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RESEARCH ARTICLE

Physiological evidence that nitrate use positively correlates with internal phosphorus utilization efficiency and phosphorus uptake efficiency in rice (*Oryza sativa* L.)

Yoshiaki Ueda · Matthias Wissuwa

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Abstract

Purpose Phosphorus (P) is one of the plant nutrients most frequently deficient in soils. Under such condition, adoption of P-efficient crops is desirable to maintain agricultural production and avoid heavy reliance on fertilizer application. Previous studies reported significant genotypic difference in internal P use efficiency (PUE) in rice, but key physiological processes remain poorly understood. We aimed at revealing novel key factors that affect PUE.

Methods Rice seedlings were cultivated with different nitrogen (N) sources, and PUE and root traits were characterized. In addition, genotypes that differ in P efficiency were grown under different P supply and growth, gene expression and nutrient uptake were analyzed.

Results Addition of nitrate to P-deficient plants improved PUE compared with ammonium-only plants. Maximum root length of P-inefficient plants was significantly shorter in the presence of ammonium compared with nitrate-only plants under low P supply, while the difference was absent in P-efficient plants. Under low P supply, P-efficient genotypes had lower ratio of ammonium/nitrate accumulation in root and *AMT1;1*/*NRT1.1B* expression (encoding an ammonium and nitrate transporter, respectively) than P-inefficient plants, suggesting that PUE positively correlates with the ability to use nitrate. The ability to use nitrate also positively correlated with root efficiency in the field under low P supply, suggesting that nitrate use may positively modulate both internal P utilization and P uptake.

Conclusion This study provides physiological evidence that N metabolism is linked with PUE and suggests that strengthening the ability to use nitrate may improve P use, hence contributing to the crop production in P-impoverished soils.

Keywords Rice · Phosphorus · Nitrogen · Natural variation · Nutrient uptake

Abbreviations

PAE P acquisition efficiency
PUE Internal P use efficiency
RE Root efficiency

Introduction

Plants require various essential nutrients for their growth, most of which are absorbed by roots from

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Y. Ueda · M. Wissuwa (✉)
Crop, Livestock and Environment Division, Japan International Research Center for Agricultural Sciences, Tsukuba, Japan
e-mail: wissuwa@affrc.go.jp

the rhizosphere. Due to the sessile nature of plants, optimal soil nutrient availability is an important key to vigorous growth and successful seed setting. However, the plant-available nutrient content of agricultural soils is often not in the optimal range, causing plants to suffer from nutrient deficiencies or toxicities (Marschner 1995; Rakotoson et al. 2022). In response to such nutrient imbalances plants can alter their nutrient uptake capacity, internal distribution and metabolism (Xu et al. 2012; Ueda et al. 2021). Among the essential plant nutrients, the demand for phosphorus (P) is relatively large, since P is indispensable as building blocks for nucleic acids, lipids and other metabolites including intermediate metabolites for carbon fixation (Heuer et al. 2017). Despite its abundance, most P in soil is present in forms of low plant availability due to adsorption to soil particles or formation of insoluble salts (Nishigaki et al. 2021). Lynch (2011) estimated that 50% of worldwide soils are potentially deficient in plant-accessible P, which severely limits the productivity of plants in these regions (Andrianary et al. 2021; Rakotoson et al. 2022). Notably, P deficits in agricultural soils occur mainly in developing countries with sub-Saharan Africa being particularly affected (MacDonald et al. 2011). Amendment of these soils with P fertilizers is indeed a measure to increase agricultural productivity, but small-scale farmers in these regions often cannot afford adequate fertilizers due to limited financial resources (Niang et al. 2017; Vandamme et al. 2018). In addition, high-grade rock phosphate reserves, from which P fertilizers are produced, are expected to be depleted in the future (Vinod and Heuer 2012), and their concentration in few geographical regions is a matter of concern at present (Cooper et al. 2011). Increasing crop productivity on P-deficient soils without relying heavily on application of P fertilizers is therefore an important objective globally.

Utilization of P is determined by two factors, namely, P acquisition efficiency (PAE) and internal P use efficiency (PUE) (Rose et al. 2011; López-Arredondo et al. 2014). Thus, one countermeasure to cope with the shortage of P is to develop crop varieties with improved PAE. PAE is affected by root size and root efficiency (RE), which is defined as the amount of P taken up per root surface area. Previous studies with rice reported large genotypic differences in both root size and RE (Mori et al. 2016; Wissuwa et al. 2020), suggesting the potential to improve PAE in modern cultivars exists. Recently, rhizosphere processes were also shown to play a potentially pivotal role in P acquisition of rice. In highly weathered acidic soils with pH-dependent charge, an increase in soil pH will solubilize P and therefore increase its availability for uptake (Barrow 2017). Such rise in rhizosphere pH is expected if rice takes up excess anion over cations, as would be the case if the uptake of nitrate (NO_3^-) exceeds that of ammonium (NH_4^+) (Kuppe et al. 2022). An alternative way to improve P utilization is to develop crop varieties with high PUE. Since PUE is defined as the inverse of tissue P concentration, plants with high PUE produce more biomass per unit of absorbed P (Rose et al. 2011). Contrary to PAE that has been the focus of many previous studies, the mechanisms for PUE have been explored to a lesser extent. Modern improved rice varieties tend to have low PUE, suggesting the potential for genetic improvement (Wissuwa et al. 2015). One difficulty with screening for PUE is that plants with higher P uptake tend to have lower PUE, since increasing P uptake decreases the P deficiency stress of a plant (Rose et al. 2016). This confounding effect likely hampers the development of varieties with high PUE, since most screening systems neglect the differences of total P uptake in different accessions, under which condition genotypic differences in PUE cannot be appropriately evaluated (Rose et al. 2016). Overcoming such a confounding effect by supplying the same low quantity of P to each plant, Wissuwa et al. (2015) observed wide variation in PUE among rice accessions. In a genome-wide association study it was further shown that PUE is controlled by many small-effect loci, suggesting that several genetic and physiological mechanisms are involved in PUE (Wissuwa et al. 2015).

The analysis of the metabolome in four rice accessions contrasting in PUE revealed that certain metabolites (e.g. threonine and benzoate) were enriched in leaves of P-efficient genotypes under low P supply, suggesting that these metabolites could serve as markers to select P-efficient plants (Watanabe et al. 2020). Further investigation of changes in main leaf P pools showed that P-efficient rice accessions have lower investment of P in the lipid-P pool (Hayes et al. 2021). It has also been shown that P-efficient rice genotypes preferentially allocate P to roots (Adem et al. 2020; Hayes et al. 2021), as well as having characteristic root transcriptome (Prodhan et al. 2022),

suggesting that roots also play important roles in determining PUE.

