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Cloning and Functional Characterization of a Constitutively Expressed Nitrate Transporter Gene, *OsNRT1*, from Rice[1]

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Elucidating how rice (*Oryza sativa*) takes up nitrate at the molecular level could help improve the low recovery rate (<50%) of nitrogen fertilizer in rice paddies. As a first step toward that goal, we have cloned a nitrate transporter gene from rice called *OsNRT1*. OsNRT1 is a new member of a growing transporter family called PTR, which consists not only of nitrate transporters from higher plants that are homologs of the Arabidopsis CHL1 (AtNRT1) protein, but also peptide transporters from a wide variety of genera including animals, plants, fungi, and bacteria. However, despite the fact that OsNRT1 shares a higher degree of sequence identity with the two peptide transporters from plants (approximately 50%) than with the nitrate transporters (approximately 40%) of the PTR family, no peptide transport activity was observed when OsNRT1 was expressed in either *Xenopus* oocytes or yeast. Furthermore, contrasting the dual-affinity nitrate transport activity of CHL1, OsNRT1 displayed only low-affinity nitrate transport activity in *Xenopus* oocytes, with a K_m value of approximately 9 mM. Northern-blot and in situ hybridization analysis indicated that OsNRT1 is constitutively expressed in the most external layer of the root, epidermis and root hair. These data strongly indicate that *OsNRT1* encodes a constitutive component of a low-affinity nitrate uptake system for rice.

Nitrogen loss in fertilized crop fields is a serious environmental problem because it contributes not only to decreased diversity of vegetation and greenhouse warming, but also to pollution of drinking water sources (Frink et al., 1999). In Asia, the staple crop is rice (*Oryza sativa*), 75% of which is harvested from irrigated lowlands. Unfortunately, due to the mixture and fluctuation of the oxidative and reductive environment in rice-planted submerged soil, rice cultivars usually recover less than 50% of the nitrogen fertilizer in irrigated fields (Vlek and Byrnes, 1986; Cassman et al., 1993). Denitrification and leaching of nitrate left in flooded soils are major contributing factors to nitrogen loss (Reddy, 1982; Buresh et al., 1989; Aulakh and Singh, 1996). Understanding the mechanisms of how rice takes up nitrate and assimilates it at the genetic level is a critical step toward alleviating the nitrogen loss problem.

Intense efforts, most notably by Hasegawa and coworkers (1992), have been directed to identify genetic mutants of rice defective in nitrate acquisition or assimilation. Mutants that are either resistant (Hasegawa et al., 1992; Sato et al., 1997) or hypersensitive (Hasegawa and Ichii, 1994; Hasegawa et al., 1995) to chlorate, a nitrate analog that is toxic when reduced, were isolated. In addition, mutants were found with reduced nitrate uptake activity (Hasegawa, 1996) or that exhibited nitrogen deficiency symptoms when grown with nitrate as the sole nitrogen source (Ichii et al., 1993). However, most of the rice mutants identified so far are either defective in nitrate reductase or in the proton-ATPase that provides the proton gradient for nitrate uptake (Hasegawa et al., 1992; Ichii et al., 1993; Hasegawa, 1996; Sato et al., 1997). One mutant, M605, might be the result of a defect in nitrate transport (Hasegawa and Ichii, 1994; Hasegawa et al., 1995), but it has not been further characterized. To date, no rice nitrate transporter genes have been isolated.

In comparison, recent years have seen a flurry of reports describing the molecular cloning of nitrate transporter genes in other higher plants (for review, see Von Wiren et al., 1997; Crawford and Glass, 1998; see Daniel-Vedele et al., 1998). These newly identified genes define two sequence-distinct nitrate transporter families called NRT1 and NRT2. Results from expression and functional studies obtained at this early stage assign NRT1 to low-affinity (millimolar nitrate) and NRT2 to high-affinity (micromolar nitrate) transporters. The one exception is CHL1 (AtNRT1), which has been shown to be a dual-affinity nitrate transporter (Wang and Crawford, 1998; Liu et al., 1999). Although higher plants comprise both low- and high-affinity nitrate transport systems (encoded by *NRT1*, *NRT2*, and other yet-to-be identified gene families), physiological studies of nitrate uptake in rice have focused primarily on the high-affinity uptake phase, using micromolar levels of nitrate (Sasakawa and Yamamoto, 1978; Youngdahl et al., 1982; Hasegawa and Ichii, 1994; Raman et al., 1995; Kronzucker et al., 1999). Few studies have investigated the kinetics of low-affinity nitrate uptake in rice.

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We report the cloning and functional characterization of a nitrate transporter gene from rice, *OsNRT1*. The cloning of *OsNRT1* was made possible by searching the rice expressed sequence tag database for homologs of *CHL1* (Tsay et al., 1993). Functional characterization in *Xenopus* oocytes and in situ hybridization suggests that *OsNRT1* encodes a constitutively expressed transport system for low-affinity nitrate uptake. *OsNRT1* exhibits more functional properties in common with *AtNRT1:2*, a second Arabidopsis NRT1 gene reported recently (Huang et al., 1999), than with *CHL1*. The successful cloning of *OsNRT1* may suggest a straightforward approach to cloning other rice nitrate transporter genes, intensifying molecular studies of nitrate uptake in rice. The properties of OsNRT1 further our understanding of the NRT1 family.

MATERIALS AND METHODS

Plant Materials and Culture Conditions

Rice (*Oryza sativa* cv Nipponbare) was used in all of the experiments unless mentioned otherwise. Plants were grown hydroponically on iron mesh in modified Kimura B solution (Ehara et al., 1990) containing 180 μM $(NH_4)_2SO_4$, 109 μM KNO_3, 274 μM $MgSO_4$, 911 μM KH_2PO_4, 31 μM ferric citrate, 183 μM $Ca(NO_3)_2$, 2.5 μM H_3BO_3, 0.2 μM $MnSO_4$, 0.2 μM $ZnSO_4$, 0.05 μM $CuSO_4$, and 0.05 μM H_2MoO_4. The solution was refreshed every other day. Plants were grown under a 16-h light/8-h dark photoregime, 70% relative humidity, and a temperature of 28°C. Light was provided at approximately 250 μmol m^{-2} s^{-1}.

