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Short Communication

Constitutive Expression of a Novel-Type Ammonium Transporter *OsAMT2* in Rice Plants

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To characterize ammonium transport pathways in rice, two cDNAs with high homology to MEP/AMT2-type ammonium transporters, *OsAMT2;1* and *OsAMT3;1*, were isolated. Expression of *OsAMT2;1* in an ammonium-uptake-defective yeast mutant showed that this gene encodes functional ammonium transporters. *OsAMT2;1* was constitutively expressed in both roots and shoots irrespective of the supply of inorganic nitrogen to the medium, whereas *OsAMT3;1* expression was relatively weak. A database search with the amino acid sequence of OsAMT2;1 showed that there are 10 putative *OsAMT* genes in rice, i.e. three each for *OsAMT1*, *OsAMT2* and *OsAMT3*, respectively, and one for *OsAMT4*.

Keywords: Ammonium transporter — *AMT2* — Antibody — Functional expression — Rice.

Abbreviations: AMT, ammonium transporter; HATS; high affinity transporter system; LATS, low affinity transporter system; RACE, rapid amplification of cDNA ends; RT-PCR, reverse transcription-PCR.

The nucleotide sequences reported in this paper have been submitted to DDBJ, EMBL, GenBank under accession numbers of AB051864 for OsAMT2;1 cDNA, AB083581 for the OsAMT2;1 gene, and AB083582 for OsAMT3;1 cDNA.

The major form of nitrogen that is available for growth of rice plants in paddy fields is NH_4^+, requiring NH_4^+ transport systems at the root plasma membrane. Also in plant metabolism NH_4^+ transport systems are required in several processes, which liberate and fix NH_4^+, such as the conversion of glycine to serine in the photorespiratory nitrogen cycle, the deamination of phenylalanine catalyzed by phenylalanine ammonia lyase in the phenylpropanoid pathway or the biosynthesis and catabolism of amino acids (Lea and Ireland 1999). The NH_3 molecule is a weak base that protonates rapidly to form NH_4^+ with a dissociation constant of $10^{-9.25}$ (Kleiner 1981). According to the equation proposed by Freney et al. (1985), 99.4% of the total ammonia is in the protonated form in water at pH 7.0 and 25°C, and the proportion of the ionic form increases as a function of decreasing pH. Thus, the protonated form, NH_4^+, is the major molecular species in paddy fields, as well as in the cytosol, in mitochondria, vacuoles and in the phloem and xylem sap of plants.

Physiological studies on NH_4^+ uptake in plant roots provided evidence for the existence of two transport systems for NH_4^+, a high-affinity transport system (HATS) and a low-affinity transport system (LATS) in plants (Karasawa et al. 1994, Kronzucker et al. 1996, Mäck and Tischner 1994, Wang et al. 1993). Ninnemann et al. (1994) first identified the gene encoding a high-affinity NH_4^+ transporter, AtAMT1;1, from *Arabidopsis thaliana* using functional complementation of a yeast mutant defective in NH_4^+ uptake. Since then, the isolation of further AMT1 homologs from *Arabidopsis thaliana* (*AMT1;1*, *AMT1;2*, *AMT1;3*; Gazzarrini et al. 1999), tomato (Lauter et al. 1996, von Wirén et al. 2000) and rice (von Wirén et al. 1997, Saiki et al. 2002) have shown that the AMT1 gene family in plants consists of at least three to five members. For LeAMT1;1 an NH_4^+ uniport has been demonstrated by heterologous expression in *Xenopus* oocytes, while for most of these AMT1 proteins act as functionality has been proved by complementation of NH_4^+ transport in yeast mutants (Ludewig et al. 2001, Gazzarrini et al. 1999, von Wirén et al. 2000). Except for LeAMT1;3 and AtAMT1;3, most AMT1 members were preferentially expressed in root tissues pointing to a function in NH_4^+ uptake. Recently, another type of ammonium transporter, *AMT2;1,* has been isolated from *Arabidopsis thaliana*. AtAMT2;1 is the only member in *Arabidopsis thaliana* of the MEP-like subfamily that is sequencewise more closely related to bacterial AMT and yeast MEP transporters (Sohlenkamp et al. 2000). Together with the Rhesus proteins from the animal kingdom, MEP and AMT transporters belong to the same superfamily of AMT-MEP-RH-proteins (Ludewig et al. 2001). Compared with other AMT1 genes, *AtAMT2;1* was highly expressed in shoots and in roots, leaving it open whether this

