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RESEARCH PAPER

Rice nitrate transporter OsNPF2.4 functions in low-affinity acquisition and long-distance transport

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Abstract

Plant proteins belonging to the NPF (formerly NRT1/PTR) family are well represented in every genome and function in transporting a wide variety of substrates. In this study, we showed that rice OsNPF2.4 is located in the plasma membrane and is expressed mainly in the epidermis, xylem parenchyma, and phloem companion cells. Functional analysis in oocytes showed that OsNPF2.4 is a pH-dependent, low-affinity NO_3^- transporter. Short-term $^{15}NO_3^-$ influx rate, long-term NO_3^- acquisition by root, and upward transfer from root to shoot were decreased by disruption of *OsNPF2.4* and increased by *OsNPF2.4* overexpression under high NO_3^- supply. Moreover, the redistribution of NO_3^- in the mutants in comparison with the wild type from the oldest leaf to other organs, particularly to N-starved roots, was dramatically changed. Knockout of *OsNPF2.4* decreased rice growth and potassium (K) concentration in xylem sap, root, culm, and sheath, but increased the shoot:root ratio of tissue K under higher NO_3^-. We conclude that OsNPF2.4 functions in acquisition and long-distance transport of NO_3^-, and that altering its expression has an indirect effect on K recycling between the root and shoot.

Key words: Growth reduction, low-affinity nitrate transporter, mutant, potassium recycling, overexpression, rice.

Introduction

Nitrogen (N) is one of the most important nutrients for all plants and is a component of nucleotides, amino acids, and proteins, the fundamental building bricks of life. To acquire sufficient N for growth, plants have to cope with temporal and spatial fluctuation in the availability of N sources in the soil (Xu *et al.*, 2012). Therefore, plants have developed two NO_3^- uptake systems, the high-affinity NO_3^- transport system and low-affinity NO_3^- transport system (Glass *et al.*, 1992; Crawford, 1995; Crawford and Glass, 1998; Forde, 2000), for root acquisition of NO_3^- at low (<1 mM) and high (>1 mM) external NO_3^- concentration in the medium, respectively (Glass *et al.*, 1992; Crawford and Glass, 1998).

The uptake of NO_3^- is an active process driven by H^+/NO_3^- co-transporters (Zhou *et al.*, 2000; Miller *et al.*, 2007). Several families of membrane proteins are involved in uptake, allocation, and storage of NO_3^- in plants: the NO_3^-/peptide transporter family (NRT1/PTR), NO_3^- transporter 2 family (NRT2), chloride channel (CLC) family, and slow anion channel-associated homologues (SLAC/SLAH) (Wang *et al.*, 2012; Krapp *et al.*, 2014). Plant NRT1/PTR proteins transport a wide variety of substrates and have recently been

Abbreviations: ANOVA, analysis of variance; eGFP, enhanced green fluorescent protein; GUS, β-glucuronidase; IRRI, International Rice Research Institute; K, potassium; N, nitrogen; OX, overexpressed; qRT-PCR, quantitative real-time PCR; RT-PCR, reverse transcription-PCR; WT, wild type.

renamed NPF (Leran *et al.*, 2014). In *Arabidopsis*, several N uptake and transport systems have been identified. Five members belonging to the NPF and NRT2 families, AtNPF6.3 (NRT1.1, CHL1), AtNPF4.6 (NRT1.2), AtNRT2.1, AtNRT2.2, and AtNRT2.4, show the function of NO_3^- uptake in roots (Tsay *et al.*, 1993; Wang *et al.*, 1998; Huang *et al.*, 1999; Liu *et al.*, 1999; Cerezo *et al.*, 2001; Filleur *et al.*, 2001; Orsel *et al.*, 2004; Li *et al.*, 2007; Kiba *et al.*, 2012). Among these, AtNPF6.3 is a bidirectional and dual-affinity nitrate transporter (Tsay *et al.*, 1993; Liu *et al.*, 1999; Leran *et al.*, 2013). Moreover, AtNPF6.3 also plays a role as a NO_3^- sensor for the primary NO_3^- response (Ho *et al.*, 2009) and in auxin uptake at low NO_3^- concentration (Krouk *et al.*, 2010). AtNPF4.6 is a low-affinity nitrate transporter (Huang *et al.*, 1999) and also an abscisic acid importer (Kanno *et al.*, 2012). AtNRT2.1, AtNRT2.2, and AtNRT2.4 are high-affinity nitrate transporters (Orsel *et al.*, 2004; Okamoto *et al.*, 2006; Kiba *et al.*, 2012). The NO_3^- transport activity of all AtNRT2 family members except AtNRT2.7 requires a partner protein, AtNAR2.1 (AtNRT3.1), in a heterologous system (Kotur *et al.*, 2012).

Some members of the NPF (NRT1/PTR) family are not directly involved in NO_3^- uptake from the external medium. AtNPF6.2 (NRT1.4) is expressed in the leaf petiole and regulates leaf NO_3^- homeostasis (Chiu *et al.*, 2004). AtNPF2.7 (NAXT1) and AtNPF7.3 (NRT1.5) are both involved in NO_3^- efflux (Segonzac *et al.*, 2007; Lin *et al.*, 2008), and AtNPF7.3 functions in loading NO_3^- from xylem parenchyma cells into the xylem (Lin *et al.*, 2008). AtNPF7.2 (NRT1.8), which is phylogenetically close to AtNPF7.3, is expressed predominantly in xylem parenchyma cells within the vasculature for unloading of NO_3^- from xylem sap (Li *et al.*, 2010). AtNPF2.13 (NRT1.7) is expressed in the phloem of the leaf minor vein and is responsible for NO_3^- remobilization from source to sink tissues (Fan *et al.*, 2009). AtNPF2.9 (NRT1.9), AtNPF1.2 (NRT1.11), and AtNPF1.1 (NRT1.12) are all expressed in phloem companion cells, and AtNPF2.9 facilitates NO_3^- loading in root phloem to balance the NO_3^- in the root and shoot (Wang and Tsay, 2011), whereas AtNPF1.1 and AtNPF1.2 are both involved in xylem-to-phloem transport to redistribute NO_3^- into developing leaves (Hsu and Tsay, 2013). AtNPF2.12 and AtNRT2.7 are both expressed in the seeds, and AtNPF2.12 acts to deliverNO_3^- from maternal tissue to the developing embryo (Almagro *et al.*, 2008), whereas AtNRT2.7 plays a specific role in nitrate loading into the vacuole during seed maturation (Chopin *et al.*, 2007).

Rice is a staple food crop and grown in both upland and flooded paddy soil. Rice roots develop abundant aerenchyma cells under flooding condition, which allows oxygen to be transported from the aerial parts to the roots and released to the rhizosphere, where microbially mediated nitrification converts ammonium (NH_4^+) to NO_3^- on the root surface (Li *et al.*, 2008). The highest NO_3^- concentration in flooded soil can reach 1.72 ± 0.42 mM (Eguchi *et al.*, 2009). Therefore, in paddy soil, the amount of NO_3^- taken up by rice roots may be comparable with the amount of NH_4^+ (Luo *et al.*, 1993; Kronzucker *et al.*, 2000; Kirk and Kronzucker, 2005).

In the rice genome, at least five genes belong to NRT2 (Feng *et al.*, 2011) and 80 genes to the NPF family (Tsay *et al.*, 2007). Among the five *OsNRT2* genes, *OsNRT2.1* and *OsNRT2.2* share an identical coding region sequence with different 5′- and 3’-untranscribed regions. *OsNRT2.3a* and *OsNRT2.3b* are derived from the alternative splicing of *OsNRT2.3* (Feng *et al.*, 2011; Yan *et al.*, 2011), and OsNRT2.3a is involved in long-distance NO_3^- transport from root to shoot at low NO_3^- concentration (Tang *et al.*, 2012). The NO_3^- uptake activity of OsNRT2.1, OsNRT2.2, and OsNRT2.3a requires a partner protein, OsNAR2.1 (Feng *et al.*, 2011; Yan *et al.*, 2011). Several members of the transporters belonging to the NPF family have also been characterized in rice. OsNPF8.9 (NRT1.1) is a low-affinity nitrate transporter and is constitutively expressed in the most external layer of the roots, epidermis, and root hairs (Lin *et al.*, 2000). OsNPF6.5 (OsNRT1.3) is a putative nitrate transporter and its promoter responds to drought stress (Hu *et al.*, 2006). OsNPF7.3 (OsPTR6) is a peptide transporter (Ouyang *et al.*, 2010). For OsNPF4.1 (OsSP1) and OsNPF8.20 (OsPTR9), no nitrate or peptide transport has been observed (Li *et al.*, 2009; Fang *et al.*, 2013), but OsNPF4.1 affects the panicle size of rice (Li *et al.*, 2009), while OsNPF8.20 dramatically affects ammonium uptake and grain yield (Fang *et al.*, 2013). Recently, we observed that overexpression of *OsNPF7.3* (*OsPTR6*) could increase rice growth through increasing ammonium transporter expression and glutamine synthetase activity (Fan *et al.*, 2014). However, none of the NPF members was functionally characterized in terms of NO_3^- acquisition and mobilization in rice.

Here, we report the expression patterns and physiological function of OsNPF2.4 belonging to cluster IV of the NPF family in rice. We found that OsNPF2.4 is a pH-dependent, low-affinity nitrate transporter and involved in direct acquisition of NO_3^- by the roots from high external NO_3^- solution. Potassium (K)-coupled NO_3^- upward transport from roots to shoots, as well as redistribution of NO_3^- from source leaves to sink organs, is linked to the activity of OsNPF2.4. Increased NO_3^- supply to N-deficient rice could not significantly eliminate the defects of total N uptake and growth in T-DNA insertional *osnpf2.4* knockout mutants. We conclude that OsNPF2.4 plays an important role in maintaining NO_3^--mediated growth and development in rice.

Materials and methods

Plant growth conditions

Seeds were sterilized and germinated to give uniform seedlings as described previously (Chang *et al.*, 2009). One-week-old seedlings were transferred to plastic boxes containing 7 l of International Rice Research Institute (IRRI) nutrient solution [1.25 mM NH_4NO_3 or $(NH_4)_2SO_4$ or $Ca(NO_3)_2$, 0.3 mM KH_2PO_4, 0.35 mM K_2SO_4, 1 mM $CaCl_2$, 1 mM $MgSO_4$, 0.5 mM Na_2SiO_3, 20μM EDTA-Fe, 9 μM $MnCl_2$, 20 μM H_3BO_3, 0.77 μM $ZnSO_4$, 0.32 μM $CuSO_4$, and 0.39μM $(NH_4)_6Mo_7O_{24}$, pH 5.5]. For K-free treatment, 0.3 mM KH_2PO_4 and 0.35 mM K_2SO_4 were replaced by 0.3 mM NaH_2PO_4 and 0.35 mM Na_2SO_4. Dicyandiamide (7 μM, $C_2H_4N_4$) was mixed into the nutrient solutions to inhibit nitrification. All seedlings were grown in a greenhouse with 16 h light (30 °C)/8 h dark (22 °C), and the relative humidity was approximately 70%. The nutrient solution was refreshed every 2 d.

