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Expression Patterns of Nine Ammonium Transporters in Rice in Response to N Status*[1]

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ABSTRACT

Nitrogen use efficiency (NUE) was very low in China and a loss of as much as 70% of the applied nitrogen fertilizers was reported in high-yielding rice fields. In order to investigate the molecular basis of high-affinity ammonium transport or uptake into rice (*Oryza sativa* L.), we analyzed the expression profiles of nine ammonium transporters (AMT), three each of *OsAMT1*, *OsAMT2* and *OsAMT3*, at two different N requirement stages (young seedling stage and tillering stage) of rice growth as well as the changes in these expression patterns according to external N status using real-time reverse transcription polymerase chain reaction (RT-PCR). The results suggested that the nine *OsAMT* genes were expressed in different organs of rice plants, including mature roots, new roots, stems, old leaves and new leaves and that the expression patterns were organ specific and independent of the positions of the corresponding proteins in the phylogenetic tree. *OsAMT1;1*, *3;2* and *3;3* were expressed in the roots and shoots, primarily old leaves, *OsAMT1;2* and *1;3* mainly in the roots, and *OsAMT2;1*, *2;2*, *2;3* and *3;1* mainly in the shoots, primarily in new leaves, and relatively more in the stems than other genes. The expression patterns at the two different N requirement stages were the same; however, at the tillering stage with greater N requirements, the *OsAMTs* transcript levels were greater than those at the young seedling stage with low N requirements. N starvation for 48 h up-regulated *OsAMT1;1*, *1;2*, *3;1*, *3;2*, *3;3* and down-regulated *OsAMT1;3* mRNA abundance. Following N starvation, NH_4^+ and NH_4NO_3 re-supply down-regulated *OsAMT1;2* and *3;3* and up-regulated *OsAMT1;3*, whereas NO_3^- re-supply down-regulated *OsAMT1;1* and *1;2*. These suggested that the organ-specific expression pattern of *OsAMT* could be regulated by N requirement and external N status.

Key Words: external N level, gene expression, growth stage, N requirement, plant organ

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INTRODUCTION

Genes that encode ammonium transporters (AMT) have been identified in several plant species, such as *Arabidopsis thaliana* (Ninnemann *et al.*, 1994; Gazzarrini *et al.*, 1999; Sohlenkamp *et al.*, 2000, 2002; Loqué and von Wirén, 2004), *Lycopersicon esculentum* (Lauter *et al.*, 1996; von Wirén *et al.*, 2000), *Lotus japonicus* (Salvemini *et al.*, 2001; Simon-Rosin *et al.*, 2003; D'Apuzzo *et al.*, 2004), *Brassica napus* (Pearson *et al.*, 2002), and *Oryza sativa* (Sonoda *et al.*, 2003; Suenaga *et al.*, 2003). All plant AMT proteins investigated so far are located in the plasma membrane, suggesting that their role is in ammonium acquisition by plant cells (Ludewig *et al.*, 2002, 2003; Sohlenkamp *et al.*, 2002; Simon-Rosin *et al.*, 2003; Loqué *et al.*, 2006; Yuan *et al.*, 2007b). The physiological roles of *Arabidopsis* AMT genes in plants in mutant studies indicate that *AtAMT1;1*, *AtAMT1;3* and *AtAMT1;5* are highly expressed in N-deficient rhizodermal cells, including root hairs, and contribute additively to approximately 70%–80% of the high-affinity ammonium uptake capacity in roots (Loqué *et al.*, 2006; Yuan *et al.*, 2007b). *AtAMT1;2* appears to be responsible for uptake of ammonium that has entered root tissues by an apoplastic transport route (Yuan *et al.*, 2007a) and *AtAMT1;4* mediates ammonium uptake across the plasma membrane of pollen (Yuan *et al.*, 2009). In rice, at least 12 genes are predicted to encode AMT proteins (Li *et al.*, 2009). Heterologous expressions of *OsAMTs* in yeast cells showed that *OsAMT1s* and *OsAMT2;1* exhibit ammonium transport activity (Sonoda *et al.*,

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2003; Suenaga *et al.*, 2003). However, few studies have reported the involvement of other members of the AMT gene family in rice other than the three genes in the *OsAMT1* family (Suenaga *et al.*, 2003).

The transcript levels of *AtAMT1;1* and *OsAMT1;1* parallel their high-affinity transport system activity in roots, indicating that they are primarily responsible for NH_4^+ uptake (Gazzarrini *et al.*, 1999; Kumar *et al.*, 2003). The expression level of AMT may be regulated by N starvation, N type, or N level, but studies of AMT in rice have presented inconsistent results for different varieties, growth conditions, and experimental setups (Kumar *et al.*, 2003; Sonoda *et al.*, 2003). In addition, all studies to date have been on young seedlings and small plants, which have an undeveloped root structure and low N requirement; however, the expression pattern of *OsAMT* genes on tillering and bigger plants, which have more developed roots with a higher N requirement, is unknown. Furthermore, expression patterns of the *OsAMT2* and *OsAMT3* families in response to N have not been studied.

In order to gain better understanding of the molecular basis of the expression pattern of *OsAMT* genes, we adopted a rice cultivar with high nitrogen use efficiency, Guidan 4 (japonica rice). The biomass and N accumulation of this rice cultivar was markedly elevated with growth in seedlings (Shi *et al.*, 2010) including young seedling stage and tillering stage. We monitored the expression pattern of nine *OsAMT* genes (three members each of the *OsAMT1*, *2*, and *3* families) in rice plants at two different N requirement stages (young seedling stage and tillering stage) and their responses to external N status, in order to know well the expression pattern of *OsAMTs* to regulate ammonium transport or uptake using corresponding genes available under corresponding external conditions.

MATERIALS AND METHODS

Growth conditions and treatments

Rice (*Oryza sativa* L. cv. Guidan 4) seeds were sterilized in 1% (v/v) NaClO for 20 min and allowed to imbibe for 48 h in sterile distilled water. Seeds were grown hydroponically in tap water for another 5 d, and then transferred to modified Kimura nutrient solution (0.5 mmol L^{-1} NH_4NO_3, 0.18 mmol L^{-1} $KH_2PO_4{\cdot}2H_2O$, 0.18 mmol L^{-1} KCl, 0.37 mmol L^{-1} $CaCl_2{\cdot}2H_2O$, 0.55 mmol L^{-1} $MgSO_4{\cdot}7H_2O$, 20 μmol L^{-1} $FeSO_4{\cdot}7H_2O$-EDTA, 50 μmol L^{-1} H_3BO_3, 9 μmol L^{-1} $MnCl_2{\cdot}4H_2O$, 0.3 μmol L^{-1} $CuSO_4{\cdot}5H_2O$, 0.7 μmol L^{-1} $ZnSO_4{\cdot}7H_2O$, and 0.1 μmol L^{-1} $H_2MoO_4{\cdot}4H_2O$) before treatment. Because co-provision of NH_4^+ and NO_3^- may improve rice growth (Chen *et al.*, 1998; Kronzucker *et al.*, 1999), NH_4NO_3 was used in the pre-culture stage. Nitrification was inhibited by addition of 5.89 mg L^{-1} $C_2H_4N_4$ (Shanghai Chemical Co., China). The solution pH was adjusted to 5.5 by adding diluted HCl or NaOH, and nutrient solutions were aerated and changed every three days. Rice plants were cultivated in a growth chamber with a 16 h/8 h day/night light cycle, light intensity of 350 μmol m^{-2} s^{-1}, 60% relative humidity, and temperature of 26±1 °C.

