

Functional Characterization and Expression Analysis of a Gene, *OsENT2*, Encoding an Equilibrative Nucleoside Transporter in Rice Suggest a Function in Cytokinin Transport¹

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We identified four genes for potential equilibrative nucleoside transporters (ENTs) from rice (*Oryza sativa*; designated *OsENT1* through *OsENT4*). Growth analysis of budding yeast (*Saccharomyces cerevisiae*) cells expressing *OsENTs* showed that *OsENT2* transported adenosine and uridine with high affinity (adenosine, $K_m = 3.0 \mu\text{M}$; uridine, $K_m = 0.7 \mu\text{M}$). Purine or pyrimidine nucleosides and 2'-deoxynucleosides strongly inhibited adenosine transport via *OsENT2*, suggesting that *OsENT2* possesses broad substrate specificity. *OsENT2*-mediated adenosine transport was resistant to the typical inhibitors of mammalian ENTs, nitrobenzylmercaptapurine ribonucleoside, dilazep, and dipyridamole. The transport activity was maximal at pH 5.0 and decreased slightly at lower as well as higher pH. In competition experiments with various cytokinins, adenosine transport by *OsENT2* was inhibited by isopentenyladenine riboside (iPR). Direct measurements with radiolabeled cytokinins demonstrated that *OsENT2* mediated uptake of iPR ($K_m = 32 \mu\text{M}$) and trans-zeatin riboside ($K_m = 660 \mu\text{M}$), suggesting that *OsENT2* participates in iPR transport in planta. In mature plants, *OsENT2* was predominantly expressed in roots. The *OsENT2* promoter drove the expression of the β -glucuronidase reporter gene in the scutellum during germination and in vascular tissues in germinated plants, suggesting a participation of *OsENT2* in the retrieval of endosperm-derived nucleosides by the germinating embryo and in the long-distance transport of nucleosides in growing plants, respectively.

Nucleotides participate in various biochemical processes in plants (Stasolla et al., 2003). They are essential precursors for nucleic acids, as well as metabolites participating in bioenergetic processes and in the synthesis of polysaccharides, phospholipids, and glycolipids (Ross, 1981). Furthermore, they are essential constituents of cytokinin (CK), a plant hormone controlling growth and development (Mok, 1994). During de novo nucleotide biosynthesis, nucleosides do not occur as intermediates, but appear during nucleotide turnover in the salvage pathway (Stasolla et al., 2003). Plant cells harbor enzymes allowing nucleoside salvage, and some of the genes encoding these enzymes have been identified and characterized (Stasolla et al., 2003). The enzymes that act on adenine and adenosine have also attracted the attention of researchers interested in CKs because these enzymes are involved in the metabolism of this group of phytohormones (Mok and Mok, 2001). For instance, adenine phosphoribo-

syltransferase (APT) converts free-base CKs into the corresponding nucleotides (Schnorr et al., 1996). *Arabidopsis thaliana* mutants lacking an APT gene are male sterile and are impaired in CK metabolism (Moffatt et al., 1991; Gaillard et al., 1998).

Nucleosides are readily taken up into plant cells. Pollen cells of petunia (*Petunia hybrida*) are able to uptake exogenous nucleosides via both an active transport system and a facilitated diffusion (Kamboj and Jackson, 1984, 1985, 1987). Cotyledons separated from castor bean (*Ricinus communis*) efficiently import endosperm-derived secretion products, including purine and pyrimidine bases, nucleosides, and AMP, but not ATP (Kombrink and Beevers, 1983). Furthermore, when [³H]dihydrozeatin riboside and [³H]trans-zeatin riboside (tZR) were supplied to soybean (*Glycine max*) explants, they were incorporated and immediately metabolized into the corresponding nucleotides (Singh et al., 1988).