Isolation and Sequencing of OsNT1 cDNA

The rice expressed sequence tag (EST) clone *RICR2778* was identified by a homology search using the protein sequence of CHL1 (Tsay et al., 1993). The insert of *RICR2778*, obtained from the Rice Genome Center (Rice Genome Research Program, Japan), was subcloned into vector pBluescriptSK+ (Stratagene, La Jolla, CA) and sequenced by serial deletions of the cDNA in both directions (Sambrook et al., 1989) and by the dideoxy-chain termination method (Sanger et al., 1977) using the Sequenase 2.0 kit (United States Biochemical, Cleveland). The missing part of the cDNA at the 5′ end was rescued by rapid amplification of 5′ cDNA ends (5′-RACE) with two primers of *RICR2778* (RC2, 5′-GAATTGTACAGTACTTCCCC-3′, nt 473 to nt 492, and RC4, 5′-TTCTGAGAAGAGACTGGATCTGTCC-3′, nt 589 to nt 613, in the reverse direction). The RC4 primer was used to synthesize the first strand of the cDNA using the 5′-RACE kits (Gibco-BRL, Gaithersburg, MD). This first strand was tailed with a stretch of 15 cytidines by terminal deoxytransferase. The new 5′ sequence was then amplified by Pfu DNA polymerase (Stratagene) in a thermal cycler (Hybaid, Middlesex, UK) with the RC2 primer and an anchor primer provided in the kit. The obtained fragments were cloned into *Sma*I-restricted pBluescriptK-SII− and confirmed by sequencing. The full-length *OsNRT1* clone, designated as *pSK-OsNRT1F*, was constructed by replacing the 5′ end of *pRICR2778* with the *Bam*HI-*Nde*I fragment of the 5′-RACE product.

DNA and RNA Gel Blotting Analysis

Genomic DNA was isolated from rice tissues using urea extraction buffer (Sheu et al., 1996). Genomic DNA (10–15 μg) was digested with the restriction enzyme and fractionated in a 0.8% (w/v) agarose gel. For RNA gel blotting, total RNA was isolated from rice tissue with the TRIZOL reagent (Gibco-BRL, Grand Island, NY). Total RNA (15–25 μg) was fractionated in a 1% (w/v) agarose gel circulated with a 10 mM sodium phosphate buffer, pH 6.5. The DNA or RNA was then transferred overnight by capillary with 10× SSC (1.5 M NaCl and 0.17 M sodium citrate) to Hybond-N nylon membrane (Amersham International, Buckinghamshire, UK). The membrane was hybridized overnight with a ^{32}P-labeled, 1.7-kb, *Sal*I-*Not*I-restricted *OsNRT1* cDNA probe at 65°C in hybridization buffer containing 5× SSC, 0.1% (w/v) SDS, 5× Denhardt's solution, and 25 μg/mL fragmented salmon sperm DNA. The blots were also hybridized with ^{32}P-labeled exon 1 of the rice nitrate reductase 1 (*nia1*) gene (obtained by PCR amplification) (Choi et al., 1989) as a positive control for nitrate induction and a 1.4-kb *Eco*RI fragment of the rice actin 1 gene (McElroy et al., 1990) as an internal loading control. The blots were washed sequentially with 2× SSC and 0.1% (w/v) SDS for 30 min at room temperature; 2× SSC and 0.1% (w/v) SDS for 30 min at 65°C; 0.2× SSC and 0.1% (w/v) SDS for 30 min at 65°C.

Yeast Transformation and Growth Assays

The *OsNRT1* cDNA fragment excised with *Bam*HI at both ends from *pSK-OsNRT1F* was blunted with Klenow DNA polymerase and inserted into a *Bst*XI-restricted, Klenow-filled pFL61 vector (Minet et al., 1992) to create *pOsNRT1(S)* (with *OsNRT1* in the sense orientation) or *pOsNRT1(AS)* (with *OsNRT1* in the antisense orientation) downstream of the phosphoglycerate kinase (PGK) promoter. *pOsNRT1(S)* and *pOsNRT1(AS)* were transformed into yeast strain PB1X-2AΔ (*MAT*a *ura3-52 leu2-3 lys1-1 his4-32 ptr2::LEU2*) grown in YEPG medium (1% [w/v] yeast extract, 2% [w/v] peptone, and 2% [w/v] Glc) (Perry et al., 1994) according to the method previously reported by Gietz and Schiestl (1995). Yeast transformants were selected on minimal medium composed of 2% (w/v) Glc, 1× yeast nitrogen base (YNB) without amino acids, and 2% (w/v) agar supplemented with 80 μM His and 80 μM Lys.

Growth assays were performed to assess the ability of yeast transformants containing *pOsNRT1(S)* and *pOsNRT1(AS)* to use the dipeptide (His-Leu) as a source of His in meeting auxotrophic requirements. The dipeptide medium consisted of minimal medium supplemented with auxotrophic requirements minus the amino acid components (His) of the added dipeptide (80 μM His-Leu). PB1X-2AΔ and PB1X-2AΔ (pYES2), a transformant containing the pYES2 vector, were used as negative controls, and PB1X-2AΔ (pJP9) expressing the *Saccharomyces cerevisiae* peptide transporter PTR2p (Perry et al., 1994) was used as a posi-

tive control. Strains were grown in minimal medium (except for PB1X-2AΔ which was grown in YEPG broth) at 30°C overnight and harvested by centrifugation. The cells were washed twice with sterilized distilled water, and were resuspended at a titer of 2×10^8 cells mL^{-1}. A 5-μL aliquot of each dilution, 2×10^8, 2×10^7, and 2×10^6 cells mL^{-1}, was applied to the dipeptide medium to achieve 10^6, 10^5, and 10^4 cells, and incubated at 30°C. Growth of each strain was scored at 48 h.

Functional Analysis of OsNT1 Expressed in *Xenopus* Oocytes

The full-length *OsNRT1* cDNA was subcloned into the oocyte expression vector *pGEMHE* containing the 5′-UTR and 3′-UTR of the *Xenopus* β-globin gene (Liman et al., 1992) to enhance protein expression in oocytes. Capped mRNA was transcribed from the linearized plasmid in vitro using a kit (mMESSAGE mMACHINE, Ambion, Austin, TX). Oocytes were isolated and injected with 50 ng of cRNA as described previously (Tsay et al., 1993). Measurements were made in solutions of: (a) 230 mM mannitol, 0.3 mM $CaCl_2$, 5 mM 2-(*N*-morpholino)-ethanesulfonic acid (MES), and 10 mM Tris-MES at different ratios to yield the pH indicated, and (b) 220 mM mannitol, 0.3 mM $CaCl_2$, and 10 mM Tris-MES at the pH indicated plus HNO_3, Gly-Gly, His-Gly, Ala-His, Ala-Asp, or His at the concentration indicated. For the current-voltage (I-V) curve measurement, oocytes were clamped at −60 mV and assayed from −20 to −140 mV in 20-mV step for 300 ms each. For K_m measurement, oocytes were voltage-clamped to −60 mV or clamped at −60 mV and assayed from −30 to −150 mV in 30-mV step for 300 ms each and exposed to different concentrations of nitrate. Measurements were recorded with a 486-based microcomputer using the AXOTAPE and pCLAMP programs (Axon Instruments, Foster City, CA). The high- and low-affinity uptake assay of *OsNRT1*-injected oocytes determined by HPLC analysis was performed as described previously (Liu et al., 1999).

