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Over-expression of *OsPTR6* in rice increased plant growth at different nitrogen supplies but decreased nitrogen use efficiency at high ammonium supply

Xiaorong Fan*, Dan Xie, Jingguang Chen, Haiyan Lu, Yanling Xu, Cui Ma, Guohua Xu

State Key Laboratory of Crop Genetics and Germplasm Enhancement, MOA Key Laboratory of Plant Nutrition and Fertilization in Low-Middle Reaches of the Yangtze River, College of Resources and Environmental Sciences, Nanjing Agricultural University, Nanjing 210095, PR China

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ABSTRACT

Nitrogen (N) plays a critical role in plant growth and productivity and PTR/NRT1 transporters are critical for rice growth. In this study, *OsPTR6*, a PTR/NRT1 transporter, was over-expressed in the *Nipponbare* rice cultivar by *Agrobacterium tumefaciens* transformation using the ubiquitin (Ubi) promoter. Three single-copy T_2 generation transgenic lines, named OE1, OE5 and OE6, were produced and subjected to hydroponic growth experiments in different nitrogen treatments. The results showed the plant height and biomass of the over-expression lines were increased, and plant N accumulation and glutamine synthetase (GS) activities were enhanced at 5.0 mmol/L NH_4^+ and 2.5 mmol/L NH_4NO_3. The expression of *OsATM1* genes in over-expression lines showed that the *OsPTR6* over expression increased *OsAMT1.1*, *OsATM1.2* and *OsAMT1.3* expression at 0.2 and 5.0 mmol/L NH_4^+ and 2.5 mmol/L NH_4NO_3. However, nitrogen utilisation efficiency (NUE) was decreased at 5.0 mmol/LNH_4^+. These data suggest that over-expression of the *OsPTR6* gene could increase rice growth through increasing ammonium transporter expression and glutamine synthetase activity (GSA), but decreases nitrogen use efficiency under conditions of high ammonium supply.

1. Introduction

Nitrogen (N) plays a critical role in plant growth and productivity, as it is required for the synthesis of many essential molecules including nucleic acids (DNA and RNA), amino acids and proteins [1,2]. Plants have evolved multiple transport systems for N uptake from the soil as well as for intra- and intercellular reallocation of N containing compounds [3]. N can be taken up not only in the form of inorganic N, such as nitrate and ammonium, but also in the form of organic N, such as amino acids, small peptides or even protein [4–6].

In higher plants, there are two types of nitrate transporters, known as *NRT1s* (Nitrate Transporter 1 family) and *NRT2s* (Nitrate Transporter 2 family). *NRT2s* are high-affinity nitrate transporters, while most *NRT1s* are low-affinity nitrate transporters, with the exception of *CHL1* (*AtNRT1.1*), which is a dual-affinity nitrate transporter [7–10]. *CHL1* (*AtNRT1.1*), the first-identified nitrate transporter, belongs to the large *NRT1s* transporter family of *Arabidopsis* and functions as a dual-affinity transporter regulated by phosphorylation [11–15]. It may be a nitrate sensor in plants with sensitivity over a wide range of concentrations and involved in nascent organ development [16,17]. *AtNRT1.2* was cloned and characterised as a constitutive component of low-affinity uptake expressed before and after nitrate exposure; its transcripts were present primarily in root hairs and the epidermis [18]. Characterisation of *AtNRT1.4* revealed the special role of the petiole in nitrate homeostasis [19]. *AtNRT1.5* mediates efflux in xylem loading for root-to-shoot transport of nitrate [20], and *AtNRT1.6* is responsible for nitrate remobilisation from older to younger leaves [21]. *AtNRT1.7* delivers nitrate for seed development [22], while *AtNRT1.8* is involved in nitrate removal from xylem sap and mediates cadmium tolerance [23]. *AtNRT1.9* in root companion cells facilitates the loading of nitrate into the root phloem and enhances downward nitrate transport in roots [24]. *LeNRT1.2*, a nitrate-inducible gene in tomato, is root-specific and localises to root hairs, while transcripts of the constitutively expressed *LeNRT1.1* gene are found throughout the root [25]. *BnNRT1.2*, isolated from *Brassica napus*, can transport both nitrate and histidine [26].

Some members of NRT1s are nitrate transporters while others are peptide transporters. Two types of small peptide transporters,

* Corresponding author. Tel.: +86 25 84396238; fax: +86 25 84396238.
E-mail address: xiaorongfan@njau.edu.cn (X. Fan).

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known as PTRs (peptide transporters) and OPTs (oligopeptide transporters), have been identified. OPTs are tetra/pentapeptide transporters, while PTRs are di/tripeptide transporters. As *NRT1s* and PTRs belong to the same family, kown as NRT1(PTR) [16,27]. Several plant members of the PTR/NRT1 family have been identified; these exhibit various functions, including transportation of substrates such as nitrate, di-/tripeptides, auxin or carboxylates [16,28,29]. The abundance of di-/tripeptide transporters suggests that they play diverse and important roles in plant growth and development. Possible substrates for these transporters include glutathione, gamma-glutamyl peptides, hormone-amino acid conjugates, phytosulfokine, peptide-like compounds and peptide phytotoxins [27].

The first plant peptide transporter, *AtPTR2*, was isolated by complementation of a yeast histidine transport-deficient mutant with an *Arabidopsis* cDNA library [30]. *AtPTR2* is a di-/tripeptide transporter in *Arabidopsis* [31] and is expressed in most plant tissues, with high levels in green siliques, roots and young seedlings [32]. Transgenic *Arabidopsis* expressing the *AtPTR2* cDNA in an antisense orientation exhibited a delay in flowering and an arrest in seed development [33]. *AtPTR1* recognises not only a wide spectrum of naturally occurring di- and tripeptides, but also the modified tripeptide, phaseolotoxin, and substrates lacking peptide bonds. *AtPTR1* is expressed throughout the plant in vascular tissue, indicating a role in long-distance transport [34]. The apparent affinity of *AtPTR1* for Ala-Ala, Ala-Lys and Ala-Asp is pH-dependent and decreases with decreasing proton concentrations [35]. *AtPTR5*, localised at the plasma membrane, mediates high-affinity transport of dipeptides to supply peptides to maturing pollen, developing ovules and seeds. Over-expression resulted in enhanced shoot growth and increased N content [35,36]. *AtPTR3* is a salt stress and wound-induced peptide transporter, and both jasmonate (JA) and salicylic acid (SA) are involved in its regulation [37,38]. *AtPTR4* and *AtPTR6*, members of subgroup II of the *PTR*/*NRT1* family and localised in the tonoplast, show distinct expression patterns [39]. While *AtPTR4* is expressed in the plant vasculature, *AtPTR6* is highly expressed in pollen and during senescence [39]. The barley peptide transporter gene, *HvPTR1*, had high tissue- and developmental stage-specific expression. Transport activity of *HvPTR1* is regulated by phosphorylation in response to rising levels of amino acids in germinating grain [40–42]. Functional di-/tripeptide transporters also were reported in *Nepenthes* (*NaNTR*1, [43]), faba bean (*VfPTR1*, [44]) and *Hakea actites* (*HaPTR4*, [45]).

In rice, few members of the *NRT1s* family have been functionally verified. *OsNRT1.1* encodes a constitutive transport system and is expressed in the most external layers of the root, epidermis and root hair [46]. *OsNRT1.3* is induced by drought [47]. *SP1*, a putative PTR gene, determines panicle size and has high expression in the phloem of young panicle branches [48]. Phylogenetic analysis suggested *SP1* to be a nitrate transporter; however, neither nitrate nor transport activity of other compounds was observed [48]. Eight peptide transporters, *OsPTR1*, *OsPTR2*, *OsPTR3*, *OsPTR4*, *OsPTR5*, *OsPTR6*, *OsPTR7* and *OsPTR8*, were investigated in a yeast ptr2 mutant strain and their expression patterns in plants were evaluated. Only *OsPTR6* transports Gly-His and Gly-His-Gly and shows substrate selectivity for di/tripeptides; however, the other seven proteins did not transport the five tested di-/tripeptides [49]. It was well reviewed by Masclaux-Daubresse et al. [50] that peptide transporter was involved in leaf N remoblisation and grain filling, therefore we foretasted that *OsPTR6* expression might also enhance Gly-His and Gly-His-Gly transport in rice [49] and improve the amino acid transformation and NUE in rice plant.