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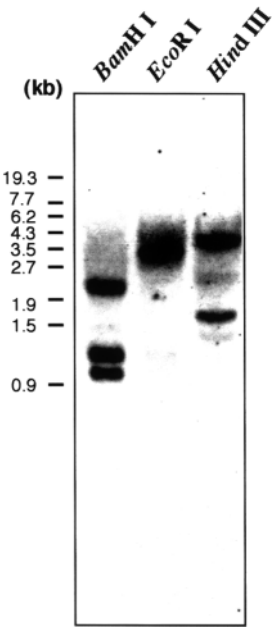

Fig. 1 DNA gel blot analysis of *OsAMT2* in the rice genome. Genomic DNA (10 μg) prepared from the shoots of 26-day-old seedlings was digested with *Bam*HI, *Eco*RI or *Hin*dIII. The digested DNA was electrophoresed through a 1% agarose gel, denatured, transferred to a nylon membrane, and probed with a 666 bp *OsAMT2*-EST (accession no. AU070277). The numbers at the left refer to the positions of DNA molecular markers in kilobases.

type of AMT might be related to the intra- or intercellular transport of NH_4^+ within or between adjacent cells. To better understand the organisation of NH_4^+ transport processes in rice, we isolated one AMT2 homolog and a more distantly related AMT, designated *OsAMT3;1*, from rice and investigated their functionality and transcriptional regulation.

An EST clone from rice leaf blades (AU070277; 666 bp long), which is homologous to *AtAMT2* (Sohlenkamp et al. 2000), was kindly provided by the Rice Genome Projects in the Ministry of Agriculture, Forestry and Fisheries, Tsukuba, Japan. To estimate the gene copy number of the *AtAMT2* homologs in rice, DNA gel blot of genomic DNA was performed using low stringent hybridization conditions. Rice genomic DNA digested with *Bam*HI, *Eco*RI or *Hin*dIII yielded a few signals (Fig. 1) when the 666-bp-long EST clone was used as a radioactive probe. The fact that there is no cleavage site for any restriction enzyme within the EST clone, suggests the presence of a few genes in rice, which are homologous to *AMT2;1* in *Arabidopsis*.

The full-length of the cDNA (DDBJ/EMBL/GenBank accession number: AB051864) for *OsAMT2;1* was cloned using 5′- and 3′-RACE using Marathon cDNA Amplification Kits (Clontech, CA, U.S.A.) against rice root poly(A)$^+$ RNA. The cloned cDNA is 2,040 bp long and the putative open reading frame (1,461 bp) encodes a 486 amino acid protein with a molecular mass of 51.4 kDa. Since there was a stop codon at 213 bp upstream from the putative start codon, we decided that the open reading frame was complete. A genomic clone for *OsAMT2;1* (5,153 bp) was also obtained by PCR and inverse PCR for the 5′-upstream region using a rice genomic DNA as a template. The complete nucleotide sequence can be obtained from the DNA data bank under accession no. AB083581. Alignment of the genomic and cDNA clone of *OsAMT2;1* showed that *OsAMT2;1* consists of three exons separated by two introns (Fig. 2). The nucleotide sequence of the coding region of *OsAMT2;1* was completely identical to that of the open reading frame in the cDNA of *OsAMT2;1*.

When homology search using the deduced amino acid sequence of *OsAMT2;1* was carried out against the database on DDBJ/EMBL, we found in total 10 homologs including two additional members of putative *OsAMT2*, i.e. AP003252 (P0446G04.19) and AP003252 (P00446G04.22). A phylogenetic analysis showed that *OsAMT2;1* clusters within the same branch as *AtAMT2* (Fig. 3). By contrast, four genes, i.e. AP003235 (P0039A07.11), AC104487 (location: 86401–89940), AP004775 (location: 29161–31980) and AC091811