In Situ Hybridization

Plants were grown in modified Kimura B solution for 1 month, then shifted to a medium with 5 mM KNO_3 for 12 h before harvesting root tissues. The root tissues were fixed, dehydrated, embedded in Paraplast, and sectioned as described previously (Huang et al., 1999) with the following modifications: (a) in xylene:Paraplast mixture (3:1, 1:1, 1:3) for 1 h each instead of 30 min, (b) pure melted Paraplast at 62°C for 2 h three times.

Full-length *OsNRT1* without the poly(A^+)-tail was synthesized by PCR using primer RC7 (5′-CCGGATCCAT GGACTCCTCATACC-3′) and primer RC8 (5′-CCTCTAG AGCAACACAATTGTCC-3′), and cloned into *Sma*I-restricted pBluescript SII− vector to give plasmid *pOsNRT1*(−*A*), which was confirmed by sequencing. A ^{33}P-labeled *OsNRT1* antisense RNA probe was synthesized using T7 RNA polymerase (Promega, Madison, WI) from *Eco*RI-linearized *pOsNRT1*(−*A*), and a sense RNA probe was synthesized using T3 RNA polymerase (Promega) from *Xba*I-linearized *pOsNRT1*(−*A*). Root sections were hybridized to hydrolyzed RNA probes and washed as described previously (Huang et al., 1999). Slides were exposed for 2 months.

RESULTS

Cloning and Sequence Analysis of OsNRT1

As described in the experimental procedures, a full-length *OsNRT1* clone was constructed by rescuing, using 5′-RACE, the missing sequences of *RICR2778*, a rice EST clone that was initially identified by its homology with the sequence of the Arabidopsis nitrate transporter gene *CHL1*. The final clone obtained had an insert of 1,950 bp. Genomic Southern analysis indicated that *OsNRT1* is a single-copy gene, because only one hybridized band was detected when the genomic DNA was digested with *Bgl*II, *Bam*HI, *Eco*RI, or *Xho*I (Fig. 1, lanes 1–3 and 5), and two bands were found when the genomic DNA was restricted with *Hin*dIII (Fig. 1, lane 4), which has a cutting site in the cDNA of *OsNRT1*.

Sequencing the *OsNRT1* cDNA clone revealed a 1,755-bp open reading frame for a 584-amino acid protein with a predicted molecular mass of 64 kD (Fig. 2A). Hydropathy analysis of the deduced amino acid sequence suggests that, similar to the predicted topologies of previously identified NRT1 proteins, OsNRT1 contains 12 putative transmembrane domains with a long hydrophilic loop separating the two groups of six transmembrane domains (Fig. 2B). OsNRT1 shares significant sequence identity (30%–50%) with members of a growing proton-dependent transporter

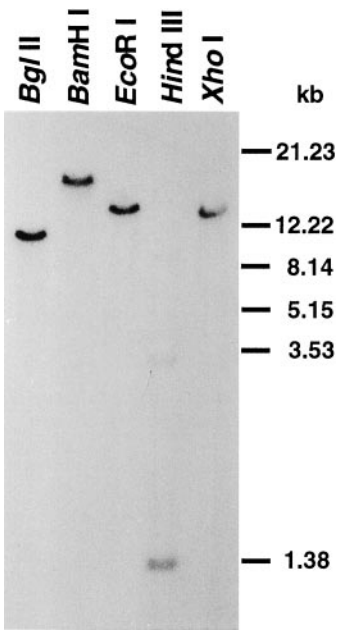

Figure 1. Genomic DNA-blot analysis of *OsNRT1* gene. Genomic DNA (10 μg), isolated from the rice cv Nipponbare, was digested with *Bgl*II, *Bam*HI, *Eco*RI, *Hin*dIII, and *Xho*I, fractionated in a 0.8% (w/v) agarose gel, transferred, and hybridized with radiolabeled full-length (1.7 kb) *OsNRT1* cDNA probes.

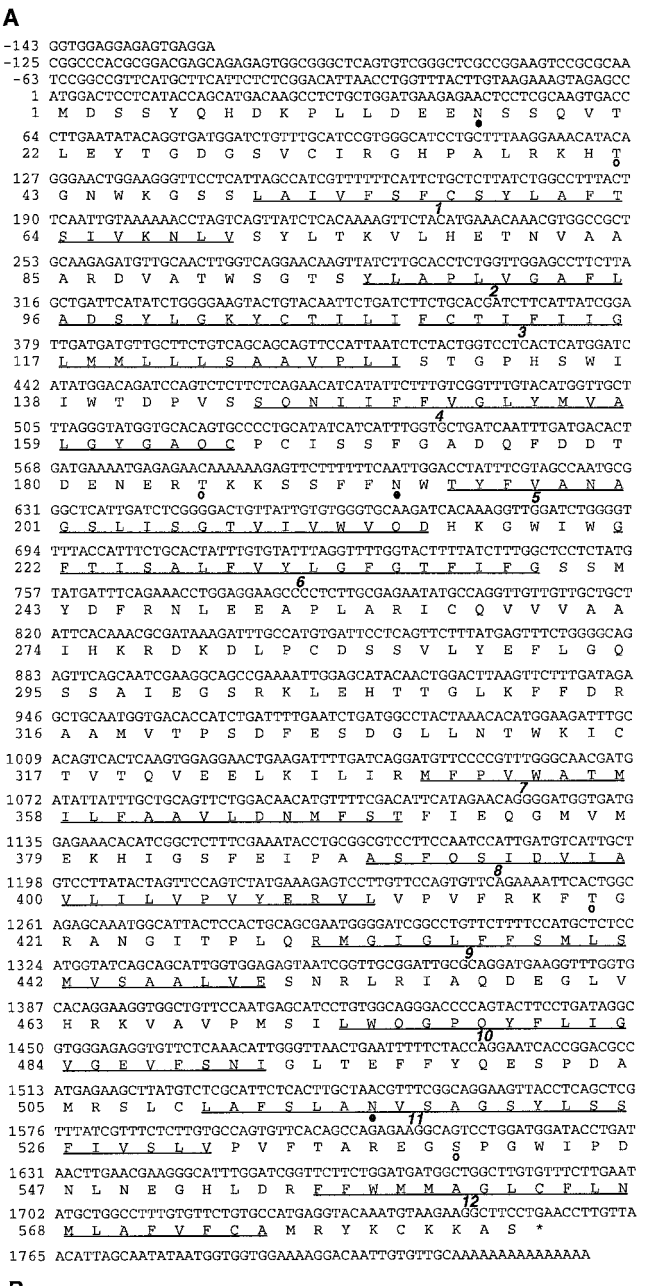

Figure 2. Sequence of *OsNRT1* cDNA and hydropathy profile of OsNRT1. A, Nucleotide sequence of *OsNRT1* cDNA and the deduced amino acid sequence of the OsNRT1 protein (accession no. AF140606). *OsNRT1* cDNA (1,950 bp) contains an open reading frame starting at position 144. ●, Potential *N*-linked glycosylation sites. ○, Consensus phosphorylation sites. The putative transmembrane regions are underlined and numbered. B, Hydropathy analysis of the predicted sequence of OsNRT1. Hydropathy profile of the OsNRT1 protein was determined by the method of Kyte and Doolittle (1982) using a window of 19 amino acid residues.

