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Promoter analysis of *OsAMT1;2* and *OsAMT1;3* implies their distinct roles in nitrogen utilization in rice

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We previously reported two rice ammonium transporter (OsAMT) genes, *OsAMT1;2* and *OsAMT1;3*, which show root-specific expression at the vegetative stage. To further understand the functional differences between the two genes, we used the *promoter::gusA* reporter gene to investigate their detailed expression patterns. We show that in transgenic rice plants carrying individual 4-kb *promoter::gusA* fusion genes, the expression of the two genes was confined to roots during the entire plant life cycle, suggesting the housekeeping roles of the two transporters in rice. Detailed observation of the above transgenic lines further shows that *OsAMT1;3* was preferentially expressed in the apices of seminal and lateral roots under nitrogen-deficient conditions, and was repressed when supplied with ammonium. By contrast, *OsAMT1;2* expression was induced most intensely in the root elongation zone under nitrogen-sufficient conditions. These observations suggest that the two genes have distinct roles in nitrogen utilization: *OsAMT1;3* appears to function as a nitrogen sensor and *OsAMT1;2* as an assimilator. In contrast to 4-kb promoters, *gusA*-reporter expression was not detected in the roots under both nitrogen-deficient and -sufficient conditions when using individual 2-kb promoter sequences proximal to transcription initiation sites for the two genes, indicating that distal 2-kb promoter sequences are essential for the root-specific expression and conditional responsiveness to nitrogen of *OsAMT1;2* and *OsAMT1;3* genes.

Key Words: Promoter, OsAMT1;2, OsAMT1;3, Root, Ammonium uptake.

Introduction

The ability of roots to take up nutrients at a rate that matches seasonal and diurnal changes in plant growth rates is fundamental for efficient plant development. Since soil nutrient availability is of high heterogeneity, and nutrient uptake conditions are influenced by various factors, such as drought, plants are expected to meet frequent nutrient deficiencies during their life cycle. Therefore, to direct morphological and physiological responses to nutrient deficiencies, plants require nutrient transport systems with high flexibility regarding internal nutrient demand, external substrate availability, and the spatial distribution of nutrient sources within the exploitable soil volume. On the other hand, plants require sensing systems scanning the external substrate concentration in the rooted area and signaling to the plant in which direction a further development of root system could be most promising (Loque and von Wiren 2004). High coordination between these two systems is thus expected to be of great importance for nutrient uptake.

Inorganic nitrogen is the mineral nutrient required in the largest amounts, and ammonium is the preferred form of nitrogen for anaerobic soil-grown rice plants. Since excess accumulation of ammonium tends to inhibit plant growth (Kronzucker *et al.* 2001), plants must develop flexible transport systems to adjust cellular ammonium levels that vary not only in response to the uptake of external ammonium but also to intercellular amino acid metabolism. So far, studies on ammonium uptake have identified ammonium transporters (AMT) from various plant species such as *Arabidopsis thaliana* (Gazzarrini *et al.* 1999, Sohlenkamp *et al.* 2000, 2002), *Lotus japonicus* (Salvemini *et al.* 2001, Simon-Rosin *et al.* 2003), *Lycopersicon esculentum* (Lauter *et al.* 1996, von Wiren *et al.* 2000), *Brassica napus* (Pearson *et al.* 2002), and rice (Kumar *et al.* 2003, Suenaga *et al.* 2003). These identified AMTs were phylogenetically classified into the AMT1 or AMT2/MEP subfamily, and regulate ammonium transport at transcriptional and post-transcriptional levels (Loque and von Wiren 2004); however, the detailed expression pattern of these *AMTs* in various plant organs

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remains largely unclear.