Nitrogen starvation (N-deficient treatment) involved transferring plants to a no-N solution and was performed for 48 h on 3-week-old (young seedling) or 6-week-old (tillering) rice plants. A control (N-sufficient treatment) was also performed using modified Kimura solution with 0.5 mmol L^{-1} NH_4NO_3 as the N source. Samples of whole roots, mature roots (1–6 cm), new roots (0–1 cm), stems, new leaves and old leaves were collected from the N-sufficient (0.5 mmol L^{-1} NH_4NO_3) and N-deficient treatments. At the same time, P- and K-deficient treatments were performed for 48 h on young seedlings. Following 48 h of N starvation, the young seedlings were transferred to solutions with different proportions of ammonium/nitrate (100:0, 50:50, 0:100), with a total N level of 1 mmol L^{-1} for 2 h as an N re-supply treatment. Whole root samples in the P- and K-deficient and N re-supply treatments were collected. All samples were snap-frozen in liquid nitrogen, and stored at −80 °C. Each treatment was triplicated, and the whole experiment was repeated at least twice.

RNA isolation and analyses

Total RNA from different samples was extracted using TRIzol (Takara, Japan) according to the manufacturer's instructions, followed by DNase I (RNAase-free) treatment to remove residual genomic DNA. RNA was quantified by measuring optical density (OD) at 230 (OD_{230}), 260 (OD_{260}), and 280 (OD_{280}) nm with a UV/Visible spectrophotometer (Helios, Thermo Spectronic, UK). Only RNA samples with an OD_{260}/OD_{280} ratio (an indication of protein contamination) between 1.7 and 1.9 and an OD_{260}/OD_{230} ratio (an indication of reagent contamination) greater than 2.0 were used for the analysis. To verify RNA quality, 10 μg total RNA from each sample was subjected to agarose-formaldehyde gel electrophoresis using standard protocols, stained with ethidium bromide, and visualized under ultra-violet (UV) light. The presence of intact

28S and 18S rRNA bands was used as a criterion of RNA integrity. RNA concentration (ng μL^{-1}) was also calculated as $OD_{260} \times 40 \times$ dilution factors. cDNA was prepared from 5 μg total RNA according to the manufacturer's instructions (Clontech, USA) and then diluted 1:10 for real-time reverse transcription polymerase chain reaction (RT-PCR).

Real-time RT-PCR reactions and mRNA level analysis

Primer pairs were designed using Primer 5 software (PRIMER-E, Ltd., UK) (Table I). To ensure that the primers amplified the right cDNA segment, each pair of primers was checked in the BLAST program against the rice genomic sequence available in the NCBI database. If possible, the primers were designed to span intron sequences to detect any genomic contamination. The diluted cDNA samples were used as a template for real-time PCR analysis, using the Opticon Monitor2 System and software (Bio-Rad, USA) according to the method of Xu and Shi (2006). PCR reactions used the following parameters: 1 min at 95 °C, 40 cycles of 30 s at 95 °C, 45 s at 60 °C, and 45 s at 72 °C in 96-well optical reaction plates. The quality of PCR products was visually inspected using electrophoresis; the generation of a single band of the expected size was taken as the criterion for specificity. The identity of PCR products was confirmed by direct DNA sequencing. The relative mRNA levels of the individual *OsAMT* genes in different RNA samples were computed with respect to an internal standard, *OsActin*, a housekeeping gene, to normalize for variance in the quality of RNA and the amount of input cDNA. *β-tubulin* was used as a control gene to normalize the RT-PCR results. At least two different RNA isolations and cDNA syntheses were used for quantification for each treatment, and each cDNA was measured in duplicate.

The copy number of each gene was determined using the Opticon Monitor 2.02 software (Bio-Rad, USA). *OsActin* mRNA was defined as 100 relative expression units (REU). The expression level of all genes corresponded to the ratio of the copy number of cDNA of the studied gene to the copy number of *OsActin* multiplied by 100 REU.

Generation of transgenic rice plants

The promoters of *OsAMT1;2* were amplified using the specific primers (forward: 5′-AAGCTTGCAAGGATGCGAGGAGATAC-3′; reverse: 5′-ATGGATCCAGCCAAGTGTGGCAAGGT-3′), subcloned into pDrive (Qiagen, Germany), and then placed in a plant expressing vector containing the β-glucuronidase (GUS) gene. Transgenic rice plants were generated by Agrobacterium-mediated transformation of rice calli (*Oryza sativa* L. cv. Guidan 4) according to the protocol of electrotransformation. Plants were regenerated from transformed calli by selecting for hygromycin resistance. Regenerated transgenic rice plants were grown in a greenhouse at 28 °C. Three transgenic lines, P3, P6 and P10, were randomly selected and propagated, and the seeds were harvested separately for identification by real-time RT-PCR. Seeds from the transgenic rice plants were used to grow rice plants for fluorometric GUS assay.

Fluorometric GUS assay

The transgenic lines selected were grown in the presence of 0.5 mmol L^{-1} NH_4NO_3 for 4 weeks and then transferred to a nutrient solution with or without N for 48 h. Then, one root from each seedling was sampled and dyed with X-Gluc. Fluorometric analysis

TABLE I

Genes and gene-specific primers used for the real-time reverse transcription polymerase chain reaction analysis