family called PTR or POT (Paulsen and Skurray, 1994; Steiner et al., 1995) that comprise not only nitrate transporters from higher plants but also peptide transporters from bacteria, fungi, animals, and higher plants (Fig. 3). No significant homology with members of the NRT2 high-affinity nitrate transporter family (Daniel-Vedele et al., 1998) was detected.

Substrate Specificity of OsNRT1

To determine whether *OsNRT1* encodes a nitrate transporter or a peptide transporter, in vitro-synthesized *OsNRT1* complementary RNAs were microinjected into *Xenopus* oocytes. Two to 3 d after injection, these oocytes were incubated in a mannitol buffer of pH 7.4, voltage clamped at −60 mV, and then perfused with substrate solution at pH 5.5. The shift of pH from 7.4 to 5.5 elicited little current in both *OsNRT1*- and water-injected oocytes (bottom row of Table I), whereas the inward current change elicited by nitrate in *OsNRT1* cRNA-injected oocytes was approximately 200 times larger than those observed in the water-injected controls (top row of Table I). In contrast, *OsNRT1*-injected oocytes and water-injected controls showed little or no inward current at −60 mV in the presence of the neutral dipeptide Gly-Gly, a positively charged dipeptide Ala-Lys, or a negatively charged dipeptide Ala-Asp.

The substrate specificity of OsNRT1 remained unchanged at lower membrane potentials. Inward currents elicited by nitrate in *OsNRT1* cRNA-injected oocytes became larger at more negative membrane potentials, but little or no current was elicited by neutral or charged dipeptides at all of the membrane potentials tested (Fig.

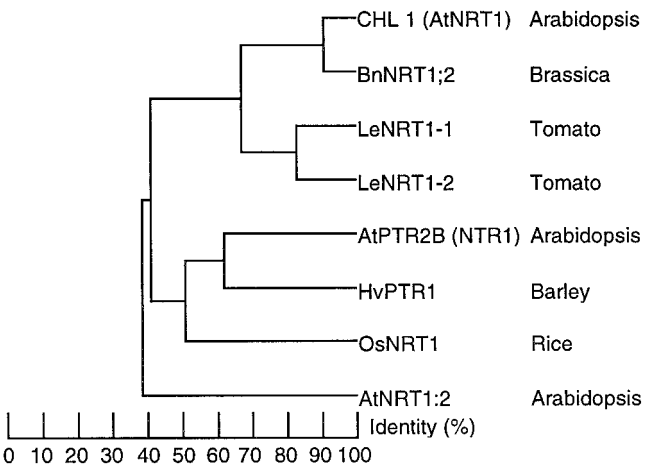

Figure 3. Phylogenetic tree of OsNRT1, CHL1, and its homologs in higher plants. The phylogram was created using the Growtree program of the GCG package (version 9.1, Genetic Computer Group, Madison, WI). CHL1 (AtNRT1) and AtNTR1:2 are nitrate transporters from Arabidopsis (Tsay et al., 1993; Huang et al., 1999), BnNRT1;2 is a nitrate/His transporter from *B. napus* (Muldin and Ingemarsson, 1995; Zhou et al., 1998), LeNRT1-1 and LeNRT1-2 are from tomato (Lauter et al., 1996), AtPTR2B is an Arabidopsis peptide transporter (Frommer et al., 1994; Rentsch et al., 1995; Song et al., 1996), and HvPTR1 is a barley peptide transporter (West et al., 1998).

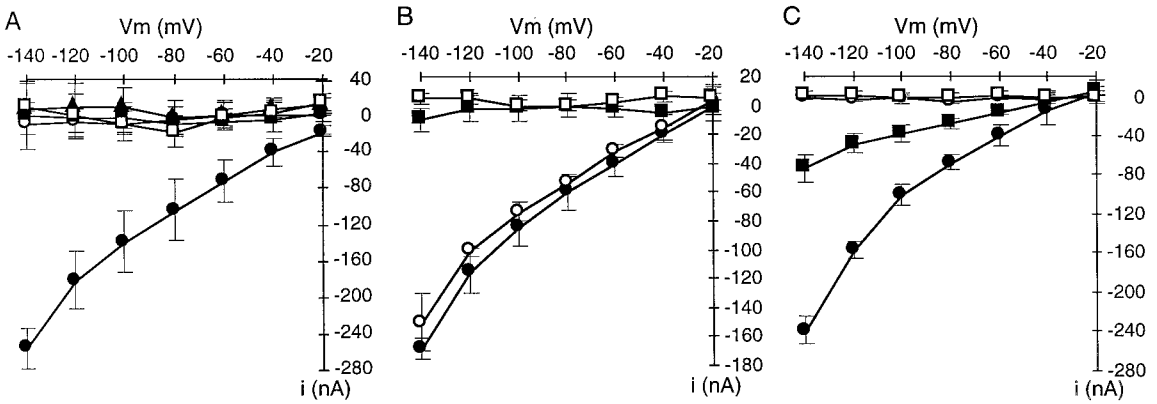

Figure 4. The I-V difference relationship of *OsNRT1* expressed in *Xenopus* oocytes. Currents presented here are the difference between measurements conducted in the presence and absence of substrate. Currents elicited at the end of 300-ms pulses were plotted as the function of the voltage. A, Voltage dependence of nitrate-elicited currents in *OsNRT1* cRNA-injected oocytes. The *OsNRT1*-injected oocyte was exposed to 10 mM nitrate (●), 10 mM dipeptides Gly-Gly (○), 10 mM His-Gly (■), 10 mM Ala-His (□), and 10 mM Ala-Asp (▲) at pH 5.5. The oocyte was voltage clamped from −60 mV to a voltage between −20 and −140 mV for 300 ms at −20-mV increments. Each data point represents the average current obtained from four independent oocytes isolated from three different frogs. B, His and nitrate uptake. *OsNRT1*-injected oocytes were exposed to 10 mM nitrate at pH 5.5 (●), 10 mM His at pH 5.5 (■), 10 mM nitrate plus 10 mM His at pH 5.5 (○), and 10 mM His at pH 8.5 (□). Each data point represents the average current obtained from three independent oocytes. C, pH dependence of the nitrate-elicited current in *OsNRT1*-injected oocytes. ●, Current elicited by nitrate at pH 5.5. ■, Current elicited by nitrate at pH 7.4. ○, Current elicited by nitrate in a water-injected oocyte at pH 5.5. □, Current elicited by nitrate in a water-injected oocyte at pH 7.4. Shown here are average currents elicited in three *OsNRT1*-injected oocytes from three donor frogs.