Although plant cells are capable of absorbing nucleosides, our knowledge of the mechanism of the process is limited. In general, nucleoside transporters have been categorized into concentrative nucleoside transporters (CNTs) and equilibrative nucleoside transporters (ENTs), which share no structural similarity (Hyde et al., 2001; Acimovic and Coe, 2002; Cabrita et al., 2002). The CNT system catalyzes the active transport of nucleosides against their concentration gradients. Typical CNT proteins possess 12 to 13 predicted transmembrane (TM) helices and are either sodium or proton

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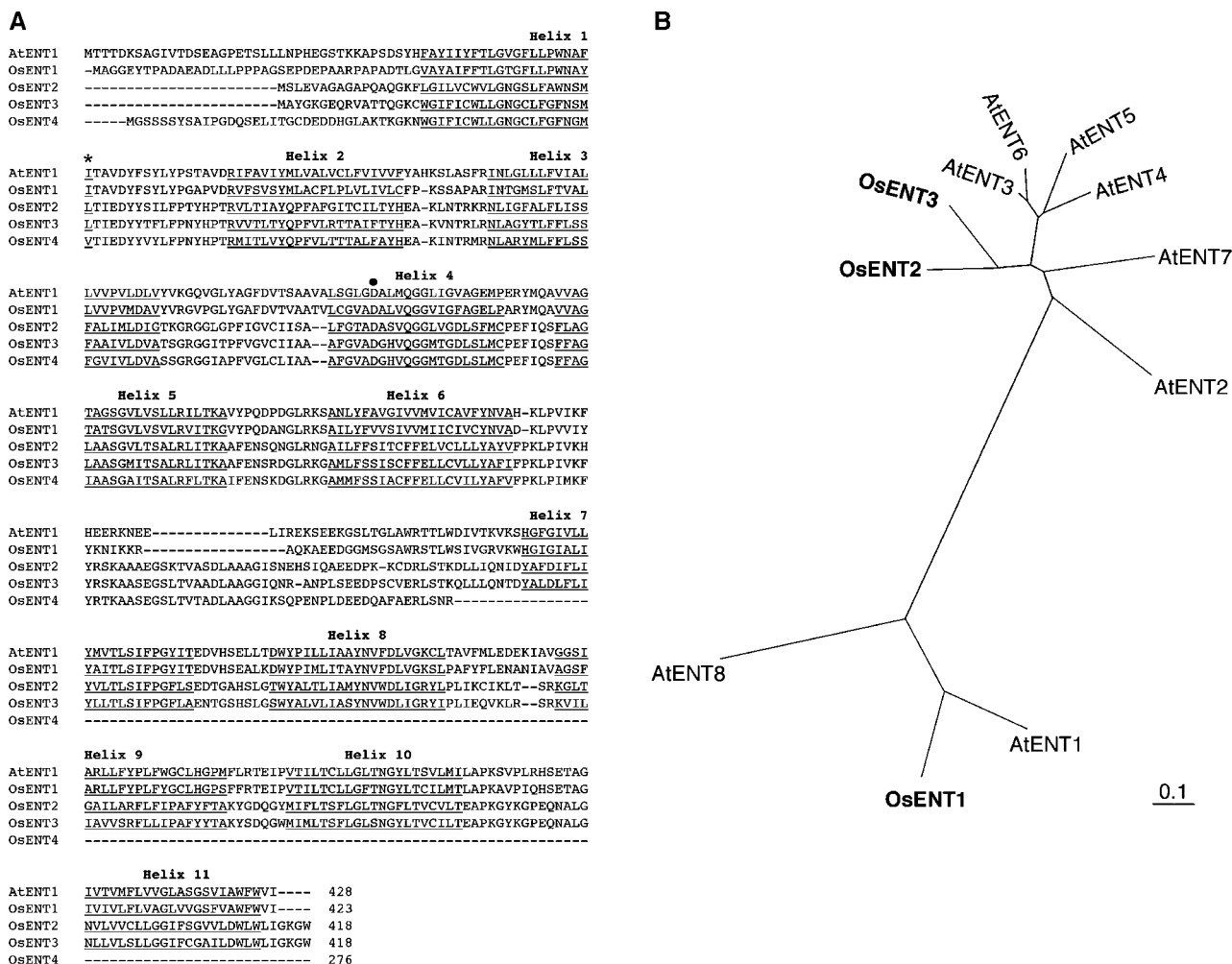
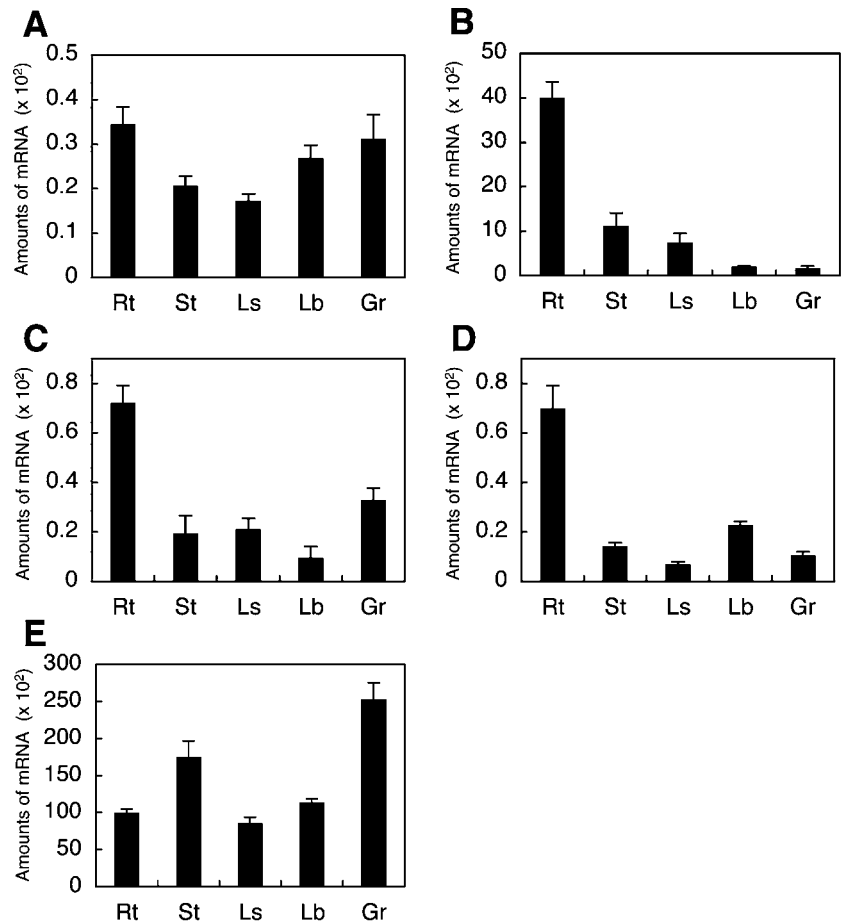