In this study, we report the production and characterisation of a rice PTRs transporter, *OsPTR6*. By *A. tumefaciens* transformation using the ubiqutin (Ubi) promoter, the over-expression of *OsPTR6* was tested in the *Nipponbare* rice cultivar to increase plant N use efficiency (NUE), which is essential for the development of sustainable agriculture [51]. The expression of low affinity nitrate transporter (*OsNRT1.1*), high affinity nitrate transporters (*OsNRT2.1* and *OsNRT2.2*), and high affinity ammonium transporters (*OsAMT1.1 OsAMT1.2* and *OsAMT1.3*), growth, N content, glutamine synthetase activity (GSA),and NUE characteristics in response to high and low nitrate and ammonium supplies were investigated in these transgenic rice plants.

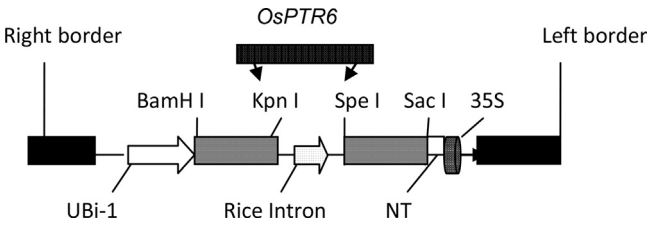

Fig. 1. Schematic diagram of the pUbi-*OsPTR6* expression vector for rice transformation. *OsPTR6* cDNA was inserted in place of the rice intron in pTCK303 by means of *Kpn*I and *Spe*I sites in the pUbi-*OsPTR6* plasmid.

2. Materials and methods

2.1. Cloning and sequence analysis of OsPTR6

The *OsPTR6* clone was obtained from *Oryza sativa L. ssp. japonica cultivar-group* cDNA clone: J033041C01 (KOME; http://cdna01.dna.affrc.go.jp/cDNA/) and registered in the DDBJ under the accession number AK101480. The full *OsPTR6* sequence was inserted into the Lambda-FLC plasmid vector [52]. The nucleotide sequence was identified using the NCBI BLAST software (http://www.ncbi.nlm.nih.gov/BLAST). The bioinformatics tools at the website (http://www.expasy.org) were used to analyse the deduced protein. The mature protein localisation site was predicted by PSORT (http://psort.ims.u-tokyo.ac.jp). Sequence alignment was performed using the DNAMAN software version 5.2.2 with default parameters. Transmembrane topology models were predicted using on-line tools (http://www.cbs.dtu.dk/services/TMHMM/).

2.2. Construction of vectors and rice transformation

The complete ORF of the *OsPTR6* gene was inserted into the Lambda-FLC plasmid vector using the following primers: forward (5′-AGGTACCATACCGGCGGCAAT-3′) and reverse (5′-AACTAGTGCTGTCAGCCCATCAAACCT-3′). *KpnI* and *SpeI* restriction enzyme sites were added at the 5′-end of the forward and reverse primers, respectively, to facilitate cloning into the expression vector pTCK303–Ubi [53]. The PrimeSTAR HS DNA Polymerase (TaKaRa Biotechnology Co., Ltd, Dalian, China) was used to amplify the *OsPTR6* gene; the PCR parameters were 95 °C for 5 min followed by 30 cycles of 98 °C for 10 s, 68 °C for 2 min and 72 °C for 10 min. The PCR products were cloned into the pMD18-T vector (TaKaRa Biotechnology Co., Ltd., Dalian, China) after gel extraction. The target fragment in the clone was digested with *KpnI* and *SpeI* and then introduced into pTCK303–Ubi. The constructed plasmid was named Ubi-pTCK303-*OsPTR6* (Fig. 1), and confirmed by sequencing.

The construct was then transformed by the *A. tumefaciens* (strain EHA105) method as described by Ai *et al.* [54]. Rice (*Oryza sativa L cv. Nipponbare*) embryonic calli were induced on N6 media and transformation was performed by *Agrobacterium*-mediated co-cultivation. After 50 mg/L hygromycin (Roche, USA) screening, T_0-generation transgenic plants were grown for seed. The T-DNA insertion numbers were tested in T_0- and T_1-generation transgenic plants by real-time quantitative PCR [55,56]. Three low-copy-number lines were selected and designated OE1, OE5 and

Table 1
OsPTR6 and *OsNRT* genes analysed by semi RT-PCR and their primer sequences, annealing temperatures and product sizes.

Gene	GenBank accession number	Primer sequences (5′ to 3′)		°C	Product length (bp)
		Forward	Reverse		
OsPTR6	AB008519	ATGAAATCATCGTGGCCCAGC	TACGAACACCGGGGTGAGGT	55	339
OsNRT1.1	AK109733	GAATTGTACAGTACTTCCCC	TTCTGAGAAGAGACTGGATCTGTCC	55	450
OsNRT2.1	AB008519	CACGGTGCAAGTCTCAAG	GGTATAAATGCCTCTCCC	50	316
OsNRT2.2	AK109733	TGGAACATTTGGATCCTCC	CCATGACGACATACTCTAG	53	438
OsAMT1.1	AF289477	GGTCATCTTCGGGTGGGTCA	CGTGCCGTGTCAGGTCCAT	55	321
OsAMT1.2	AF289478	GAAGCACATGCCGCAGACA	GACGCCCGACTTGAACAGC	55	224
OsAMT1.3	AF289479	GCGAACGCGACGGACTA	GACCTGTGGGACCTGCTTG	55	347
OsActin	AB047313	GGAACTGGTATGGTCAAGGC	AGTCTCATGGATAACCGCAG	55	250

OE6. The copy numbers in the T_2 generation of the OE1, OE5 and OE6 lines were confirmed by Southern blotting (DIG High Prime DNA Labelling and Detection Starter Kit I; http://www.roche.com/index.htm). The fragment of the coding sequences of the Hyg genes labelled with digoxigenin was used as a probe, which was prepared by PCR according to the supplier's instructions (Roche, http://www.roche.com/index.htm).

2.3. RT-qPCR analyses

The sterilised seeds were germinated on a plastic support netting (mesh, 1 mm^2) mounted in plastic containers for 1 week and then cultured with 0.1 or 2.5 mmol/L $Ca(NO_3)_2$, 0.1 or 2.5 mmol/L $(NH_4)_2SO_4$ and 2.5 mmol/L NH_4NO_3 as a nitrogen source nutrient solution at pH 5.5 for 1 day. Total RNA was extracted from 100 mg of 8-day-old rice using whole roots and shoots with TRIzol reagent (Invitrogen, Carlsbad, CA, USA). Total RNA concentrations were determined by UV spectrophotometry (Eppendorf, Biophotometer, Germany). Total RNA (2 μg) from each sample were used as templates for the first-strand cDNA synthesis with an oligo (dT)-18 primer, which was performed using M-MLV reverse transcriptase (Fermentas, Foster City, CA, USA) according to the manufacturer's manual. PCR amplification was performed using Taq DNA polymerase (Fermentas, Foster City, CA, USA) for target genes and *OsActin*. RT-PCR was performed using the gene-specific primers shown in Table 1. The PCR parameters for the detection of *OsActin*, *OsPTR6*, *OsNRT1.1*, *OsNRT2.1*, *OsNRT2.2 OsAMT1.1*, *OsAMT1.2* and *OsAMT1.3* were 95 °C for 5 min, followed by 94 °C for 30 s, 55 °C for 30 s and 72 °C for 30 s, ending with 72 °C for 5 min.

