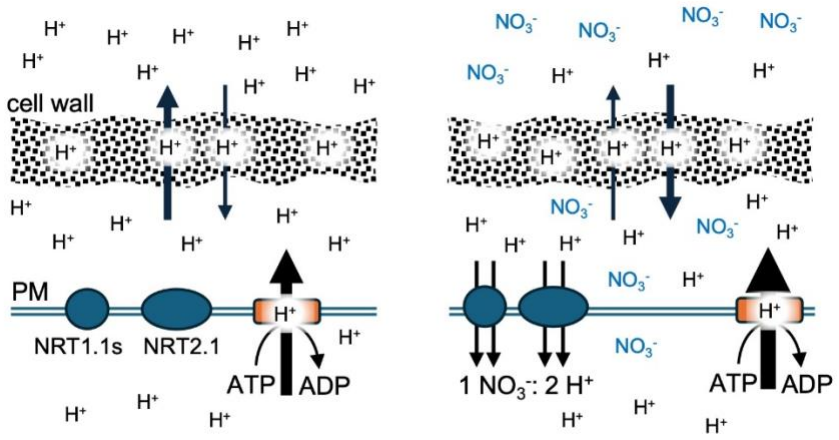

Figure 4. A model for nitrate uptake in hypoxic plants. (left) Without aeration and in the absence of nitrate the rhizosphere and apoplast of immersed plants are acidified through the activity of H^+-ATPases that serve to maintain cytosolic pH during hypoxia. (right) When nitrate is added to the media, the activation of NRT1.1 and NRT2.1 symporters uptake both nitrate and protons from the apoplast, eventually leading to the alkalization of the rhizosphere. Nitrate/H+ uptake into the cytoplasm initially triggers an overcompensation of the H^+-ATPase, which offsets H^+ uptake, leading to a decrease in pH_{apo}.

Acknowledgments

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Author contributions

QX, JW, YC and WJ performed experimental work and data analysis. JDM and AM supervised and co-directed the research. QX, AJM, FL and JDM conceived the experiments and wrote the article.

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Data availability

The data that support the findings of this study are available from the corresponding author upon request.

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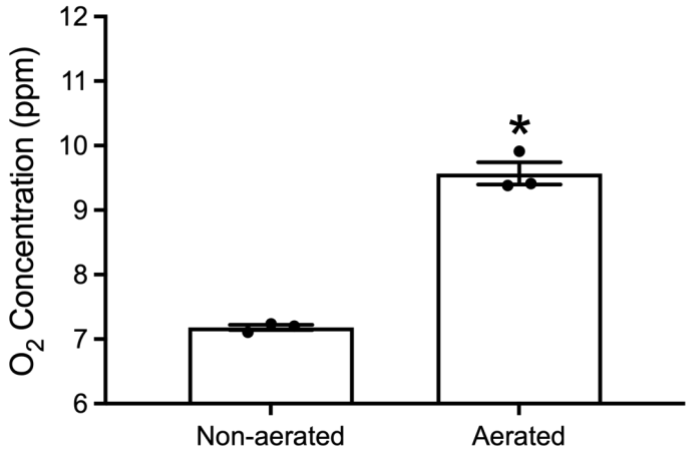

Fig. S1 The effect of gentle aeration on dissolved oxygen levels in FP medium. *P<0.05, Students' t-test was used.