Figure 1. Structural features of OsENTs. A, Multiple alignment of the predicted amino acid sequences of four OsENTs with that of AtENT1. Putative transmembrane helices are underlined. The asterisk highlights the amino acid in plant ENTs that corresponds to residue 33 of hENT1 and hENT2. The black dot marks the Gly residue at the 154 position of hENT1. B, Phylogenetic relationship of OsENT1, OsENT2, OsENT3, and AtENTs. Amino acid alignment of ENTs was performed using the ClustalW program at the DDBJ Web site. Bar = 0.1 amino acid substitutions per site.

symporters (Cabrita et al., 2002). Genes for CNTs have been identified in a variety of bacteria, *Caenorhabditis elegans*, *Drosophila melanogaster*, and several mammalian species (Cabrita et al., 2002). However, structurally homologous genes have not been found in *Arabidopsis thaliana* and rice (*Oryza sativa*). On the other hand, ENT proteins typically exhibit 11 predicted TMs and catalyze the transport of nucleosides down their concentration gradients (Hyde et al., 2001; Cabrita et al., 2002). Genes for ENT family proteins have been identified in a variety of eukaryotes such as *C. elegans* and yeast (*Saccharomyces cerevisiae*), various parasites such as *Trypanosoma brucei brucei* and *Leishmania donovani*, mammals, and higher plants (Hyde et al., 2001; Acimovic and Coe, 2002; Cabrita et al., 2002). The *Arabidopsis* genome harbors eight ENT homologous genes (*AtENTs*), out of which *AtENT1*, *AtENT3*, *AtENT4*, *AtENT6*, and *AtENT7* have been biochemically characterized (Li and Wang, 2000; Möhlmann et al.,