2.4. Plant materials and growth conditions

Wild-type (WT cv. *Nipponbare*) and mutant (T_2 generation) rice seeds were surface-sterilised with 10% (v/v) H_2O_2 for 30 min and rinsed thoroughly with deionised water. The sterilised seeds were germinated on a plastic support netting (mesh 1 mm^2) mounted in plastic containers for one week. Uniform seedlings were selected and then transferred to a tank containing 8 L of IRRI nutrient solution at pH 5.5. To inhibit nitrification, 7 μmol/L dicyandiamide (DCD-$C_2H_4N_4$) was mixed into all solutions. The solution was refreshed every 2 days.

Seedlings were selected and cultured with 0.1 or 2.5 mmol/L $Ca(NO_3)_2$, 0.1 or 2.5 mmol/L $(NH_4)_2SO_4$ and 2.5 mmol/L NH_4NO_3 as a nitrogen source nutrient solution at pH 5.5. Other major and minor elements were supplied with IRRI nutrient solution containing 0.3 mmol/L KH_2PO_4, 0.35 mmol/L K_2SO_4, 1 mmol/L $CaCl_2$, 1 mmol/L $MgSO_4$, 0.5 mmol/L Na_2SiO_3, 20 μmol/L EDTA-Fe, 9 μmol/L $MnCl_2$, 20 μmol/L H_3BO_3, 0.77 μmol/L $ZnSO_4$, 0.32 μmol/L $CuSO_4$ and 0.39 μmol/L $(NH_4)_6Mo_7O_{24}$. Plants were grown in a growth chamber (Thermoline Scientific Equipment Pty. Ltd., Smithfield, Australia) at 30 °C during the day and 22 °C during the night with a 16-h light/8-h dark regime. The light intensity was 400 μmol m^{-2} s^{-1} and the relative humidity was 65–70%. Each experiment was replicated three times using 15 plants. Both the wild-type and the over-expression seedlings were grown for 30 days in the culture solution for nitrogen treatment. The plant height, root length and total fresh weight were measured every 10 days with 4 replicates. Relative growth rate (RGR) was calculated from plant length data obtained from 0, 10, 20 and 30 d after germination, i.e. $RGR = (\ln L2 - \ln L1)/(t2 - t1) \times 100\%$

2.5. GSA measurement

Fresh shoots were harvested with four replicates after 30 days' growth under different N supply conditions at 9:00 AM. Ground samples were extracted with 0.5 mmol/L EDTA and 50 mmol/L K_2SO_4. The homogenates were centrifuged at 20,000 × *g* for 20 min. The clear filtrate (1.2 mL) was added to a centrifuge tube, followed by 0.6-mL imidazole–HCl (pH 7.0, 0.25 mol/L), 0.4 mL sodium glutamate (pH 7.0, 0.3 mol/L), 0.4-mL ATP-Na (pH 7.0, 15 mmol/L), 0.2 mL $MgSO_4$ (0.5 mol/L) and 0.2 mL hydroxylamine (1 mol/L). After the mixture was incubated at 25 °C for 20 min, the reaction was terminated by adding 0.8-mL acidic $FeCl_3$, [24% (w/v) trichloroacetic acid and 10% (w/v) $FeCl_3$ in 18% HCl]. Production of γ-glutamylhydroxamate was measured with a spectrophotometer at 540 nm. One unit of GS activity was defined as the amount of enzyme catalysing the formation of 1 μmol γ-glutamylhydroxamate min^{-1} at 25 °C [57].

2.6. Biomass, total N measurement and NUE calculation

Fresh plants of wild type and transgenic lines were harvested with four replicates after 30 days' growth under different N supply conditions at 9:00 AM and then heated at 105 °C for 30 minutes. Afterwards shoot and root were dried at 75 °C for 3 days respectively. The dry weight was recorded as biomass. Root to shoot ratio was calculated as root dry weight divided by shoot dry weight. Total nitrogen content was measured according to the Kjeldahl method [57]. The accumulation of plant nitrogen was calculated as shoot nitrogen content multiplied by shoot biomass plus root nitrogen content multiplied by root biomass and NUE was expressed as the whole-plant dry biomass relative to N accumulation [58].

2.7. Statistical analysis of data

All the data collected were tabulated and analysed for significant differences using statistical software (SPSS 13.0; SPSS Inc., IL).

3. Results

3.1. Confirmation of transgenic plants by PCR, Southern blotting and RT-PCR

The segregation population of T_0-generation transgenic plants transformed with *OsPTR6* was selected with 50 mg/L hygromycin;

121 lines were obtained. Copy numbers for T_0 and T_1 were confirmed by real-time PCR; three lines (OE1, OE5, OE6) with low copy numbers were selected. The T_2 segregating population of the OE1, OE5 and OE6 lines was further characterised by Southern blotting and RT-PCR. Southern blotting of *BamHI* and *HindIII* digests was performed using the WT as the negative control and the empty plasmid vector transgenic as the positive control. All test plants for OE1, OE5 and OE6 had positive hybridisation signals, while the negative control did not. The copy number of the T_2 generation was one (Fig. 2a). RT-PCR analysis was performed using the WT as a control; the expression in the OE1, OE5 and OE6 transgenic lines was increased (Fig. 2b). These results indicate that the *OsPTR6* gene was over-expressed and integrated into the genomes of the transgenic lines.

3.2. Expression patterns of OsPTR6 in WT and the T_2 generation of OE1 in response to nitrate and ammonium

RT-qPCR was performed to determine the expression pattern of the *OsPTR6* gene in WT and the T_2 generation of transgenic line OE1 grown under different N supply conditions. The *OsActin* gene was used as a reference for comparison. *OsPTR6* expression was detected primarily in the roots of the WT, with almost no transcripts in the shoots (Fig. 3a); in OE1 *OsPTR6* expression was detected in roots and shoots (Fig. 3a). The WT and OE1 were grown in the presence of several NO_3^- and NH_4^+ concentrations. *OsPTR6* expression in WT roots was increased at 0.2 and 5.0 mmol/L NO_3^- and 2.5 mmol/L NH_4NO_3 compared with 0.2 and 5.0 mmol/L NH_4^+ (Fig. 3a). However, in WT shoots, *OsPTR6* expression was increased at 0.2 and 5.0 mmol/L NH_4^+ and 5.0 mmol/LNO_3^- compared to all other conditions (Fig. 3a). These results indicate that *OsPTR6* was up-regulated by NO_3^- in WT roots, but a different regulatory mechanism was active in shoots. In OE1 plants, the expression of *OsPTR6* was unchanged under all N supply conditions.

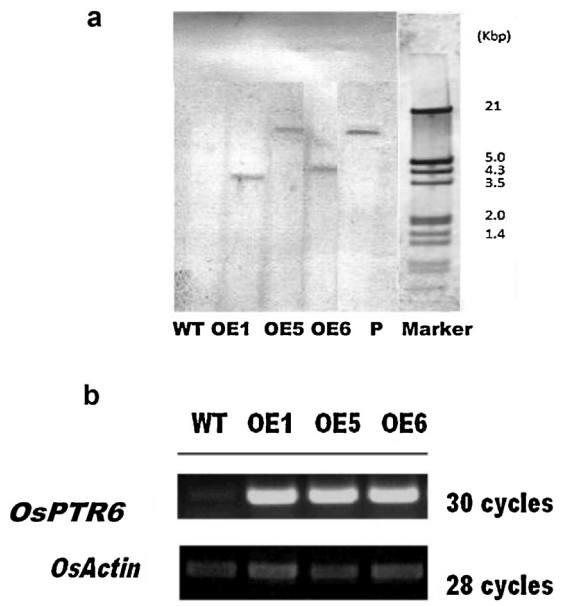

Fig. 2. Molecular characterisation of transgenic plants harbouring the pUbi-*OsPTR6* construct. (a) Determination of copy numbers in transgenic rice plants OE1, OE5 and OE6 by Southern blotting. WT indicates wild-type. OE1, OE5 and OE6 indicate transgenic rice lines. Southern blotting of *BamH*I and *Hind*III digests was performed using the WT as the negative control and the empty plasmid vector transgenic as the positive control; (b) the expression of *OsPTR6* in WT and transgenic rice roots by RT-PCR.