Gene	Primer used		Length	GenBank accession number
	Forward (5′-3′)	Reverse (5′-3′)		
			bp	
OsAMT1;1	GTCATCTTCGGGTGGGTCAGCT	TTCGCTGTGACGTCGTTCGTTC	282	AF289477.1
OsAMT1;2	ATGGCGACGTGCTTGGACAG	CGAACACGTTGGTGAGCATG	232	AF289478.1
OsAMT1;3	GCAAGGAGTACGTGGACCAGA	AGATGCGCAGCAATCCCAGCT	180	AF289479.1
OsAMT2;1	GCGTTCGTGATCGCGTGGA	TAGAGCTGGATGGTGACGC	269	AB051864.1
OsAMT2;2	GCTCTTCGTCGTCGTGTGGA	TACAGCTGAATCGTGACTCCTC	275	AP003252.4
OsAMT2;3	ATTGCCCGATCCCGAACATG	CTCCCGTCCTCGTCGTCTCC	200	AP003252.4
OsAMT3;1	CTCCCGCAGACGACGCAGTT	GCCGACGGTGTAGGAGAAGGTG	225	AB083582.1
OsAMT3;2	CTCACCTTCTCCTACACCGTC	ACCCCATCCATAGTAACCCTG	228	AC104487.3
OsAMT3;3	GCTGGCGCACTATTTGTCA	CATTCTGTGTCACTCCTACA	229	AP004775.3
OsActin	CTTCATAGGAATGGAAGCTGCGGGTA	CGACCACCTTGATCTTCATGCTGCTA	197	AB047313.1
β-tubulin	TGCCTCCAAGGATTTCAAGTCTGC	TTGTAAGGTTCCACCACGGTATCAG	167	X79367

of GUS activity was performed as described by Jefferson *et al.* (1987), using 4-methylumbelliferyl-β-D-glucuronide as a substrate. Protein concentrations in the extracts were measured according to Bradford (1976). GUS activity is presented as nmol 4-methylumbelliferone (4-MU) mg^{-1} protein min^{-1}.

Statistical analysis

The data were analyzed using the statistical software program SPSS version 10.0. Significant differences between treatments were determined by one-way analysis of variance (ANOVA) and post hoc comparisons were carried out using Tukey's multiple range test at $P < 0.05$.

RESULTS

$^{15}NH_4^+$ influx in rice roots under N deficiency

To examine the time of N deficiency for inducing optimum ammonium transport systems, we grew rice plants hydroponically without an N source for a range of time, and measured influxes of $^{15}NH_4^+$ using 50 μmol L^{-1} ^{15}N-labeled $(NH_4)_2SO_4$. The maximum high-affinity $^{15}NH_4^+$ influx appeared after 48-h N deficiency (Fig. 1). The changes in *AtAMT1;1* and *OsAMT1;1* transcript levels paralleled those of high-affinity transport system activity following changes in N supply to plants (Gazzarrini *et al.*, 1999; Kumar *et al.*, 2003).

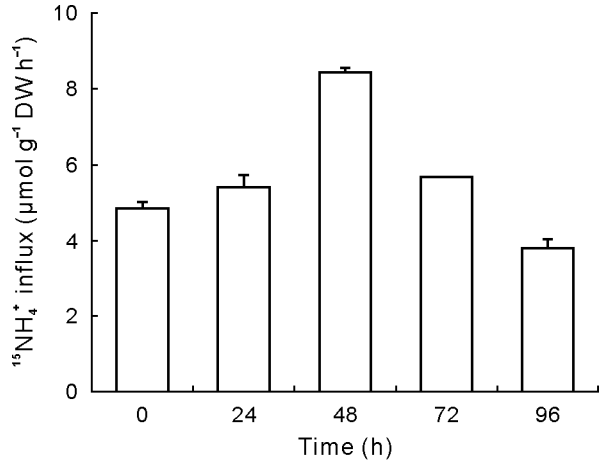

Fig. 1 Time dependence of $^{15}NH_4^+$ influx into plant roots by the high-affinity transport system. Plants were grown in the presence of 0.5 mmol L^{-1} NH_4NO_3 for 4 weeks and then transferred to nutrient solution without nitrogen for 96 h. Root $^{15}NH_4^+$ influx was measured with 50 μmol L^{-1} ^{15}N-labelled $(NH_4)_2SO_4$ at 24, 48, 72, and 96 h. The values are means ± standard deviations ($n = 3$). DW = dry weight.

OsAMT gene family in rice

Twelve protein sequences encoding *OsAMTs* were obtained from the GenBank database. The phylogenetic tree (Fig. 2) showed that *OsAMT* gene family fell into two major groups. The first group, including three genes each of *OsAMT2* and *OsAMT3*, one of *OsAMT4*, and two of *OsAMT5*, had 55.6%–72.5% homology to *AtAMT2*, and the other group, including three genes of *OsAMT1*, had 72.4%–78.3% homology to *AtAMT1;1*. Furthermore, the phylogenetic tree places *OsAMT1;1*, *1;2* and *1;3* together, *OsAMT2;1*, *2;2* and *2;3* together, *OsAMT3;1*, *3;2* and *3;3* together, and *OsAMT5;1* and *AMT5;2* together. These results revealed the evolutionary relationships of *OsAMT* proteins in rice.

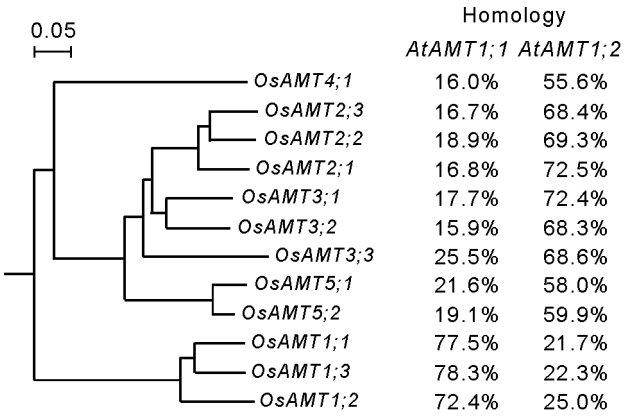

Fig. 2 A phylogenetic tree of protein sequences encoded by *OsAMT* gene family of rice. The GeneBank accession numbers for the *OsAMTs* except *OsAMT4;1* (AC091811), *OsAMT5;1* (NC_008405) and *OsAMT5;2* (NC_008404), are listed in Table I. The dendrogram was generated with the DNAMAN software (version 6.0, Lynnon Biosoft Company, USA) using the neighbour-joining method.