Reverse transcription (RT)-PCR and quantitative real-time (qRT)-PCR

Total RNA extraction from different rice tissues and RT-PCR using specific primers for *OsNRT2.4* (Supplementary Table S2 at *JXB* online) and internal standard gene *OsActin* was performed by following the protocol described by Feng *et al.* (2011). For qRT-PCR analysis, a relative quantification method using a single-colour real-time PCR detection system (StepOnePlus Real-time PCR System; Applied Biosystems,) was used to evaluate quantitative variation among the replicates. The amplification of *OsActin* was used as an internal control to normalize all expression data. All primers used for qRT-PCR are listed in Supplementary Table S3 at *JXB* online.

Transient expression of OsNPF2.4 in rice protoplasts

To construct the OsNPF2.4–enhanced green fluorescent protein (eGFP) fusion proteins, *OsNPF2.4* cDNA was amplified by PCR using forward primer 5′-tccaagcttTCGGTCGCTTCGCTTCTA-3′ and reverse primer 5′-ggagaattcTTCACTTGCATCTGCGTTGGC-3’. *Hind*III and *Eco*RI restriction sites (underlined) were introduced, respectively, into each primer. The amplified cDNA was cloned in front of the eGFP-coding region in the middle vector pSAT6A-eGFP-N1. To construct the eGFP–OsNPF2.4 fusion protein, *OsNPF2.4* cDNA was amplified by PCR using forward primer 5′-agaaagcttGTGGTGGTTGGTGAGAGAGG-3′ and reverse primer 5’-agggaattcACCACCCAAACGAACAAACT-3′. *Hind*III and *Eco*RI restriction sites (underlined) were introduced, respectively, into each primer. The amplified *OsNPF2.4* cDNA was cloned into the middle vector pSAT6-eGFP-C1 (no termination codon) behind eGFP. The two middle vectors were cut by the PI-*Psp*I and cloned in frame into the expression vector pRCS2-ocs-nptII. Transient expression of OsNPF2.4 in rice protoplasts and fluorescence microscopy imaging observation were carried out following previously described procedures (Tang *et al.*, 2012).

Construction of an OsNPF2.4 *promoter fusion with β-glucuronidase (GUS) and GUS staining*

An upstream 1.2 kb genomic fragment of the *OsNPF2.4*-encoding region was amplified by PCR using forward primer 5′-gcttaattaaCAAATTTCAAACCTTTTGGCACCAG-3′ (with a *Pac*I restriction site, underlined) and reverse primer 5′-ccgggcgcgccCCTCTCTCACCAACCACCACCTCTG-3′(with an *Asc*I restriction site, underlined). The product was cut with *Asc*I and *Pac*I and ligated into the pS1aGUS-3 vector. GUS staining analysis was performed as described previously (Feng *et al.*, 2011). To differentiate the expression of *OsNPF2.4* in different cell types, GUS-stained root and leaf blade were embedded in Spurr resin and sectioned (5 µm thick). The sections were visualized using a colour CCD camera (Olympus).

Functional analysis of OsNPF2.4 in Xenopus laevis *oocytes*

The *OsNPF2.4* cDNA was subcloned as a *Bgl*II–*Spe*I fragment into the oocyte expression vector pT7Ts, linearized using *Xba*I, and the cRNA was transcribed *in vitro* using an Ambion mMessage mMachine®T7 kit. Oocytes were injected with 50 ng of *OsNPF2.4* cRNA as described previously (Tsay *et al.*, 1993; Feng *et al.*, 2013). The oocytes were incubated in ND96 solution with antibiotics (96 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$, 1.8 mM $CaCl_2$, 5 mM HEPES, 50 µg ml^{-1} of gentamycin,100 µg ml^{-1} of streptomycin, pH 7.4). Two-electrode voltage clamp analysis was performed as described previously (Huang *et al.*, 1999). NO_3^- influx and efflux measurements were performed as described elsewhere (Lin *et al.*, 2008; Feng *et al.*, 2013).

To obtain the affinity value (K_m) of OsNPF2.4 for nitrate in oocytes, OsNPF2.4 cRNA-injected oocytes were incubated in solutions containing different levels of $Na^{15}NO_3$ for 1.5 h at pH 5.5, dried at 70 °C to a constant weight and the ^{15}N in the oocytes measured using a continuous-flow isotope ratio mass spectrometer coupled to a C-N elemental analyser (ANCA-GSL MS; PDZ Europa).

Identification of OsNPF2.4 T-DNA insertional mutants

Two independent T-DNA insertion mutants, namely *npf2.4-1* and *npf2.4-2*, were obtained from RiceGE in Korea (http://signal.salk.edu/cgi-bin/RiceGE). Genomic DNA was extracted using the SDS method from rice leaf blade of the wild type (WT) and the mutants, and Southern blot analysis was performed as described previously (Jia *et al.*, 2011) to verify the two independent mutants. Two-round PCR analysis using the primers supplied by RiceGE was performed to identify the T-DNA insertion homologue.

Generation and identification of OsNPF2.4 overexpression rice lines

The open reading frame of *OsNPF2.4* was amplified using forward primer 5’-tccggtaccTCGGTCGCTTCGCTTCTA-3′ (with a *Bgl*HI restriction site, underlined) and reverse primer 5′-aggactagtACCACCCAAACGAACAAACT-3′ (with an *Spe*I restriction site, underlined) by PCR and ligated into the ubiquitin promoter of pTCK303 vector. The construct was obtained and transformed into rice callus using *Agrobacterium tumefaciens* (strain EHA105) followed a previously described procedure (Upadhyaya *et al.*, 2000). A total of 21 individual transgenic lines were obtained to verify the levels of *OsNPF2.4* overexpression. Three independent lines, OX1, OX2, and OX3, with one or two copies of the T-DNA insertion, were used for further analyses.

Analyses of total Kjeldahl N, nitrate, and K concentrations in different organs and in xylem sap

The root, culm and leaf sheath, and leaf blade of each individual rice plant were separated for assay of Kjeldahl N (total organic and amino-N), nitrate, and K concentrations as described previously (Cai *et al.*, 2012; Tang *et al.*, 2012). To measure the nitrate and K concentration in xylem sap, we followed the method described by Tang *et al.* (2012): 2-months-old seedlings of WT and *npf2.4* mutants after 4 d of N starvation were transferred to containers supplied with 0.25 mM NO_3^- or 2.5 mM NO_3^- and the shoot was cut about 4 cm above the root–shoot junction for collecting xylem sap for 2 h. In a parallel experiment, the seedlings were transferred to a container with 2.5 mM NO_3^- and 1 mM K^+ for 5 d first, then 2.5 mM NO_3^- and 0.3 mM K^+ for another 2 d, followed by further removal of both N and K for 2 or 4 h before collecting the xylem sap.

Determination of nitrate influx rate, accumulation, and nitrate redistribution from the oldest leaf blade using $^{15}NO_3^-$

The nitrate influx rate was assayed using $^{15}NO_3^-$ as described previously (Delhon *et al.*, 1995). Rice seedlings were grown in IRRI nutrient solution for 3 weeks and then N starved for 4 d. The seedlings were first transferred to 0.1 mM $CaSO_4$ for 1 min, then to the IRRI nutrient solution containing either 0.25 mM or 2.5 mM $^{15}NO_3^-$ (atom % ^{15}N:$^{15}NO_3^-$, 40%) for 10 min, and finally to 0.1 mM $CaSO_4$ for 1 min before sampling.

To determine the nitrate redistribution from the oldest leaf of WT and *npf2.4* mutants to N-starved roots and the youngest leaf, 1-week-old seedlings were grown in IRRI nutrient solution containing 1 mM NH_4^+ for 17 d and then in 0 mM N for 4 d; the oldest leaf blade of each plant was then wiped gently with a sponge and incubated in solution containing 5 mM $Ca(^{15}NO_3)_2$ at 9:00–11:00 and 15:00–17:00 giving a total of 4 h. At this stage, the oldest leaves of the WT and mutants were developmentally very similar (see Fig. 5A, B). The root, culm, and leaf sheath, and the first (youngest) leaf blade from the top were sampled at 24 h after ending the ^{15}N treatment for the assay of ^{15}N distribution. After grinding in liquid N, the powder was

well dried at 70 °C. The concentration of ^{15}N was analysed using an isotope ratio mass spectrometer (Host model: DELTA V Advantage Isotope Ratio Mass Spectrometer; external device: flash EA1112 HT Elemental Analyzer; Thermo Fisher Scientifc).

Statistical analysis

Data were analysed by analysis of variance (ANOVA) using the SPSS 10 program (SPSS Inc.). Different letters on the histograms between the transgenic plants and the WT and/or different treatments indicate a statistically significant difference at $P \leq 0.05$.

Results

Expression of OsNPF2.4 responds to N and K supply and is dependent on tissue and development stage

Rice OsNPF2.4 belongs to clade IV of the NPF family, sharing the highest (about 46–48%) amino acid identity with AtNPF2.9, -2.10, and -2.11 among all functionally characterized plant NPF transporters (Supplementary Fig. S1 at *JXB* online). As shown in Fig. 1, *OsNPF2.4* was expressed much more abundantly in aerial parts than in roots. In roots, *OsNPF2.4* expression could be increased by 33% by supply of high NO_3^- (2.5 mM) (Fig. 1A) and decreased by 79% by K deficiency (Fig. 1B). In shoots, *OsNPF2.4* was expressed most abundantly in the high NO_3^--supplied youngest leaf blade (Fig. 1C, D). N starvation could enhance *OsNPF2.4* expression in the older (third to fifth) leaf blades and sheaths (Fig. 1C, D). In addition, K deficiency downregulated expression of *OsNPF2.4* in the culm and leaf sheath but upregulated its expression in the leaf blade (Fig. 1B).

OsNPF2.4 is localized in multiple cell types in both vegetative and reproductive organs

To determine the subcellular localization of OsNPF2.4, eGFP fused at either the N or C terminus to OsNPF2.4 was transiently expressed in rice protoplasts under the control of the cauliflower mosaic virus 35S promoter. The green fluorescence of OsNPF2.4–eGFP or eGFP–OsNPF2.4 was observed at the protoplast plasma membrane compared with the diffuse cytoplasmic localization of the eGFP control (Fig. 2), confirming that OsNPF2.4 is a plasma membrane-localized transporter.