4A). These data are indicative of *OsNRT1* encoding an electrogenic nitrate transporter.

To further confirm that OsNRT1 is not a peptide transporter, *OsNRT1* was introduced into yeast mutant auxotrophic for His in both the sense (S) and antisense (AS) orientations. Figure 5 shows that PB1X-2A Δ (*pOsNRT1* [*S*]) and PB1X-2AΔ (*pOsNRT1* [*AS*]) yeast transformants exhibited no growth on the dipeptide medium of His-Leu after 48 h of incubation, indicating that these two yeast transformants were unable to use His-Leu as a source of amino acid His. In contrast, PB1X-2AΔ (pJP9), a positive control expressing the *S. cerevisiae* peptide transporter PTR2p (Perry et al., 1994), showed heavy growth in 48 h. The negative controls, PB1X-2AΔ (−) and PB1X-2AΔ (pYES2), on the same dipeptide medium exhibited no growth. A significant number of plant membrane proteins expressed in yeast function properly at the plasma membrane of yeast (for review, see Rentsch et al., 1998). *OsNRT1* mRNA was expressed at a high level in the yeast transformant, as evidenced from RNA gel blotting analysis (data not shown). Therefore, it is likely that the observed no growth of PB1X-2AΔ (*pOsNRT1 [S]*) on the dipeptide medium is due to the lack of dipeptide uptake of OsNRT1.

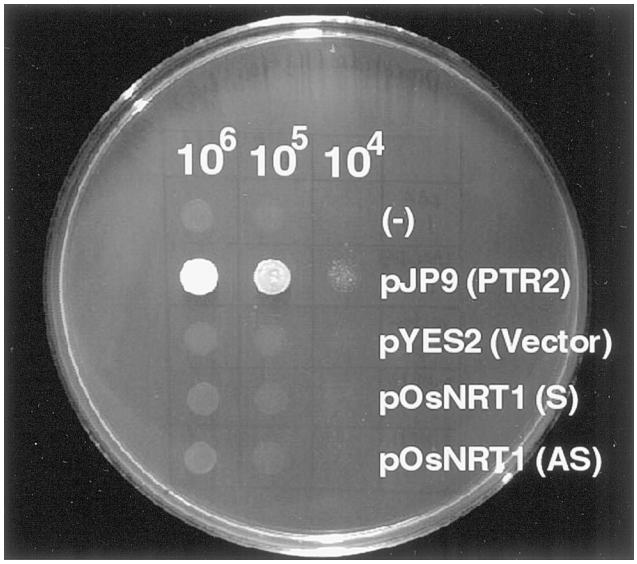

Figure 5. Growth assays of *OsNRT1*-transformed dipeptide-auxotrophic mutant yeast. *pOsNRT1(S)* and *pOsNRT1(AS)* yeast transformants were grown for 48 h on dipeptide medium (His-Leu) to assess their ability to use dipeptide (His-Leu) as a source of amino acid. (−), PB1X-2AΔ negative control; pJP9 (PTR2p), pYES2 (Vector), pOsNRT1 (S), and pOsNRT1 (AS), PB1X-2AΔ transformed with the plasmid of pJP9, pYES2, pOsNRT1 (S), and pOsNRT1 (AS), respectively.

In addition to nitrate and peptide, His uptake activity has been reported for members of the PTR family. For example, PHT1, isolated from rat brain, is a peptide and His transporter (Yamashita et al., 1997), and the *Brassica napus* nitrate transporter BnNRT1;2 exhibits significant His transport activity, especially at pH 8.5 (Zhou et al., 1998). However, at the membrane voltages tested here from −20 to −140 mV, oocytes injected with *OsNRT1* cRNA did not respond to His at either pH 5.5 or 8.5 (Table I; Fig. 4B). Moreover, the currents elicited by nitrate at pH 5.5 in *OsNRT1*-injected oocytes were the same whether or not His was present in the external solution (Fig. 4B). Based on these analyses of *OsNRT1* in *Xenopus* oocytes and yeast cells, we can conclude that *OsNRT1* encodes a nitrate transporter with no (or little) peptide and His transport activity.

Table I. *Functional analysis of OsNRT1 by whole-cell current measurements of injected oocytes*

Substrate (10 mM)[a]	pH[a]	*OsNRT1* mRNA[b]	Water[b]
		(nA)	*(nA)*
NO_3^-	5.5	−40.6 ± 7.0	0.2 ± 1.9
		n^c = 11	n = 12
Gly-Gly	5.5	2.1 ± 7.6	2.0 ± 3.5
		n = 8	n = 14
Ala-Lys^+	5.5	−6.2 ± 6.9	−4.2 ± 5.6
		n = 5	n = 11
Ala-Asp^-	5.5	−6.8 ± 9.7	−4.7 ± 4.4
		n = 5	n = 10
Histidine	5.5	−2.9 ± 4.6	1.4 ± 3.3
		n = 11	n = 8
–	5.5	−1.5 ± 4.3	−0.6 ± 2.8
		n = 11	n = 9

[a] Measurements were made in a solution containing 230 mM mannitol, 0.3 mM $CaCl_2$, 5 mM MES, and 5 mM Tris (pH 7.4), then perfused with solution containing 10 mM substrate indicated, 220 mM mannitol, 0.3 mM $CaCl_2$, and 10 mM MES/Tris at pH 5.5 or with control solution (bottom row) containing 230 mM mannitol, 0.3 mM $CaCl_2$, and 10 mM MES/Tris at pH 5.5. [b] Inward current changes elicited by the substrate indicated on the left are shown. Measurements were made 2 to 4 d after injection. [c] *n*, Number of oocytes tested. The data are shown in averages ± SD.

pH Dependence of Nitrate-Elicited Currents

When *OsNRT1*-injected oocytes were voltage-clamped at −60 mV and then exposed to 10 mM nitrate at different pH values for six independent *OsNRT1*-injected oocytes, the currents elicited by nitrate at pH 7.4 were only approximately 34% ± 17% of the currents elicited by nitrate at pH 5.5. The currents elicited at pH 8.4 were further reduced to −2% ± 14% of the currents elicited at pH 5.5. Similar results were obtained in the I-V measurements (Fig. 4C): 10 mM nitrate elicited larger currents at pH 5.5 than at pH 7.4 in *OsNRT1*-injected oocytes at each of the membrane potentials (from −20 to −140 mV) tested. The I-V curves of Figure 4C indicated that the currents elicited at pH 7.4 were approximately 30% to 40% of the currents elicited at pH 5.5. In contrast, similar treatments at pH 5.5 or 7.4 elicited no more than 5-nA currents in water-injected oocytes (Fig. 4C). The positive inward current of *OsNRT1*-injected oocytes in response to negatively charged nitrate and the pH dependence of the current elicited suggest that OsNRT1 functions as a proton-coupled nitrate cotransporter, with the ratio of proton to nitrate being larger than one.