2001; Li et al., 2003; Wormit et al., 2004). When heterologously expressed in yeast cells, these five *AtENTs* transport purine or pyrimidine nucleosides with broad substrate specificity. The *AtENTs* commonly have low sensitivity against typical inhibitors of mammalian ENT proteins such as nitrobenzylmercaptapurine ribonucleoside (NBMPR), dilazep, and dipyrindamole (Möhlmann et al., 2001; Li et al., 2003; Wormit et al., 2004). The pH dependency of the adenosine transport activity differs between the *AtENTs* (Wormit et al., 2004). It has been suggested that *AtENT1*, *AtENT3*, and *AtENT6* are localized in the plasma membrane (Li and Wang, 2000; Li et al., 2003; Wormit et al., 2004). In *Arabidopsis* suspension cells, the transcripts of *AtENT1*, *AtENT3*, *AtENT4*, *AtENT6*, and *AtENT8* accumulated under nitrogen deprivation and following application of fluorouracil and methotrexate, two inhibitors of de novo nucleotide synthesis. This suggested that some *AtENTs* may be involved in the supply of substrates to the

Figure 2. Accumulation patterns of *OsENT* transcripts in various rice organs. Total RNA prepared from various organs was subjected to quantitative real-time PCR. A, *OsENT1*; B, *OsENT2*; C, *OsENT3*; D, *OsENT4*; E, *Act1*. Rt, Root; St, stem; Ls, leaf sheath; Lb, leaf blade; Gr, grain. Amounts of mRNA are given as the copy number of *OsENT* mRNA/ng total RNA. Real-time PCR was performed three times; values shown are means \pm sd. *Actin1* was used as an internal standard.



salvage pathway of nucleotide synthesis (Li et al., 2003). Although valuable information on the transport properties and the expression patterns of *AtENTs* has become available recently, their physiological roles remain to be elucidated, especially regarding their possible involvement in CK transport. Moreover, it is unfortunate that current knowledge of plant ENTs is restricted to *Arabidopsis*.

Here, we report the isolation of four *ENT* genes from rice designated *OsENT1* through *OsENT4*. We demonstrate that *OsENT2* is predominantly expressed in the vasculature and that its gene product can transport a wide spectrum of nucleosides, including nucleoside-type CKs. Potential functions of *OsENT2* during rice development and participation in CK nucleoside transport are discussed.

RESULTS

Isolation of Members of the *ENT* Gene Family in Rice

To identify rice *ENT* genes, a BLAST search was performed in rice genome databases using the amino acid sequence of *AtENT1* as a query. Four *ENT* genes were found and designated *OsENT1*, *OsENT2*, *OsENT3*, and *OsENT4*. The genes are distributed over

two chromosomes (chromosome 8, *OsENT1*; chromosome 7, *OsENT2*, *OsENT3*, and *OsENT4*). The bacterial artificial chromosome clone (accession no. AP005125) on chromosome 7 contains a tandem repeat of three related *OsENTs* (*OsENT2*, *OsENT3*, and *OsENT4*). The cDNA clones of all *OsENTs* were found in the database of full-length cDNA clones from japonica rice at the Knowledge-based Oryza Molecular biological Encyclopedia (KOME; <http://cdna01.dna.affrc.go.jp/cDNA>): *OsENT1* (accession no. AK059439), *OsENT2* (AK102045, AK058524), *OsENT3* (AK101098), and *OsENT4* (AK065096). We obtained the cDNA clones of *OsENT2* and *OsENT4* from the Rice Genome Resource Center (www.rgrc.dna.affrc.go.jp). The *OsENT1* cDNA clone deposited in the database at KOME appeared truncated, while the *OsENT3* cDNA clone was not available from any of the above sources. Therefore, we isolated the cDNA clones of *OsENT1* and *OsENT3* by reverse transcription-PCR.

The cDNA clones of *OsENT1*, *OsENT2*, and *OsENT3* contained reading frames of 423, 418, and 418 amino acids, respectively (Fig. 1A). These three *OsENTs* possessed 11 putative TMs (Fig. 1A). On the other hand, the *OsENT4* cDNA clone contained a reading frame of 276 amino acids (Fig. 1A). Comparisons of the nucleotide sequence of the *OsENT4* cDNA clone