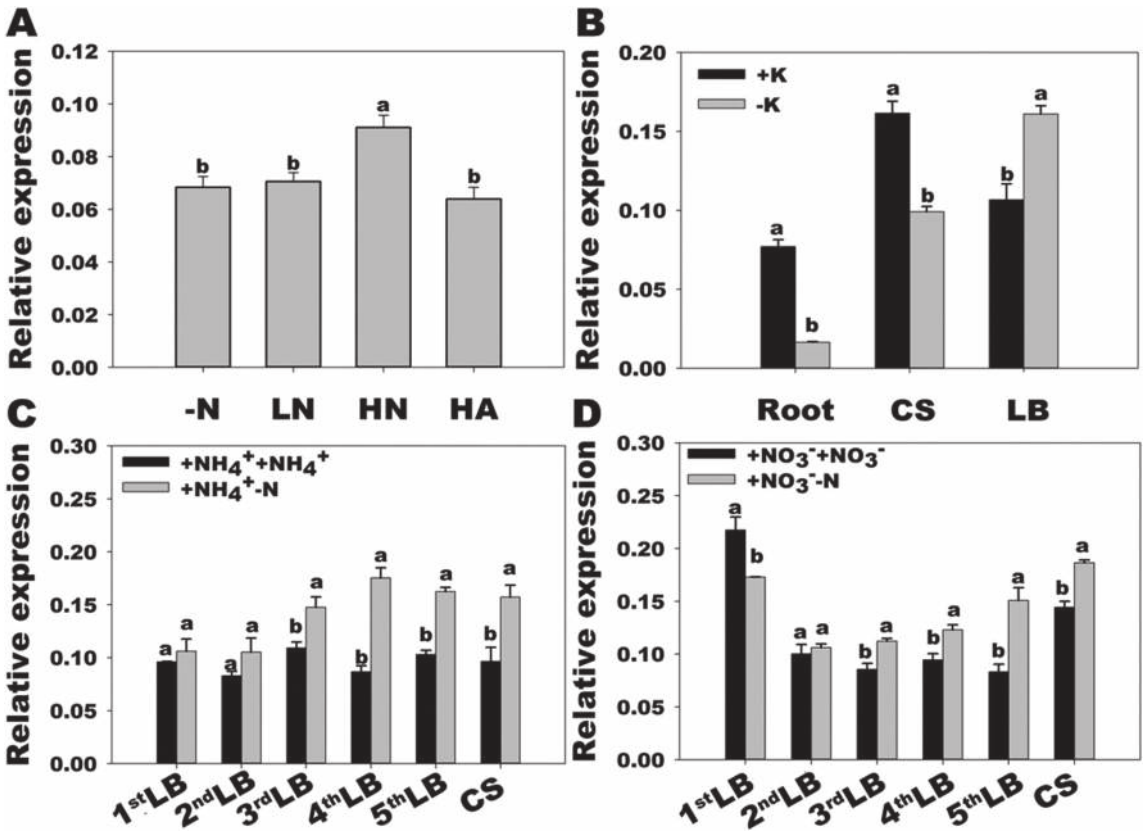

Fig. 1. Effects of N and K supply on transcriptional expression of *OsNPF2.4* in rice. (A) Effects of N supply on transcriptional expression of *OsNPF2.4* in rice root. The seedlings were fi!CursorStart!!CursorEnd!rst grown in IRRI nutrient solution containing 1 mM NH_4^+ and 1 mM K^+ for 2 weeks, then moved to N-free solution for 4 d and further resupplied with NH_4^+ or NO_3^- or continuous N-free solution for 6 h. –N, 0 mM N; LN, 0.25 mM NO_3^-; HN, 2.5 mM NO_3^-; HA, 2.5 mM NH_4^+. (B) Effects of K supply on transcriptional expression of *OsNPF2.4* in rice. The seedlings were first grown in IRRI nutrient solution containing 1 mM NH_4^+ and 1 mM K^+ for 2 weeks, then moved to IRRI nutrient solution containing 1 mM NH_4NO_3 and 1 mM K^+ or K^+-free solution for 3 d. (C, D) The seedlings were first grown in IRRI nutrient solution for 6 weeks, then supplied with either 2.5 mM NH_4^+ (C) or 2.5 mM NO_3^- (D) for 1 week, and then either a continuous supply of the same NH_4^+ ($+NH_4^+ + NH_4^+$) or NO_3^- ($+NO_3^- + NO_3^-$) or with removal of N ($+NH_4^+$ –N or $+NO_3^-$ –N) for 2 d. The values [mean±standard error (SE)] are the transcriptional expression level of *OsNPF2.4* relatively to *OsActin* (internal standard control) by qRT-PCR. Significant differences are indicated with different letters ($P \leq 0.05$, one-way ANOVA). CS, culm and leaf sheath; 1stLB, 2ndLB, 3rdLB, 4thLB, and 5thLB represent the first, second, third, fourth, and fifth leaf blade from the top, respectively.

To analyse the expression pattern in different rice tissues, the 1.2 kb upstream region of the *OsNPF2.4*-coding region was fused to the GUS reporter gene and transformed into rice (cv. Nipponbare). As shown in Fig. 3, *OsNPF2.4* was expressed throughout the entire root except the tip (Fig. 3A–C), the vasculature of the root–shoot junction, the leaf sheath and blade, the anther, the germinated seed, and the seed hull (Fig. 3F–L). Cross-sectional analysis showed that *OsNPF2.4* was expressed mainly in the root epidermis, xylem parenchyma, and phloem companion cells (Fig. 3D, E) and in the leaf phloem cells (Fig. 3I).

OsNPF2.4 is a pH-dependent, low-affinity nitrate transporter in Xenopus *oocytes*

To determine the affinity of OsNPF2.4 for NO_3^-, we used a well-characterized dual-affinity nitrate transporter, AtNPF6.3 (CHL1), as a control for parallel analysis in oocytes. As shown in Fig. 4A and B, *CHL1* cRNA-injected oocytes exhibited a significant increase in NO_3^- uptake in both 0.25 and 10 mM NO_3^- at pH 5.5 and 7.4, and the uptake at pH 5.5 was higher than that at pH 7.4, confirming a previous report that AtNPF6.3 is a pH-dependent nitrate transporter (Liu *et al.*, 1999). *OsNPF2.4* cRNA-injected oocytes showed a 2.5-fold increase in NO_3^- uptake when compared with water-injected oocytes bathed in 10 mM NO_3^- at pH 5.5 (Fig. 4A). However, when *OsNPF2.4* cRNA-injected oocytes were incubated in 0.25 mM NO_3^- at pH 5.5 or in 0.25 or 10 mM NO_3^- at pH 7.4, the cells showed no NO_3^- uptake activity difference from the water-injected controls (Fig. 4A, B). Inward currents that responded to changes in membrane potential were also elicited by 10 mM NO_3^- for *OsNPF2.4* cRNA-injected oocytes at pH 5.5 (Fig. 4C), but not by four different dipeptides that were tested (Supplementary Fig. S8A at *JXB* online). The K_m of OsNPF2.4 affinity for NO_3^- was calculated from the net NO_3^- accumulation of the oocytes incubated with different concentrations of ^{15}N-labelled NO_3^-, and was estimated as 3.1 ± 0.7 mM (Fig. 4D).

A T-DNA insertional knockout of OsNPF2.4 inhibits rice growth and development, and is not compensated by an increased nitrate supply

To determine the function of *OsNPF2.4* in rice, two independent T-DNA insertion lines were isolated and used in

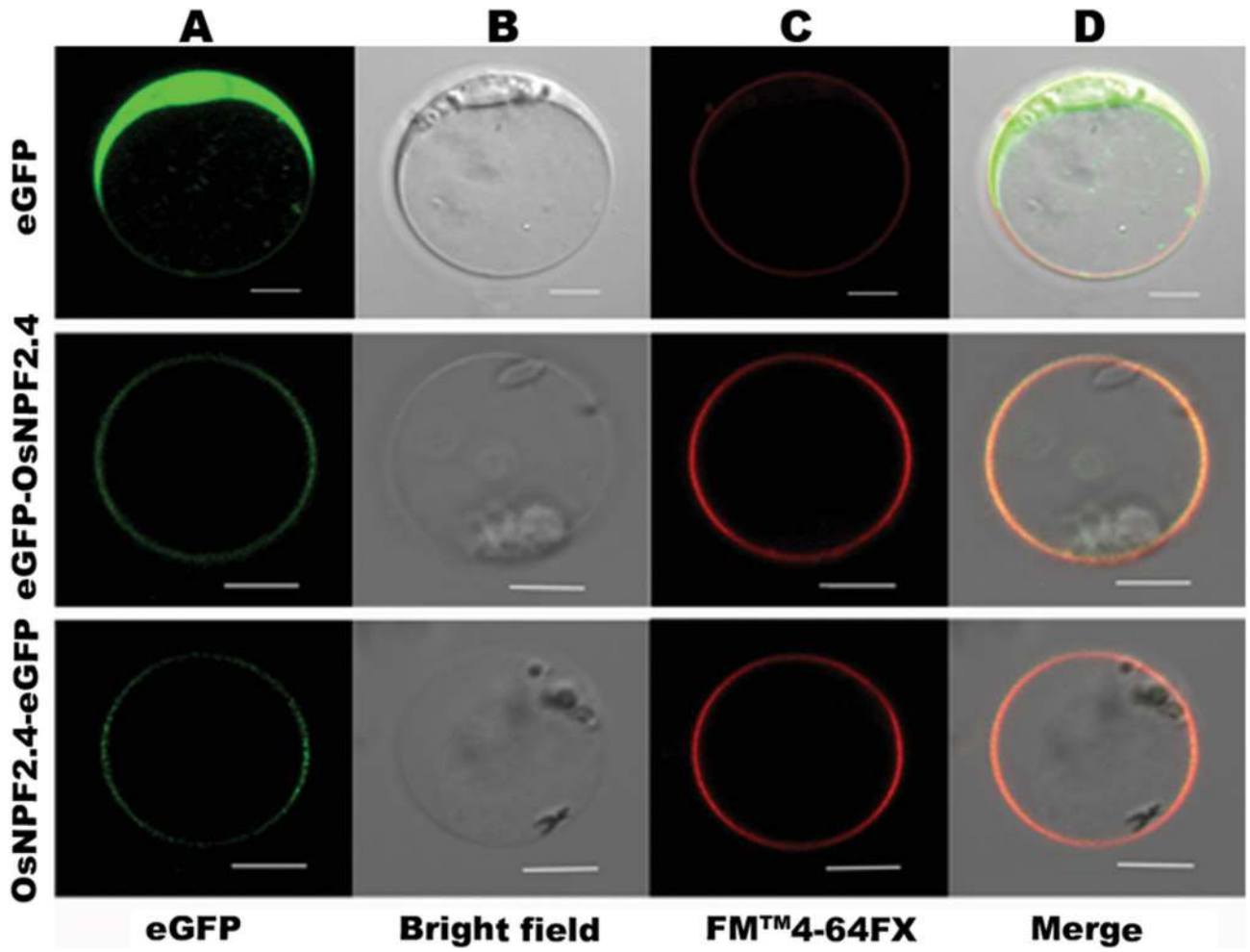

Fig. 2. Subcellular localization of OsNPF2.4 and GFP fusion in rice protoplast. (A) Fluorescence images. (B) Bright-field images. (C) FM4-64FX dye images. (D) Overlapping images of (A), (B), and (C). Row 1 is a protoplast expressing eGFP used as a control. Rows 2 and 3 are protoplasts expressing eGFP–OsNPF2.4 and OsNPF2.4–eGFP, respectively. FM4-64FX is a membrane-selective fluorescent vital dye. Bars, 10 μm. (This figure is available in colour at *JXB* online.)