Furthermore, the expression of other nitrate transporter components, *OsNRT1.1* and *OsNRT2.2* was obviously down-regulated in OE1 root compared with WT in all N condition (Fig. 3b and d). However, in WT shoot, *OsNRT1.1* was significant increased at 0.2 mmol/L NH_4^+, 0.2 mmol/L NO_3^- and 2.5 mmol/L NH_4NO_3 compared with

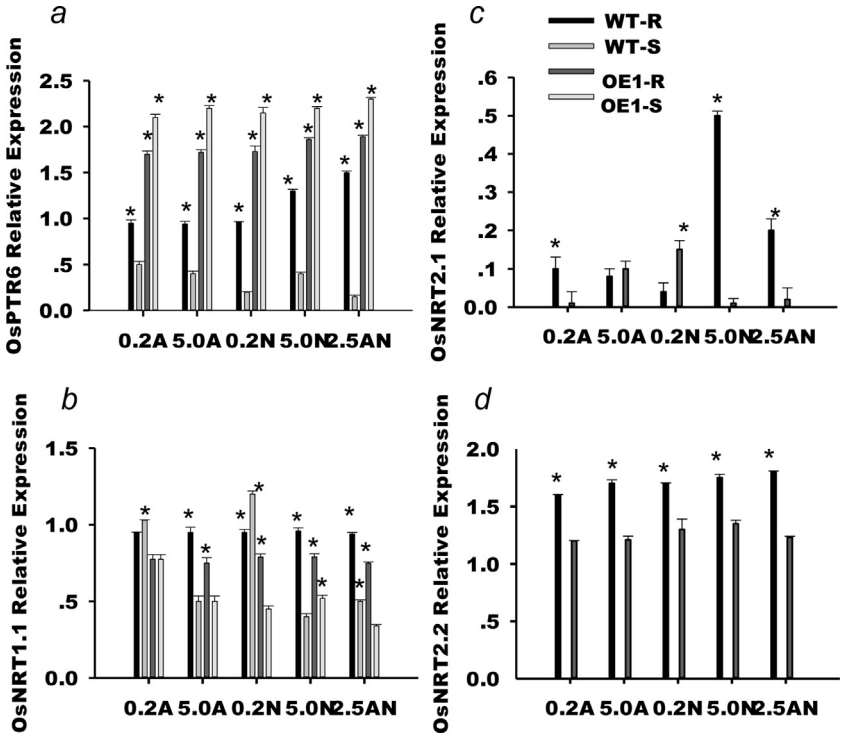

Fig. 3. Relative expression analysis of related nitrate transporter genes in the WT and OE1 under different N supplies. (a) *OsPTR6*; (b) *OsNRT1.1*; (c) *OsNRT2.1*; (d) *OsNRT2.2*. The treatments were labelled as follows: 0.2 mmol/L NH_4^+ (0.2 A), 5.0 mmol/L NH_4^+ (5.0 A), 0.2 mmol/L NO_3^- (0.2 N), 5.0 mmol/L NO_3^- (5.0 N) and 2.5 mmol/L NH_4NO_3 (2.5 AN). *Significant difference at the 0.05 probability level according to LSD test ($n = 4$) estimated by one-tailed ANOVA between WT and an over-expression line (OE1); data are means ± SE.

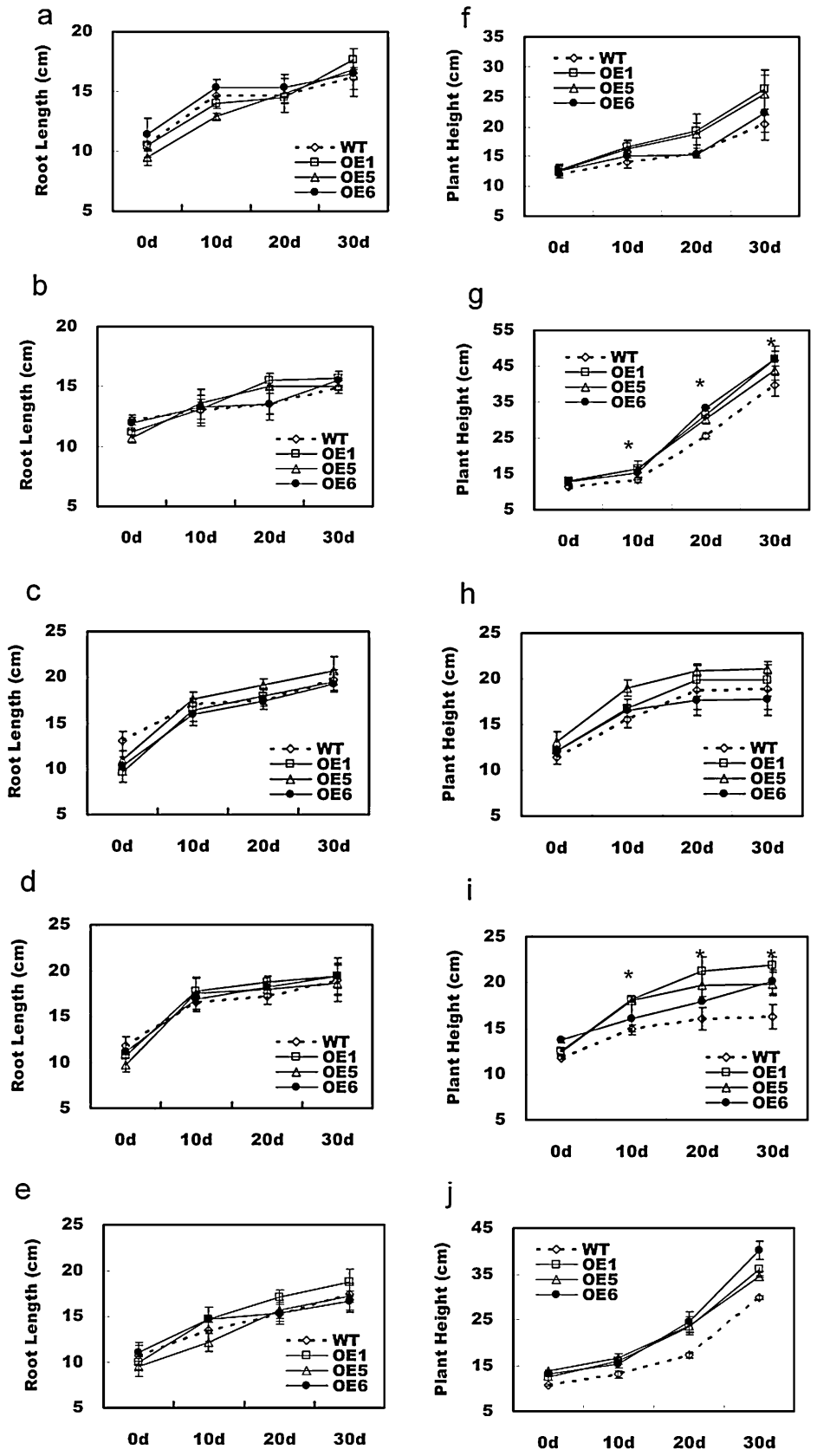

Fig. 4. Plant height and root length of WT and T_2 transgenic lines under different N supplies. (a) Root length at 0.2 mmol/L NO_3^-; (b) Root length at 5.0 mmol/L NO_3^-; (c) Root length at 0.2 mmol/L NH_4^+; (d) Root length at 5.0 mmol/L NH_4^+; (e) Root length at 2.5 mmol/L NH_4NO_3; (f) Plant height at 0.2 mmol/L NO_3^-; (g) Plant height at 5.0 mmol/L NO_3^-; (h) Plant height at 0.2 mmol/L NH_4^+; (i) Plant height at 5.0 mmol/L NH_4^+; (j) Plant height at 2.5 mmol/L NH_4NO_3. *Significant difference at the 0.05 probability level according to LSD test ($n = 4$) estimated by one-tailed ANOVA between WT and over-expression lines (OE1, OE5, OE6); data are means ± SE.