Nitrogen (N) is another important essential element for plant growth with a strong impact on crop productivity (Erisman et al. 2008; Saito et al. 2019). Under normal physiological conditions, plants take up N either as the ammonium or nitrate ion from soils. Nitrate taken up is converted to ammonium enzymatically via nitrate reductase and nitrite reductase, after which ammonium is incorporated into glutamate to produce glutamine with the help of glutamine synthetase (Xu et al. 2012). Whether N is taken up mainly as nitrate or ammonium affects many physiological aspects of plants, such as root morphology, metabolite contents and gene expression patterns (Patterson et al. 2010; Meier et al. 2020; Tian et al. 2021). Nitrate also plays a role as a signaling molecule (Crawford 1995), bridging N and P signaling pathways (Kiba et al. 2018; Maeda et al. 2018; Hu et al. 2019; Medici et al. 2019; Ueda et al. 2020a) and enabling coordinated modulation of N and P uptake and adaptation to diverse nutrient conditions. In the presence of nitrate, rice SPX4 interacts with a nitrate transporter NRT1.1B and is subject to degradation via a proteasomal pathway. The degradation of SPX4 releases NIN-LIKE PROTEIN (NLP) 3 and PHOSPHATE STARVATION RESPONSE (PHR) 2 that were originally bound with SPX4, and free NLP3 and PHR2 activate the transcription of nitrate-related and P deficiency-inducible genes, respectively (Hu et al. 2019). A second link between nitrate and P deficiency signaling is via the nitrate-inducible NIGT1 family transcription factors that repress the expression of *SPX* family genes in Arabidopsis. Since SPX proteins negatively regulate P starvation response, their repression would actually enhance P starvation responses and P uptake (Ueda et al. 2020a). In rice and Arabidopsis, these molecular linkages affect PAE in the presence of nitrate by promoting P starvation responses and thereby the expression of P transporter genes that enhance P uptake (Hu et al. 2019; Medici et al. 2019; Ueda et al. 2020a). These recently discovered links between nitrate and the P starvation response may affect various processes related to P uptake, redistribution and utilization but since experiments were conducted in mutants or genetically engineered plants, it is unknown to what extent natural genotypic variation in preference for nitrate exists and could be exploited for crop improvement under P deficiency, and how such differences may affect PAE and PUE.

In this study, our objectives were to 1) examine to what extent the N form supplied would affect PUE, 2) test whether genotypic differences in preference for nitrate or ammonium exist, and 3) examine the potential relationship between N form taken up and P efficiency (i.e. PUE and PAE). To these aims, we used groups of rice genotypes with contrasting PUE and PAE, and characterized their gene expression pattern and physiological parameters in relation to N form supplied under P replete and deficient levels.

Materials and methods

Plant materials and growth conditions

Five rice genotypes used in the current study were previously shown to contrast in PUE, as well as RE in the field with IR64 and Taichung Native being P-inefficient, while DJ123, Mudgo, and Yodanya are P-efficient (Wissuwa et al. 2015; Mori et al. 2016; Adem et al. 2020; Watanabe et al. 2020; Hayes et al. 2021). Seeds were sterilized with 5% NaClO solution for 5 min, rinsed with tap water and germinated in a petri dish on moist tissue paper at 28 °C in darkness. Germinated seeds were transferred to a tray with a stainless steel mesh bottom in contact with 8 L of deionized water below, and grown for 3 d in darkness. Afterwards, seedlings were grown in natural light in a glasshouse with Yoshida nutrient solution (Yoshida et al. 1976); initially with very diluted solution (0.1 × Ca, 0.15 × Fe, 0.05 × K, 0.05 × Mg, and 0.05 × micronutrient (Mn, B, Cu, Zn and Mo)). The solution was refreshed every 2–3 d until transplanting. After further 9 d, 2 seedlings of the same genotype were transferred to non-transparent 1-L bottle containing 0.2 × P-free Yoshida nutrient solution. After 3 d, 800 µg and 400 µg of P (as NaH_2PO_4), corresponding to 400 and 200 µg of P per plant, was added to each bottle of the high P and low P treatments, respectively. Until 43–44 d after germination, the concentration of nutrient solution was increased gradually (Fig. S1). P was periodically added to each bottle to a total amount of 8,000 µg P (high P) or 800 µg P (low P). The full strength (1x) P-free Yoshida solution contained 1.42 mM NH_4NO_3, 0.5 mM K_2SO_4, 1 mM $CaCl_2$,1 mM $MgSO_4$, 36 µM

Fe-EDTA, 9 µM $MnCl_2$, 18.5 µM H_3BO_3, 0.16 µM $CuSO_4$, 1.5 µM $ZnSO_4$ and 0.07 µM $(NH_4)_6Mo_7O_{24}$. Ammonium-only plants received the same molarity of $(NH_4)_2SO_4$ instead of NH_4NO_3. Nitrate-only plants received twice the molar ratio of KNO_3 instead of NH_4NO_3. pH of the solution was adjusted to 5.5–6.0 twice a week with potassium silicate solution (for NH_4NO_3- and ammonium-only plants) or with hydrochloric acid (for nitrate-only plants).

Plants were harvested 46–48 d after germination. Shoot and root dry weight were measured after drying at 70 °C for > 3 d. For gene expression analysis, whole root samples were flash-frozen by liquid N and stored at -80 °C until the analysis.

N uptake analysis

Plants at 41 d after germination were used for the analysis. Prior to the N uptake experiment, plants were preincubated with fresh 1 × Yoshida solution containing 2 mM MES-KOH (pH 5.7) for 2 d to stabilize pH, since pH changes may affect the rate of ammonium and nitrate uptake (Fried et al. 1965). The preincubation solution contained 0 and 100 µM P for low and high P treatment, respectively. For the uptake analysis, roots were briefly washed by 0.1 mM $CaSO_4$ and immediately soaked into 1 × Yoshida nutrient solution containing 2 mM MES-KOH (pH 5.7), where 20% of either of NO_3^- or NH_4^+ of NH_4NO_3 was replaced with $^{15}NO_3^-$ or $^{15}NH_4^+$ (Shoko Science Co., Ltd). The P concentration of the solution was 0 and 100 µM for low and high P treatment, respectively. After 1 h, roots were washed with 0.1 mM $CaSO_4$ and immediately separated from shoot. Samples were dried at 70 °C for > 3 d, pulverized into a fine powder, and subjected to isotope ratio mass spectrometer (Delta V Advantage, Thermo Fisher Scientific) connected with an elemental analyser (Flash2000, Thermo Fisher Scientific).