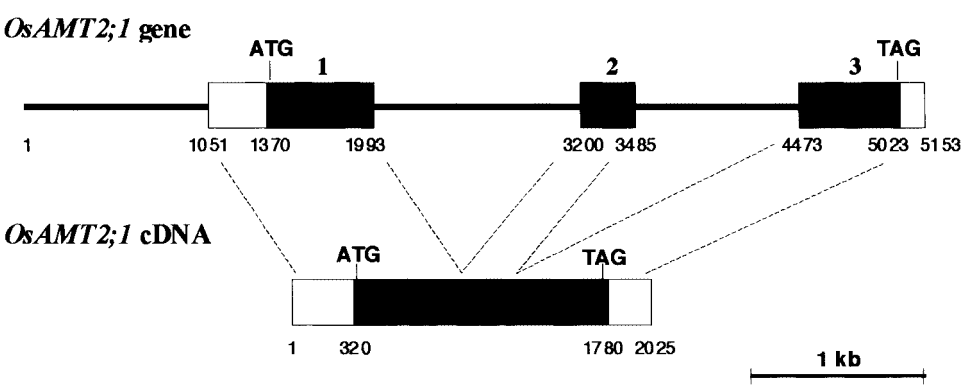

Fig. 2 Schematic comparison of the structure of *OsAMT2;1* and its cDNA. Exons are indicated by boxed regions whereas introns and the 5′-non-transcribed region are represented by lines. The open boxes of exons correspond to the untranslated sequences. The exon number and the nucleotide number are indicated above and below the boxes, respectively. Bar = 1 kb.