OE1 except 5.0 mmol/L NH_4^+ and 5.0 mmol/L NO_3^- (Fig. 3b). The expression of *OsNRT2.1* is inconsistent that it was significantly decreased in OE1 root at 0.2 mmol/L NH_4^+,5.0 mmol/L NO_3^- and 2.5 mmol/L NH_4NO_3 while significantly increased at 5.0 mmol/L NH_4^+ and 0.2 mmol/L NO_3^- (Fig. 3c).

3.3. Over-expression of OsPTR6 significantly enhanced rice growth

Plant height, root length and dry weight were measured every 10 days with 0.2 and 5.0 mmol/L NH_4^+, 0.2 and 5.0 mmol/L NO_3^- and 2.5 mmol/L NH_4NO_3 used as N sources. There was no significant difference in root length between the WT and the transgenic lines (Fig. 4a–e), while transgenic plants were significantly taller than the WT under 5.0 mmol/L NO_3^- (Fig. 4g) and 5.0 mmol/L NH_4^+ (Fig. 4i) throughout the 30 days of cultivation. For example, on day 30, the height of OE1 and OE5 plants was increased by 34% and 21%, respectively, compared with the WT at 5.0 mmol/L NO_3^-, and by 80% and 79%, respectively, at 5.0 mmol/L NH_4^+. Transgenic plant height did not significantly differ from that of WT plants at 2.5 mmol/L NH_4NO_3, 0.2 mmol/L NH_4^+ or 0.2 mmol/L NO_3^-, with the exception of OE5 at 0.2 mmol/L NO_3^- and OE1 at 0.2 mmol/L NH_4^+, which were taller than WT plants on day 10.

The relative root grow rate showed that the roots of transgenic lines were grown quicker than WT in the first 10 days in all N conditions except in 0.2 mmol/L NH_4^+ and later on the root growth did not show a similar pattern in different N supples (Fig. S1a–e). The shoot growth rate data did not show an obvious significant different pattern in all plants (Fig. S1f–j).

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After 30 days of cultivation, the shoot dry weight of all transgenic plants was significantly greater than that of WT plants in all N supply conditions (Fig. 5a). Especially at 5.0 mmol/L NH_4^+, shoot biomass of OE1, OE5 and OE6 were significantly increased by 90%, 29% and 118%, respectively, compared with the WT. The root dry weight of all transgenic plants was also increased only in high N supply, but not at 0.2 mmol/L NH_4^+ and NO_3^- (Fig. 5b), However at 5.0 mmol/L NH_4^+, root biomass of OE1, OE5 and OE6 was still significantly increased by 83%, 29% and 104%, respectively, compared with the WT. For WT plant, the root/shoot ratio was changed in different N supplies, but for transgenic plants the significant change was not observed at the same experiment (Fig. 5c). However, it was interesting that in 0.2 mmol/LNO_3^- the root/shoot ratio of all transgenic lines showed a remarkable decrease compared with WT.

3.4. Over-expression of OsPTR6 enhanced N accumulation at 5.0 mmol/LNH_4^+ and 2.5 mmol/L NH_4NO_3

To clarify the cause of the enhanced growth in transgenic plants, we measured total N contents in WT and transgenic plants. The shoot total N content in all transgenic plants was significantly higher than that of WT plants with 5.0 mmol/L NH_4^+. After 30 days' growth, the shoot total N content of transgenic lines OE1, OE5 and OE6 were ca. 95, 88 and 91 mg/g, respectively, whereas that of WT was 74 mg/g, with 5.0 mmol/L NH_4^+ (Fig. 6a). In contrast, there was no significant difference in the shoot total N content between the WT and transgenic lines under all other N treatments, with the exception of OE6 at 2.5 mmol/L NH_4NO_3 (Fig. 6a). As shown in Fig. 6b, with 5.0 mmol/LNH_4^+ the root N content of OE1, OE5 and OE6 was increased significantly compared to the WT; at 0.2 mmol/L NO_3^- the increase in the three lines was 11–23% over that of the WT. No significant difference was found in the root N content for all other N treatments, with the exception of the OE1 line, the root N content of which was significantly higher at

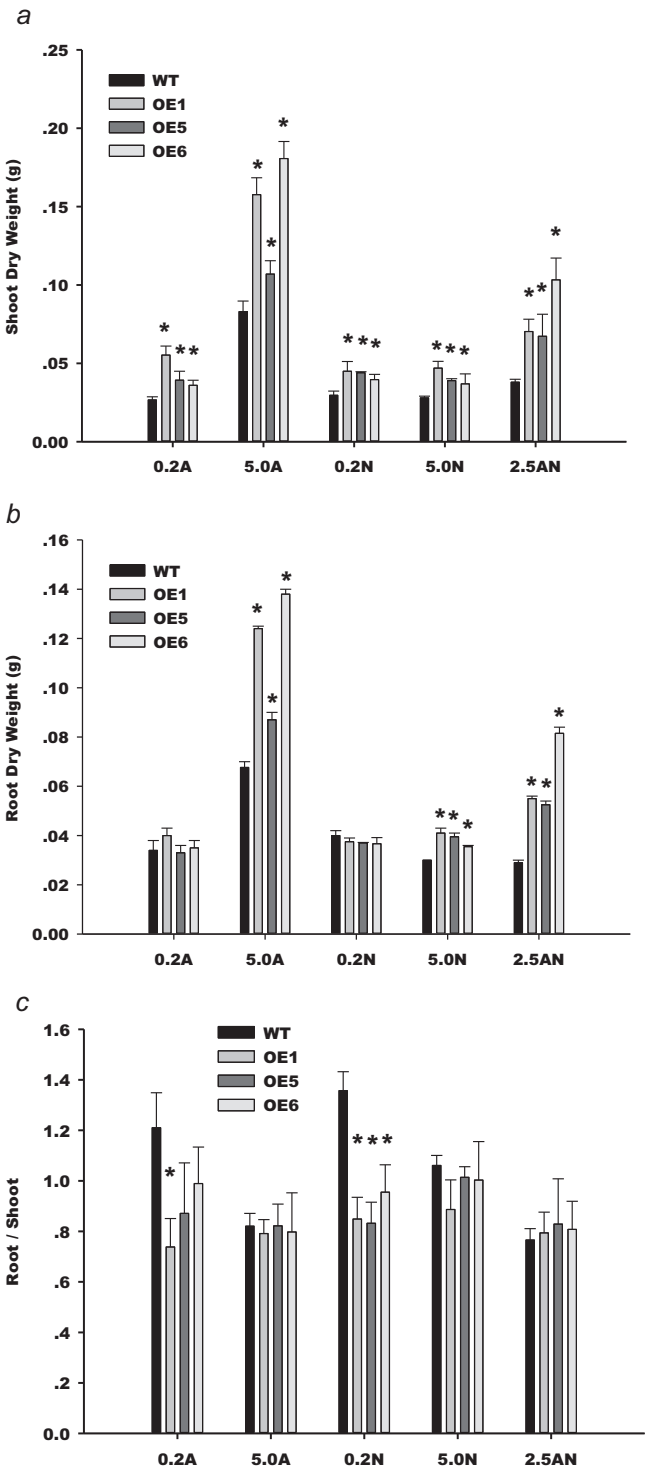

Fig. 5. Dry weight and root/shoot of WT and transgenic lines with different N supplies. (a) Root dry weight; (b) shoot dry weight; (c) root/shoot. root/shoot = root dry weight/shoot dry weight. The treatments were labelled as 0.2 mmol/L NH_4^+ (0.2 A), 5.0 mmol/L NH_4^+ (5.0 A), 0.2 mmol/L NO_3^- (0.2 N), 5.0 mmol/L NO_3^- (5.0 N) and 2.5 mmol/L NH_4NO_3 (2.5 AN). *Significant difference at the 0.05 probability level according to LSD test ($n = 4$) estimated by one-tailed ANOVA between WT and over-expression lines (OE1, OE5, OE6); data are means ± SE.