Expression pattern of OsAMT genes in rice

To investigate the expression pattern of *OsAMT* genes, relative expression units (REUs) were measured for the nine *OsAMT* genes in different organs of rice at two different N requirement stages of rice growth, the young seedling and tillering stages. To avoid bias in real-time PCR analysis, a control gene, *β-tubulin*, was used as a reference in different organs of different treatments. Ideally, expression of this control gene should be uniform in all organs studied and under all experimental conditions. The results showed that there was no significant change in the expression of *β-tubulin* in different organs under N sufficiency and N deficiency conditions (Fig. 3). As shown in Fig. 4, all nine *OsAMT* genes were expressed and exhibited similar patterns of expression at the two growth stages, although the absolute transcript levels were different between the two stages. *OsAMT1;1*, *3;2* and *3;3* were expressed in the roots and shoots, mainly old leaves, and only slightly in the stems, *OsAMT1;2* and *1;3* mainly in the roots,

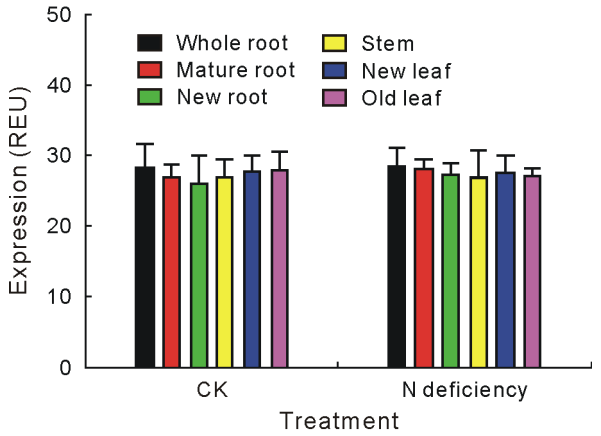

Fig. 3 Expression of *β-tubulin* in rice seedlings under N sufficiency (control, CK) and 48-h N deficiency conditions. Relative expression units (REU) were calculated and normalized with respect to *OsActin* mRNA (100 REU). The values are means ± standard deviations ($n = 4$).

and *OsAMT2;1*, *2;2*, *2;3* and *3;1* mainly in the shoots, primarily new leaves, and relatively more in the stems than other genes. At the tillering stage, the transcript levels of most *OsAMTs* increased significantly, as compared to those at the seedling stage (Fig. 4). The expression levels of *OsAMT1;1*, *OsAMT3;2* and *OsAMT3;3* were enhanced in the roots and leaves at tillering stage, by about 2.0 folds, except that *OsAMT3;2* showed a 3.3-fold decrease in the old leaves at tillering stage. The expression level of *OsAMT1;2* was enhanced greatly in the roots, by 12.6 folds in the whole roots, 7.1 folds in the mature roots, and 22.9 folds in new roots, and was also increased in the new leaves by 4.1 folds. *OsAMT1;3* showed decreased expression levels in the roots, by 2.5 folds in the mature roots and 3.3 folds in the new roots, and increased expression levels by 2.8 folds in the stems and 2.3 folds

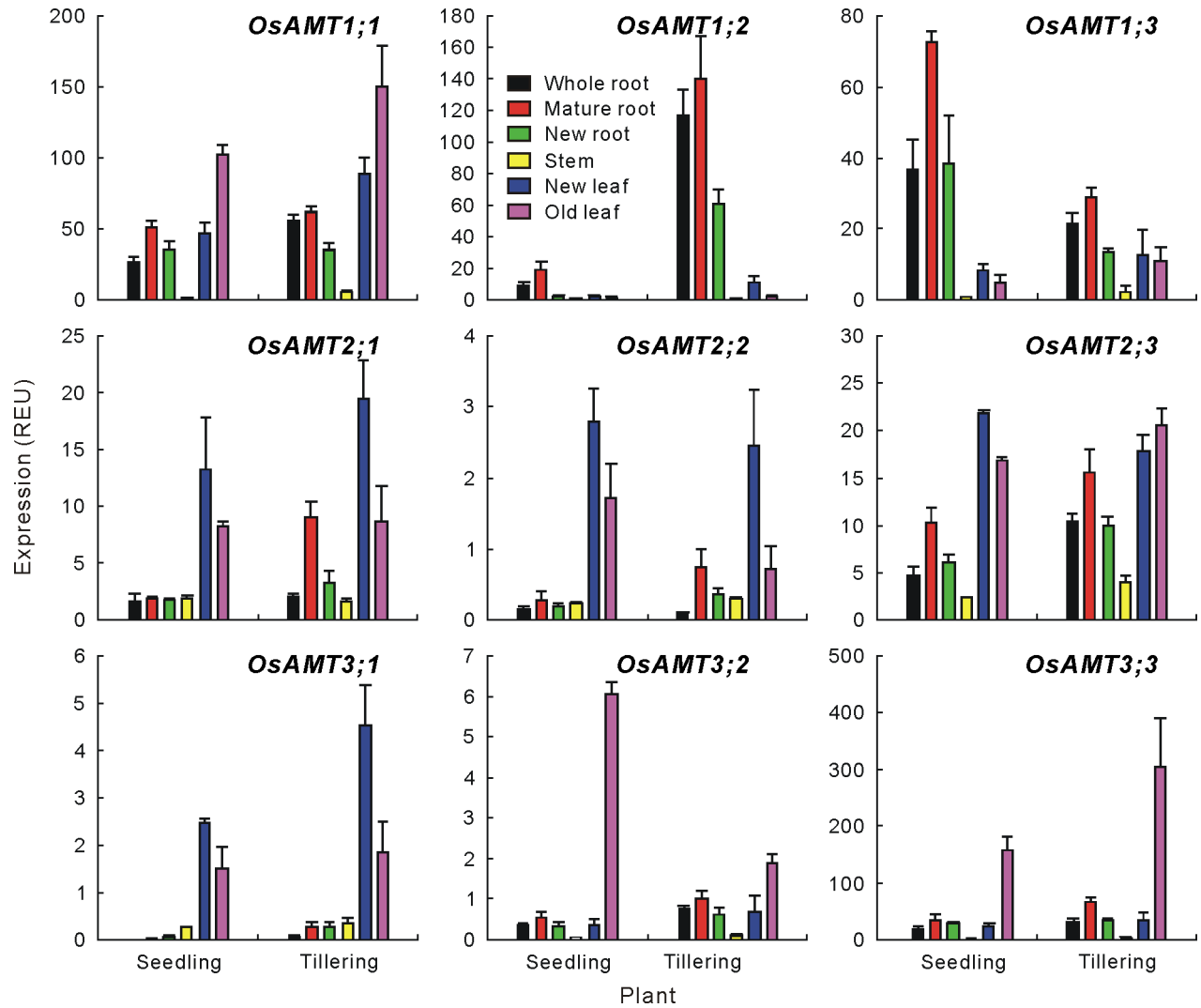

Fig. 4 Expression of *OsAMT* gene family members in different organs of young seedlings (3 weeks old) and tillering (6 weeks old) rice plants. Relative expression units (REU) were calculated and normalized with respect to *OsActin* mRNA (100 REU). The values are means ± standard deviations ($n = 4$).

in the old leaves. The expression of *OsAMT2;1* and *2;2* increased significantly in the mature roots by 4.8 and 2.6 folds, respectively. *OsAMT2;3* expression increased by 2.2 folds in the new roots. *OsAMT3;1* expression increased significantly in the roots, irrespectively of the root segments, by 6.7, 11.2, and 3.5 folds in the whole roots, mature roots, and new roots, respectively. The transcript levels of the nine genes for the two different growth stages are summarized in Table II. In general, the expression levels of *OsAMT1;1*, *1;2*, *1;3* and *3;3* appeared to be high, those of *OsAMT2;1* and *2;3* were moderate, and those of *OsAMT2;2*, *3;1* and *3;2* were very low.