Analysis of mineral content

Dried shoot and root samples were cut into small pieces with scissors. Around 100 mg of dried samples were subjected to wet-acid digestion using 8 mL of HNO_3 and $HClO_4$ mixture (3:1 [v/v]) as previously described (Wang et al. 2017). P concentration in the digest was analyzed by the molybdenum-blue method using a standard curve (Murphy and Riley 1962). Total iron (Fe) was extracted from pulverized dried whole root samples with 1 M sodium dithionite solution with gentle shaking at 450 rpm for 2 min at room temperature by a bead beater (Hartmann and Asch 2018). The supernatant was obtained after centrifugation at 16,000 g for 5 min and an aliquot of 100 µL was mixed with the assay solution (120 mM ascorbic acid, 8.5 mM 2,2'-bipyridyl, 70 mM acetate buffer [pH 5.0]) (Waters and Troupe 2012). The mixture was incubated at 37 °C for 1 h and the absorbance at 520 nm was recorded. Standard curve was prepared using a dilution series of Fe standard solution (Fujifilm WAKO Pure Chemical). N concentrations were measured from pulverized dry samples during the mass spectrometry analysis.

Gene expression analysis

Frozen whole root samples were ground into a fine powder with chilled mortar and pestle. Total RNA was extracted using the ISOSPIN Plant RNA kit (Nippon Genetics) according to the manufacturer's instructions including DNase treatment. cDNA was synthesized using the PrimeScript RT Master Mix (Perfect Real Time) (Takara Bio Inc.). Quantitative real-time PCR (qPCR) was carried out using the CFX96 Real-Time PCR Detection System (Bio-Rad) and TB Green *Premix Ex Taq* II (Tli RNaseH Plus) (Takara Bio Inc.). Quantification was based on the standard curve method as previously described using *OsC3H38* as the internal standard (Adem et al. 2020). Since the presence of polymorphisms may affect the efficiency of qPCR, gene-specific primers (Table S1) were designed following two criteria: 1) no polymorphism in the annealing site, and 2) no more than 3 bp of indels in the amplified fragment. Polymorphisms were searched for on the RiceVarMap website (http://ricevarmap.ncpgr.cn/, last accessed in February 2022) (Zhao et al. 2015).

Data analysis

The ANOVA was conducted to compare genotype means while differences between group means (i.e. high- and low-PUE groups) were tested by the Wilcoxon's rank sum test at $\alpha = 0.05$.

Results

Effect of N source on biomass production, P concentration, PUE and root elongation

To reveal the effect of N sources on PUE, P-inefficient *indica* cultivar Taichung Native (Wissuwa et al. 2015) was grown in the presence of both nitrate and ammonium ions (1:1 ratio, as NH_4NO_3) or sole ammonium ($(NH_4)_2SO_4$). N sources did not affect shoot biomass production (Fig. 1a,b), but the presence of nitrate increased root biomass significantly under the low P condition (Fig. 1c) and this resulted in lower root P concentrations (Fig. 1d,e). PUE based on total plant biomass was positively affected by the presence of nitrate and this was more pronounced in the low P treatment (Fig. 1f).

The N-form supplied furthermore affected root growth patterns, especially maximum root length. All genotypes had longest roots when supplied with nitrate only and the addition of ammonium had an inhibitory effect. However, it was only P-inefficient IR64 and Taichung Native which significantly reduced root length in the ammonium-nitrate treatment compared with nitrate-only treatment, which