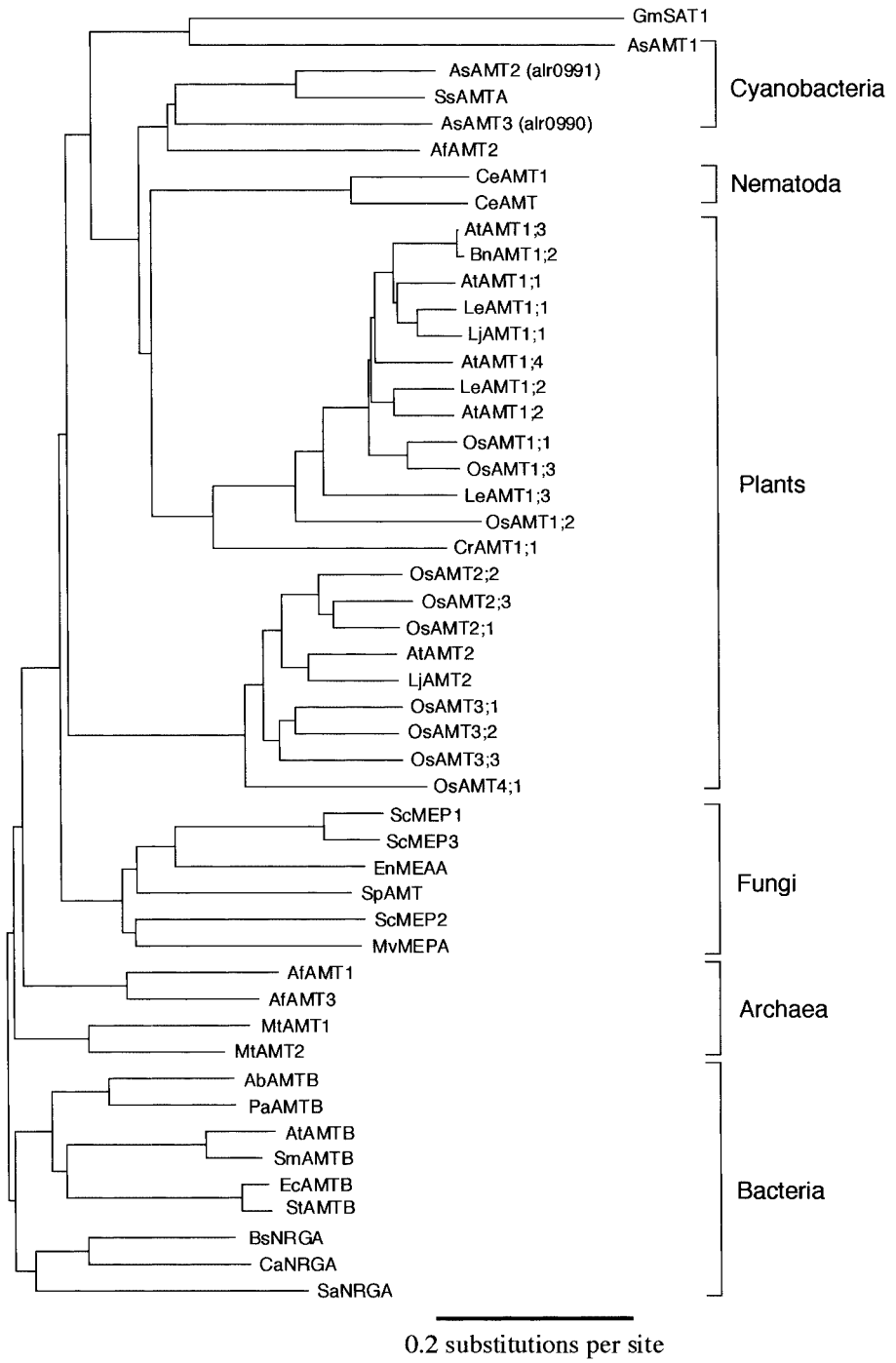

Fig. 3

(OSJNBa0069E14.17), were clearly separated from the branch of plant *AMT2* transporters and were therefore designated as OsAMT3;1 to OsAMT3;3 (AP003235, AC104487, AP004775) and OsAMT4;1 (AC091811), respectively. Genes for *OsAMT2* to *OsAMT4* form a small group, which is more closely related to AMTs in prokaryotes and MEP-type ammonium transporters from yeast.

A cDNA for *OsAMT3;1* was obtained by RT-PCR against a rice-root poly(A)$^+$ RNA. A 1,577-bp-long clone (*OsAMT3;1*, accession no. AB083582) consisted of a putative open reading frame (1,497 bp) encoding 499 amino acids with a molecular mass of 53.7 kDa. The amino acid sequences deduced from the nucleotide sequences of the cDNAs for *OsAMT2;1* and *OsAMT3;1* were 71% and 66% identical to that of AtAMT2,

respectively. However, OsAMT2;1 and OsAMT3;1 showed only 20–25% sequence identity with proteins of the AMT1 family, including OsAMT1.

A yeast mutant complementation test was used to determine whether the protein encoded by the *OsAMT2;1* cDNA encodes a functional NH_4^+ transporter. The yeast strain 31019b is deleted in three endogenous NH_4^+ transporter genes (*MEP1*, *MEP2*, *MEP3*) and unable to grow on medium containing < 5mM NH_4^+ as the sole nitrogen source (Marini et al. 1997). Transformation of this strain with the yeast expression vector p416MET25 (Mumberg et al. 1994) harboring an *OsAMT2;1* cDNA in *Spe*I and *Hin*dIII sites under the control of an *O*-acetyl homoserine sulfydrylase promoter failed to express a functional OsAMT2;1 protein. An obvious difference in the cDNA sequence of the cDNAs successfully complementing the yeast mutant (Sohlenkamp et al. 2000, von Wirén et al. 1997, von Wirén et al. 2000) and of our *OsAMT2;1* cDNA was the lack of an adenine nucleotide at the position −3 upstream from the translation start codon. This adenine nucleotide is important for the stability of the translation product in yeast and other eukaryotes (Kozak 1999). After adding this base at the right position, OsAMT2;1 functionally complemented the yeast mutant, when 5 mM NH_4^+ was supplied to the agar medium (Fig. 4). When 1 mM NH_4^+ was used, however, OsAMT2;1 was not able to grow, unlike when OsAMT1;1 or OsAMT1;2 were used. This result shows that *OsAMT2;1* cDNA encodes a functional AMT and suggests that it might be an NH_4^+ transporter with different biochemical properties. In contrast, AtAMT2 was able to complement yeast mutants when 0.5 mM NH_4^+ was present in the medium (Sohlenkamp et al. 2000).