Regulation of OsAMTs expression by N status

The high-affinity ammonium transport system influx of $^{15}NH_4^+$ was rapidly up-regulated by N starvation for 48 h. The total transcript levels of nine *OsAMT* genes were increased in the whole roots of the young rice seedlings (3 weeks old); however all genes responded differently to 48 h of N deficiency. The expression of *OsAMT1;1* increased by 2.1 folds and that of *OsAMT1;2* increased by 7.8 folds, while *OsAMT1;3* expression decreased by 3.4 folds. The transcript levels of *OsAMT2;1*, *2;2* and *2;3* were relatively stable and those of *OsAMT3;1*, *3;2* and *3;3* were induced by 3.5, 3.5 and 2.9 folds, respectively.

Moreover, when the young seedlings were transferred to solutions that were deficient in P or K for 48 h, only the transcription levels of *OsAMT1;3* and *3;3* in the whole roots were significantly modified. P starvation up-regulated *OsAMT1;3* expression and K deficiency down-regulated *OsAMT3;3* expression (Table III). This indicated that the transcript levels of *OsAMTs* were regulated specifically by N-deficiency stress, but not by other macronutrient stresses. Following N starvation for 48 h, N was re-supplied to the young rice seedlings by provision of nutrient solutions containing either no N, 0.5 mmol L^{-1} $(NH_4)_2SO_4$, 0.5 mmol L^{-1} NH_4NO_3, or 1 mmol L^{-1} KNO_3 as the sole N source, and roots and shoots were harvested separately after 2 h for analysis of mRNA accumulation of the nine *OsAMT*s in the roots. Overall, only one gene (*OsAMT1;3*) was significantly up-regulated in response to N (NH_4^+ and NH_4NO_3) re-supply. The transcript levels of three genes (*OsAMT1;1*, *1;2* and *3;3*) decreased after N re-supply: *OsAMT1;1* only by NO_3, *OsAMT1;2* by NH_4^+, NH_4NO_3 and NO_3^-, and *OsAMT3;3* only by NH_4^+. The mRNA abundance of *OsAMT1;2* was reduced greatly, by 8.8 and 5.4 folds upon re-supply of NH_4NO_3 and NO_3^-, respectively, consistent with the previous results (Li and Shi, 2006). Thus, the transcript of *OsAMTs* was influenced by specific N deficiency and the expression of *OsAMTs* was influenced by re-supply of N in the same manner as N starvation.

Expression of OsAMT1;2-GUS induced by N starvation

As mentioned above, the expression of *OsAMT1;2*

TABLE II

Expression of *OsAMT* gene family members in different organs of young seedling (3 weeks old) rice plants and the fold changes of expression of the tillering (6 weeks old) compared to young seedling rice plants

Stage	Plant organ[a)]	Gene								
		OsAMT1;1	*OsAMT1;2*	*OsAMT1;3*	*OsAMT2;1*	*OsAMT2;2*	*OsAMT2;3*	*OsAMT3;1*	*OsAMT3;2*	*OsAMT3;3*
		Expression (REU)[b)]								
Young seedling	WR	26.0	9.3	36.6	1.6	0.1	4.8	0.0	0.3	18.8
	MR	51.2	19.6	72.8	1.9	0.3	10.3	0.0	0.5	33.6
	NR	35.4	2.6	38.7	1.7	0.2	6.1	0.1	0.3	28.8
	S	1.9	1.0	0.8	1.9	0.2	2.4	0.3	0.1	1.5
	NL	46.0	2.8	8.3	13.2	2.8	21.9	2.5	0.4	24.5
	OL	101.5	1.4	4.7	8.2	1.7	16.9	1.5	6.1	157.1
		Fold change[c)] *of expression compared to the young seedling stage*								
Tillering	WR	2.1[d)]	12.6[d)]	−1.7	1.2	−1.4	2.2[d)]	6.7[d)]	2.2[d)]	1.7
	MR	1.2	7.1[d)]	−2.5[d)]	4.8[d)]	2.6[d)]	1.5	11.2[d)]	1.9	2.0[d)]
	NR	1.0	22.9[d)]	−3.3[d)]	1.9	2.0[d)]	1.6	3.5[d)]	1.9	1.2
	S	3.0[d)]	1.3	2.8[d)]	−1.2	1.2	1.6	1.3	1.8	2.5[d)]
	NL	1.9	4.1[d)]	1.6	1.5	−1.1	−1.2	1.8	1.9	1.5
	OL	1.5	1.7	2.3[d)]	1.0	−2.5[d)]	1.2	1.2	−3.3[d)]	1.9

[a)] WR = whole roots; MR = mature roots; NR = new roots; S = stem; NL = new leaves; OL = old leaves.
[b)] Relative expression units (REU) were calculated and normalized with respect to *OsActin* mRNA (100 REU).
[c)] Calculated by dividing the expression of an *OsAMT* gene in an organ at the tillering stage by that of the young seedling stage.
[d)] Significantly different changes ($P \leq 0.05$) as well as changes greater than 2 folds as compared with the seedling stage.

TABLE III

Fold changes[a)] in transcript levels of *OsAMT* genes in the whole roots of the young seedling (3 weeks old) rice plants grown under nutrient deficiency relative to the control with N supply at 0.5 mmol L^{-1} NH_4NO_3 and those under re-supply of 1 mmol L^{-1} different N forms following N deficiency relative to the control without N re-suply

Gene	Nutrient deficiency			Re-supply after N deficiency		
	N	P	K	NH_4^+	NH_4NO_3	NO_3^-
OsAMT1;1	2.1[b)]	1.4	1.0	1.2	−1.2	−2.7[b)]
OsAMT1;2	7.8[b)]	−1.6	−1.4	−2.6[b)]	−8.8[b)]	−5.4[b)]
OsAMT1;3	−3.4[b)]	2.3[b)]	1.5	2.9[b)]	2.1[b)]	1.5
OsAMT2;1	1.3	1.1	−1.2	1.3	1.4	−1.5
OsAMT2;2	−1.1	−1.1	−1.1	−1.7	−1.8	−1.2
OsAMT2;3	−1.1	−1.1	−1.1	−1.7	−1.8	−1.2
OsAMT3;1	3.5[b)]	1.1	−1.1	1.6	−1.1	1.2
OsAMT3;2	3.5[b)]	−1.6	−1.8	1.8	−1.5	−1.5
OsAMT3;3	2.9[b)]	−1.5	−2.0[b)]	−4.7[b)]	−2.7[b)]	1.3

[a)] Calculated by dividing the gene transcript level of each treatment by that of the control.
[b)] Significantly different changes ($P \leq 0.05$) as well as changes greater than 2 folds as compared to the control.

was induced steeply by high N requirement and N starvation, the regulation of expression by N starvation was examined with β-glucuronidase (GUS) used as a reporter gene. As shown in Fig. 5, *OsAMT1;2* could direct the GUS reporter gene in rice seedling roots and the GUS activities were significantly enhanced by N starvation in transgenic rice seedling roots.