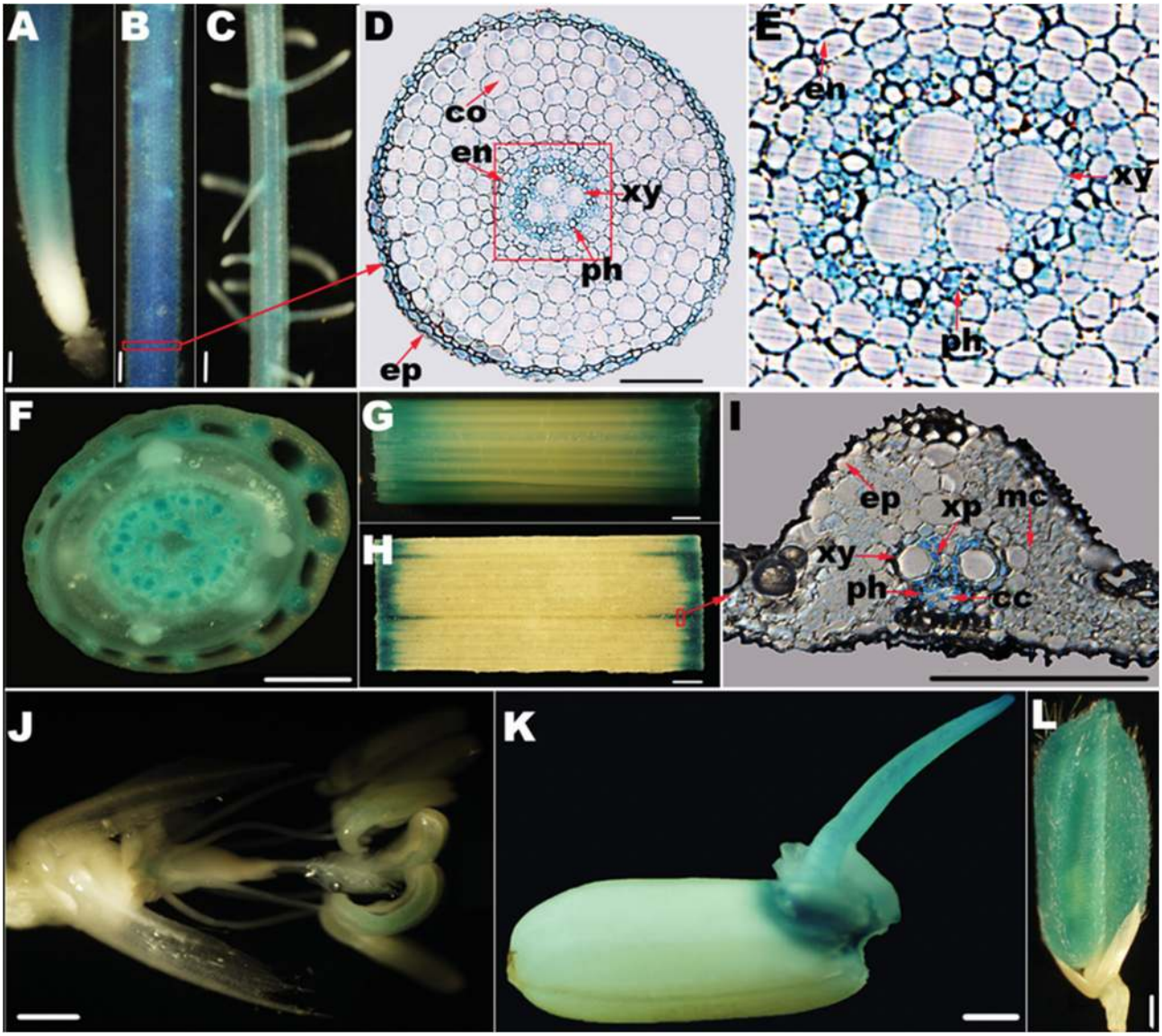

Fig. 3. Expression of *OsNPF2.4pro*:GUS in transgenic rice. (A) Root tip. (B) Lateral root initiation zone (0.5–2 cm from root tip). (C) Root lateral branching zone (4–5 cm from root tip). (D) Cross-section of the root. (E) Amplified (3×) cross-section of the red box in (D). (F) Root–shoot junction. (G) Sheath. (H) Blade. (I) Cross-section of the blade. (J) flower. (K) Three-day-old germinated seeds. (L) Seed hull. ep, Epidermis; en, endodermis; co, cortex; mc, mesophyll cells; xy, xylem; ph, phloem; xp, xylem parenchyma; cc, companion cell. GUS activity was not detected in the negative control (WT) and pictures are not shown. Bars, 100 μm (A–D, I); 1 mm (F–H, J–L). (This figure is available in colour at *JXB* online.)

this study (Supplementary Fig. S2A at *JXB* online). In the *npf2.4-1* and *npf2.4-2* mutants, the second and third exons of *OsNPF2.4* were inserted with a single copy of T-DNA, respectively (Supplementary Fig. S2A, C). The *OsNPF2.4* transcripts were not detected by RT-PCR analysis, showing that they were null mutants (Supplementary Fig. S2B). In comparison with the WT, both *npf2.4* mutants grown in the soil and applied with 1 mM NH_4NO_3 had a shorter height and panicle length (Supplementary Fig. S2D), a lower seed-setting rate, and much lower yield (Supplementary Table S1 at *JXB* online).

In order to determine the contribution of OsNPF2.4 to the acquisition of NO_3^- and growth at different levels of NO_3^- supply, we conducted hydroponics experiments. Uniform rice seedlings of the WT and the two *npf2.4* mutants were selected and transferred to IRRI nutrient solution containing 1 mM NH_4^+ as the N source for 3 weeks, and then grown with 0.25 or 2.5 mM NO_3^- for 1 week. The dry weight of the root, culm and sheath, and blade of *npf2.4* mutants were 75–82% that of the WT supplied with 0.25 mM NO_3^- (Fig. 5A, C) and 60–70% of the WT with 2.5 mM NO_3^- (Fig. 5B, D) as the sole N source, respectively. It was notable that an increase in NO_3^- supply from 0.25 to 2.5 mM increased growth of the WT by 23–26%, but it did not significantly improve the growth of root, culm and sheath, or leaf blade of the mutants (compare Fig. 5C and D). Another independent experiment with *osnpf2.4* mutants treated with 0.5 and 5 mM NO_3^- further confirmed that N deficiency caused a growth limitation that could not be rescued by raising the N supply level in *osnpf2.4* mutants (Supplementary Fig. S3 at *JXB* online). In contrast, there was no significant difference in root and shoot growth between *osnpf2.4* mutants and the WT with high (2.5 mM) ammonium

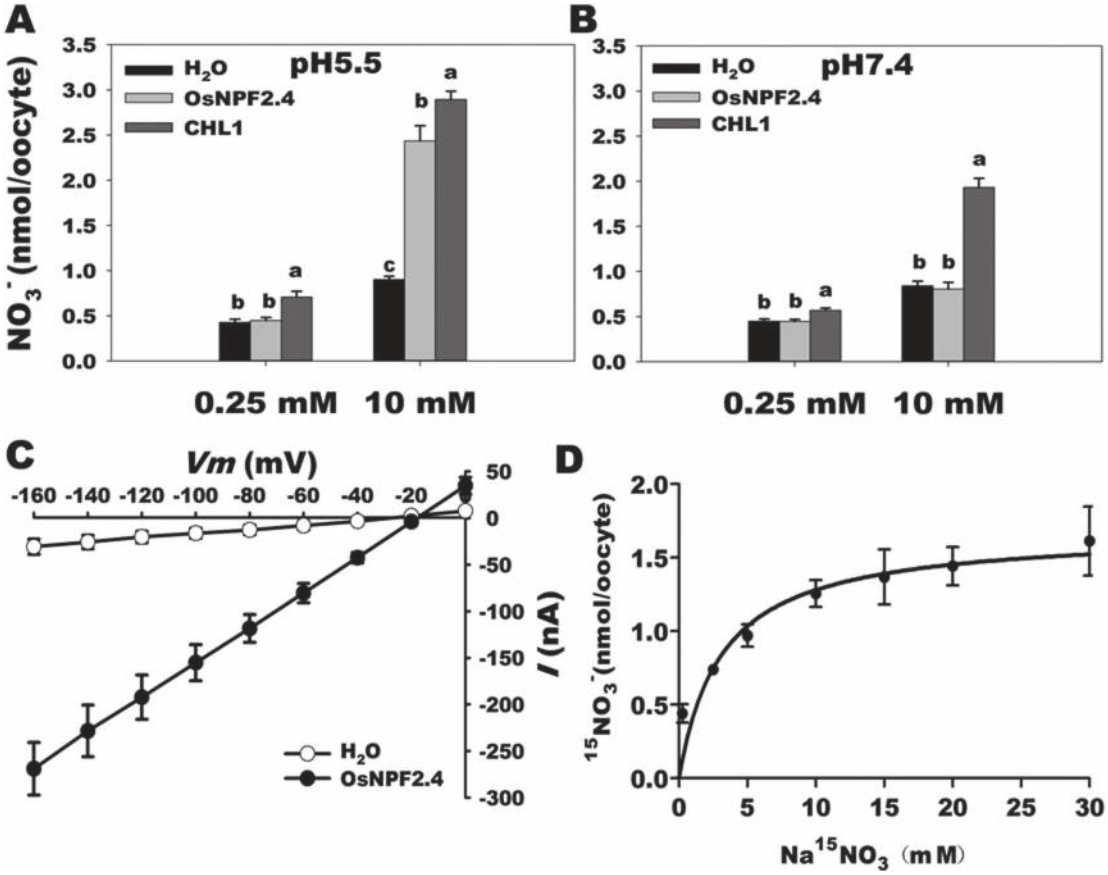

Fig. 4. Nitrate uptake activity of OsNPF2.4 in oocytes. (A, B) High- and low-affinity nitrate uptake activity of *OsNPF2.4* and *CHL1* cRNA-injected oocytes determined using 0.25 and 10 mM nitrate at pH 5.5 (A) and pH 7.4 (B). (C) Current–voltage curves of oocytes expressing OsNPF2.4. The *I–V* curves shown were recorded from *OsNPF2.4*-injected oocytes, which were treated with 10 mM nitrate or without any N resource at pH 5.5. (D) Nitrate uptake kinetics of OsNPF2.4 in oocytes. *OsNPF2.4* cRNA was injected into oocytes, which were incubated in different solutions with different concentrations of $Na^{15}NO_3$ for 1.5 h at pH 5.5 to determine ^{15}N accumulation. The values are means±SE (n=5–7 oocytes for every concentration). Different letters indicate significant differences between the water, *OsNPF2.4 and CHL1* cRNA-injected oocytes of the same treatments ($P \leq 0.05$, one-way ANOVA). Three batches of oocytes were used for each test.

as the only N source in the presence of a nitrification inhibitor (Supplementary Fig. S4 at *JXB* online).