Low-Affinity Nitrate Transport of OsNRT1

To determine the nitrate affinity of OsNRT1, *OsNRT1*-injected oocytes voltage-clamped at −60 mV were exposed to different concentrations of nitrate at pH 5.5. As shown in Figure 6A, the amplitudes of the inward positive currents elicited by nitrate at pH 5.5 were concentration dependent. For 14 oocytes tested, the K_m values obtained by fitting the current-concentration curves to the Michaelis-Menten equation were in the range of 7.2 to 12.6 mM, with an average of 9.1 ± 1.8 mM. At the membrane potentials tested, from −30 to −150 mV, the K_m values for nitrate measured at pH 5.5 were voltage independent, with an average of 8 to 9 mM (Fig. 6B).

We have recently shown that CHL1 is a dual-affinity nitrate transporter, but its Arabidopsis homolog AtNRT1:2 is a low-affinity nitrate transporter (Huang et al., 1999; Liu et al., 1999). Therefore, it was important to determine whether OsNRT1 could also function as a high-affinity nitrate transporter. The same batch of *OsNRT1*-injected oocytes was incubated with either 10 mM or 250 μM of nitrate for low- and high-affinity uptake measurement, respectively. The measurements were carried out with

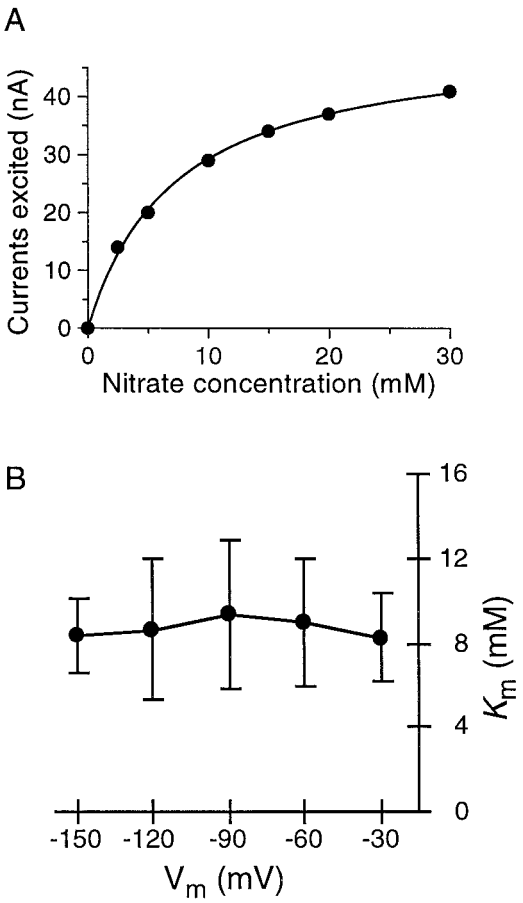

Figure 6. Kinetics of nitrate-evoked currents in *OsNRT1*-injected oocytes. A, Concentration dependence of nitrate-elicited currents in a single *OsNRT1*-injected oocyte. The oocyte was voltage-clamped at −60 mV, and the inward currents elicited by nitrate at pH 5.5 were plotted as a function of the external nitrate concentration. For this particular experiment, the K_m value calculated by fitting to the Michaelis-Menten equation using the nonlinear least-squares method in the Origin 5.0 program (Microcal Software, Northampton, MA) was 7.3 ± 0.4 mM. The average K_m calculated from 14 independent oocytes was 9.1 ± 1.8 mM. B, Voltage dependence of K_m for nitrate determined at pH 5.5 in four *OsNRT1*-injected oocytes. K_m values were determined by fitting I-V curves to the Michaelis-Menten equation at each voltage. Shown here are average values of K_m calculated from four individual oocytes isolated from two different frogs.

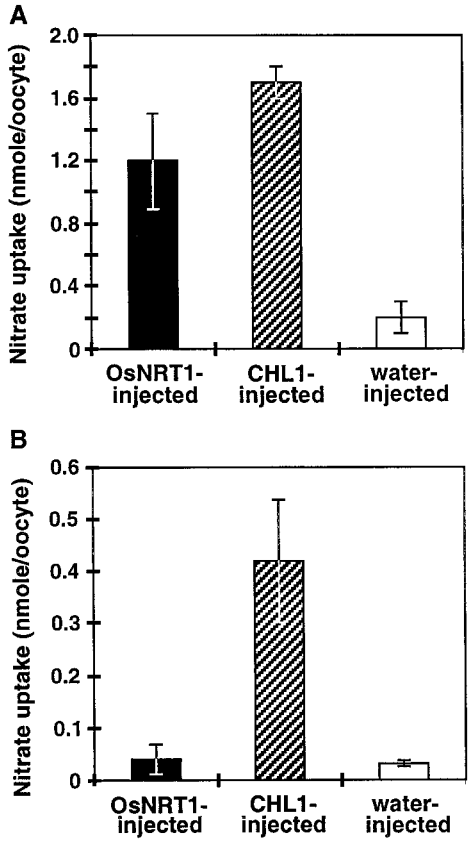

Figure 7. Nitrate uptake analysis of *OsNRT1*-injected and *CHL1*-injected oocytes. A, Low-affinity nitrate uptake activities of *OsNRT1*-injected and *CHL1*-injected oocytes determined with 10 mM NO_3^- at pH 5.5. Oocytes were incubated with 10 mM NO_3^- at pH 5.5 for 3 h, then assayed for the retained nitrate by HPLC. B, High-affinity nitrate uptake activities of *OsNRT1*-injected and *CHL1*-injected oocytes determined with 0.25 mM NO_3^- at pH 5.5. Oocytes were incubated with 0.25 mM NO_3^- at pH 5.5 for 3 h, then the amount of nitrate depleted from the medium was determined by HPLC. Each data point represents the average value obtained from the measurements of three batches, each consisting of five oocytes. Similar results were obtained with oocytes isolated from three different frogs.