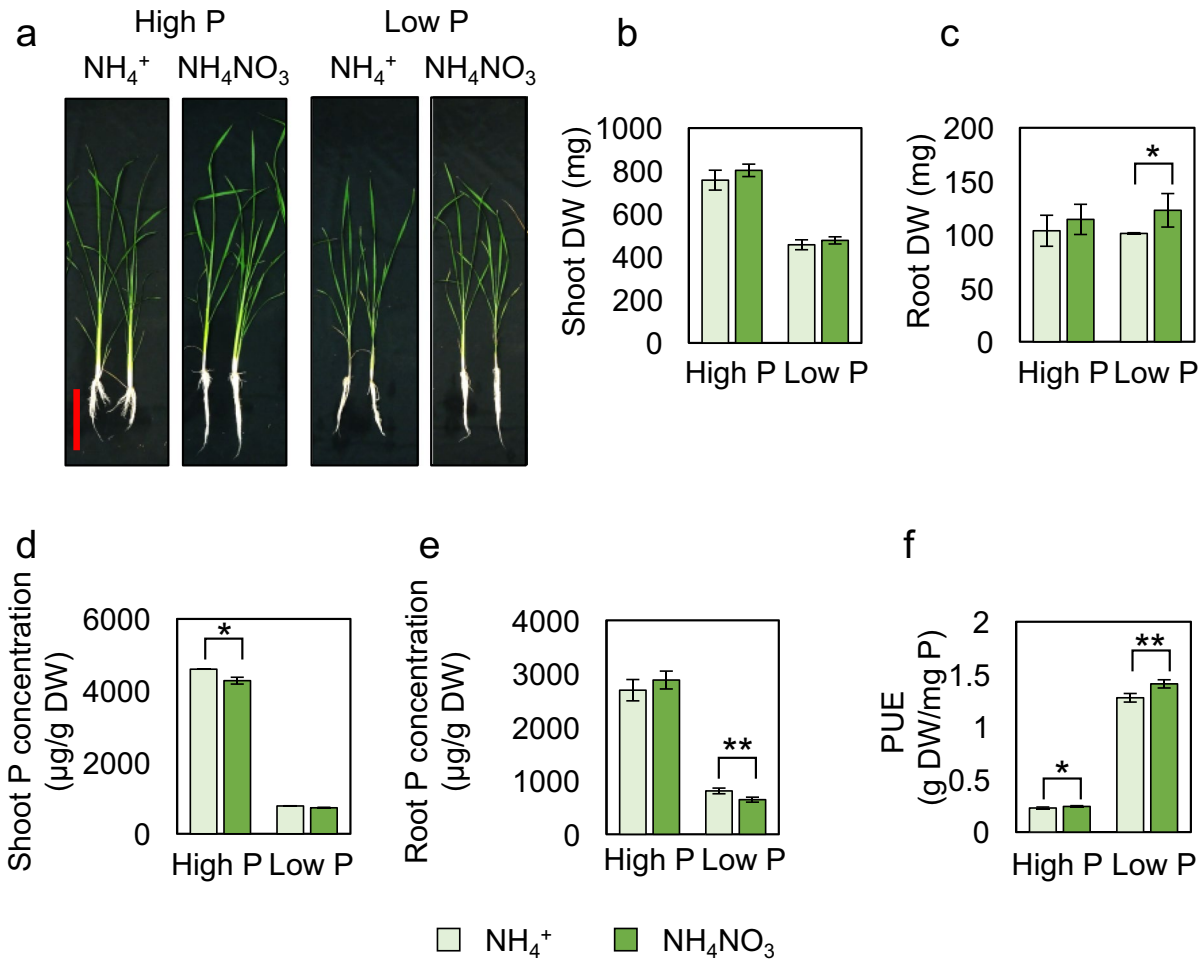

Fig. 1 Effect of different N sources on biomass production and internal P use efficiency. **a** Representative Taichung Native plants grown with $(NH_4)_2SO_4$ as sole N source (NH_4^+) or with NH_4NO_3, under high or low P supply. All the photos are in the same scale. The scale bar indicates 20 cm. **b**, **c** Dry weight of shoot (**b**) and root (**c**). **d**, **e** Concentration of total P in shoot (**d**) and root (**e**). **f** Internal P use efficiency. For **b**-**f**, data represent mean ± S.D. ($n = 4$). Significant differences between the two N conditions are indicated using asterisks (*, $P < 0.05$; **, $P < 0.001$; two-tailed Student's *t*-test)

made the contrast between the groups more evident under this condition (Fig. 2a,b).

Gene expression patterns of P response and N-related genes

To further investigate potential link between N use and PUE, we carried out gene expression analyses in roots. P deficiency-inducible marker genes *IPS1* and *PT6* were strongly up-regulated by the low P condition (Fig. 3a) but more so in the P-inefficient group (IR64 and Taichung Native) compared to the P-efficient group (Mudgo, Yodanya, and DJ123). This indicated that higher PUE was not simply caused by a stronger general P deficiency response.

Next, we tested the expression of genes mediating N-P interaction; the transcription factor *NLP3*, *NIGT1* and a suppressor of P deficiency signaling *SPX4*. Gene expression analysis in roots revealed that, even though *SPX4* showed slightly higher expression in P-efficient group, this pattern was absent in another set of genotypes, suggesting that these previously established molecular links are likely not the determinant for PUE (Fig. 3b; Fig. S2).

Ammonium is mainly taken up by three members of the AMT1 family (AMT1;1, AMT1;2 and AMT1;3) at the high-affinity range (Konishi and Ma 2021). Nitrate uptake is suggested to be mediated by NRT1.1A, NRT1.1B, NRT2.3, NPF2.4 and NRT1.5A under various physiological conditions (Wang et al. 2020a). Among the *AMT1* family genes, the expression pattern of *AMT1;1* and *AMT1;2* was very similar across samples ($R = 0.94$), and both exhibited significantly lower expression in P-efficient genotypes compared to P-inefficient ones ($P < 0.01$) (Fig. 4a). The expression of N deficiency-inducible *AMT1;3* (Ferreira et al. 2015; Konishi and Ma 2021) did not show a contrast between P-efficient and -inefficient genotypes (Fig. 4a). On the other hand, one of the examined nitrate transporters, *NRT1.1B*, showed higher expression in P-efficient genotypes even under high P supply, and this trend remained under low P supply (Fig. 4b). The other major nitrate transporter genes that contribute to nitrate uptake (*NRT1.1A*, *NRT2.3*, *NPF2.4*, and *NRT1.5A*) did not exhibit significant genotypic differences under low P supply (Fig. 4b).

Since plants cannot directly utilize nitrate and ammonium ions, uptake of these ions should be followed by assimilation into amino acids (Fig. S3a). The expression of two nitrate assimilation-related genes *NR1* (encoding a nitrate reductase) and *NIR* (encoding a nitrite reductase), which are involved in reduction of nitrate to ammonium, were significantly higher in the P-efficient group ($P < 0.01$), whereas ammonium assimilation-related genes, *GS1;2* (encoding a cytosolic glutamine synthetase) and *NADH-GOGAT* (encoding a NADH-dependent glutamate synthase), had significantly lower expression in the

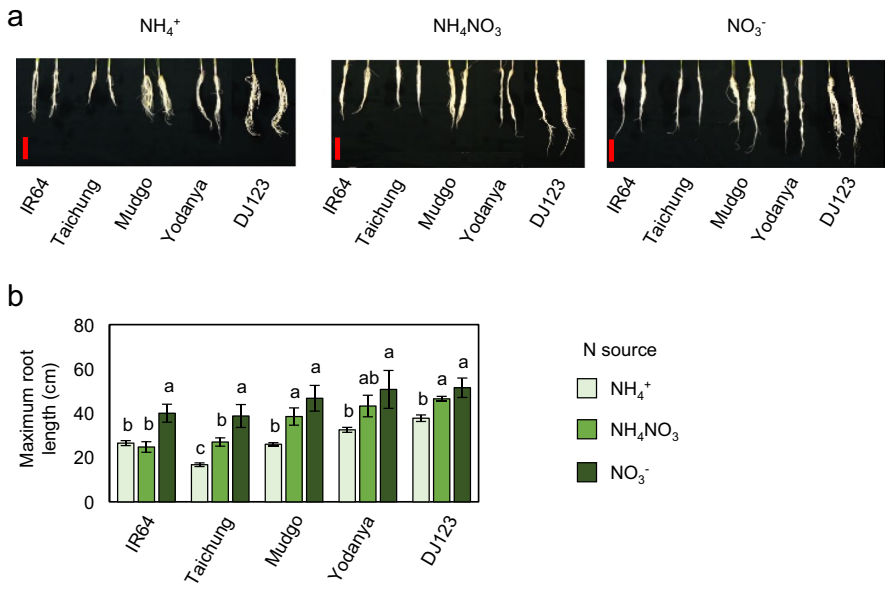

Fig. 2 Examination of ammonium and nitrate-related phenotypes. **a** Roots of plants grown with $(NH_4)_2SO_4$ (left panel), NH_4NO_3 (middle panel) or KNO_3 (right panel) as N source under low P supply. Scale bar indicates 10 cm. **b** Maximum root length of plants grown under the same condition as **a**. Data represent mean ± S.D. ($n = 4$). Significant differences were determined among 3 conditions in each genotype, followed by Tukey's HSD test, and are indicated by different lowercase letters