Low-affinity nitrate acquisition of roots is decreased by knockout and increased by overexpression of OsNPF2.4

To determine the effect of *OsNPF2.4* gene knockout on root NO_3^- influx, short-term NO_3^- acquisition was analysed by exposing the N-starved roots to 0.25 or 2.5 mM $^{15}NO_3^-$ for 10 min. Compared with the WT, both *osnpf2.4* lines showed significantly lower 2.5 mM $^{15}NO_3^-$ influx rate into roots, while no difference in the 0.25 mM $^{15}NO_3^-$ influx rate was observed (Fig. 6A).

In longer-term NO_3^- treatments, however, the knockout mutation of *OsNPF2.4* decreased total NO_3^- accumulation at both lower (0.25 mM) and higher (2.5 mM) NO_3^- levels, although the total Kjeldahl N concentration in *osnpf2.4* mutants was similar to that in the WT (Fig. 6B–E). The difference in both NO_3^- concentrations and total N per plant between the mutants and WT was larger at the higher NO_3^- concentration (Fig. 6B, C, F, G). It is interesting that increasing the NO_3^- supply from 0.25 to 2.5 mM for 7 d increased the total N accumulation by 20–35% in the root, culms and sheath, and blade of the WT, but increased the total N much less in the mutants (compare Fig. 6F and G), indicating the important role of OsNPF2.4 in maintaining NO_3^--mediated growth in rice.

To further characterize the role of OsNPF2.4 in root NO_3^- acquisition, overexpression (OX) lines were generated by transforming the *OsNPF2.4* construct directed by the ubiquitin promoter into Dongjin, the parental cultivar of the *osnpf2.4* knockout lines. Three independent *OsNPF2.4-OX* lines, OX1–OX3, were detected by Southern blot and qRT-PCR analyses (Supplementary Fig. S5 at *JXB* online) and selected for further analysis. We found that after just 1 d of provision of 2.5 mM NO_3^-, the NO_3^- content of the *OsNPF2.4-OX* lines was significantly increased in the root compared with the WT, but not in the shoot (Fig. 7A). When the NO_3^- supply was extended to 7 d, both the root and shoot NO_3^- concentrations were largely increased (Fig. 7B). However, the *OsNPF2.4-OX* lines had a similar dry weight as the WT when they were treated with a 2.5 mM NO_3^- supply for 1 week (Supplementary Fig. S6 at *JXB* online).

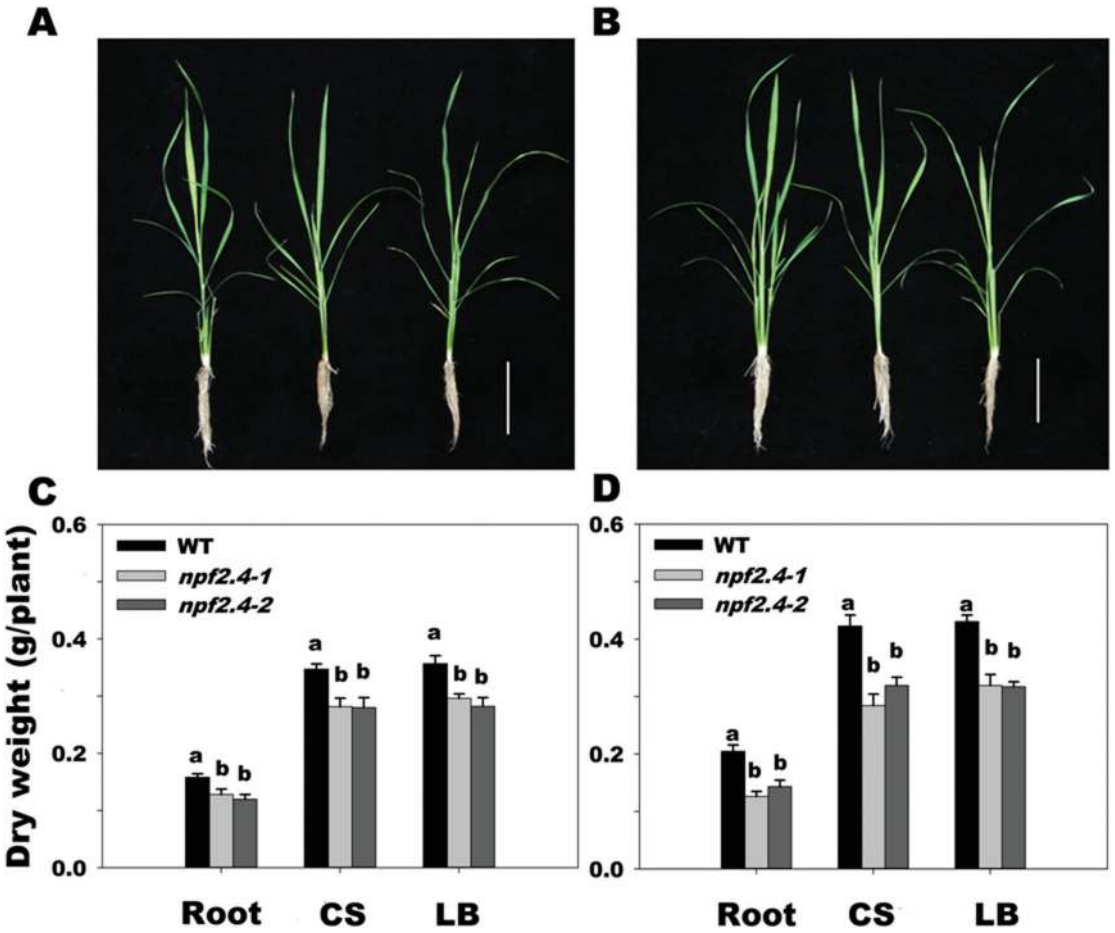

Fig. 5. Comparison of growth and biomass dry weight of *npf2.4* mutants and WT at different nitrate supply levels. The seedlings were first grown in IRRI solution containing 0.5 mM $(NH_4)_2SO_4$ for 3 weeks. After removal of the N supply in the culture solution for 4 d, the seedlings were resupplied with 0.25 mM NO_3^- (A, C) or 2.5 mM NO_3^- (B, D) for 7 d. The values are means±SE. Different letters indicate significant differences between the WT and *npf2.4* mutants of the same treatments ($P \leq 0.05$, one-way ANOVA). Bars, 10 cm. CS, culm and leaf sheath; LB, leaf blade. (This figure is available in colour at *JXB* online.)

Knockout of OsNPF2.4 impairs K-coupled nitrate upward transport from the root to the shoot

In plants, NO_3^- taken up by roots is predominantly transported to the shoot by the xylem for assimilation, and this process needs K^+ as a partner for charge balance (Ben-zioni *et al.*, 1970, 1971). To explore whether OsNPF2.4 is involved in NO_3^- transport from the root to the shoot, we collected the xylem exudates of *osnpf2.4* mutants and WT supplied with 0.25 or 2.5 mM NO_3^-. Both NO_3^- and K^+ concentrations in the xylem sap between WT and *osnpf2.4* mutants were similar with a 0.25 mM NO_3^- supply; however, they showed about 42 and 27% lower concentrations in the mutants than in the WT under 2.5 mM NO_3^- supply (Fig. 8A, B). In contrast to the WT, increasing the NO_3^- supply from 0.25 to 2.5 mM did not significantly increase the NO_3^- and K^+ concentrations in the xylem sap of the mutants (Fig. 8A, B).

In order to exclude the effect of root NO_3^- acquisition on NO_3^- and K^+ export from the root to the shoot, we temporally removed both NO_3^- and K^+ in the culture solution at 2 and 4 h before sampling the xylem sap. Interestingly, NO_3^- transport rate in the xylem of the mutants was only about 45% of that in WT at 2 h, while it did not show a difference at 4 h, probably due to decreased residual NO_3^- in the root (Fig. 8C). Meanwhile, the K^+ transport rate in the xylem of the mutants was also significantly lower than that of the WT (Fig. 8D). Interestingly, the xylem sap K^+ was increased in the WT but decreased in the *osnpf2.4* mutants as the time was extended from 2 to 4 h (Fig. 8D).

We also measured the long-term effect of *OsNPF2.4* knockout on K^+ uptake and distribution. As shown in Fig. 9A and C, the *osnpf2.4* mutants and WT had similar K^+ concentrations and distribution ratios between the shoot and root at a 0.25 mM NO_3^- supply level. Increasing the NO_3^- from 0.25 to 2.5 mM under the same sufficient K^+ provision (1 mM) condition increased the K^+ concentration largely in the root and culm and sheath for the WT but not for the mutants (compare Fig. 9A and B). *OsNPF2.4* knockout decreased the K^+ concentration in the root and culm and sheath (Fig. 9B), but increased the shoot:root ratio of K^+ content (Fig. 9D), suggesting that the lower amounts of K^+ recycled back from the shoot to the root in the mutants compared with the WT.

Knockout of OsNPF2.4 impairs nitrate redistribution from old leaves to N-starved root and young leaves

Since *OsNPF2.4* was detected in phloem cells of both leaves and roots (Fig. 3E, I), we analysed the effect of *OsNPF2.4*