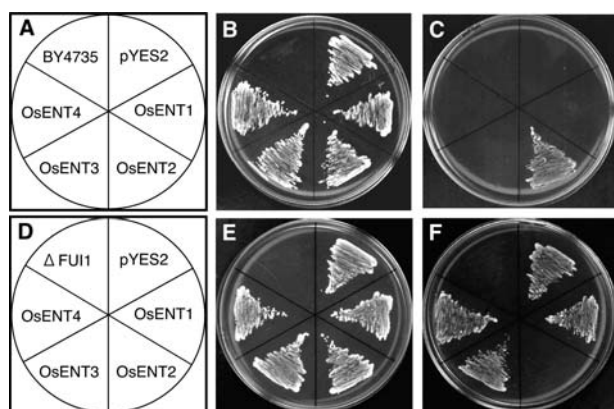


Figure 3. Growth analysis of yeast cells expressing OsENTs. Growth of yeast cells harboring expression plasmids containing *OsENT1*, *OsENT2*, *OsENT3*, *OsENT4*, or controls (BY4735; BY4735 plain recipient cells, Δ FUI1; Δ FUI1 plain recipient cells, pYES2); cells transformed with empty vector pYES2). A, Schematic representation of BY4735 strains used in B and C. B and E, Growth on synthetic minimal medium lacking uracil but containing Gal (20 g L⁻¹). C, Growth on synthetic minimal medium lacking uracil and adenine but containing adenosine (150 μ M) and Gal. D, Schematic representation of Δ FUI1 strains used in E and F. F, Growth on synthetic minimal medium lacking uracil but containing fluorouridine (1 mM) and Gal. Cells were grown for 2 to 3 d at 30°C.

with that of the bacterial artificial chromosome clone (AP005125) revealed that *OsENT4* carried a stop codon in the eighth exon. Thus, *OsENT4* may be a pseudogene, at least in the Nipponbare cultivar, or it may encode a truncated form of typical ENT. *OsENT1* exhibited 65% and 45% amino acid sequence identity with AtENT1 and AtENT8, respectively. *OsENT2* and *OsENT3* shared 71% amino acid sequence identity. Phylogenetic analysis indicated that *OsENT2* and *OsENT3* are more highly homologous to a group of Arabidopsis ENTs consisting of AtENT1 through AtENT7 than to AtENT1 or AtENT8 (Fig. 1B).

Expression Patterns of *OsENTs* in Mature Rice Plants

To evaluate levels of *OsENT* expression in different organs, the accumulation of *OsENT* transcripts was analyzed by quantitative real-time PCR using RNA samples extracted from various organs of mature rice plants. The transcripts of *OsENTs* were detected in all organs tested, but their distribution patterns differed (Fig. 2). Transcripts of *OsENT2*, *OsENT3*, and *OsENT4* accumulated predominantly in roots, whereas that of *OsENT1* did not show any pronounced preference. Accumulation levels of the *OsENT2* transcript were significantly higher than those of the other *OsENTs* in all organs tested, suggesting that *OsENT2* is the dominant form of the rice ENT family.