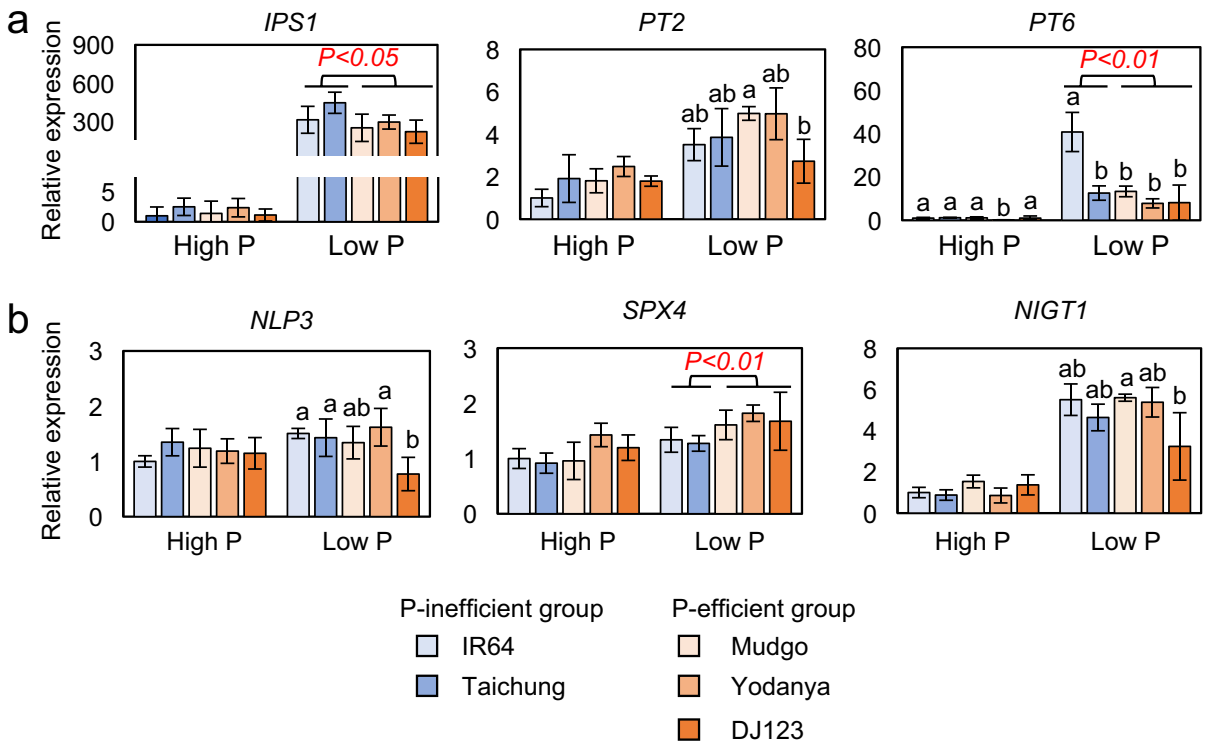

Fig. 3 Expression of P and N signaling-related genes in roots. The expression of P-inducible marker genes (**a**) and genes involved in N-P signaling (**b**) are shown for 5 genotypes under high and low P supply. Data represent mean ± S.D. ($n = 4$). Significant differences among 5 genotypes in each P condition were determined using one-way ANOVA, followed by Tukey's HSD test, and are indicated by different lowercase letters. Significant differences between the P-efficient and –inefficient group were determined by Wilcoxon's rank sum test, and the resultant P values are shown in red letters if $P < 0.05$

P-efficient group ($P < 0.01$ and < 0.05, respectively) (Fig. S3b).

Uptake analysis of N sources

N uptake patterns were examined using ^{15}N-labeled ammonium or nitrate. Under low P supply, the accumulation of ammonium-derived N in roots was significantly lower in the P-efficient group (Fig. 5a), whereas nitrate-derived N accumulation in roots was significantly higher in the P-efficient genotypes (Fig. 5b). Similar trend was observed for the uptake of these ions on whole-plant basis, where P-efficient genotypes absorbed more nitrate than P-inefficient genotypes (Fig. S4). As a result, the ratio of ammonium/nitrate uptake in roots was consistently higher in P-inefficient genotypes (Fig. 5c). Accumulation of ammonium-derived N in root exhibited a positive correlation with the expression of *AMT1;1* and this correlation was very strong ($R = 0.97$, $P < 0.01$) under low P supply (Fig. 5e). Accumulation of nitrate-derived N showed a positive correlation with the expression of *NRT1.1B*, irrespective of P supply ($R = 0.96$, $P < 0.001$) (Fig. 5f). Similar results were observed in an additional experiment using 8 genotypes (Fig. S5), suggesting that the expression of *AMT1;1* is a good indicator for ammonium uptake in roots under low P supply, while expression of *NRT1.1B* predicts nitrate uptake in roots irrespective of the P conditions. Consistent with above data, the ratio of *AMT1;1*/*NRT1.1B* expression was also high in the P-efficient group, showing a positive correlation with the actual uptake ratio, especially under the low P supply ($R = 0.61$ for high and 0.86 for low P supply) (Fig. 5d).