Steady-state accumulations of mRNA of *OsAMT2;1* and for *OsAMT3;1* were compared following the extraction of total RNA from roots and shoots of 26-day-old rice seedlings. As described previously, mRNA for NADH-GOGAT in rice roots at this stage markedly accumulated within 3 h following the supply of 1 mM NH_4Cl to nitrogen-prestarved plants (Hirose et al. 1997). Northern blot analysis showed that a single band of a

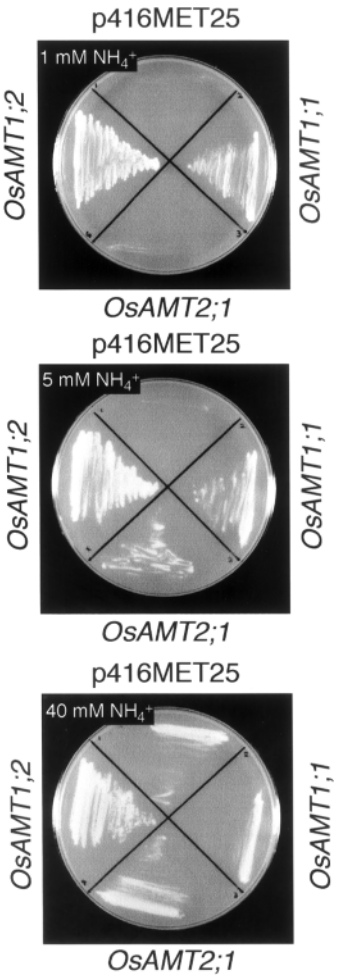

Fig. 4 Growth of the yeast mutant 31019b (Freney 1985) expressing *OsAMT2;1* on minimal medium containing either 1 mM (top), 5 mM (middle) or 40 mM NH_4^+ (bottom) as sole nitrogen source in the presence of 2% (w/v) glucose. Positive controls are the mutant expressing *OsAMT1;1* or *OsAMT1;2*, whereas the negative control is the mutant expressing the p416MET25 vector alone.

Fig. 3 The unrooted phylogenetic tree of the deduced amino acid sequences of the ammonium transporters. The tree was calculated by the neighbor-joining method found in the CLUSTAL W (Thompson et al. 1994) and PHYLIP programs (Felsenstein, J., PHYLIP (Phylogeny Inference Package) ver. 3.5c., Department of Genetics, University of Washington, Seattle, U.S.A.). The length of the bar represents 0.2 substitutions per site. The information of the deduced amino acid sequences of ammonium transporters was obtained from the GenBank and EMBL databases. The accession numbers used were: OsAMT1;1 (*Oryza sativa*), AF289477; OsAMT1;2, AF289478; OsAMT1;3, AF289479; OsAMT2;1, AB051864; OsAMT2;2, AP003252; OsAMT2;3, AP003252; OsAMT3;1, AB083582; OsAMT3;2, AC104487; OsAMT3;3, AP004775; OsAMT4;1, AC091811; AtAMT1;1 (*Arabidopsis thaliana*), X75879; AtAMT1;2, AF083036; AtAMT1;3, AF083035; AtAMT1;4, AL161573; AtAMT2, AF182039; LeAMT1;1 (*Lycopersicon esculentum*), X92854; LeAMT1;2, X95098; LeAMT1;3, AF118858; LjAMT1;1 (*Lotus japonicus*), AF182188; LjAMT2, AF187962; BnAMT1;2 (*Brassica napus*), AF306518; GmSAT1 (*Glycine max*), AF069738; CrAMT1;1 (*Chlamydomonas reinhardtii*), AY058211; ScMEP1 (*Saccharomyces cerevisiae*), X77608; ScMEP2, Z71418; ScMEP3, U40829; SpAMT, AL136235; MvMEPA (*Microbotryum violaceum*), AF159568; EnMEAA (*Emericella nidulans*), AY049706; CeAMT (*Caenorhabditis elegans*), U53338; CeAMT1, U53338; AsAMT1 (*Anabaena sp*. PCC7120), AF196328; AsAMT2 (alr0991), AP003584; AsAMT3 (alr0990), AP003584; SsAMTA (*Synechococcus* sp. PCC7002), U36388; AbAMTB (*Azospirillum brasilense*), AF005275; PaAMTB (*Pseudomonas aeruginosa*), AE004941; AtAMTB (*Agrobacterium tumefaciens*), AE009223; SmAMTB (*Sinorhizobium meliloti*), AL591793; EcAMTB (*Escherichia coli*), U40429; StAMTB (*Salmonella typhimurium*), AE008717; BsNRGA (*Bacillus subtilis*), Z82987; CaNRGA (*Clostridium acetobutylicum*), AE007583; SaNRGA (*Staphylococcus aureus*), AP003135; AfAMT1 (*Archaeoglobus fulgidus*), AE001036; AfAMT2, AE000982; AfAMT3, AE000982; MtAMT1 (*Methanobacterium thermoautotrophicum*), AE000846; MtAMT2, AE000846.