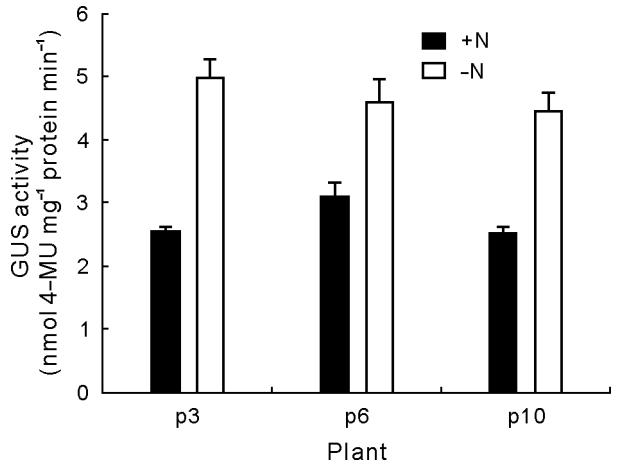

Fig. 5 β-glucuronidase (GUS) activity of *OsAMT1;2* promoter-GUS in response to N starvation. Transgenic rice plants (p3, p6 and p10) were grown in the presence of 0.5 mmol L^{-1} NH_4NO_3 for 4 weeks and then transferred to nutrient solutions with (+N) or without (−N) nitrogen for 48 h. Total proteins were extracted from the roots and GUS activity was measured by fluorometric assay. Values are means ± standard deviations ($n = 3$). 4-MU = 4-methylumbelliferone.

DISCUSSION

The expression patterns of gene family members are not completely in agreement with the phylogenetic relationships of gene families (Orsel *et al.*, 2002; Mladek *et al.*, 2003; Xu and Shi, 2006). Our results also showed that the expression pattern of *OsAMT* family members in response to external N status was not strictly related to the position of the corresponding proteins within the phylogenetic tree (Fig. 4, Table II). In one case, all nine *OsAMTs* in three families were expressed in the whole roots, new roots, mature roots, stems, old leaves, and new leaves. However, preferential expression differed within each family, where *OsAMT1;1*, *3;2* and *3;3* were expressed in the roots and shoots, primarily old leaves, and only slightly in the stems, *OsAMT1;2* and *1;3* mainly in the roots, *OsAMT2;1*, *2;2*, *2;3* and *3;1* in the shoots, primarily the new leaves, and relatively more in the stems than other genes. This suggests that *OsAMTs* have undergone different molecular evolution that has influenced protein sequences in rice.

Plants have evolved to regulate their transport systems depending on nutritional conditions and developmental requirements. Rice has 12 AMT transporters that mediate high-affinity ammonium uptake. Such a large family may suggest that each member has a specific role in a specific period or that there is considerable functional redundancy. At different growth stages, the demand for N and the role of each transporter in N transport or uptake differ. Comparing the tillering stage to the young seedling stage, the transcript level of most of *OsAMTs* was significantly up-regulated, except that of *OsAMT1;3* which was down-regulated. At the tillering stage, *OsAMT1;1*, *1;2*, *2;3* and *3;2* showed increased expression in whole roots, *OsAMT1;2*, *2;2* and *3;1* in the new roots, and *OsAMT1;2*, *2;1*, *2;2*, *3;1* and *3;3* in the mature roots, as compared to the young seedling stage. *OsAMT1;2* showed greatly increased (22.9 folds) expression in the roots, suggesting that it may serve a vital function in NH_4^+ transport or uptake within the plant because the

demand for N at the tillering stage is greater than that at the young seedling stage. The expression of *OsAMT1;3* decreased in the roots but increased in the leaves; therefore, it might function in ammonium recycling during leaf senescence or photorespiration. These results indicated that with greater N requirements rice needed enhanced expression of *OsAMTs* and specific functions of the particular *OsAMT* genes.

The expression of *OsAMTs* also exhibited diverse regulation patterns in response to external N status. *OsAMT1;1* was expressed in the roots and shoots and its expression increased by 2.1 folds in response to N deficiency. *OsAMT1;2* was mainly expressed in the roots and its expression increased by 7.8 folds by N deficiency. *OsAMT1;3* was mainly expressed in the roots. The GUS activity was enhanced significantly in the transgenic rice plant roots, which indicated that the promoter of *OsAMT1;2* responded to N starvation. These results are in agreement with those of the study by Sonoda *et al.* (2003), where a northern blot analysis showed that *OsAMT1;1* showed constitutive expression in the shoots and roots and is little affected by N starvation, *OsAMT1;2* is a root-specific transporter, whereas *OsAMT1;3* displays limited expression as a root-specific and N deficiency-inducible transporter. However, the expression of *OsAMT1;1* and *1;2* in the roots of indica rice was rapidly up-regulated by the change from high N supply to low N and the change of *OsAMT1.2* was approximately 50% less than that of *OsAMT1.1* (Kumar *et al.*, 2003). The difference in the expression pattern of *AMT1* genes in rice observed between this study and the previous reports may be attributed to the use of different rice genotypes (Shi *et al.*, 2010) and that the expression might be regulated by the balance between ammonium and nitrate both in the external medium and inside the plant. This study first detailed the expression patterns of the *OsAMT2* and *3* families in a single experiment and reinforced previous reports on *OsAMT* expression profiles. *OsAMT2;1, 2;2* and *2;3*, which were expressed in the roots and shoots, primarily in new leaves, were constitutive, irrespective of the supply of external N. *OsAMT3;1*, *3;2* and *3;3* were expressed in the roots and shoots and their transcript in the roots was up-regulated by N deficiency. Moreover, P and K starvation displayed different regulation pattern from N deficiency and the effect was only on a limited number of genes. Following N starvation for 48 h and subsequent re-supply of N for 2 h, the expression of *OsAMT1;1* was reduced only by nitrate, and no significant up-regulation was found for ammonium. The expression of *OsAMT1;2* was down-regulated by external N status, irrespective of the form of N supplied, and to an even larger extent by nitrate compared to ammonium, consistent with the previous results (Sonoda, 2003; Li and Shi, 2006). The expression of *OsAMT1;3* was significantly up-regulated in response to N re-supply and enhanced to a significant extent by ammonium, which was consistent with the study of Sonoda (2003). The expression of *OsAMT3;3* decreased when NH_4^+ was present. These results indicated that the transcription level of *OsAMTs* genes was regulated by external N status and the effect of external N status on the expression regulation was specific. However, expression studies only give hints to gene function.