We previously reported three members of the *AMT1* gene family in rice (*OsAMT1;1-1;3*, Sonoda *et al.* 2003a, 2003b). Our studies suggested that the three genes have distinct expression patterns, i.e., constitutive expression in shoots and roots for *OsAMT1;1*, root-specific and ammonium-inducible expression for *OsAMT1;2*, and root-specific and nitrogen-repressible expression for *OsAMT1;3*. Since both *OsAMT1;2* and *OsAMT1;3* show root-specific expressions, this raises the question of their roles in nitrogen utilization. To further characterize functional differences between the two root-specific genes, we used the *promoter::gusA* reporter gene to analyze the detailed expression patterns of the two genes in various plant organs under ammonium-sufficient and ammonium-deficient conditions, respectively. Our results suggest that OsAMT1;2 acts mainly to assimilate ammonium from the nitrogen-rich area, while OsAMT1;3 appears to function as an ammonium-sensing protein.

Materials and Methods

Promoter::gusA fusion constructs and their transformation into rice

Genomic DNA fragments bearing individual 4-kb and 2-kb 5′-upstream sequences of the coding regions for *OsAMT1;2* (accession No. AK107204) and *OsAMT1;3* (accession No. AF289478) were amplified by genomic PCR using gene-specific primer pairs listed in Table 1. For easier cloning, appropriate recognition sequences for *Sbf*I, *Xba*I and *Nco*I enzymes were attached to the above primers. The amplified fragments carrying the putative promoters were first introduced into the pGEM-T Easy cloning vector according to the technical manual of the pGEM-T Easy Vector System from Promega (Madison, WI, USA). Sequences of the promoter inserts were then verified by DNA sequecing. The 2-kb and 4-kb promoter fragments of *OsAMT1;2* were generated by double digestion of the corresponding plasmids with *Sbf*I and *Nco*I, and ligated individually into *Sbf*I and *Nco*I sites of a binary vector pSMAHdN627-M2GUS (Nakamura *et al.* manuscript in preparation; T-DNA structure of the vector is shown in Fig. 1A). The 2-kb and 4-kb promoter fragments of *OsAMT1;3*, generated by double digestion of the appropriate plasmids with *Sbf*I and *Xba*I, were similarly cloned into pSMAHdN627-M2GUS. Schematic illustrations of the constructed *promoter::gusA* fusion genes are shown in Fig. 1B. The four chimeric *promoter::gusA* constructs were individually transformed into *Agrobacterium tumefaciens* strain EHA105 (Hood *et al.* 1993) by electroporation, and then introduced into rice (*Oryza sativa* cv. Nipponbare) using a rapid and highly efficient *Agrobacterium*-mediated transformation method (Toki *et al.* 2006). Selection of transgenic regenerants (T1 generation) was conducted in the presence of 30 mg/L hygromycin.

Plant growth conditions

For adult stage investigation, transgenic plants (T1 generation) were further transferred to 1/5000 Wagner pots containing nutrient solution with 1 mM $(NH_4)_2SO_4$ or in soil culture. Plants were grown to maturity under conditions of continuous light, 60% relative humidity and 30°C, and the culture solution was changed every two days. The following organs were sampled at the flowering stage: blade and sheath of the flag leaf, first and second internodes, rachis, spikelet, root apical and maturation region.

Transgenic seeds (T2 generation) from individual transgenic lines were sterilized in 1% (v/v) NaClO solution for 20 min, and then washed thoroughly with sterile distilled water. The seeds were first grown hydroponically in tap water for one week, and the seedlings were further grown for two weeks with nitrogen-deficient nutrient solution as described (Sonoda *et al.* 2003a). To investigate GUS activity during the vegetative stage, half of the seedlings were transferred to nutrient solutions with or without 1 mM $(NH_4)_2SO_4$. After 12 h treatment, roots and shoots were sampled for GUS assay.