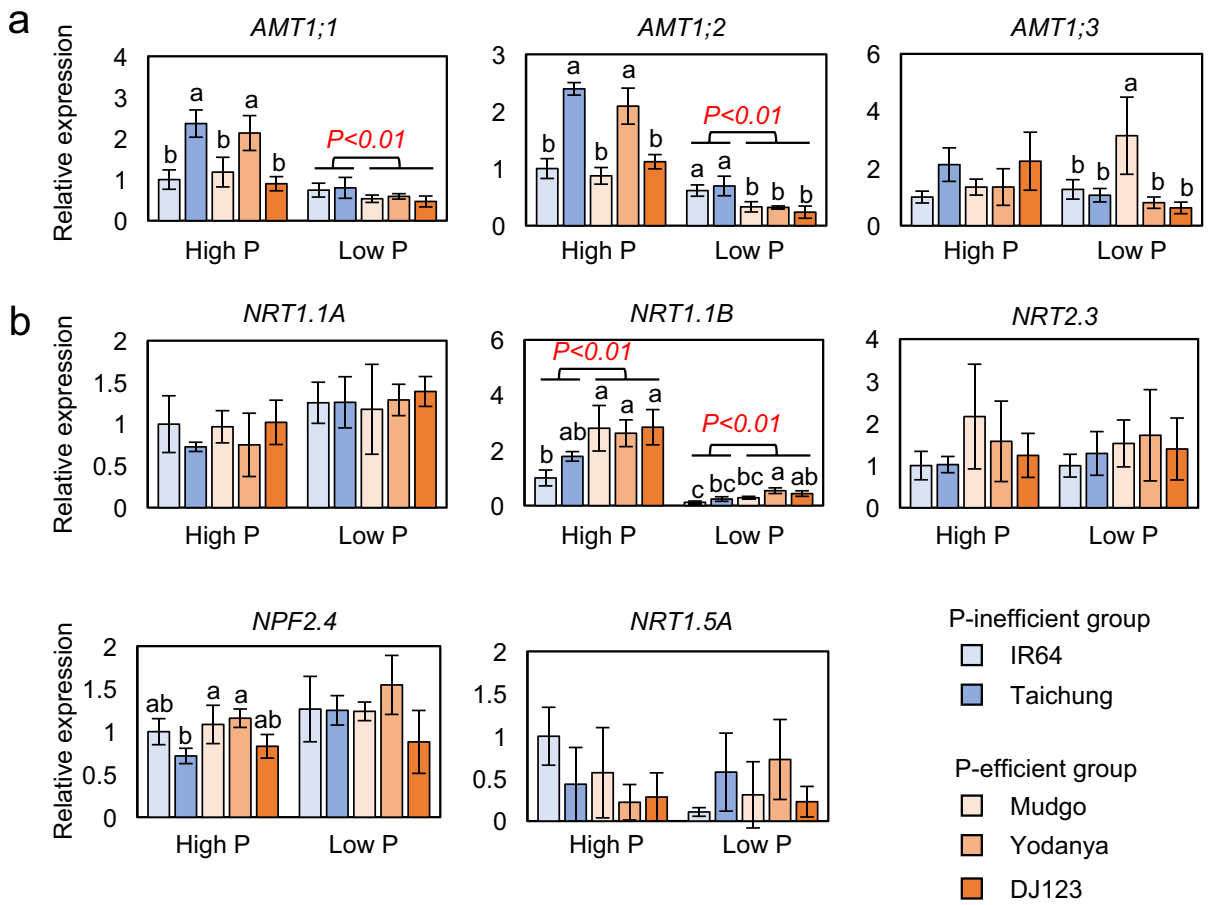

Fig. 4 Expression of N uptake-related genes in roots. The expression of genes involved in the uptake of ammonium (**a**) and nitrate (**b**) are shown for 5 genotypes under high and low P supply. Data represent mean ± S.D. ($n = 4$). Significant differences among 5 genotypes in each P condition were determined using one-way ANOVA, followed by Tukey's HSD test, and are indicated by different lowercase letters. Significant differences between the P-efficient and –inefficient group were determined by Wilcoxon's rank sum test, and the resultant P values are shown in red letters if $P < 0.05$

Correlation of N uptake pattern with root efficiency

In addition to the effect of N form provided on PUE, we also investigated a possible association with PAE. The 5 genotypes used in the current study had previously been grown in a P-deficient field and their RE had been estimated (Mori et al. 2016). Accumulation of nitrate-derived N measured here in low P supply was closely correlated ($R = 0.91$, $P < 0.05$) with the field RE estimate (Fig. 6) but no association was detected based on nitrate uptake in high P supply.

Discussion

The two most important plant nutrients are N and P and recent studies have indicated cross-talk between N and P nutrition exists that affects how plants fine-tune their response to the relative scarcity of either nutrient (Kiba et al. 2018; Maeda et al. 2018; Hu et al. 2019; Medici et al. 2019; Ueda et al. 2020a). Previously suggested physiological factors for P efficiency involve P translocation (recycling) and substitution of phospholipid by sulfolipids or galactolipids (Adem et al. 2020; Hayes et al. 2021;