It is still unclear whether the expression level of these genes is related to uptake function associated with ammonium transport within the plant, although direct evidence from insertion of T-DNA to disrupt *AtAMT1;1 (amt1;1:T-DNA)* demonstrates a 30% decrease in high-affinity ammonium influx compared to the wild type (Kaiser *et al.*, 2002; Loqué *et al.*, 2006). Moreover, a double insertion line for *AtAMT1;1* and *AtAMT1;3* showed that the additive contribution of both transporters led to 60%–70% lower transport activity than in the wild type (Loqué *et al.*, 2006). Additionally, a quadruple mutant *amt1;1 amt1;2 amt1;3 amt2;1* line (*qko*) showed severe growth depression under ammonium supply and maintained only 5%–10% of wild type high-affinity ammonium uptake capacity (Yuan *et al.*, 2007a). On the other hand, overexpression of *OsAMT1;1* in rice retarded plant growth and led to ammonium toxicity in tissues due to the lack of a concomitant increase in ammonium assimilation (Hoque *et al.*, 2006), and net uptake of NH_4^+ was greater than in the wild type (Kumar *et al.*, 2006). This suggested that the expression level of a gene may in fact reflect the gene function in the plant.

The results of the present study demonstrated that the expression of *OsAMT1;2* was induced by 22.9 folds in the new roots at the tillering stage as compared to the young seedling stage (Fig. 4, Table II). In addition, it was up-regulated (7.8-fold increase) at the young seedling stage in response to N starvation for 48 h. A 1 516-bp promoter of *OsAMT1;2* was able to drive GUS and was significantly up-regulated by N starvation. These results suggested that *OsAMT1;2* was an N sensor as in a previous report (Yao *et al.*, 2008). The existence of N-inducible AMTs tempts speculation that these genes may have greater transport capacity than their N-stable counterparts. Characterising AMT promoters specific to the N deficiency response, such as the *HvPht1* promoter (Schünmann *et al.*, 2004), and generation of mutants by T-DNA insertion, by RNAi,

or by over-expression may help to define in detail their functions in ammonium uptake or transport.

In such a complex system, the allocation of individual *OsAMT* to functions at the organ level is difficult but should be the first step. Firstly, *OsAMT* genes representing different expression patterns within the organs tested increased the complexity of the *OsAMT* gene family at the functional level. Secondly, the analysis of null or negative-dominant mutants, such as insertion of a T-DNA to disrupt *AtAMT1;1*, or the generation of mutant lines with multiple insertions such as *qko* (Yuan *et al*, 2007a) in the *OsAMT* genes, is necessary to elucidate the functions fulfilled by individual *OsAMT* gene. Finally, it is not enough to only elucidate the function of genes at the transcript level but characterization at the protein level will also be required in the future.

CONCLUSIONS

We used real-time RT-PCR to analyse in detail the expression patterns of the three *OsAMT* gene families in rice plants at young seedling and tillering stages and to describe how these genes respond to external N supply at the young seedling stage. The results demonstrated that the organ-specific expression pattern of *OsAMT1, 2* and *3* families was not related to the position of corresponding proteins in the phylogenetic tree. N requirement and external N status regulated the expression level of *OsAMTs*. High N requirement and N starvation up-regulated the expression of *OsAMT1;2*, and down-regulated the expression of *OsAMT1;3*.

REFERENCES

Bradford, M. M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72**: 248–254.

Chen, J. G., Cheng, S. H., Cao, W. X. and Zhou, X. 1998. Involvement of endogenous plant hormones in the effect of mixed nitrogen sources on growth and tillering in wheat. *J. Plant Nutr.* **21**: 87–97.

D'Apuzzo, E., Rogato, A., Simon-Rosin, U., Alaoui, H. E., Barbulova, A., Betti, M., Dimou, M., Katinakis, P., Marquez, A., Marini, A. M., Udvardi, M. K. and Chiurazzi, M. 2004. Characterization of three functional high-affinity ammonium transporters in *Lotus japonicus* with differential transcriptional regulation and spatial expression. *Plant Physiol.* **134**: 1763–1774.

Gazzarrini, S., Lejay, L., Gojon, A., Ninnemann, O., Frommer, W. B. and von Wirén, N. 1999. Three functional transporters for constitutive, diurnally regulated, and starvation-induced uptake of ammonium into Arabidopsis roots. *Plant Cell.* **11**: 937–947.

Hoque, M. S., Masle, J., Udvardi, M. K., Ryan. P. R. and Upadhyaya, N. M. 2006. Over-expression of the rice OsAMT1-1 gene increases ammonium uptake and content, but impairs growth and development of plants under high ammonium nutrition. *Funct. Plant Biol.* **33**: 153–163.

Jefferson, R. A., Kavanagh, T. A. and Bevan, M. W. 1987. GUS fusions: Beta-glucuronidase as a sensitive and versatile gene fusion marker in higher plants. *EMBO J.* **6**: 3901–3907.

Kaiser, B. N., Rawat, S. R., Siddiqi, M. Y., Masle, J. and Glass, A. D. M. 2002. Functional analysis of an Arabidopsis T-DNA "knockout" of the high-affinity NH_4^+ transporter $AtAMT_{1;1}$. *Plant Physiol.* **130**: 1263–1275.

Kronzucker, H. J., Siddiqi, M. Y., Glass, A. D. M. and Kirk, G. J. D. 1999. Nitrate ammonium synergism in rice. A subcellular flux analysis. *Plant Physiol.* **119**: 1041–1046.

Kumar, A., Kaiser, B. N., Siddiqi, M. Y. and Glass, A. D. M. 2006. Functional characterization of *OsAMT1.1* overexpression lines of rice, *Oryza sativa*. *Funct. Plant Biol.* **33**: 339–346.

Kumar, A., Silim, S. N., Okamoto, M., Siddiqi, M. Y. and Glass, A. D. M. 2003. Differential expression of three members of the AMT1 gene family encoding putative high-affinity NH_4^+ transporters in roots of *Oryza sativa* subspecies indica. *Plant Cell Environ.* **26**: 907–914.

Lauter, F. R., Ninnemann, O., Bucher, M., Riesmeier, J. W. and Frommer, W. B. 1996. Preferential expression of an ammonium transporter and of two putative nitrate transporters in root hairs of tomato. *Proc. Natl. Acad. Sci. USA.* **93**: 8139–8144.