GUS activity investigation

To investigate the expression patterns of *OsAMT1;2* and *OsAMT1;3* and the activities and degrees of intensity of the putative promoters, the expression of *gusA* in the samples described above was assayed with 5-bromo-4-cgloro-3-indolyl glucuronide (X-Gluc) as a substrate. Briefly, the samples were immersed in phosphate buffer (50 mM $NaPO_4$, 20% methanol, 0.1% Triton X-100, 5 mM DL-dithiothreitol) containing 1 mM X-Gluc and placed under a mild vacuum

Table 1. Primers used in this study

Promoter	Primer	Sequence (5′→3′)[a]
OsAMT1;2 4-kb promoter	AMT1;2p4F1	ATGCCCTGCAGGCGGCCTATTATGACCGCTTG
	AMT1;2p4R2	ATGCCCATGGTCGCCGCCTCGACGCGCGCTCAACACAGACTGT
OsAMT1;2 2-kb promoter	AMT1;2p2F3	ATGCCCTGCAGGTCACGTGTTTGATCATTCGTC
	AMT1;2p2R4	ATGCCCATGGTCGCCGCCTCGACGCGCGCTCAACACAGACTGT
OsAMT1;3 4-kb promoter	AMT1;3p4F1	ATGCCCTGCAGGATGGTCCATGGCAACTAGCC
	AMT1;3p4R2	ATGCTCTAGAGTGGCAAGGTTTTGTGCTGC
OsAMT1;3 2-kb promoter	AMT1;3p2F3	ATGCCCTGCAGGCAGAGTACTCAGTAGTCAGC
	AMT1;3p2R4	ATGCTCTAGAGTGGCAAGGTTTTGTGCTGC

[a] Underlined bases indicate the restriction sites for *Sbf*I, *Xba*I and *Nco*I attached to the respective primers.

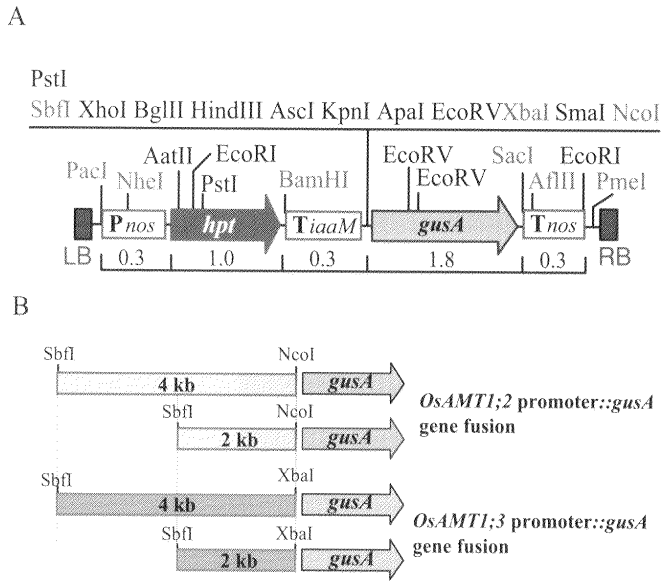

Fig. 1. Construction of *promoter::gusA* chimeric genes for *OsAMT1;2* and *OsAMT1;3*. (A) T-DNA structure of the binary vector pSMAHdN627-M2GUS. Restriction enzyme sites for *Sbf*I, *Xba*I and *Nco*I, located in the multiple cloning site, are shown in gray. (B) Schematic representation of *promoter::gusA* fusion genes. Putative promoter regions of *OsAMT1;2* (accession No. AK107204) and *OsAMT1;3* (accession No. AF289478) were amplified by genomic PCR using gene-specific primer pairs listed in Table 1 and were checked by DNA sequencing.

for 15 min. After being incubated overnight at 37°C, the samples were photographed under a microscope MZ FLIII (Leica, Wetzlar, Germany) equipped with a digital camera (Penguin 600CL by Pixera Corporation, San Jose, CA, USA).