5.0 mmol/L NO_3^- compared with the WT. Biomass and N content increased with 5.0 mmol/LNH_4^+ supply, and considerably greater total N accumulated than under the other conditions; the over-expression lines exhibited significantly greater accumulation than the WT. At 2.5 mmol/L NH_4NO_3, the biomass of transgenic lines

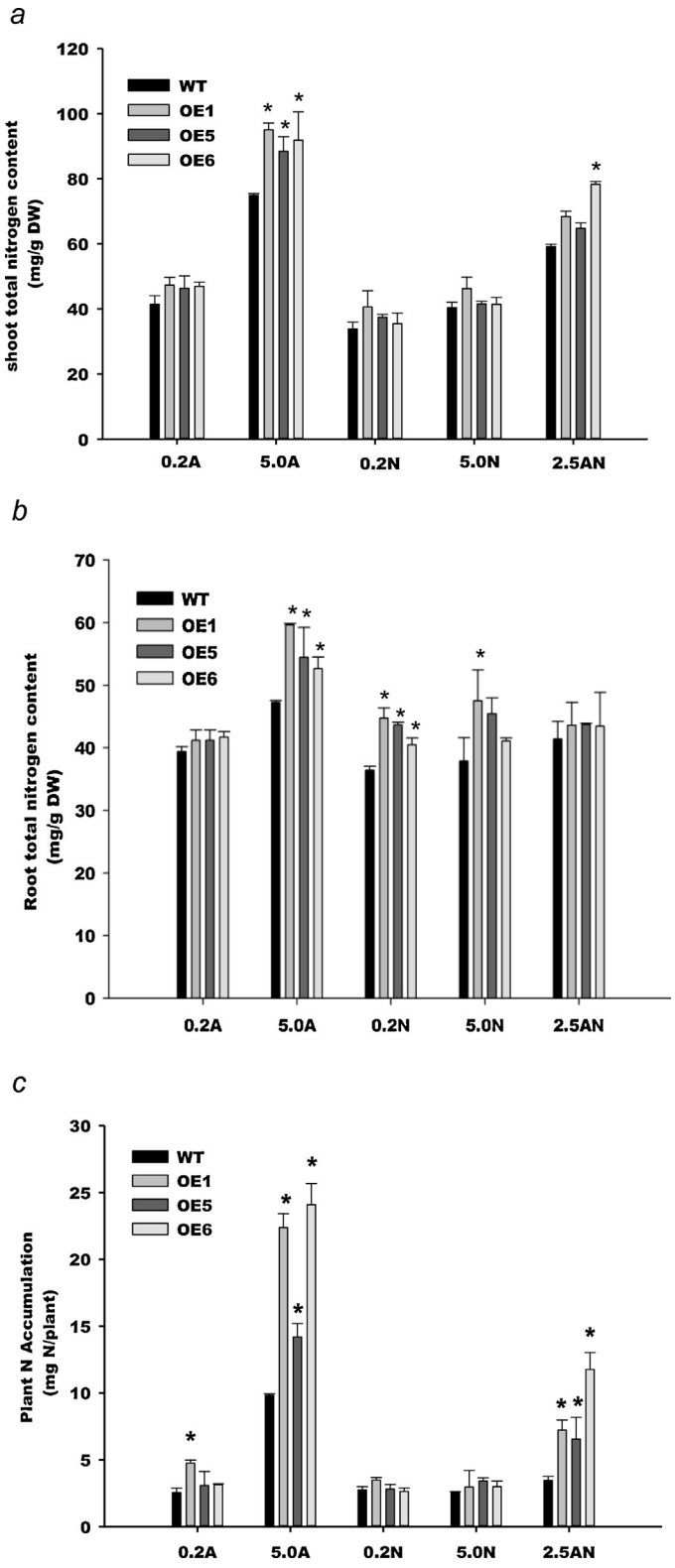

Fig. 6. Plant total nitrogen content and nitrogen accumulation of WT and transgenic lines with different N supplies. (a) Shoot nitrogen content; (b) root nitrogen content; (c) plant nitrogen accumulation. The treatments were labelled as 0.2 mmol/L NH_4^+ (0.2 A), 5.0 mmol/L NH_4^+ (5.0 A), 0.2 mmol/L NO_3^- (0.2 N), 5.0 mmol/L NO_3^- (5.0 N) and 2.5 mmol/L NH_4NO_3 (2.5 AN). Plant nitrogen accumulation = shoot nitrogen content × shoot dry weight + root nitrogen content × root dry weight. *Significant difference at the 0.05 probability level according to LSD test ($n = 4$) estimated by one-tailed ANOVA between WT and over-expression lines (OE1, OE5, OE6); data are means ± SE.

increased remarkably compared to the WT, even though the actual N content did not increase. The total plant N (shoot nitrogen content multiplied by shoot biomass plus root nitrogen content multiplied by root biomass) increased significantly compared with the WT (Fig. 6c). These results suggest that *OsPTR6* over-expression enhances the uptake and accumulation of N under high NH_4^+ supply.

3.5. Over-expression of OsPTR6 increased rice GSA at 5.0 mmol/LNH_4^+ and 2.5 mmol/L NH_4NO_3

To investigate the mechanisms underlying total N accumulation in transgenic plants, we measured GS activity in WT and transgenic plants. The shoot GSA of all transgenic plants—with the exception of OE5—was significantly greater than that of WT plants under 5.0 mmol/L NH_4^+ and 2.5 mmol/L NH_4NO_3 supply (Table 2). The over expression of OsPTR6 increased GSA by 22–31% compared to the WT at 5.0 mmol/L NH_4^+ (Table 2). However, under the 0.2 mmol/L NH_4^+ and NO_3^- conditions, GSA did not show significant changes (Table 2).

3.6. Over-expression of OsPTR6 increased rice the expression of OsAMT1 genes in 0.2 and 5.0 mmol/LNH_4^+ and 2.5 mmol/L NH_4NO_3

In order to understand the reasion why OsPTR6 over-expression plant could grow better than WT under ammonium supply, we tested the expression of *OsAMT1.1*, *OsAMT1.2* and *OsAMT1.3* in all three over-expression lines' root. In OE1, OE5 and OE6 lines, the expression of all three genes of *OsAMT1* was increased compared with WT in low and high ammonium conditions and 2.5 mmol/L NH_4NO_3. In low and high nitrate conditions, the expression of OsAMT genes in over-expression lines showed no significantly different from WT.

The expression of OsAMT1 genes in WT roots was decreased in high ammonium treatment, This effect of high ammonium on *OsAMT1* genes was also reported by [59]. In our experiment, for WT root, the expression of *OsAMT1.1* was reduced to 33% in 5.0 mmol/L NH_4^+ as 0.2 mmol/L NH_4^+; *OsAMT1.2* was reduced to 35% and *OsAMT1.3* was reduced to 50%. However when *OsPTR6* was over expressed, the expression of *OsAMT1* genes did not decreased as much as WT. For OE1 line, the expression of *OsAMT1.1* was decreased to 70% in 5.0 mmol/LNH_4^+ as 0.2 mmol/L NH_4^+; *OsAMT1.2* was down to 66% and *OsAMT1.3* was down to 81%. For OE6 line, the expression of *OsAMT1* genes almost did not show the decrease pattern, except *OsAMT1.2* was down to 72% in 5.0 mmol/LNH_4^+ as 0.2 mmol/L NH_4^+. For OE5 line, the decrease of *OsAMT1* expression was to about 50% for *OsAMT1.1*, 44% for *OsAMT1.2* and 70% for *OsAMT1.3* in 5.0 mmol/L NH_4^+ as 0.2 mmol/L NH_4^+, which was still less than WT.

Furthermore *OsAMT1* gene expression was increased by the mixture of nitrate and ammonium, compared with 5.0 mmol/L NH_4^+ in all rice plants. The similar result was also found in other rice cultivars [60]. However the increase range of *OsAMT1* gene expression in WT was much more than over-expression lines. All these *OsAMT1* expression data suggested the nitrogen feedback regulation in *OsPTR6* over-expression lines was significantly different from WT.