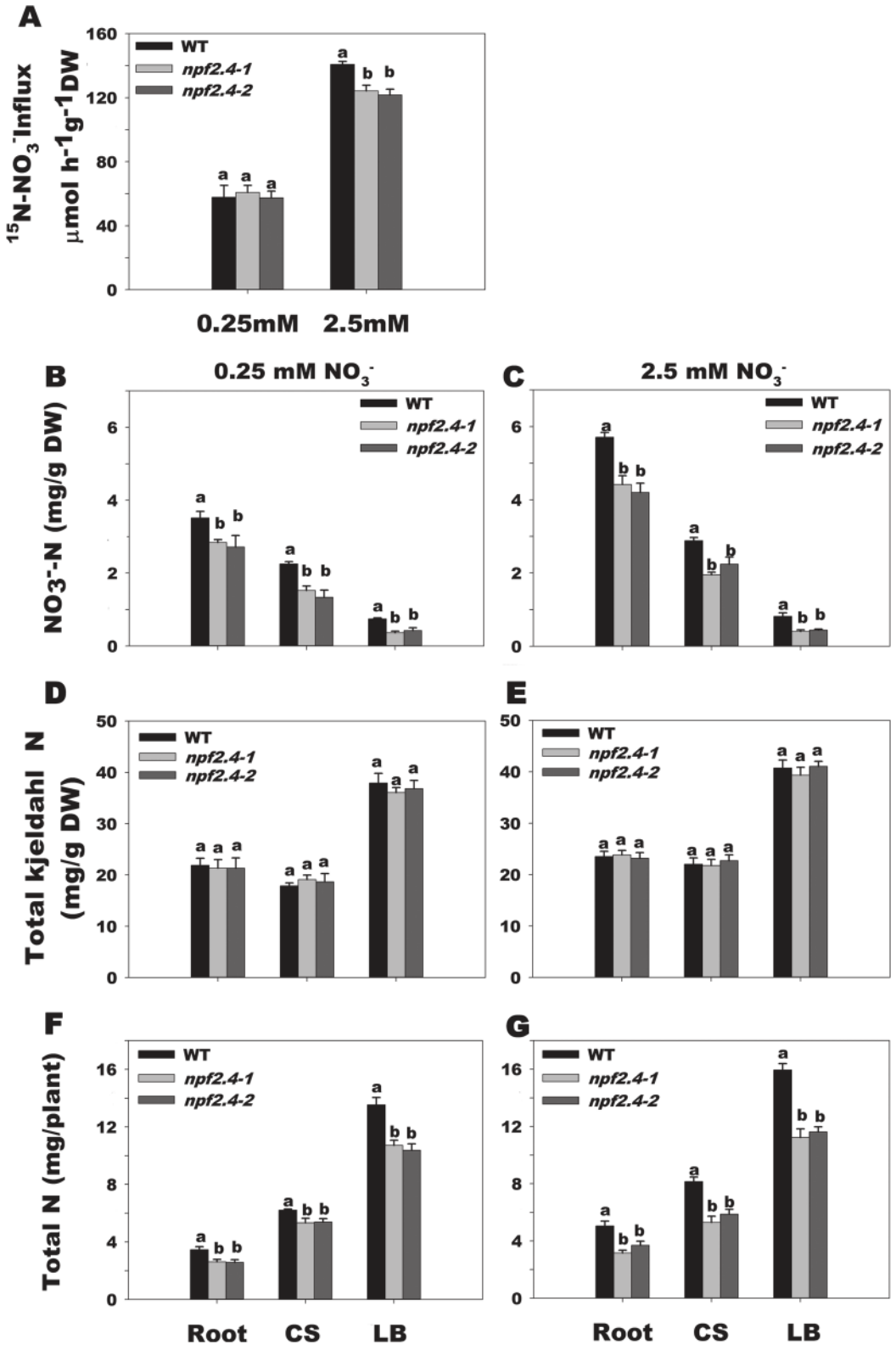

Fig. 6. Nitrate influx rate in roots and nitrate concentration, total Kjeldahl N concentration, and total N uptake of *npf2.4* mutants and the WT at different nitrate supply levels. (A) Nitrate influx rate in the roots. Uniform seedlings were grown in IRRI nutrient solution containing 1 mM NH_4^+ for 3 weeks and then the N supply was removed for 4 d. The nitrate influx rate in the N-starved roots was measured by provision of either 0.25 or 2.5 mM $^{15}NO_3^-$ in the culture solution for 10 min. (B–G) Extractable nitrate concentration (B, C), total Kjeldahl N concentration (D, E), and total N uptake per plant (F, G). The seedlings were grown in IRRI solution containing 0.5 mM $(NH_4)_2SO_4$ for 3 weeks. After removal of the N supply in the solution for 4 d, the seedlings were resupplied with 0.25 or 2.5 mM NO_3^- for 7 d. (B, C) NO_3^- -N concentration of the WT and mutants treated with 0.25 mM NO_3^- (B) or 2.5 mM NO_3^- (C). (D, E) Total Kjeldahl N concentration of the WT and mutants treated with 0.25 mM NO_3^- (D) or 2.5 mM NO_3^- (E). (F, G) Total N content per plant supplied with 0.25 mM NO_3^- (F) or 2.5 mM NO_3^- (G). The values are means±SE. Different letters indicate significant differences between the WT and *npf2.4* mutants of the same treatments ($P \leq 0.05$, one-way ANOVA). DW, dry weight; CS, culm and leaf sheath; LB, leaf blade.

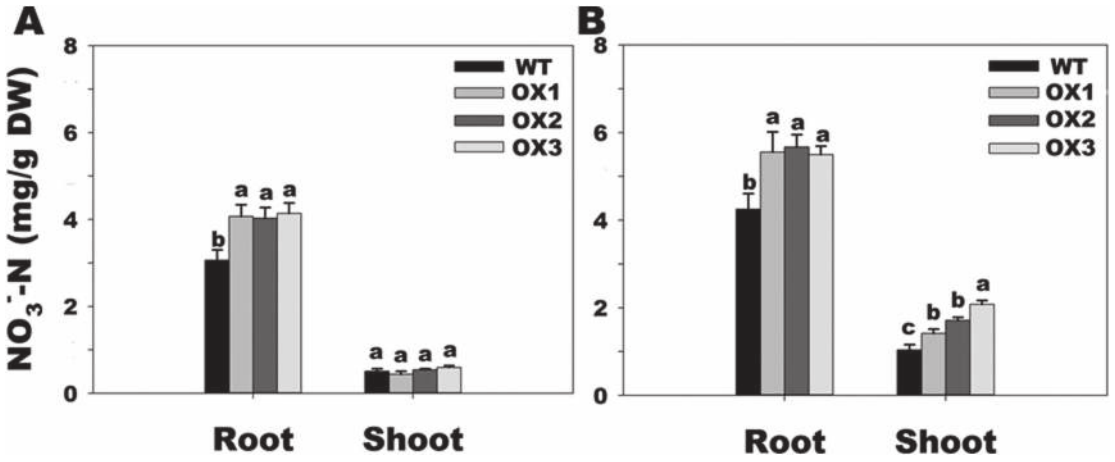

Fig. 7. Effect of *OsNPF2.4* overexpression on NO_3^- -N concentration in the root and shoot of rice. The seedlings were grown in IRRI nutrient solution containing 1 mM NH_4NO_3 for 6 weeks. After removal of the N supply in the solution for 5 d, the seedlings were resupplied with 2.5 mM NO_3^- for another 1 d (A) or 7 d (B). The values are means±SE. Significant differences between the *npf2.4* mutants and WT are indicated with different letters ($P \leq 0.05$, one-way ANOVA). DW, dry weight.

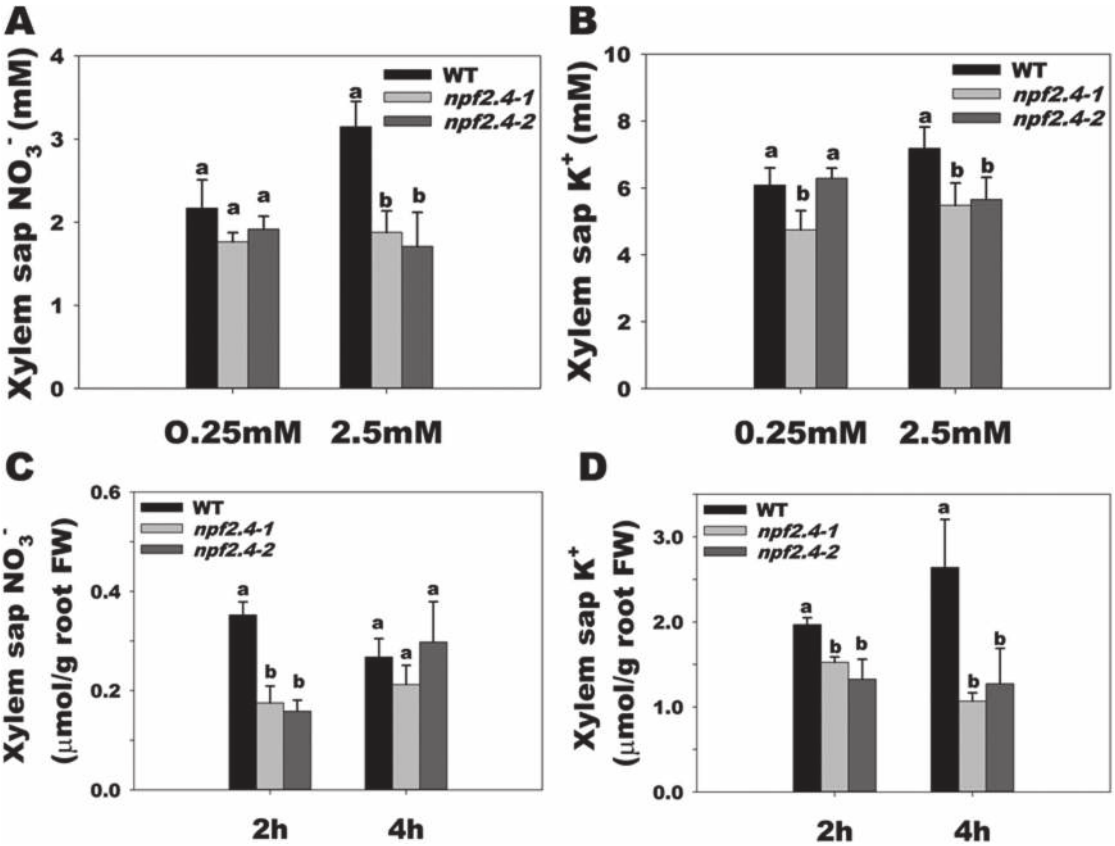

Fig. 8. Nitrate and K^+ concentration in xylem sap of *npf2.4* mutants and WT at different nitrate supply levels. The seedlings were grown in IRRI solution containing 0.5 mM $(NH_4)_2SO_4$ for 2 months. (A, B) Two-month-old seedlings were grown in N-free solution for 4 d before being transferred to a container supplied with 0.25 or 2.5 mM NO_3^- to collect the xylem sap for 2 h. (A) NO_3^- concentration in xylem sap. (B) K^+ concentration in xylem sap. (C, D) Two-month-old seedlings were grown in 2.5 mM NO_3^- and 1 mM K^+ for 5 d and then in 2.5 mM NO_3^- and 0.3 mM K^+ for 2 d, and then transferred to a container without NO_3^- or K^+ for 2 or 4 h before collecting the xylem sap for 2 h. (C) NO_3^- concentration in xylem sap. (D) K^+ concentration in xylem sap. The values are means±SE. Significant differences between the *npf2.4* mutants and WT are indicated with different letters ($P \leq 0.05$, one-way ANOVA).

knockout on the redistribution of $^{15}NO_3^-$ from the source leaf to the sink organs of N-starved rice. The oldest leaf blades of WT and *osnpf2.4* mutant seedlings were fed in the same manner and with the same amount of $^{15}NO_3^-$ solution for 4 h during the day. As shown in Fig. 10A, the concentration of ^{15}N distributed into the root, culm and sheath, and youngest (first) leaf blade, and the total amount of ^{15}N accumulated in these organs of the mutants was about 3- to 5-fold lower than those of the WT. Moreover, the relative redistribution ratio of ^{15}N was significantly decreased in the root, but increased