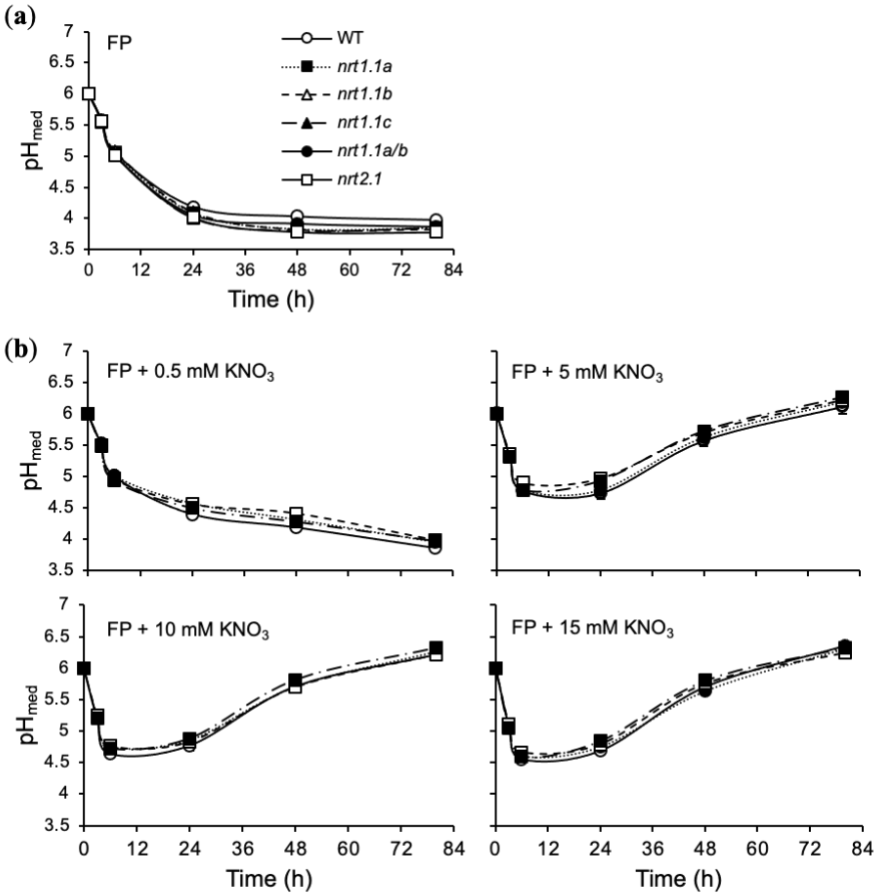

Fig. S2 NRT1.1 single mutants do not greatly affect immersion-induced media acidification during nitrate uptake *M. truncatula*. (a) M. truncatula seedlings grown on nitrate-free FP agar plates for one week were transferred to either FP or (b) FP with varying concentrations of KNO_3 and pH_{med} was monitored. n=3, with 5 plants per biological replicate. Error bars indicate SEM.

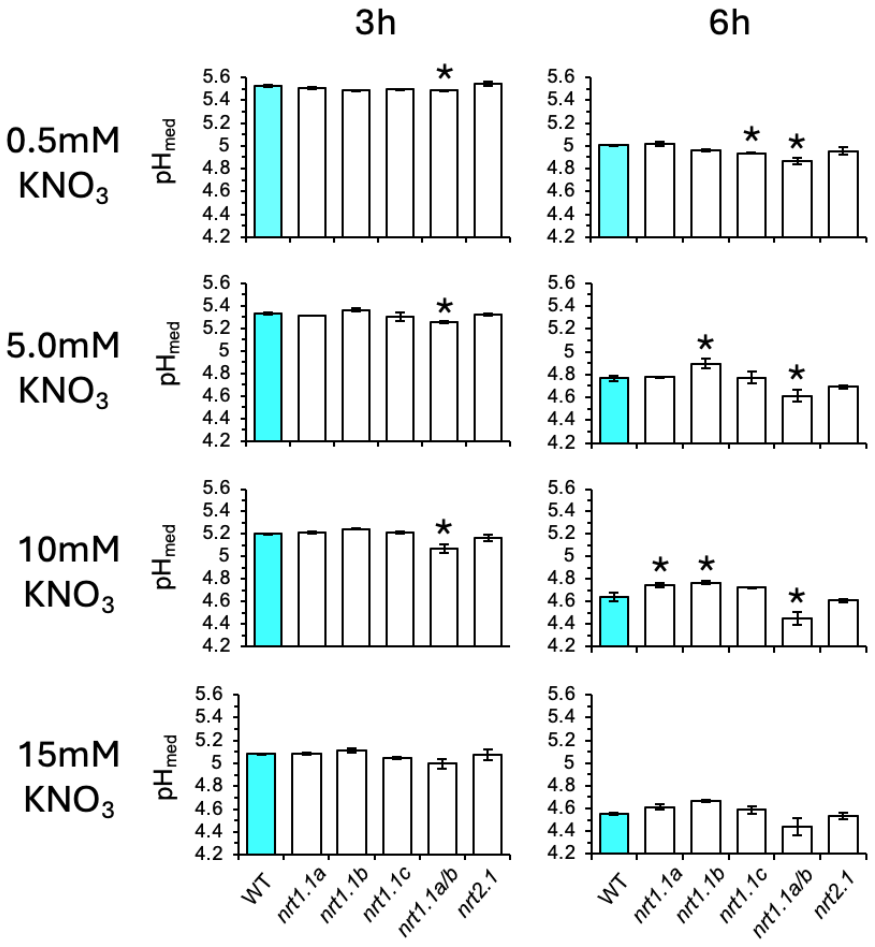

Fig. S3 Effects of *M. truncatula* seedlings on pH_{med} 3 and 6 h after addition of varying amounts of KNO_3. Data from Fig. 1. Dunnett's test for mutants vs WT, *p<0.05, n=3.