Results

Root-confined expression of OsAMT1;2 and OsAMT1;3

We previously reported three *OsAMT1s* in rice (Sonoda *et al.* 2003a). RT-PCR analysis revealed that at the vegetative stage, both *OsAMT1;2* and *OsAMT1;3* show root-specific and ammonium-regulated expressions, while *OsAMT1;1* shows constitutive expression in both roots and shoots. To understand further the spatial and temporal expressions of *OsAMT1;2* and *OsAMT1;3*, we extended our research to the reporter gene approach by fusing the putative 4-kb promoter fragments of the two *OsAMT1* genes to the *gusA* reporter, and generated about 50 independent transgenic plants for each *OsAMT1-4kb::gusA* construct. Histochemical GUS (reporter) assay found that intense GUS staining was localized in seminal and lateral roots of *OsAMT1;2-4kb::gusA* transgenic plants (Fig. 2G, H). No GUS signals, however, were detected in the aerial parts of these plants, including the leaf, stem, internode, spikelet, and rachis (Fig. 2A–F) as well as the wild type (data not shown). *OsAMT1;3-4kb::gusA* plants showed intense GUS activity across the whole root system, including seminal and lateral roots (Fig. 3G, H), but no GUS staining was observed in

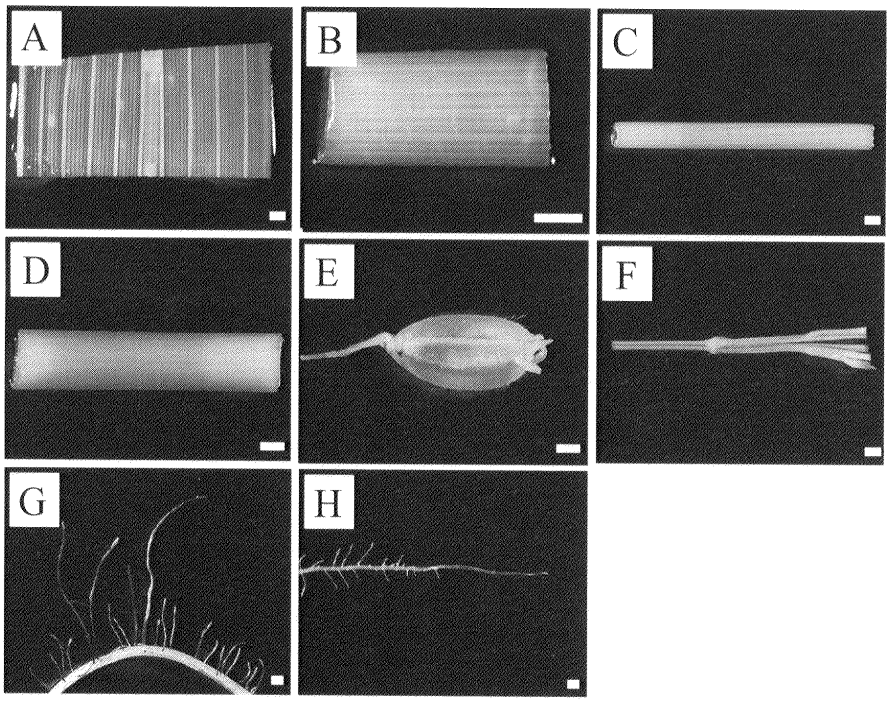

Fig. 2. Spatial expression of *OsAMT1;2-4kb::gusA* reporter gene. Samples were taken at the flowering stage from transgenic plants bearing *OsAMT1;2-4kb::gusA* that were cultured with solutions containing 1 mM $(NH_4)_2SO_4$ or soil culture. (A) Leaf blade of the flag leaf. (B) Leaf sheath of the flag leaf. (C) First internode. (D) Second internode. (E) Spikelet. (F) Rachis. (G) Root maturation region. (H) Apical region of the seminal root. Bars = 1 mm.