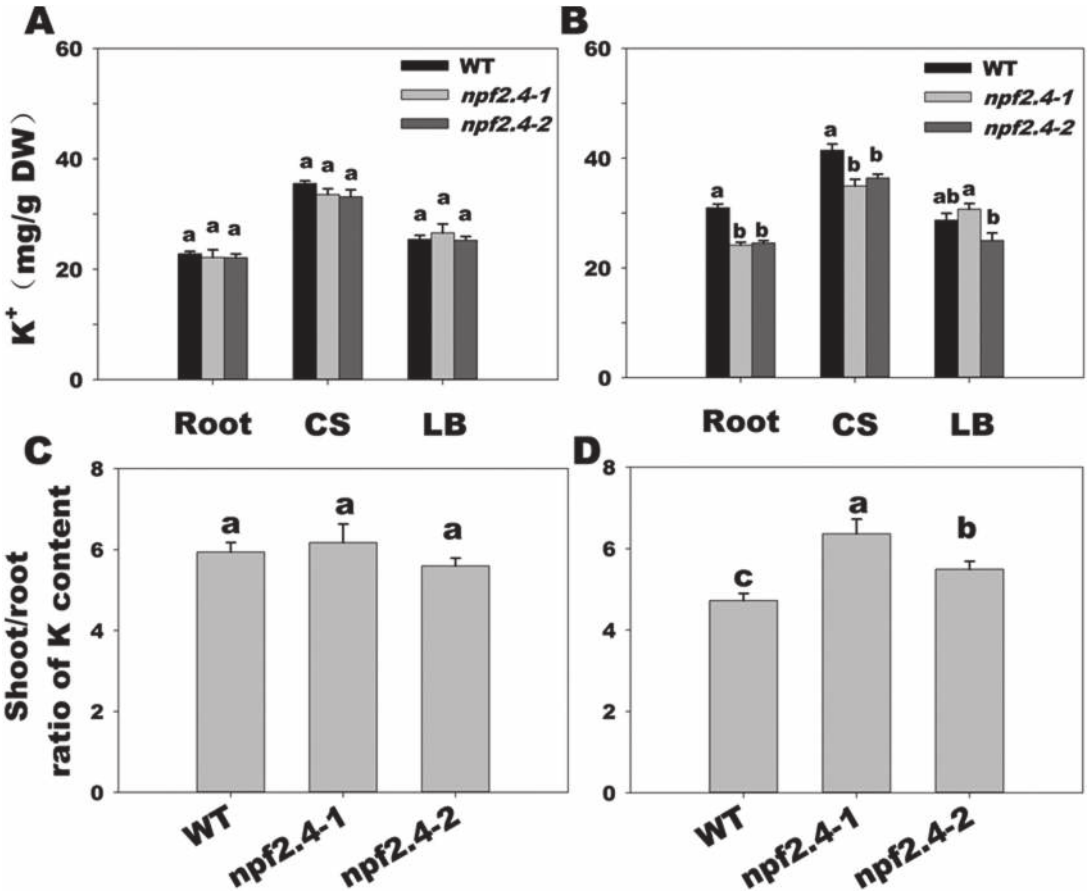

Fig. 9. Effect of *OsNPF2.4* knockout on K^+ concentration and distribution between the root and shoot grown at different nitrate supply levels. The seedlings were grown in IRRI solution containing 0.5 mM $(NH_4)_2SO_4$ for 3 weeks. After removal of the N supply in culture solution for 4 d, the seedlings were resupplied with 0.25 mM NO_3^- (A, C) or 2.5 mM NO_3^- (B, D) for 7 d to determine the K^+ concentration (A, B) and distribution ratio between the shoot and root (C, D). The shoot:root ratio of K^+ content is the total K^+ content in the shoot including the culm and sheath and leaf blade, divided by the total K^+ content in the root. Data are means±SE of four replicates for each line. Significant differences between the *npf2.4* mutants and WT are indicated with different letters ($P \leq 0.05$, one-way ANOVA). DW, dry weight; CS, culm and leaf sheath; LB, leaf blade.

in the culm and leaves of the mutants in comparison with the WT (Fig. 10B). These data indicated that *OsNPF2.4* knockout prevented the redistribution of N largely from the oldest leaf through the phloem to the source organs, particularly to N-starved roots.

Discussion

OsNPF2.4 is a pH-dependent low-affinity nitrate transporter facilitating nitrate uptake by the root

The putative transporters belonging to NPF show diverse functions in rice (Lin *et al.*, 2000; Li *et al.*, 2009; Ouyang *et al.*, 2010; Fang *et al.*, 2013), but few of the total 80 members have been identified for their NO_3^- transport activity. In the present study, we first demonstrated that OsNPF2.4 is a pH-dependent, low-affinity nitrate transporter facilitating NO_3^- uptake in rice by providing five types of evidence in both heterogeneous systems and in rice. First, we showed in oocytes that OsNPF2.4 could function in NO_3^- influx with an affinity value (K_m) of 3.1±0.7 mM at pH 5.5 (Fig. 4A–D). Secondly, *OsNPF2.4* was expressed in the epidermis of roots (Fig. 3D), the outermost cell layer, which is first in contact with external NO_3^-, and upregulated by an increase in the NO_3^- supply (Fig. 1A). Thirdly, knockout of *OsNPF2.4* impaired root NO_3^- acquisition (short-term $^{15}NO_3^-$ influx rate in N-starved roots) only at high, and not at low, NO_3^- supply (Fig. 6A). Fourthly, increasing NO_3^- as the sole N source from 0.25 to 2.5 mM could not significantly increase the total N uptake (Fig. 6F, G) and improve the growth of both root and shoot of *osnpf2.4* mutants (Fig. 5C, D). Lastly, overexpression of *OsNPF2.4* could improve NO_3^- uptake by roots supplied with 2.5 mM (Fig. 7).

Several NPF members have been identified as participating in nitrate transport in roots. *AtNPF6.3* encoding a NO_3^--inducible dual-affinity nitrate transporter is expressed mainly in the epidermal cells of young root (root tip) and in the endodermis and cortical cells of mature root (Tsay *et al.*, 1993; Huang *et al.*, 1996; Liu *et al.*, 1999). In contrast, another dual-affinity nitrate transporter isolated from *Medicago truncatula*, MtNPF6.8 (MtNRT1.3), is downregulated by the addition of NO_3^- to the root (Morère-Le Paven *et al.*, 2011). Among the known constitutive low-affinity nitrate transporter genes, *AtNPF4.6* is expressed primarily in the root hairs and epidermis (Huang *et al.*, 1999), and *AtNPF7.2* and *AtNPF7.3* are expressed in pericycle cells adjacent to the protoxylem and parenchyma cells of the xylem (Lin *et al.*, 2008; Li *et al.*,

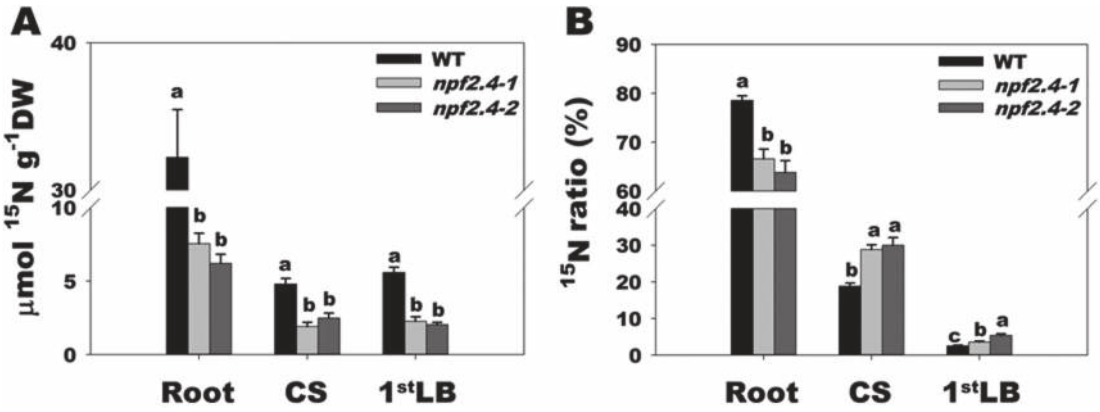

Fig. 10. $^{15}NO_3^-$ tracing assay for redistribution of nitrate-N from the oldest leaf blade to the youngest leaf blade, the sheath and culm, and the root in *npf2.4* mutants and WT. The seedlings were supplied with 1 mM NH_4^+ for 17 d and 0 mM N for 4 d, and the oldest leaf blade with a similar size in each seedling of WT and *npf2.4* mutant was incubated in solution containing 5 mM $Ca(^{15}NO_3)_2$ for 4 h (9:00–11:00 and 15:00–17:00). The root, culm and leaf sheath, and first (youngest) leaf blade from the top were sampled at 24 h after stopping the ^{15}N treatment for the assay of ^{15}N distribution. (A) ^{15}N concentration and (B) ^{15}N distribution ratio in the root, culm and leaf sheath, and first leaf blade from the top. The sum of total ^{15}N content accumulated in the root, culm and leaf sheath, and first leaf blade was defined as 100% in each individual plant. The values are means±SE of five replicates for individual WT or mutant plants of the same treatment. Significant differences between the *npf2.4* mutants and WT are indicated with different letters ($P \leq 0.05$, one-way ANOVA). DW, dry weight; CS, culm and leaf sheath; 1st LB, first leaf blade from the top.

2010). *AtNPF2.9* is expressed exclusively in the companion cells of root (Wang and Tsay, 2011). The different expression patterns suggested complementary roles of multiple NPF members participating in nitrate acquisition and allocation in the roots, and explained why OsNPF2.4 knockout only partially impaired root nitrate uptake (Fig. 6A). Phylogenetic tree analysis revealed AtNPF2.9, the *Arabidopsis* phloem-loading glucosinolate and nitrate transporter, to be a close relative of OsNPF2.4 (Supplementary Fig. S1 at *JXB* online).

It has been reported that *OsNPF8.9* is expressed constitutively in the epidermis of rice root and root hairs with little or no expression in the shoot (Lin *et al.*, 2000). We observed that *OsNPF2.4* knockout enhanced *OsNPF8.9* expression in the root (Supplementary Fig. S7 at *JXB* online).The K_m value for NO_3^- uptake derived for OsNPF8.9 in oocytes is 9.1 ± 1.8 mM (Lin *et al.*, 2000), almost 3-fold higher than that for OsNPF2.4 (Fig. 4D). Since flooding paddy soil contains NO_3^- concentrations of less than 2 mM (Eguchi *et al.*, 2009; our unpublished data), it is tempting to speculate that OsNPF2.4 might play a more important role in NO_3^- uptake than OsNPF8.9 for rice grown in paddy soil. Such a hypothesis needs to be proved by examining the effect of single and double knockouts of *OsNPF8.9* and *OsNPF2.4* genes on NO_3^- acquisition and growth in a future study. As nitrate is more generally available in aerobic soil, the phenotypes of these nitrate transporter mutants may be more obvious in this culture system.

As the major facilitator superfamily members that use the proton electrochemical gradient to drive substrate uptake into the cell (Miller *et al.*, 2007; Tsay *et al.*, 2007), many plant NPF members show stronger activity in transporting NO_3^- at a lower pH range. For example, the net NO_3^- uptake and current elicited by NO_3^- in oocytes expressing AtNPF6.3, AtNPF4.6, or OsNPF8.9 at pH 7.4 was about 30–60% of that at pH 5.5 (Fig. 4A, B) (Tsay *et al.*, 1993; Huang *et al.*, 1999; Li *et al.*, 2010). However, OsNPF2.4 seems more sensitive to pH in the medium, as it showed high activity for NO_3^- uptake at an apoplastic or vacuole pH range (~pH 5.5) (Fig. 4A), while it completely lost such function at alkaline pH (~pH 7.4) (Fig. 4B), supporting the role of OsNPF2.4 in acquisition of nitrate by roots, particularly in relatively low pH soil.