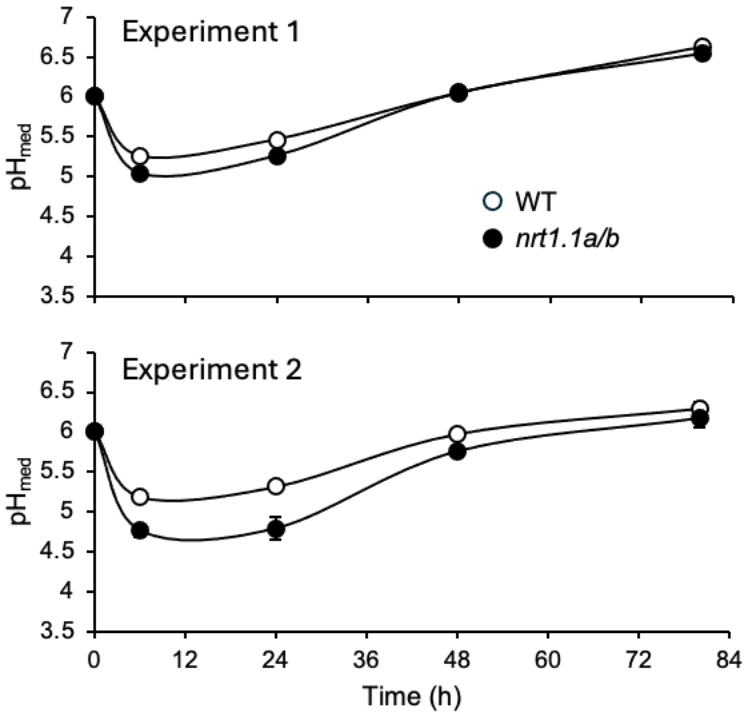

Fig. S4 The affect of pH_{med} *nrt1.1a/b* on pH_{med} during nitrate uptake is stable across experiments. *M. truncatula* seedlings were germinated on nitrate-free FP agar plates and grown for one week, then transferred to either FP, or FP with 5 mM KNO_3 and pH_{med} was monitored. n=3, with 5 plants per biological replicate. Error bars indicate SEM.

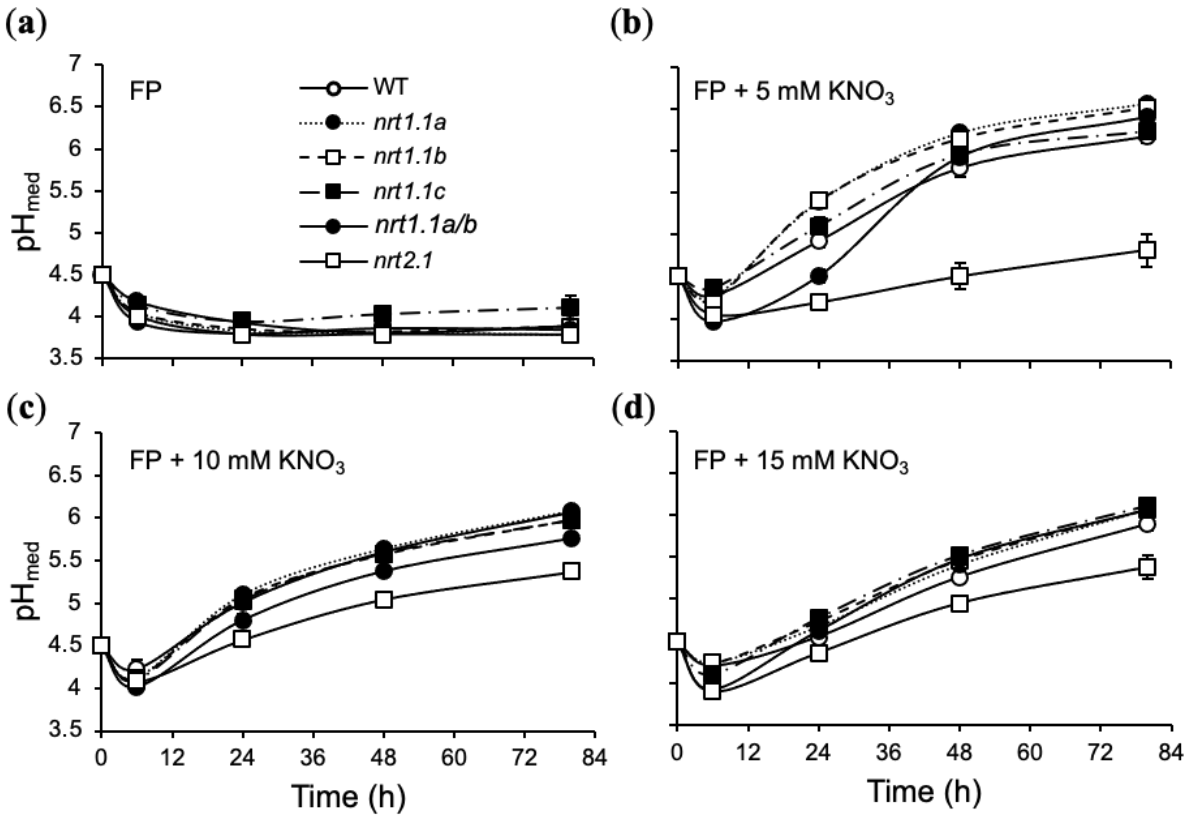

Fig. S5 NRT1.1A/B and NRT2.1 affect pH_{med} during nitrate uptake when using an initial pH of 4.5. *M. truncatula* seedlings were grown on nitrate-free FP agar plates for one week and then transferred to either FP (a), or FP with 5 (b), 10 (c), 15 mM (d) KNO_3 and media pH was monitored. For all experiments the media was adjusted to an initial pH of 4.5. Error bars indicate SEM. n=3, with 5 plants per biological replicate.