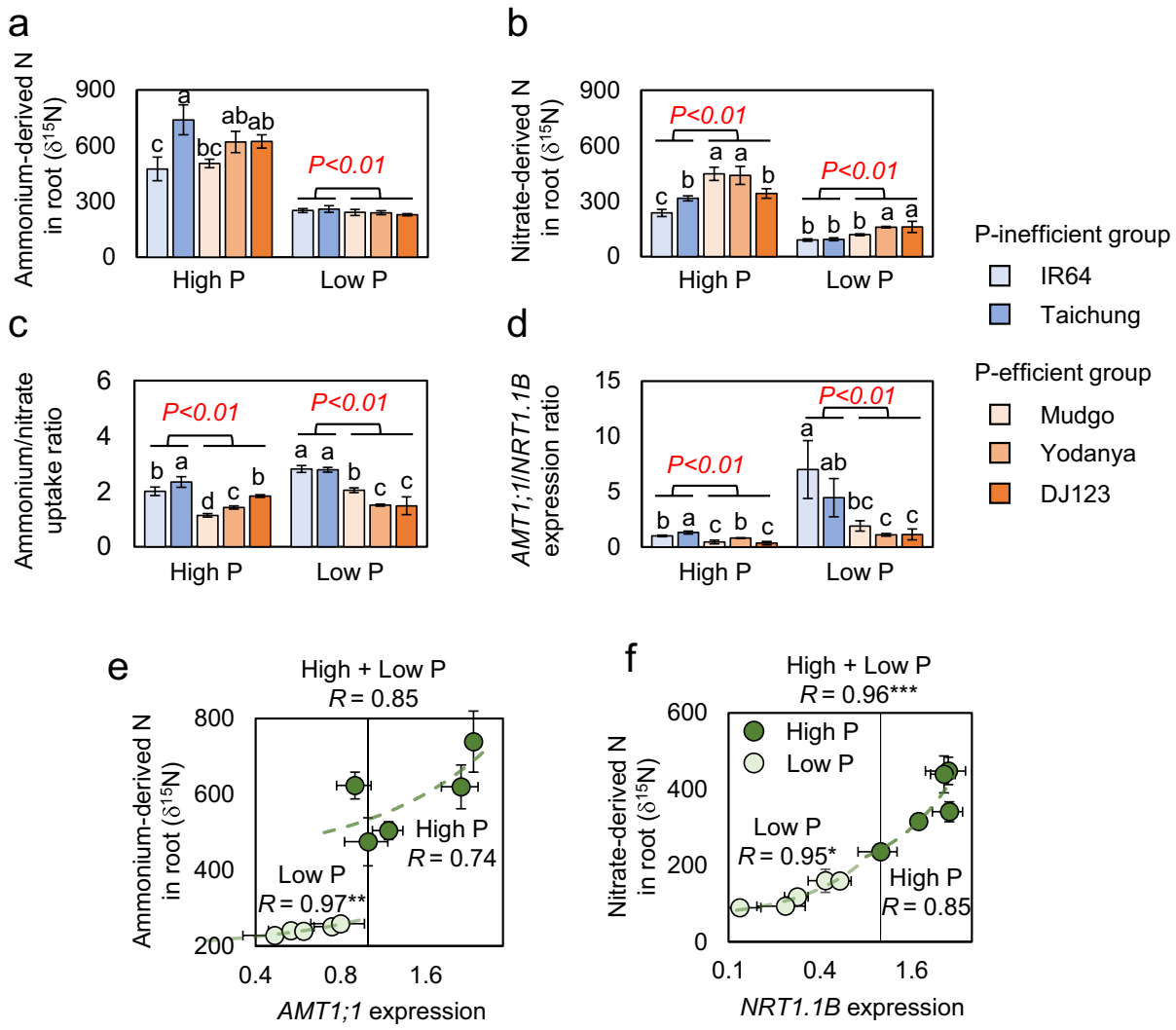

Fig. 5 N source uptake patterns in P-inefficient and –efficient genotypes. **a**,**b** Enrichment of absorbed ^{15}N in root. The value for ammonium-derived N (**a**) and nitrate-derived N (**b**) are shown. **c** Ratio of ammonium/nitrate uptake in roots. **d** Ratio of *AMT1;1*/*NRT1.1B* expression in roots. **e**,**f** Correlation between the expression of *AMT1;1* and ammonium-derived N (**e**) and between the expression of *NRT1.1B* and nitrate-derived N (**f**) in root. Asterisks indicate significant correlation (*, $P<0.05$; **, $P<0.01$; ***, $P<0.001$). Data represent mean ± S.D. ($n=4$). In **a**-**d**, significant differences among 5 genotypes in each P condition were determined using one-way ANOVA, followed by Tukey's HSD test, and are indicated by different lowercase letters. Significant differences between the P-efficient and –inefficient group were determined by Wilcoxon's rank sum test, and the resultant *P* values are shown in red letters if $P<0.05$

Prodhan et al. 2022), which are regulated as part of the P deficiency response pathway (Liu et al. 2010). The magnitude of the P deficiency response is affected by the availability of nitrate (Medici et al. 2019; Ueda et al. 2020a), suggesting a potential link between N use and P deficiency. A second line of evidence for the link between P efficiency and N metabolism in rice was provided by the study of Watanabe et al. (2020) that detected signature N-related metabolites in leaves of P efficient but not inefficient rice genotypes. Here our objective was to determine whether the N form supplied affected the efficiency of P use and related parameters in rice genotypes with contrasting P efficiency.

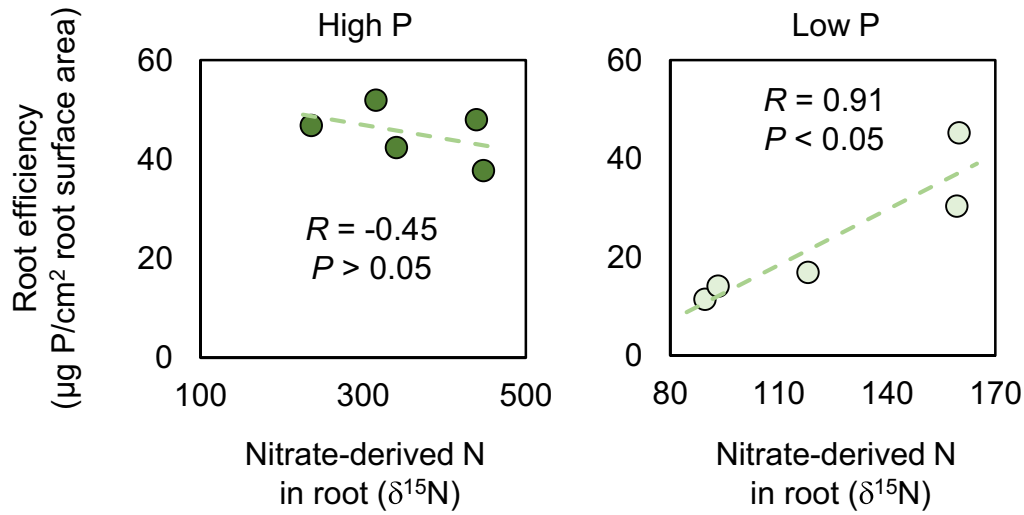

Fig. 6 Correlation of nitrate uptake and root efficiency. The data for root efficiency (Mori et al. 2016) was plotted against the values for nitrate uptake (Data from Fig. 5b). Plot was made separately for high (left panel) and low P (right panel) conditions. Correlation coefficients and resultant *P* values are indicated

Genotypic differences in preference for N form

Clear genotypic differences were observed for the the capacity to use nitrate by roots, with two P inefficient varieties (IR64 and Taichung native) accumulating almost 3 times as much ammonium than nitrate, while the ratio was 1.5–2 for P efficient ones. We assume that this differential N use pattern is not confounded by genotypic differences in the speed of nutrient depletion: in our experiment, total N uptake per plant was typically < 8 μmol/ h and < 0.8 μmol/h under high and low P supply, respectively. These values correspond to < 0.6 mmol and < 0.06 mmol total N uptake over 3 d, which is far less than the total amount of N contained in the hydroponic solution (2.8 mmol total N in 1 L of solution). The differences in use of N form were matched by the relative gene expression ratio of ammonium versus nitrate transporters in both groups (Fig. 5d). Under low P supply the higher ammonium to nitrate uptake ratio in roots of the P inefficient group was not caused by differences in ammonia uptake between groups but by significantly higher nitrate uptake in P efficient genotypes (Fig. 5a,b). This higher nitrate uptake was associated with higher expression of the nitrate transporter *NRT1.1B* in the P efficient group, whereas the inefficient group showed higher expression of ammonium transporters. Previous studies have suggested the presence of within- and inter-species differences in ammonium and nitrate preference (Falkengren-Grerup 1995; Ueda et al. 2020b). It was reported that plants adapted to acidic soils tend to prefer ammonium, suggesting that the preference may reflect the growth environment of the variety (Falkengren-Grerup 1995). Besides, the availability of N form under different growth environment may also affect the preference; under lowland conditions, where the redox potential of soil is low, ammonium is more prevalent, while nitrate is more prevalent under upland conditions (Wang et al. 1993; Crawford and Glass 1998). Upland-adapted plants may have acquired the ability to use nitrate more than lowland-adapted plants, since rapid uptake of nitrate before leaching may benefit plants. In addition, genetic engineering of N signaling pathway and N transporters leads to altered levels of ammonium and nitrate uptake, substantially shifting the preference of N forms (Wang et al. 2020b; Zhang et al. 2022). Considering more frequent occurrence of P deficiency (Ponnamperuma 1972) and more availability of nitrate under upland conditions, our current finding could be more relevant with improvement of plants under P-deficient upland conditions. It is currently unknown if the higher preference for nitrate in P-efficient plants reflects adaptation of these genotypes to specific growth environment or is caused by characteristic N metabolism patterns.