OsNPF2.4 is a low-affinity NO_3^- transporter in oocytes (Fig. 4) and its mutation did not significantly alter the short-term $^{15}NO_3^-$ influx rate of unit weight roots (Fig. 6A); however, the *osnpf2.4* mutants in comparison with the WT accumulated less nitrate and total N when grown in 0.25 mM NO_3^- solution (Fig. 6B, D). The lower root concentrations of N in *osnpf2.4* mutants do not easily fit with the loss of function of OsNPF2.4 in phloem loading and long-distance transport. Interestingly, we found that both *OsNAR2.1* and *OsNRT2.1* encoding the high-affinity NO_3^- transporters were downregulated in the roots of low NO_3^--supplied *osnpf2.4* mutants relative to the WT (Supplementary Fig. S9A, C at *JXB* online). Since expression of *OsNAR2.1* and *OsNRT2.1* in rice roots is not sensitive to NO_3^- supply (Feng *et al.*, 2011; Yan *et al.*, 2011), this change in the expression pattern suggests an altered sensing of the tissue nitrate status in the *osnpf2.4* mutants. The increased expression of *OsNPF8.9* (*OsNRT1.1*) under N starvation in the mutants relative to WT (Supplementary Fig. S7) also suggests altered N-status sensing in the plants. Therefore, a potential role of OsNPF2.4 in phloem-mediated signals of N status is suggested and is worthy of further investigation.

OsNPF2.4 functions in K^+-coupled long-distance transport of NO_3^-

Potassium-coupled transport of NO_3^- in plants was proposed more than 40 years ago (Ben-zioni *et al.*, 1970, 1971). Therefore, it is interesting to know if the activities of NO_3^- and K^+ transporters affect each other and alter the N and K balance. In this study, we provided several lines of evidences that OsNPF2.4 participates in long-distance K^+-linked NO_3^- transport in rice. First, *OsNPF2.4* is expressed in vascular tissue, xylem parenchyma, and phloem companion cells,

and in the vasculature of the root–shoot junction (Fig. 3F). Expression of *OsNPF2.4* was suppressed by K^+ limitation in the root and culm and sheath, but not in the leaf blade (Fig. 1B). Secondly, *OsNPF2.4* knockout decreased the NO_3^- and K^+ concentrations in the xylem sap by about 42 and 27%, respectively, when the root was provided with sufficient NO_3^- (Fig. 8A, B). Limiting the external supply of K^+ to the root resulted in the largest difference in the K^+ transport rate in the xylem between the WT and the *npf2.4* mutants (Fig. 8D). Unlike in the WT, increasing the NO_3^- supply from 0.25 to 2.5 mM did not significantly increase the NO_3^- and K^+ concentrations in the xylem sap of the mutants (Fig. 8A, B). Thirdly, knockout of *OsNPF2.4* decreased the K^+ concentration in the root, and culm and sheath, but increased the K distribution in the shoot when both NO_3^- and K^+ supplies were sufficient (Fig. 9B, D), while no such difference was observed at low NO_3^- supply (Fig. 9A, C).

In *Arabidopsis*, AtNPF7.3 is a pH-dependent, bidirectional (influx–efflux) nitrate transporter, responsible for exporting NO_3^- out of pericycle cells for xylem loading (Lin *et al.*, 2008). However, OsNPF2.4 did not show nitrate efflux activity in oocytes at lower and higher pH ranges (Fig. 4A, Supplementary Fig. S8). *AtNPF7.2* is expressed abundantly in xylem parenchyma cells (Li *et al.*, 2010), similar to *OsNPF2.4* expression in the roots (Fig. 3E). However, in contrast to *OsNPF2.4*, knockout of *AtNPF7.2* results in an increase of NO_3^- concentration in the xylem, suggesting its functions in transporting NO_3^- across the xylem parenchyma cell membrane to unload NO_3^- from the xylem sap (Li *et al.*, 2010). Furthermore, the *Arabidopsis* xylem nitrate transporter AtNPF7.2 is located in a peptide and bidirectional nitrate transporting cluster of the phylogenetic tree (Supplementary Fig. S1) that is distinct from *OsNPF2.4*. We identified that a nitrate transporter belonging to the NRT2 family, OsNRT2.3a, which is also abundantly expressed in xylem parenchyma cells, shows functions in root–shoot nitrate transport mainly at a low NO_3^- supply level (Tang *et al.*, 2012) and was upregulated in the *osnpf2.4* mutants (Supplementary Fig. S9E). Therefore, taking these data together, we suggest that OsNPF2.4 does not have an orthologous xylem loading function to AtNPF7.2 in rice.

It has been proposed that there is homeostatic balance between K^+ and NO_3^- in long-distance transport (Wang *et al.*, 2012). After transport into the leaves, NO_3^- can be stored in the vacuole or assimilated into amino acids with parallel production of malate, while K^+ can accompany malate in the phloem from the leaves to the roots (Ben-Zioni *et al.*, 1970, 1971; Lu *et al.*, 2005). In *Arabidopsis*, *AtNPF7.3* (*NRT1.5*) is downregulated by K^+ limitation and its knockout mutant showed a lower NO_3^- concentration in xylem sap and less distribution of K^+ in the shoot (Lin *et al.*, 2008). Phloem retranslocation of K^+ from the shoot to the root can contribute to xylem-transported K^+ (Lu *et al.*, 2005). In rice, a large part of shoot K^+ for accompanying malate may be recycled through the phloem to the root, resulting in higher K^+ in xylem sap under lower or no external K^+ supply conditions (Fig. 8D). In contrast, the *osnpf2.4* mutants transported less NO_3^- together with less K^+ from the root to the shoot (Figs 8 and 9), and therefore less malate was probably generated during NO_3^- assimilation in the shoot, which in turn might result in a lower amount and rate of K^+–malate recycling in phloem from the shoot to the root. This could explain why the shoot:root ratio of K^+ content was actually significantly increased at a high NO_3^- supply level in the mutants in comparison with the WT (Fig. 9D). Our results suggest that long-distance nitrate transport contributes to NO_3^- and K^+ distribution to maintain plant growth.

OsNPF2.4 is involved in nitrate redistribution from the old leaves to N-starved roots and young leaves, and in N assimilation

As a mobile element, N can be recycled within the plant from older leaves to developing tissues to sustain growth when plants encounter N deficiency. Nitrate-supplied plants may contain several millimolar NO_3^- in their phloem sap (Hocking, 1980; Hayashi and Chino, 1985; Allen and Smith, 1986; Hayashi and Chino, 1986), indicating that phloem transport of NO_3^- plays an important role in NO_3^- redistribution. It has been shown that several members of NPF and NRT2 coordinate the redistribution of NO_3^- in *Arabidopsis*. At least three NPF members, AtNPF2.13 (Fan *et al.*, 2009), AtNPF1.1, and AtNPF1.2 (Hsu and Tsay, 2013), are involved in the redistribution of NO_3^- through the shoot phloem. AtNPF2.9 is expressed in the root phloem, and also has the function of delivering NO_3^- from the xylem to the phloem and regulating root–shoot NO_3^- transport (Wang and Tsay, 2011).

We found in this study that OsNPF2.4 in rice is directly involved in the redistribution of NO_3^- from source to sink organs with several types of evidence. First, *OsNPF2.4* is expressed in the companion cells of leaf phloem (Fig. 3E). Secondly, N starvation upregulated the expression of *OsNPF2.4* in the older leaves including the culms and sheaths and downregulated it in nitrate-supplied youngest leaf blades. Noticeably, supply of NO_3^- in comparison with NH_4^+ enhanced expression, particularly in the youngest leaf blades and culm and sheaths (Fig. 1C, D). Thirdly, *osnpf2.4* mutants in comparison with the WT contained lower ^{15}N in the youngest leaf blade, culm and sheath, and the N-starved root imported from the oldest leaf blade fed by $^{15}NO_3^-$ (Fig. 10A, B). This may suggest that nitrate redistribution is affected by *OsNPF2.4* knockout, but it could also be a secondary effect due to the transport of reduced labelled ^{15}N compounds. The remarkable decline in N transported from the oldest leaf to the root relative to younger leaves in the mutants might be due to decreased K^+ recycling in the phloem from the shoot to the root (compare Fig. 9B and D).

Knockout of *OsNPF2.4* consistently decreased the concentration of NO_3^- stored in different organs under different patterns of N supply, starvation and resupply (compare Figs 6B, C and Supplementary Fig. S4); however, it did not affect the concentration of total Kjeldahl N, the assimilated ammonium, and organic N (Fig. 6D, E). The tissue storage pools of nitrate in rice, although small, may be an important indicator of N status.

Supplementary data

Supplementary data can be found at *JXB* online.

Supplementary Table S1.The yield and agronomic traits of wild type (WT) and *OsNPF2.4* T-DNA insertion mutant (*npf2.4-1* and *npf2.4-2*) grown in a paddy soil with alternative dry-flooding irrigation.

Supplementary Table S2. The primers for RT-PCR of *OsNPF2.4*.

Supplementary Table S3. Gene-specific PCR primers used for qRT-PCR.

Supplementary Table S4. Primers used for cloning in oocyte expression vector.

Supplementary Fig. S1. Phylogenetic tree of the transporter members belonging to NPF family in plants including OsNPF2.4.

Supplementary Fig. S2. T-DNA insertion sites in the sequence of *OsNPF2.4* gene and phenotype of the two *OsNPF2.4* knockout mutants.

Supplementary Fig. S3. Effects of *OsNPF2.4* mutation on growth and N uptake at low (0.5 mM) and high (5 mM) nitrate supplies.

Supplementary Fig. S4. Growth phenotype and total Kjeldahl N acquisition of *npf2.4* mutants and wild type (WT) grown in 2.5 mM NH_4^+-N solution.

Supplementary Fig. S5. Transcriptional expression level of *OsNPF2.4* and Southern blot analysis of T-DNA copy number in *OsNPF2.4* overexpression lines and wild type.

Supplementary Fig. S6. Effect of *OsNPF2.4* overexpression on dry weight of root and shoot in rice.

Supplementary Fig. S7. Relative expression intensity of *OsNPF8.9* (*OsNRT1.1*) in the roots of *npf2.4* mutants and wild type under N-starvation conditions.

Supplementary Fig. S8. Inward current elicited in *OsNPF2.4* expressed oocytes by supply of 10 mM nitrate or peptide or water at altered membrane potentials (A) and change of nitrate content in *OsNPF2.4* expressed oocytes pre-injected with KNO_3 (B) or $NaNO_3$ (C) for detecting nitrate export activity of OsNPF2.4.

Supplementary Fig. S9. Relative expression intensity of *OsNAR2s* and *OsNRT2s* in the roots of *npf2.4* mutants and wild type under low nitrate supply.

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