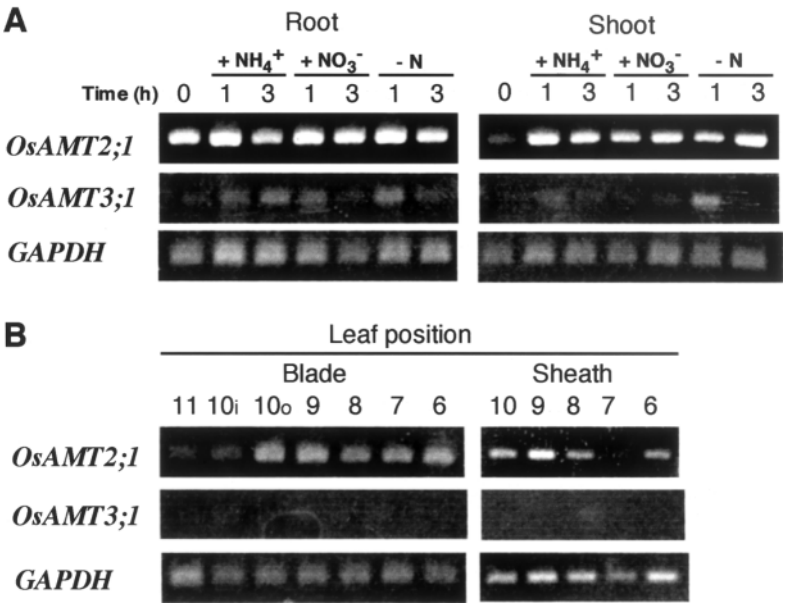

Fig. 5 RT-PCR was used to detect mRNAs for *OsAMT2;1* and *OsAMT3;1* in roots and shoots of 26-day-old rice seedlings supplemented with either 1 mM NH_4Cl, 1 mM KNO_3 or no nitrogen for 0, 1 and 3 h (A). Leaf blades and leaf sheaths from various leaf position (assigned from the bottom to the top) at the 9.5-leaf stage on the main stem were analyzed with RT-PCR (B). *GAPDH* was used as a control.

mRNA, which corresponded to the size of the *OsAMT2;1* cDNA, was detected in all extracts from both roots and shoots, and there was only a small effect of NH_4Cl being resupplied on the signal intensities (results not shown). Since accumulation of *OsAMT3;1* mRNA was not detected in Northern blot analysis, RT-PCR was performed (Fig. 5). Constitutive expression of *OsAMT2;1* was also observed in leaf blades and the leaf sheath at different position of the 6th to 11th leaf (the leaf position was assigned from the bottom to the top; Fig. 5B). Compared with the signal intensities of *OsAMT2;1*, those for *OsAMT3;1* were very weak. In contrast, expression of *OsAMT1;2* was induced by the supply of either 1 mM NH_4Cl or KNO_3 to roots (Saiki et al. 2002).

To detect the translation product of *OsAMT2;1*, a polyclonal antibody (anti-OsAMT2;1) was prepared using a synthetic peptide. In Western blot analysis, the anti-OsAMT2;1 IgG recognized 1.2 ng of the antigen. However, the affinity-purified IgG did not give any signal on immunoblots, when soluble proteins of either shoots (50 μg) or roots (25 μg), 10 μg protein of microsomal membrane fraction, or 10 μg protein of plasma membrane fraction was analyzed, the latter being prepared using aqueous two-phase partitioning with root and shoot tissues of rice seedlings (data not shown). This observation suggests that 10 μg of membrane-protein extract did not contain more than approx. 30 ng of OsAMT protein (1.2 ng times 486/20 in amino acids).