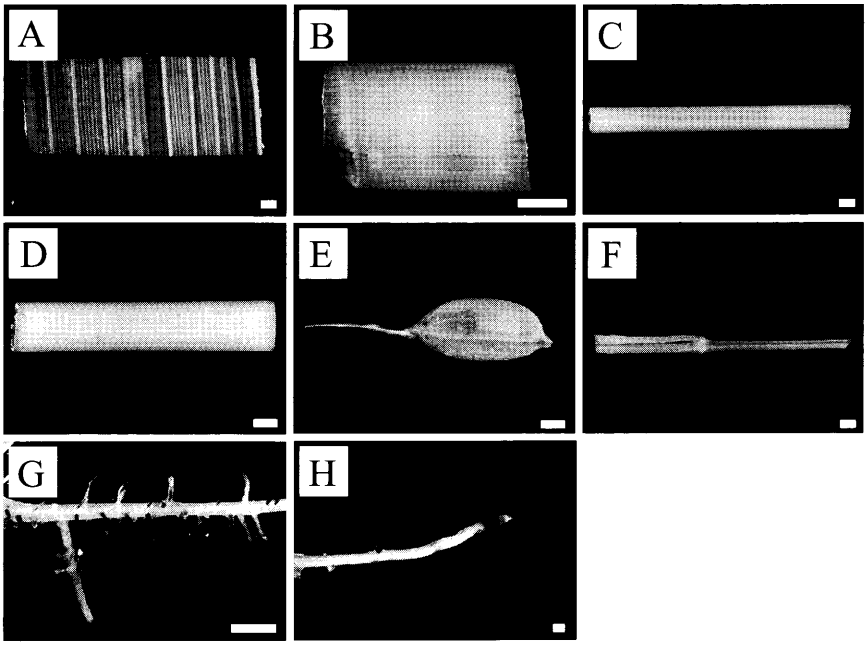

Fig. 3. Spatial expression of *OsAMT1;3-4kb::gusA* reporter gene. Samples were taken at the flowering stage from transgenic plants bearing *OsAMT1;3-4kb::gusA* that were cultured with solutions containing 1 mM $(NH_4)_2SO_4$ or soil culture. (A) Leaf blade of the flag leaf. (B) Leaf sheath of the flag leaf. (C) First internode. (D) Second internode. (E) Spikelet. (F) Rachis. (G) Root maturation region. (H) Apical region of the seminal root. Bars = 1 mm.

aerial organs at any of the developmental stages, including vegetative, booting, and flowering (Fig. 3A–F, data not shown). These results indicate that the expression of *OsAMT1;2* and *OsAMT1;3* is confined to the roots during the entire plant growth period. Furthermore, individual 4-kb promoter sequences are sufficient to confer root-specific expression profiles upon *OsAMT1;2* and *OsAMT1;3*.

Distal 2-kb promoter regions are essential to direct the expressions of OsAMT1;2 and OsAMT1;3

To further detect the locations of the regulatory sequences required to direct *OsAMT1;2* and *OsAMT1;3* expressions, we constructed *promoter::gusA* fusion genes using proximal 2-kb promoter fragments (*OsAMT1-2kb::gusA*) relative to individual translation initiation sites (Fig. 1B). In accordance with our previous gene-expression studies on *OsAMT1;2* (Sonoda *et al.* 2003a), GUS staining of the root tissues sampled from transgenic rice plants carrying respective *promoter::gusA* constructs revealed no GUS activity in the roots of *OsAMT1;2-4kb::gusA* transgenic plants under nitrogen starvation (Fig. 4A, B), whereas intense GUS staining was detected in both seminal and lateral roots when transgenic plants were treated with ammonium solution (Fig. 4C, D); however, *OsAMT1;2-2kb::gusA* transgenic plants exhibited no GUS activity in any roots under both nitrogen-starved and ammonium-supplied conditions (Fig. 4E–H).