3.7. Over-expression of OsPTR6 decreased rice NUE only at 5.0 mmol/LNH_4^+

Using the formula for NUE (N utilisation efficiency), originally defined as the dry mass productivity per unit N taken up from soil [61], as plant biomass divided by the total N accumulation [57], we calculated the NUE of transgenic and WT plants under differing N

Table 2
Plant GSA of WT and transgenic lines at 0.2 mmol/L NH_4^+, 5.0 mmol/L NH_4^+, 0.2 mmol/L NO_3^-, 5.0 mmol/L NO_3^- and 2.5 mmol/L NH_4NO_3.

	$0.2NH_4^+$	$5.0NH_4^+$	$0.2NO_3^-$	$5.0NO_3^-$	$2.5NH_4NO_3$
WT	167 ± 11.2	218 ± 15.1	152 ± 13.1	189 ± 16.6	205 ± 11.1
OE1	173 ± 11.3	279 ± 12.1*	162 ± 10.5	201 ± 17.2	243 ± 14.2*
OE5	171 ± 10.4	267 ± 13.2*	184 ± 16.0	196 ± 16.7	230 ± 12.2
OE6	181 ± 13.1	287 ± 18.4*	164 ± 14.6	204 ± 15.3	252 ± 13.8*

Note: GSA unit (mmol g^{-1} FW h^{-1}).
* Significant difference at the 0.05 probability level according to LSD test ($n = 4$) estimated by one-tailed ANOVA between WT and over-expression lines (OE1, OE5, OE6); data are means ± SE.

supply conditions during 30 days' growth. The NUE of OE1, OE5 and OE6 was decreased significantly compared with the WT when grown with a 5.0 mmol/L NH_4^+ supply (Table 3). However, under all other conditions, the NUE of all three lines did not differ from that of the WT, with the exception of OE1 where NUE decreased with 0.2 mmol/L NH_4^+.

The NUE of all plants was lower at 5.0 mmol/L NH_4^+. The NUE of WT at 5.0 mmol/LNH_4^+ was about 62–70% of the value with 0.2 mmol/LNH_4^+ and 0.2 and 5.0 mmol/L NO_3^-, and 82% of the value with 2.5 mmol/L NH_4NO_3 (Table 3). The NUE of OE6 with 5.0 mmol/LNH_4^+ was ~50–59% of that with 0.2 mmol/LNH_4^+ and 0.2 NO_3^- and 5.0 mmol/L NO_3^-, and 80% of the value with 2.5 mmol/L NH_4NO_3 (Table 3). This indicates that at 5.0 mmol/L NH_4^+, the NUE of rice plants was decreased compared with other N supply conditions, and *OsPTR6* over-expression decreased the NUE to a greater extent in transgenic lines. Interestingly, with 2.5 mmol/L NH_4NO_3 (high ammonium with nitrate treatment) the NUE of the transgenic lines did not show any decline compared to the WT. At the same time, the NUE in transgenic lines under 0.2 mmol/L and 5.0 mmol/L nitrate supply was not significantly different from that of the WT, regardless of whether a high or low concentration was used. This result showed that with a pure nitrate supply, the NUE is unaffected by *OsPTR6* over-expression, but with a high ammonium level as the sole N source, the NUE of transgenic lines is altered relative to that of the WT.

4. Discussion

To investigate the biological function of PTR/NRT1 transporters, many researchers have used reverse genetics with a deletion mutation to induce loss of function. This work focused on over-expression of a transporter to test for gain of function to investigate the potential to alter di/tripeptide transporter function in rice. According to previous study of Komarova et al. [35], over-expression of a dipeptides transporter *AtPTR5* could enhance shoot growth and increased N content in 2.5 mM peptide mixture supply under 0.1 mM NH_4NO_3 condition but no any increase of growth or N content under no N or 0.1 mM NH_4NO_3 without peptides supplies. However our data showed that the over-expression of *OsPTR6* resulted in an altered phenotype depending on the nitrogen supplies, and biomass and nitrogen content was increased and NUE was decreased under high ammonium conditions without any peptides supplies.

Transgenic *OsPT6* expression was increased in roots, but this result was complicated by the presence of endogenous *OsPTR6* transcripts, while in leaves it was obvious that the Ubi-promoter increased the expression of the *OsPTR6* gene in the transgenic lines (Fig. 3). Fraisier et al. [62] reported that the 35S-promoter-driven expression of *NpNRT2.1* was not increased in transgenic lines compared with the wild-type under 1 mmol/L nitrate supply. The transgenic expression pattern was affected by differing N supplies, such as low or high nitrate or ammonium [62]. For rice, in roots and shoots, *OsPTR6* expression driven by the Ubi promoter was increased, and there was no significant change when different N supplies were used. These results show differences between the Ubi and 35S promoter-driven expression of the *NRT1* and *NRT2* nitrate transporters (*OsPTR6* and *NpNRT2.1*) in eudicot and monocot backgrounds. These differences may be due to the different promoters used or variation in endogenous gene expression.

OsPTR6 has 4 exons and 3 introns and possesses 12 transmembrane motifs and a long hydrophilic loop between TM6 and TM7, exhibiting high similarity to *NTR1* members (*OsNRT1.1*, *AtNRT1.1*, *AtNRT1.2*, *AtNRT1.4*, *AtNRT1.5*, *AtNRT1.6* and *AtNRT1.7*), which have a known nitrate transport function. *OsPTR6* transported Gly-His and Gly-His-Gly and showed substrate selectivity for di/tripeptides [49]. When nitrate was used as the sole N source, the shoot biomass, root/shoot ratio and total N content in root were increased, but root biomass, shoot total N content, plant N accumulation and NUE showed no significant difference between the transgenic lines and the WT (Figs. 5 and 6, Table 3). It was not difficult to understand why *OsPTR6* over-expression did not increase plant N accumulation at nitrate supply (Fig. 6c), as *OsPTR6* is a di/tripeptide transporter [49]. Furthermore, over-expression of *OsNRT2.1*, a high-affinity nitrate transporter (HAT), did not increase nitrate uptake at low concentrations [63]. The failure in gain of function of *OsPTR6* and *OsNRT2.1* under nitrate supply conditions suggests that both *OsPTR6* and *OsNRT2.1* could not increase nitrate uptake when over expression. Furthermore this was also likely because the transgenic lines could not assimilate the additional nitrate, since GSA in transgenic lines did not show any increase in NO_3^- conditions compared with the WT (Table 2).

In the rice paddy soil, most nitrogen is in the form of ammonium and is the main nitrogen form taken up by rice. However as rice root releasing O_2 to rice root surface, some ammonium was transferred into nitrate by ammonium oxidation bacterial (AOB) [57]. The real N forms around rice root surface were the mixture of ammonium and nitrate. When 5.0 mM ammonium was

Table 3
NUE of WT and transgenic lines at 0.2 mmol/LNH_4^+, 5.0 mmol/L NH_4^+, 0.2 mmol/L NO_3^-, 5.0 mmol/L NO_3^- and 2.5 mmol/L NH_4NO_3.

	$0.2NH_4^+$	$5.0NH_4^+$	$0.2NO_3^-$	$5.0NO_3^-$	$2.5NH_4NO3$
WT	24.81 ± 0.55	15.95 ± 0.02	25.33 ± 0.31	22.74 ± 0.66	19.44 ± 0.63
OE1	20.12 ± 1.13*	12.59 ± 0.06*	23.66 ± 1.05	23.09 ± 0.21	17.41 ± 0.42
OE5	22.86 ± 2.10	13.70 ± 0.32*	26.84 ± 0.06	23.01 ± 0.67	18.72 ± 1.28
OE6	22.57 ± 0.02	13.44 ± 0.83*	26.42 ± 0.46	24.22 ± 0.53	17.37 ± 0.38

Note: NUE = plant dry weight/total N accumulation (g/g N).
* Significant difference at the 0.05 probability level according to LSD test ($n = 4$) estimated by one-tailed ANOVA between WT and over-expression lines (OE1, OE5, OE6); data are means ± SE.