HPLC analysis, as described previously (Liu et al., 1999). The results showed that only low-affinity (Fig. 7A) not high-affinity (Fig. 7B) nitrate uptake activity was observed in *OsNRT1*-injected oocytes. In contrast, and confirming our previous findings (Liu et al., 1999), the same batch of oocytes injected with *CHL1* exhibited significant activity in both low- and high-affinity phases of nitrate uptake. This indicates that like AtNRT1:2 of Arabidopsis, OsNRT1 is a low-affinity nitrate transporter with no apparent activities in high-affinity uptake.

Constitutive Expression of *OsNRT1* in Rice Root

As shown in Figure 8, RNA gel blotting analysis indicated that *OsNRT1* is a root-specific gene with little or no expression in the shoot. At time 0, when the plants were depleted for nitrate for 2 d, there was a low level of the mRNA of the leaf-specific nitrate reductase *nia1*. However, 4 h after the addition of nitrate, the *nia1* mRNA level increased approximately 10-fold. After 8 h, the *nia1* mRNA level experienced a negative feedback regulation and began to decrease. In contrast, the *OsNRT1* mRNA level remained relatively stable before and after nitrate induction. This indicates that like Arabidopsis *AtNRT1:2* (Huang et al., 1999) and tomato *LeNRT1–1* (Lauter et al., 1996), *OsNRT1* is a constitutive gene.

Accumulation of *OsNRT1* mRNA in Root Epidermis and Root Hair

To determine the root tissue in which *OsNRT1* is expressed, roots of 30-d-old rice were fixed, sectioned, and hybridized with ^{33}P-labeled sense and antisense transcripts of the *OsNRT1* DNA. From the exterior to the core, rice roots contain one layer of epidermal cells, followed by one layer of exodermal cells, one layer of sclerolenchymal cells, several layers of cortical cells, and then the vascular cylinder (Fig. 9; Clark and Harris, 1981). As shown in Figure 9, B and D, a high density of silver grains (which appear yellow due to the colored filter), indicating the accumulation of *OsNRT1* mRNA, was found predominantly in the epidermal cells and root hairs. As a control, similar sections hybridized with the sense probe showed only a background level of signal in all layers of the root (Fig. 9F).

DISCUSSION

In this study, a new member (and the first from rice) of the NRT1 nitrate transporter family was cloned. NRT1 is a subset of the PTR family that also includes proton-coupled oligopeptide transporters (Paulsen and Skurray, 1994; Steiner et al., 1995). Because chemically distinct compounds—nitrate, peptide, and His—have been identified as substrates for different members of the PTR family, it was critical that we clarify the substrate specificity of the new member, OsNRT1.

As shown above, significant nitrate-induced currents indicative of uptake were observed in *OsNRT1*-injected *Xenopus* oocytes, whereas no apparent uptake activity was

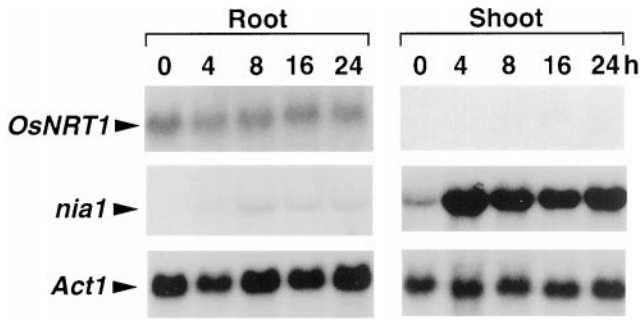

Figure 8. Northern analysis of *OsNRT1* expression. cv Nipponbare rice plants were grown in the Kimura B solution for 30 d, shifted to N-depleted Kimura B solution for 2 d, and then transferred to Kimura B solution containing 5 mM $NaNO_3$, pH 5.5, for the times indicated. Total RNA (15 μg) was fractionated in a 1% (w/v) agarose gel, transferred to nylon membrane, and hybridized with radiolabeled DNA from *OsNRT1*, *nia1*, and *Act1*.