The expression of *OsAMT1;3* has been reported to be nitrogen-repressible and up-regulated under nitrogen deficiency (Sonoda *et al.* 2003a). This was further confirmed by the following *promoter::gusA* analyses. When *OsAMT1;3-4kb::gusA* transgenic plants were cultured under nitrogen-deficient conditions, GUS staining was observed in both seminal and lateral roots (Fig. 5A, B) but could not be detected when these plants were treated with ammonium (Fig. 5C, D). In *OsAMT1;3-2kb::gusA* transgenic plants, however, no GUS staining could be observed in the roots under both nitrogen-deficient and -sufficient conditions (Fig. 5E–H). These results indicate that distal 2-kb promoter sequences are essential for conferring gene-specific expression patterns upon *OsAMT1;2* and *OsAMT1;3*.

Distinct root-specific expression pattern of OsAMT1;2 and OsAMT1;3

Plant root architecture can be divided into three different zones according to their cellular characteristics and functions: meristematic, elongation, and maturation zones. Since both *OsAMT1;2* and *OsAMT1;3* show root-specific expression patterns, we asked whether these two genes have the same expression domain. Careful comparison of the GUS staining patterns revealed that, when supplied with nitrogen, *OsAMT1;2-4kb* expression was induced most intensely in the elongation zone of both seminal and lateral roots (Figs. 2G, H, 4C, D). In the apices of these roots, GUS activity, if any, was only faintly detected (Fig. 4C), and no GUS staining was observed in the maturation region of seminal roots (Figs. 2H, 4D). In contrast, the depletion of nitrogen from *OsAMT1;3-4kb::gusA* transgenic plants gave GUS activity exclusively in the tips of both seminal and lateral roots, and GUS staining could hardly be observed in the

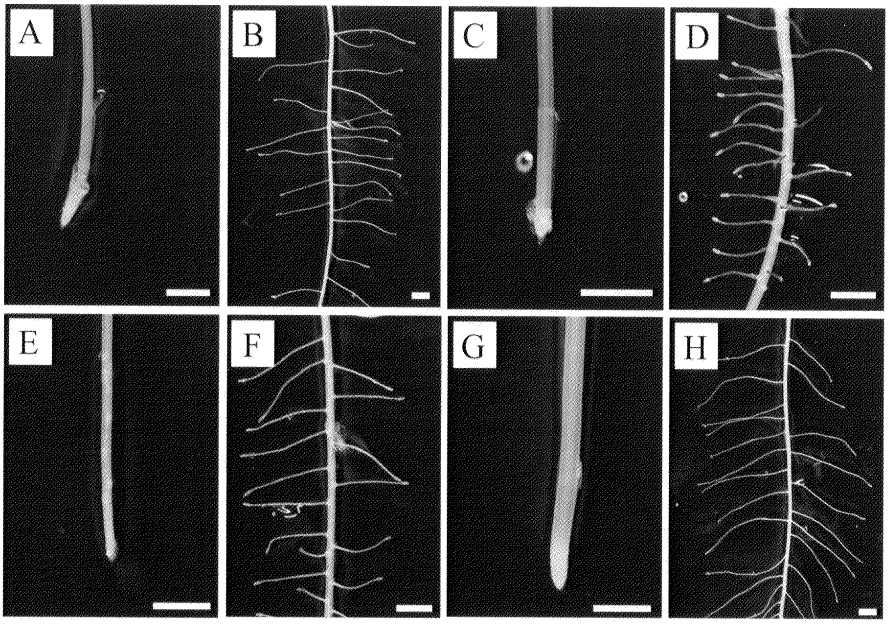

Fig. 4. Promoter analysis of nitrogen-inducible expression of *OsAMT1;2* in the roots of transgenic rice plants bearing *OsAMT1;2-4kb::gusA* (A–D) and *OsAMT1;2-2kb::gusA* (E–H) treated with or without ammonium. (A, B, E, F) Without nitrogen nutrition. (C, D, G, H) With 1 mM $(NH_4)_2SO_4$. Bars = 1 mm.