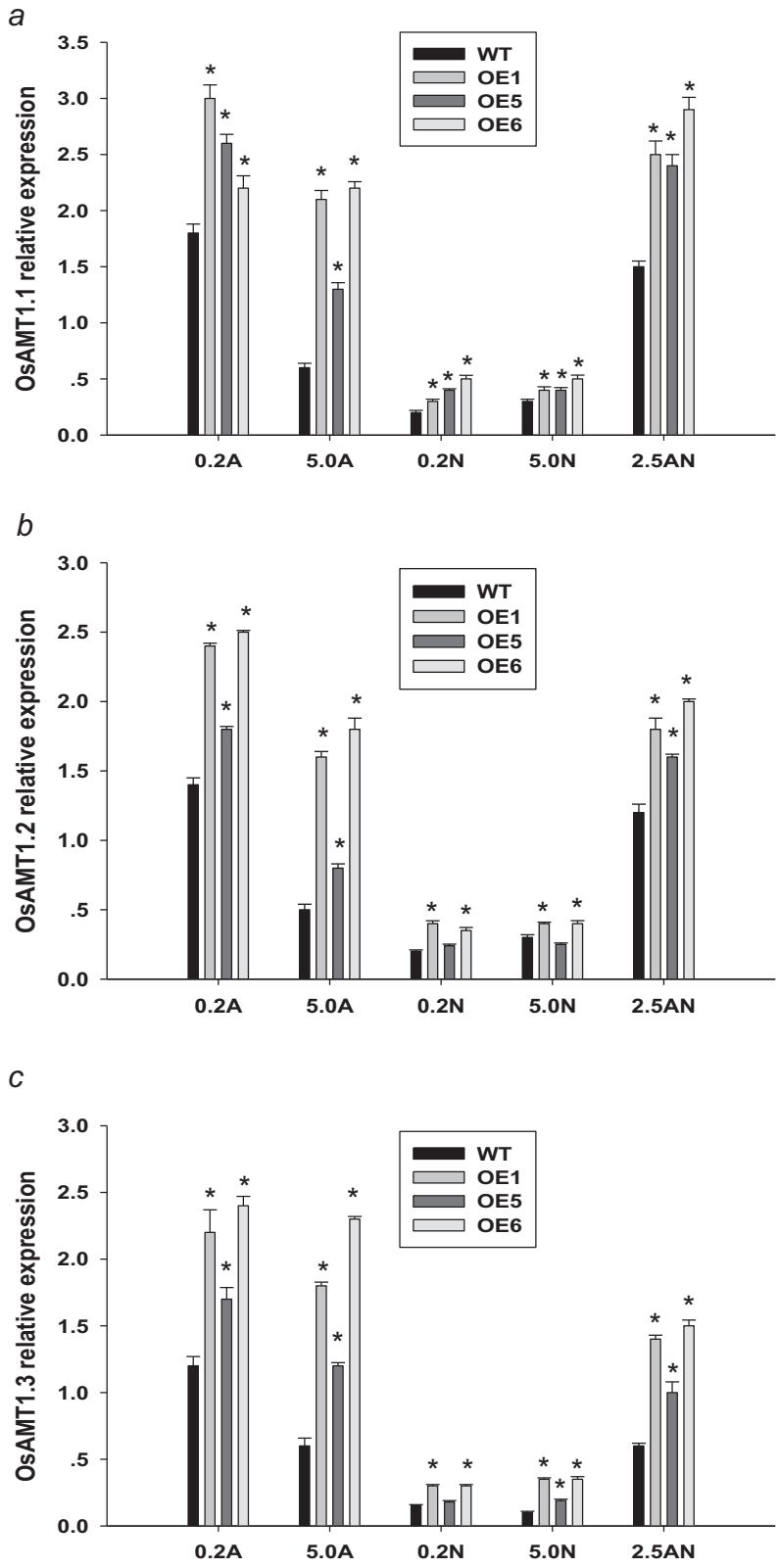

Fig. 7. Relative expression analysis of *OsAMT1* genes in the WT, OE1, OE5 and OE6 roots under different N supplies. (a) *OsAMT1.1*; (b) *OsAMT1.2*; (c) *OsAMT1.3*; The treatments were labelled as follows: 0.2 mmol/L NH_4^+ (0.2 A), 5.0 mmol/L NH_4^+ (5.0 A), 0.2 mmol/L NO_3^- (0.2 N), 5.0 mmol/L NO_3^- (5.0 N) and 2.5 mmol/L NH_4NO_3 (2.5 AN). *Significant difference at the 0.05 probability level according to LSD test ($n = 4$) estimated by one-tailed ANOVA between WT and an over-expression line (OE1); data are means ± SE.

supplied to *OsPTR6* transgenic plants, both biomass, N content, and plant N accumulation were increased in *OsPTR6* transgenic lines compared with the WT (Figs. 5 and 6). A di/tripeptide transporter could increase N accumulation under high ammonium supply conditions, which has to our best knowledge not been reported previously. Over-expression of an ammonium transporter gene, such as *OsAMT1.1*, could increase ammonium uptake in the presence of low or high ammonium levels, but plant biomass was decreased

significantly [64,65]. This suggests that excessive ammonium accumulation has negative effects for rice plant growth [64,65]. Another reason for this phenotype *may* be the low increase in the levels of ammonium metabolism enzymes to transform ammonium into amino acids. While over-expression of an amino acid synthetase gene, such as *AtASN2*, could increase asparagine content under high ammonium conditions; however, neither biomass nor nitrogen content increased under high ammonium conditions [66]. This suggests that in *AtASN2* over-expression lines, although ammonium metabolism increased, the nitrogen was not transformed into biomass. Another nitrogen uptake or use pathway must need to be up-regulated in combination with the increase in *AtASN2* activity.

In our over-expression lines, the *OsAMT1* genes were much up-regulated in high ammonium condition (Fig. 7) and did not reduce plant biomass. Rather than decreasing the biomass, *OsPTR6* over-expression lines showed increased the plant biomass at 5.0 mmol/L NH_4^+ (Fig. 5). As more di/tripeptides could be transported in *OsPTR6* over-expression lines from root to shoot or from cytosolic to vacuole like *AtPTR4* [39] and more ammonium could quickly transfer into amino acid in plant cells by GSA (Table 2), this quicker metabolism helped plant to take more ammonium through the increase of *OsAMT1* gene expression. As a result of these all, over-expression plants accumulated more nitrogen in high ammonium condition.

While as in 0.2 mmol/L NH_4^+ the biomass of OE5 and OE6 lines did not show any increase compared with WT, even though their *OsAMT1* gene expression was increased. The possible explanation was the non-change of ammonium synthesis, since GS activities did not show any increase at 0.2 mmol/L NH_4^+ (Table 2). This suggested GSA still was the key step to improve N accumulation in rice plant under ammonium condition. However, the increase in GSA at 5.0 mmol/L NH_4^+ did not result in an increase in NUE in *OsPTR6* over-expression plants and it also did not result the NUE increase in OsGS1.1, and OsGS1.2 over-expression plants [67]. Furthermore in our data we showed that under 0.2 mmol/L or 5.0 mmol/L NO_3^- treatments, a 10 or 4 folds increase of *OsPTR6* expression in OE1 shoot (Fig. 3a) could contribute a 50% or 67% increase of shoot biomass compared with WT (Fig. 5a), even though GSA did not increase significantly (Table 2). It suggested that without ammonium supply, OsPTR6 also could improve rice growth but not NUE (Table 3).

Our data demonstrate that the di/tripeptide transporter, *OsPTR6*, increases rice growth and N accumulation at 5.0 mmol/L NH_4^+ through the up-regulation on *OsAMT1* genes in roots and GSA. However NUE was decreased significantly at 5.0 mmol/LNH_4^+. Further research is required to uncover the function of *OsPTR6* transgenic lines in the presence of a mixture of ammonium and nitrate supplies.

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