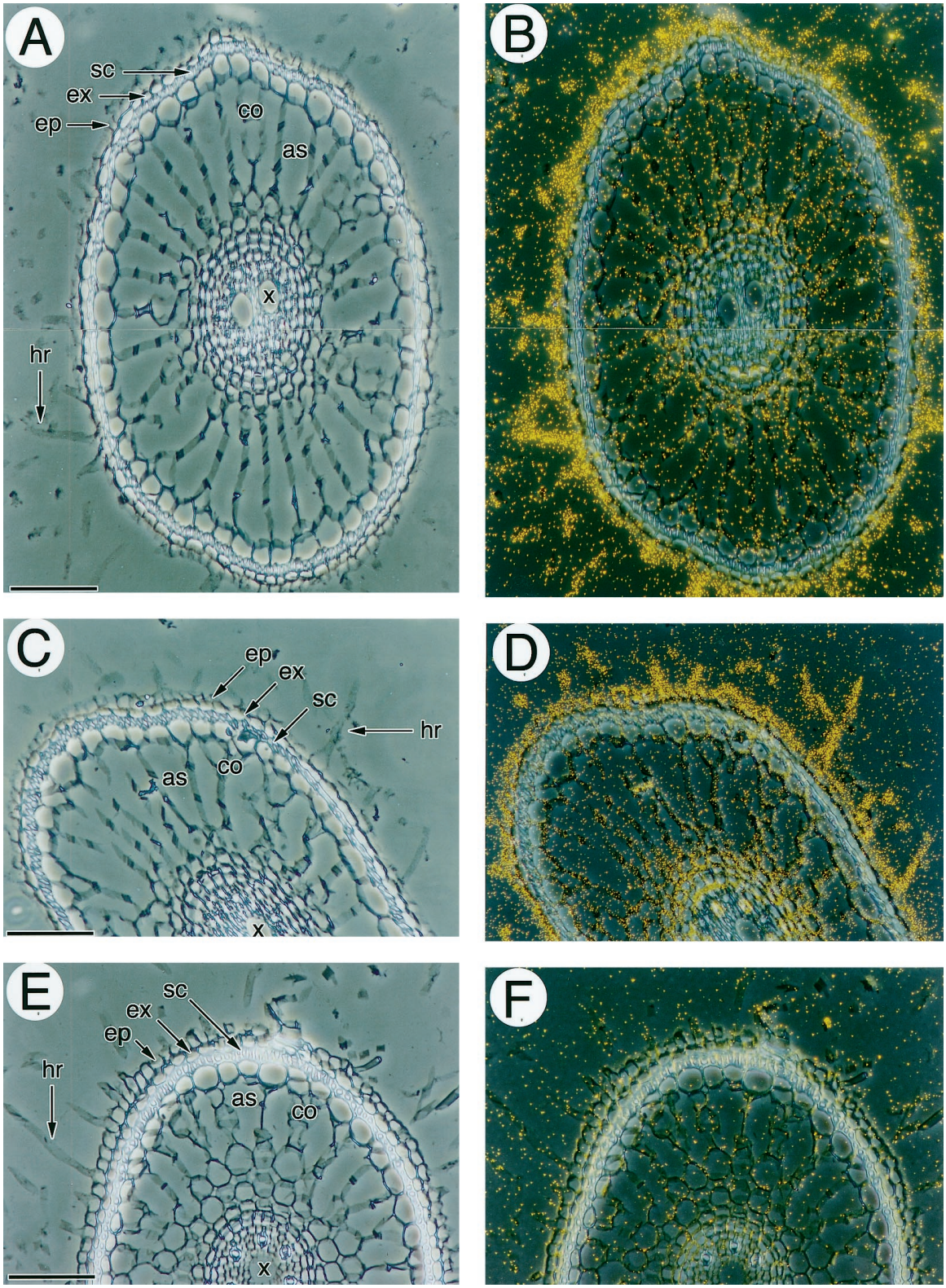

Figure 9. In situ hybridization of *OsNRT1* to rice root sections. A to D, In situ hybridization of antisense *OsNRT1* probe to a cross-section of rice root tissues. E and F, In situ hybridization of sense *OsNRT1* probes to a cross-section of rice root tissues. A, C, and E, Bright-field microscopy. B, D, and F, Double exposures using a colored filter for the dark-field exposure, causing the *OsNRT1* signals to appear yellow. ep, Epidermis; ex, exodermis; sc, sclerenchyma; co, cortex; as, air space (aerenchyma); hr, root hair; x, late metaxylem. Bars = 100 μm.

observed for various dipeptides or His (Fig. 4, A and B). Augmenting this oocyte uptake result, OsNRT1 could not complement a yeast dipeptide auxotrophic mutant, while the *S. cerevisiae* PTR2p and Arabidopsis AtPTR2B peptide transporters could (Perry et al., 1994; Steiner et al., 1994; Fig. 5). Furthermore, the dependence on membrane potential and pH of nitrate uptake activity (Fig. 4), in conjunction with expression of *OsNRT1* in the rhizodermis (Fig. 9), are consistent with the notion that OsNRT1 is a proton-dependent electrogenic nitrate transporter directly involved in uptake. The functional assays of OsNRT1, together with those of other NRT1 genes—CHL1 (Tsay et al., 1993; Huang et al., 1996) and AtNRT1:2 (Huang et al., 1999) from Arabidopsis and BnNRT1;2 from *B. napus* (Zhou et al., 1998)—provide converging evidence that the NRT1 proteins function primarily as nitrate transporters. Interestingly, this is despite OsNRT1 being more similar in sequence to two peptide transporters than to the nitrate transporters (Fig. 3). Consequently, the substrate specificity of members of the PTR family cannot be inferred from sequence comparison, leaving future research to delineate the sequence and structure components underlying the distinct substrate specificities of this transporter family.

We conducted nitrate induction experiments with different rice cultivars, including two local Japonica cultivars (cv Taikeng 9 and cv Tainung 67) and two Indica cultivars (cv Taichung Sen 10 and cv IR-36). In all of the rice cultivars tested, *OsNRT1* was expressed constitutively. In addition, in two wild rice species (*Oryza perennis* and *Oryza officinalis*), one hybridizing band was found in the RNA get blotting analysis using *OsNRT1* as a probe, and the intensity of the band remained approximately constant before and after nitrate induction (data not shown). Several studies have shown that the nitrate uptake activities of rice are repressed when ammonium is also present in the nutrient medium (Youngdahl et al., 1982; Kronzucker et al., 1999). However, our data showed no repressed expression of *OsNRT1* mRNA when ammonium was present in the assay solution (data not shown). Therefore, it might be that an ammonium inhibition effect on uptake by OsNRT1 does not occur at the transcriptional level. Alternatively, the observed repression of nitrate uptake by ammonium may be the result of inhibition of another nitrate transporter gene(s).

The oocyte uptake assays revealed significant activities of OsNRT1 in the low-affinity but not in the high-affinity range of nitrate concentrations. Kinetic measurements yielded a Michaelis-Menten K_m for nitrate of approximately 9 mM independent of the membrane potentials tested (Figs. 6 and 7; Table I). These data suggest that *OsNRT1* encodes a constitutive component for low-affinity nitrate uptake. Recent studies (Tsay et al., 1993; Huang et al., 1996, 1999; Lauter et al., 1996) seem to indicate that the NRT1 family comprises both the constitutive and the nitrate-inducible component of the low-affinity nitrate transport system of higher plants. In addition, a striking functional difference between the two components is obvious in the case of Arabidopsis: Whereas CHL1, the nitrate-inducible component, is involved in both low- and high-affinity nitrate uptake (Wang and Crawford, 1998; Liu et al., 1999), AtNRT1:2 (previously referenced as NTL1 or NRT3), the constitutive component, exhibits only low-affinity uptake activities (Huang et al., 1999; Liu et al., 1999), as does OsNRT1 (Fig. 7). The resemblance between AtNRT1:2 and OsNRT1 extends to their tissue-specific expression patterns. Both are primarily expressed in root hair and epidermis, and this expression location is independent of root age (Fig. 9; Huang et al., 1999). In contrast, as the root matures, the great majority of *CHL1* is expressed in inner layers, first in the cortex and then in the endodermis (Huang et al., 1996).

There are now several clones in the rice EST database that share higher sequence identity with *CHL1* than does *RICR2778*, the clone from which *OsNRT1* was derived. Some of these genes might encode proteins with the same properties of CHL1. On the other hand, the root structure of rice is distinctly different from that of Arabidopsis. Rice roots contain exodermis just external to one layer of sclerolenchymal cells. In addition, as rice root matures, its cortical cells develop into aerenchyma (Fig. 9A). These three structures—exodermis, sclerolenchyma, and aerenchyma—are all absent in the Arabidopsis root and they will restrict the apoplast diffusion of ions (Colmer and Bloom, 1998). The significant difference in root structure may suggest that orthologs of the nitrate transporter genes in different species will exhibit a significant variation in their physiological functions. Therefore, it will be particularly interesting to find out if a *CHL1*-like rice gene (if it exists) is similarly expressed in the inner cells (endodermis) of mature roots. In addition to *OsNRT1*, which is a member of the *NRT1* family, a rice cDNA clone belonging to the *NRT2* family has been reported (GenBank accession no. AB008519). Elucidation of the distinct roles of these nitrate transporter genes should yield considerable knowledge for genetic manipulations to lessen the demand on nitrogen fertilization.

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