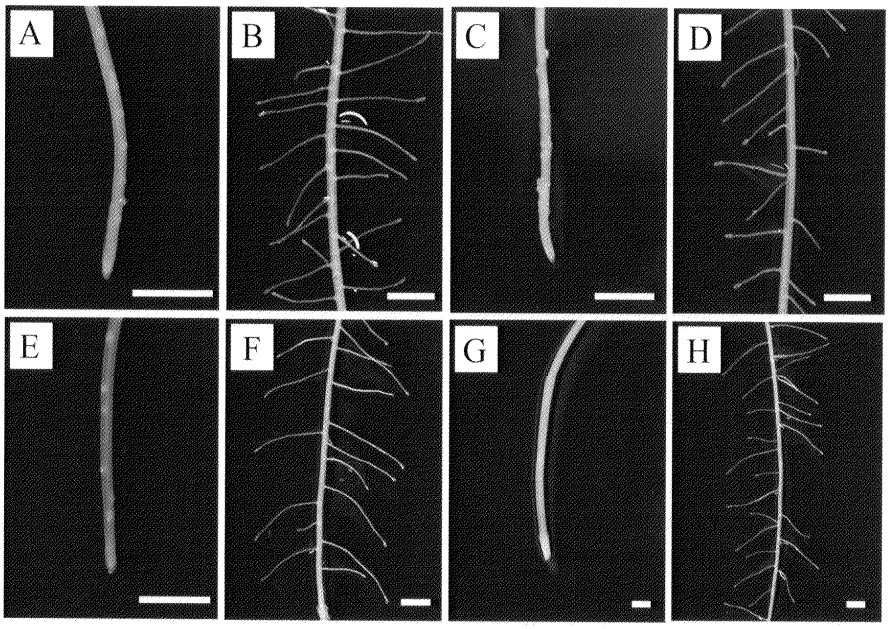

Fig. 5. Promoter analysis of nitrogen-repressible expression of *OsAMT1;3* in the roots of *OsAMT1;3-4kb::gusA* (A–D) and *OsAMT1;3-2kb::gusA* (E–H) transgenic plants treated with or without ammonium. (A, B, E, F) Without nitrogen nutrition. (C, D, G, H) With 1 mM $(NH_4)_2SO_4$. Bars = 1 mm.

elongation zone and maturation region of the roots (Fig. 5A, B). These results indicate that the two root-specifically expressed genes have different nitrogen-regulated expression patterns; thus, they should serve distinct functions in ammonium utilization.

Discussion

Using *promoter::gusA* reporter gene constructs, we further analyzed the expression patterns of the two previously reported root-expressed transporter genes, *OsAMT1;2* and *OsAMT1;3*, at various stages of plant development. We showed that the two genes are expressed exclusively in roots during the entire plant life cycle under both nitrogen-deficient and -sufficient conditions. In rice, there are at least 10 *AMT* genes which can be subdivided into 4 clades (Loqué and von Wirén 2004). Among these genes, only *OsAMT1;2* and *OsAMT1;3* show expression confined to the roots. The preferred expression of the two genes in the roots suggests that both genes are key components of the uptake systems

required for capturing nitrogen from the periphery of the rooted soil area.