The positive effect of nitrate on PUE and potential underlying mechanisms

The positive effect of nitrate on PUE was predominantly detected in roots and two potentially underlying processes shall be explored: 1) different energy costs for the uptake and assimilation of N forms, and 2) effects of N forms on root elongation. The energy requirements for the uptake and assimilation of ammonium and nitrate differ in amount and in which parts of plants the energy is consumed. The assimilation of nitrate to ammonium requires ~ 10 ATP molecules, while the assimilation of ammonium to glutamine only consumes ~ 2 ATP molecules (Rubio-Asensio and Bloom 2017). However, it should be noted that while nitrate assimilation occurs predominantly in shoot, especially in leaf blades (Rao et al. 1979), most of the absorbed ammonium is assimilated into amino acids in roots (Taylor and Bloom 1998; Hachiya and Sakakibara 2017). In addition, ammonium uptake should be accompanied by active proton extrusion and by plasmamembrane-localized H^+-ATPases to maintain proton gradient across the membrane (Zeng et al. 2012; Zhang et al. 2021). Since one molecule of ATP is used to extrude 0.8–1 molecule of H^+ (Briskin and Hanson 1992), the additional cost associated with the uptake process of ammonium by roots will at least be one extra ATP.

Thus, ammonium is energetically cheaper than nitrate but costs associated with ammonium are incurred in roots whereas those for nitrate are incurred in shoots. To what extent energy costs of N assimilation are affecting plant productivity under P deficiency is not known. Accumulation of carbohydrates in leaves of P deficient plants is a well-documented observation (Cai et al. 2012; Meng et al. 2020) that may indicate energy for nitrate assimilation in leaves may not be a limitation. However, it has been shown that starch also accumulated in roots of rice under P deficiency (Wissuwa et al. 2005) and that poses the question to what extent energy is limiting under P deficiency and whether higher energy costs associated with nitrate assimilation are inconsequential under such conditions. Further studies are needed to clarify these points. The issue of costs could be seen from a different angle. When P limits plant growth directly, costs in terms of P invested in a process may be of greater relevance and in that regard the high enzyme activity required to assimilate ammonium in roots may be a disadvantage as higher enzyme activity may require higher tissue P concentrations to maintain ribosome activity. In contrast, nitrate can be stored in vacuoles (Martinoia et al. 1981; De Angeli et al. 2006) and therefore its assimilation only incurs a cost when N is needed for further plant growth. This would be consistent with the observation that P-efficient genotypes produce "cheaper" roots, with lower content of P, Fe and N (Fig. S6) (Hayes et al. 2021).

Alternatively, preference for ammonium over nitrate may directly affect root growth and this may lead to reduced PUE in ammonium-fed rice. Previous investigations in hydroponically- and soil-grown rice plants showed that maximum root length was reduced with increasing ammonium supply (Kawata et al. 1977; Hirano et al. 2008). Inhibition of root elongation by ammonium was not caused by the ammonium ion directly, but rather by the accumulation of downstream assimilatory products (Hirano et al. 2008). The negative effect of ammonium on maximum root length was confirmed here and this effect was more pronounced in the P-inefficient group. In particular, this group was more sensitive with dual NH_4NO_3 supply, which already reduced maximum root length by 30—38% compared to 10—15% in the P-efficient group (Fig. 2b). Their higher *AMT1;1* and *AMT1;2* as well as *GS1;2* and *NADH-GOGAT* gene expression levels furthermore confirmed a highly active ammonium uptake and assimilation machinery in roots, and this relative preference for ammonium likely caused the reduced root elongation in P-inefficient genotypes.

If differences in root elongation are caused by differences in individual cell elongation, as in the case of Arabidopsis (Li et al. 2010), the resulting root is expected to have required less P per unit root length and this would explain the higher PUE observed in roots of the P-efficient group (Fig. 1f). A carry over effect of higher root PUE is that with the same amount of limiting P invested the plant can produce a larger total root system and the exploration of larger soil volumes with ensuing uptake of additional P has been identified as one main driving factor to overcome P-limitations for growth (Wissuwa et al. 2020; Gonzalez et al. 2021).

Preferential nitrate uptake and P solubilization

In highly weathered acidic soils with pH-dependent charge, an increase in soil pH will increase the P

concentration in solution and hence its plant availability (Barrow 2017). Preferential uptake of nitrate over ammonium will result in a rhizosphere pH increase and recent modeling by Kuppe et al. (2022) has shown that this can substantially improve P uptake in rice. The same study also suggested that the presence of short fine laterals enhance the uptake of solubilized P by extending the effective uptake zone around their parent roots. A relative preference for nitrate would therefore not only favor root elongation with its positive effect on P uptake but could furthermore improve RE. We investigated this possibility by associating the nitrate uptake data from Fig. 5b with estimates of RE, obtained for the same genotypes but grown on a highly P-fixing volcanic ash soil (Mori et al. 2016). Interestingly, a highly positive correlation between both traits ($r = 0.91$) was only detected under low P supply. This observation is in accordance with the fact that an increase in pH results in higher availability of phosphate in volcanic ash soil (Takahashi and Dahlgren 2016). This would suggest that genotypic differences in the preference for nitrate uptake should be explored further in the context of rhizosphere P solubilization.

Similar to the volcanic ash soil used by Mori et al. (2016), the effect of nitrate preference and subsequent pH changes on RE should be also pronounced in acidic soils, since phosphate strongly bound with iron and aluminum can be released by a small pH increases (Price 2006). This has important implications for rice breeding since the highly weathered P-deficient soils typically encountered in sub-Saharan Africa are usually acidic (Nishigaki et al. 2019). The present study presents evidence that genotypes differ for the preference for nitrate over ammonium uptake and suggests this could lead to better root elongation and higher RE. To what extent variation for this trait can be detected and exploited in the larger rice gene pool should be investigated.

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Resources, supervision, methodology, writing-review & editing: MW

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Declarations

Competing interests The authors have no competing interests to declare that are relevant to the content of this article.

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