Li, B. Z., Mike, M., Li, S. M., Li, H. Y., Zhu, S. W., Shi, W. M. and Su, Y. H. 2009. Molecular basis and regulation of ammonium transporter in rice. *Rice Sci.* **16**: 314–322.

Li, S. M. and Shi, W. M. 2006. Quantitative characterization of nitrogen regulation of *OsAMT1;1*, *OsAMT1;2*, and *OsAMT2;2* expression in rice seedlings. *Russ. J. Plant Physiol.* **53**: 837–843.

Loqué, D. and von Wirén, N. 2004. Regulatory levels for the transport of ammonium in plant roots. *J. Exp. Bot.* **55**: 1293–1305.

Loqué, D., Yuan, L. X., Kojima, S., Gojon, A., Wirth, J., Gazzarrini, S., Ishiyama, K., Takahashi, H. and von Wirén, N. 2006. Additive contribution of AMT1;1 and AMT1;3 to high-affinity ammonium uptake across the plasma membrane of nitrogen-deficient Arabidopsis roots. *Plant J.* **48**: 522–534.

Ludewig, U., von Wirén, N. and Frommer, W. B. 2002. Uniport of NH_4^+ by the root hair plasma membrane ammonium transporter LeAMT1;1. *J. Biol. Chem.* **277**: 13548–13555.

Ludewig, U., Wilken, S. and Wu, B. H., Jost, W., Obrdlik, P, Bakkoury, M. E., Marini, A. M., André, B., Hamacher, T., Boles, E., von Wirén, N. and Frommer, W. B. 2003. Homo- and hetero-oligomerization of ammonium transporter-1 NH_4^+ uniporters. *J. Biol. Chem.* **278**: 45603–45610.

Mladek, C., Guger, K. and Hauser, M. T. 2003. Identification and characterization of the *ARIADNE* gene family in Arabidopsis. A group of putative E_3 ligases. *Plant Physiol.* **131**: 27–40.

Ninnemann, O., Jauniaux, J. C. and Frommer, W. B. 1994. Identification of a high affinity NH_4^+ transporter from plants. *EMBO J.* **13**: 3464–3471.

Orsel, M., Krapp, A. and Vedele, F. D. 2002. Analysis of the NRT_2 nitrate transporter family in Arabidopsis. Structure and gene expression. *Plant Physiol.* **129**: 886–896.

Pearson, J. N., Finnegan, J. and Schjoerring, J. K. 2002. Regulation of the high-affinity ammonium transporter

(*BnAMT1;2*) in the leaves of *Brassica napus* by nitrogen status. *Plant Mol. Biol.* **49**: 483–490.

Salvemini, F., Marini, A. M., Riccio, A., Patriarca, E. J. and Chiurazzi, M. 2001. Functional characterization of an ammonium transporter gene from *Lotus japonicus*. *Gene.* **270**: 237–243.

Schünmann, P. H. D., Richardson, A. E., Smith, F. W. and Delhaize, E. 2004. Characterization of promoter expression patterns derived from Pht1 phosphate transporter genes of barley (*Hordeum vnlgare* L.). *J. Exp. Bot.* **55**: 855–865.

Shi, W. M., Xu, W. F., Li, S. M., Zhao, X. Q. and Dong, G. Q. 2010. Responses of two rice cultivars differing in seedling-stage nitrogen use efficiency to growth under low-nitrogen conditions. *Plant Soil.* **326**: 291–302.

Simon-Rosin, U., Wood, C. and Udvardi, M. K. 2003. Molecular and cellular characterization of LjAMT2;1, an ammonium transporter from the model legume *Lotus Japonicus*. *Plant Mol. Biol.* **51**: 99–108.

Sohlenkamp, C., Shelden, M., Howitt, S. and Udvardi, M. 2000. Characterization of *Arabidopsis* AtAMT2, a novel ammonium transporter in plants. *FEBS Lett.* **467**: 273–278.

Sohlenkamp, C., Wood, C. C., Roeb, G. W. and Udvardi, M. K. 2002. Characterization of Arabidopsis AtAMT2, a high-affinity ammonium transporter of the plasma membrane. *Plant Physiol.* **130**: 1788–1796.

Sonoda, Y., Ikeda, A., Saiki, S., von Wirén, N., Yamaya, T. and Yamaguchi, J. 2003. Distinct expression and function of three ammonium transporter genes (*OsAMT1;1–1;3*) in rice. *Plant Cell Physiol.* **44**: 726–734.

Suenaga, A., Moriya, K., Sonoda, Y., Ikeda, A., von Wirén, N., Hayakawa, T., Yamaguchi, J. and Yamaya, T. 2003. Constitutive expression of a novel-type ammonium transporter *OsAMT2* in rice plants. *Plant Cell Physiol.* **44**: 206–211.

von Wirén, N., Lauter, F. R., Ninnmann, O., Gillissen, B., Liu, P. W., Engels, C., Jost, W. and Frommer, W. B. 2000. Differential regulation of three functional ammonium transporter genes by nitrogen in root hairs and by light in leaves of tomato. *Plant J.* **21**: 167–175.

Xu, W. F. and Shi, W. M. 2006. Expression profiling of the 14-3-3 gene family in response to salt stress and potassium and iron deficiencies in young tomato (*Solanum lycopersicum*) roots: analysis by real-time RT-PCR. *Ann. Bot-London.* **98**: 965–974.

Yao, S. G., Sonoda, Y., Tsutsui, T., Nakamura, H., Ichikawa, H., Ikeda, A. and Yamaguchi, J. 2008. Promoter analysis of *OsAMT1;2* and *OsAMT1;3* implies their distinct roles in nitrogen utilization in rice. *Breeding Sci.* **58**: 201–207.

Yuan, L. X., Graff, L., Loqué, D., Kojima, S., Tsuchiya, Y. N., Takahashi, H. and von Wirén, N. 2009. AtAMT1.4, a pollen-specific high-affinity ammonium transporter of the plasma membrane in Arabidopsis. *Plant Cell Physiol.* **50**: 13–25.

Yuan, L. X., Loqué, D., Kojima, S., Rauch, S., Ishiyama, K., Inoue, E., Takahashi, H. and von Wirén, N. 2007a. The organization of high-affinity ammonium uptake in *Arabidopsis* roots depends on the spatial arrangement and biochemical properties of AMT_1-type transporters. *Plant Cell.* **19**: 2636–2652.

Yuan, L. X., Loqué, D., Ye, F. H., Frommer, W. B. and von Wirén, N. 2007b. Nitrogen-dependence posttranscriptional regulation of the ammonium transporter AtAMT1;1. *Plant Physiol.* **143**: 732–744.