In conclusion, unlike *Arabidopsis thaliana*, rice expresses at least two homologs of the AMT2 and AMT3 subfamilies, namely *OsAMT2;1* and *OsAMT3;1*. The *OsAMT2;1* encoded a functional ammonium transporter which was being constitutively expressed in roots and shoots irrespective of the nitrogen supply. A database search showed that a total of 10 genes for putative OsAMT were present in rice. Studies on the functions of individual putative OsAMT in the inward and outward transport of ammonium ions across the plasma membrane and organella membranes are now in progress.

Rice (*Oryza sativa* L. cv. Sasanishiki) plants were grown in hydroponic culture in a glasshouse either for 26 d or until the 9.5 leaf stage on the main stem, as described previously (Hirose et al. 1997, Nakano et al. 2000). A full-length cDNA for *OsAMT2;1* was isolated from rice-root poly(A)$^+$ RNA using the 5′- and 3′-RACE method (Marathon cDNA Amplification Kits, Clontech, CA, U.S.A.) on the basis of the sequence of a partial cDNA for OsAMT2 (rice mature leaf EST, S21159, accession no. AU070277), which was kindly provided by MAFF DNA Bank (DNA bank at the National Institute of Agrobiological Sciences, Ministry of Agriculture, Forestry and Fisheries of Japan). *GAPDH* was also provided by the Rice Genome Research Program (accession no. D16096). The following primers were used: as forward primer, 5′-AGCTCT-TCTCCCTCGAGTCCACGGT-3′; as reverse primer, 5′-GTCC-AAAAACAGTACAAACGCGCGTG-3′; and as adaptor primer, 5′-ACTCACTATAGGGCTCGAGCGGC-3′. The genomic DNA for *OsAMT2;1* was isolated by PCR and inverse-PCR using the cDNA sequence. A full-length cDNA for *OsAMT3;1* was also amplified by RT-PCR against rice-root poly(A)$^+$ RNA using information of a rice genome sequence (Rice Genome Project). Sequencing, as well as gel blot analyses of DNA and RNA, was carried out as described previously (Goto et al. 1998). To exclude PCR errors, all PCR products were produced in dupli-

cates, cloned and PCR clones were fully sequenced. Total RNA was extracted from various organs of rice plants using CTAB methods with a Sepasol-RNAI solution (Nacalai Tesque, Kyoto, Japan) according to the instruction manual. For Northern blotting, 10 μg of total RNA were separated by agarose gel electrophoresis (Hirose et al. 1997) and a DIG-labeled PCR product corresponding to a 397 bp fragment (–238 bp upstream region from the translational start codon) was used as a probe. An RNA PCR Kit Ver. 2.1 (Takara, Tokyo, Japan) was used to prepare the first strand cDNA during RT-PCR. Twenty-nine PCR cycles were performed for *OsAMT2;1* and *OsAMT2;2* but only 22 cycles for *GAPDH*. The following primers were used: 5′-TCTCACCGAACAACATCCTGCT-3′ as forward primer and 5′-TTGCCGATCTGCTTGATACC-3′ as reverse primer for *OsAMT2;1*; 5′-GTCCCGCTCTGGCTCACCTT-3′ as forward primer and 5′-CGGGGAGGAAGAGGGAGCA-3′ as reverse primer for *OsAMT3;1*; 5′-CCTTTTGCTTGCCATGGTATT-3′ as forward primer and 5′-AAGCCCATATATCAACCAGCA-3′ as reverse primer for *GAPDH*. Specificity of each primer was confirmed by PCR using a rice-root cDNA library (Goto et al. 1998) as a template.

For the functional complementation test, cDNAs for *OsAMT2;1* and *OsAMT3;1*, which had been designed to have restriction sites and Kozak sequences (Kozak 1999), were cloned into *Spe*I and *Hin*dIII sites of the yeast expression vector p416MET25 (Mumberg et al. 1994). The yeast strain 31019b (Marini et al. 1997) was transformed with the vector as described in von Wirén et al. (2000).

A polyclonal antibody raised against OsAMT2;1 was prepared as follows. A synthetic peptide consisting of 20 amino acids in the hydrophilic region at the C-terminus of the predicted protein (455–474 in the sequence with a total of 486 deduced amino acids) was fused with keyhole limpet hemocyanin and the conjugate was used as an antigen. Immunization, affinity-purification of the antibody with the antigen, and immunoblotting analysis were performed as described previously (Nakano et al. 2000).

Acknowledgments

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