Since plants are immobile, the detection of a nitrogen-rich patch in the root environment is crucial for their survival under limiting nutrient resources. Although it must involve systems for external nutrient sensing, the nature or state of the key sensor molecules remains largely elusive. So far, only a few putative nitrogen sensor proteins have been identified (Hsieh *et al.* 1998, Remans *et al.* 2006). *Arabidopsis* NRT1.1 has been identified as an upstream sensor protein of ANR1 by analyses of the detailed expression manner and the deficient mutant (Remans *et al.* 2006). The ANR1 transcriptional factor is thought to transduce the nitrate signal internally. Only four Arabidopsis nitrate transporters have been functionally characterized: the NRT1.1 as a dual-affinity transporter is involved in root nitrate influx. NRT1.1 is highly expressed in young tissue, and especially in root tips. Mutants of NRT1.1 have led to reduced lateral root elongation, which is not due to lower specific nitrate uptake activity in mutants but is associated with dramatically decreased *ANR1* expression. From these lines of evidence, Remans *et al.* (2006) concluded that NRT1.1 promotes localized root proliferation independently of any nutritional effect and indicates a role in the ANR1-dependent nitrate signaling pathway, either as a nitrate sensor or as a facilitator of nitrate influx in nitrate-sensing cells. In this study, we showed that, similar to *NRT1.1*, *OsAMT1;3* was exclusively expressed in root tips under nitrogen starvation. When supplied with ammonium, *OsAMT1;2* expression was induced most intensely in the root elongation zone, while *OsAMT1;3* expression was repressed. These results suggest that OsAMT1;2 and OsAMT1;3 have distinct roles in ammonium utilization: OsAMT1;3 is suggested to function as a nitrogen sensor and OsAMT1;2 as an ammonium assimilator, since the expression of *OsAMT1;3* is under nitrate and ammonium while that of *OsAMT1;2* is strictly under ammonium (Sonoda *et al.* 2003a). OsAMT1;3 is transcriptionally repressed by ammonium but also by nitrate to a small extent. Taken together, the possibility that OsAMT1;3 does not sense ammonium as a sole nitrogen but as internal nitrogen-related compounds, including nitrate and ammonium, cannot be ruled out. We propose that under nitrogen-deficient conditions, the expression of *OsAMT1;3* is induced by an as yet unknown upstream regulator. After entering the nitrogen-rich soil area, the root tips perceive the nitrogen-derived signal (Zhang and Forde 1998) and then the signal can repress the expression of *OsAMT1;3* in the root tips. Concomitantly, the nitrogen-derived signal induces the expression of *OsAMT1;2*, promoting nitrogen uptake from the nitrogen-rich area. In coordination with other ammonium transporters such as OsAMT1;1 and OsAMT2;1 (Sonoda *et al.* 2003a, 2003b, Suenaga *et al.* 2003), the incorporated nitrogen would be transported to leaves for photosynthesis. When nitrogen in the rooted area is exhausted, the expression of *OsAMT1;2* is down-regulated and *OsAMT1;3* is induced to direct further root development. Using this sophisticated transporter system, rice plants can utilize nitrogen efficiently to compete with their neighbors and other organisms; however, this working hypothesis must be evaluated by analyzing the knockout mutant for OsAMT1;3 as well as NRT1.1. We have not succeeded in isolating the OsAMT1;3 mutant yet. Further research is needed to clarify the function of OsAMT1;3 by using knockout mutants as well as the kinetics properties.

To feed the growing population, increasing nitrogen fertilization has been one of the major methods to improve rice production in the last 50 years; however, the high rate of nitrogen fertilization decreases soil quality by altering its physical, chemical, and biological properties. Moreover, manufacturing nitrogen fertilizer also increases global warming by emitting a large amount of CO_2 derived from the oxidation of soil organic matter (Harbinson 2001). Recently, transgenic plants containing transferred *glutamine synthetase* (*GS*) have been reported to have higher productivity and improved efficiency in nitrogen use (Miflin and Habash 2002). In this study, we showed that *OsAMT1;2* and *OsAMT1;3* are exclusively expressed in roots, and their distal 2-kb promoter sequences are sufficient for the nitrogen-specific regulation of *OsAMT1;2* and *OsAMT1;3* genes. Although further dissection of the (distal) 2-kb promoter sequences may be needed to define the regulatory elements of ammonium uptake, we could expect the possibility of using these genes/promoters to improve the efficiency of nitrogen utilization by rice plants (and related crops such as wheat, barley, maize, etc.). This could be realized by generating (or producing) transgenic lines in which these genes are over-expressed to improve their nitrogen-sensing and/or -uptake ability. Alternatively, exogenous enhancer elements could be attached to the promoters to improve their regulatory activity. The effects of these strategies on nitrogen utilization and yield, however, wait to be verified.

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