Growth Analysis of Yeast Expressing *OsENTs*

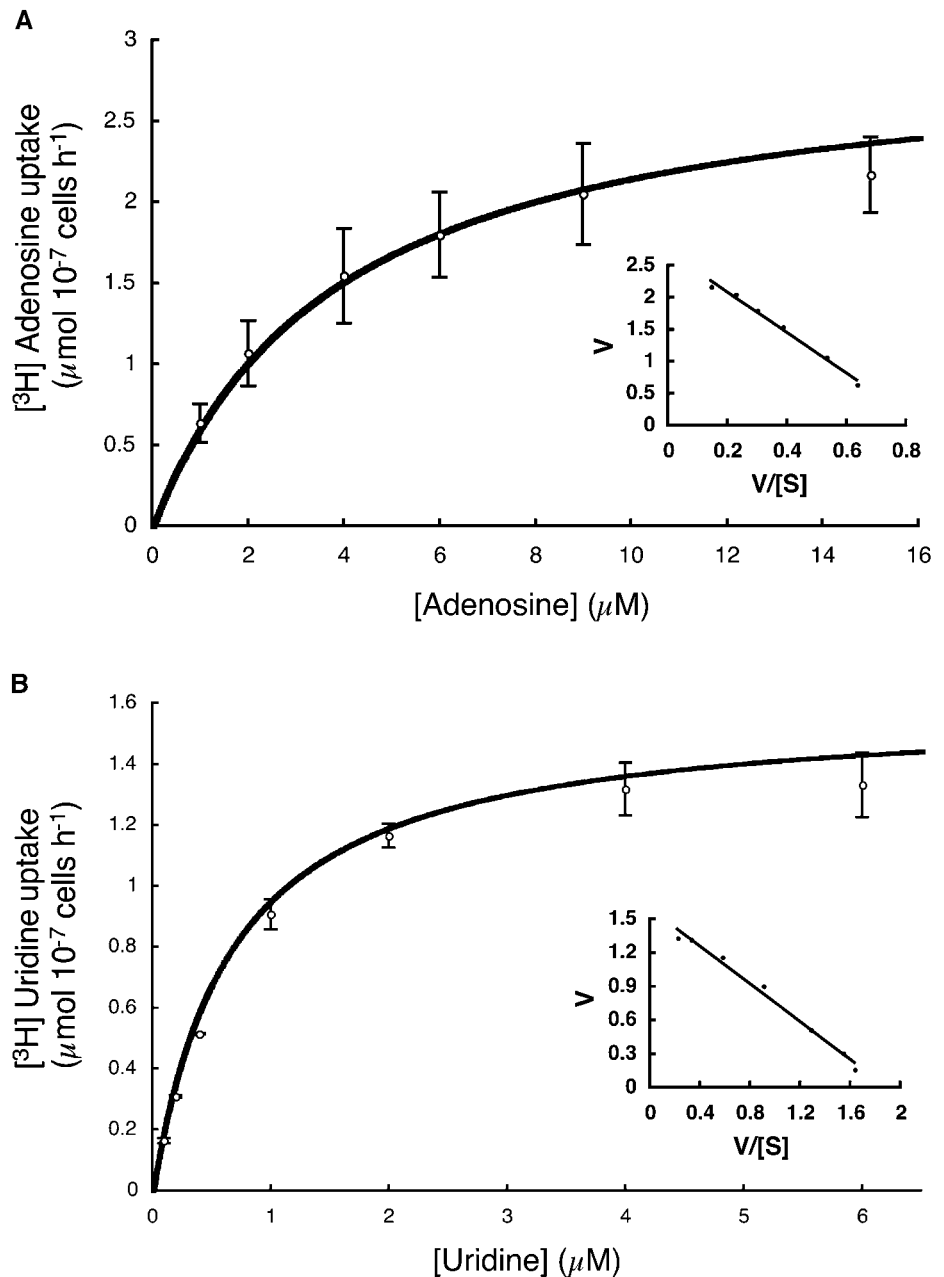
Under normal conditions, yeast cells do not uptake exogenous adenosine because of the lack of an endogenous transport system for the nucleoside and cannot

use it as a purine source (Mässer et al., 1999). Exploiting this property, adenine auxotroph *ade2* mutant strains, which are unable to synthesize adenine, have been successfully employed to evaluate the uptake of adenosine via AtENTs (Möhlmann et al., 2001; Li et al., 2003; Wormit et al., 2004). Either the presence of adenine in the medium or the endowment with a transgenic adenosine uptake system enables the yeast strain to grow in a minimal medium. The transport properties of AtENTs have also been evaluated in the yeast mutant Δ FUI1, which lacks the plasma membrane-localized uridine transporter FUI1 (Vickers et al., 2000). To examine whether *OsENTs* transport the nucleosides, we analyzed growth in the yeast mutants equipped with *OsENT* genes in the presence of either adenosine (a purine nucleoside) or fluorouridine (a toxic pyrimidine nucleoside analog). Although *OsENT4* appears to be truncated, we expressed all *OsENTs*. Figure 3, A to C, shows the growth patterns of the BY4735 (*ade2*) strain, while Figure 3, D to F, shows those of the Δ FUI1 (*fui1*) strain. In both cases, the plain recipient cells (BY4735 and Δ FUI1) could not grow on media lacking uracil, a selection substance for the pYES2 expression vector (Fig. 3, B and E). *OsENT2* was the only *OsENT* able to complement the *ade2* mutation on an adenosine-containing medium (Fig. 3C). On the other hand, although Δ FUI1 cells with the empty pYES2 vector, as well as Δ FUI1 cells expressing *OsENT1*, *OsENT3*, or *OsENT4*, were able to grow in the presence of the toxic compound fluorouridine (Fig. 3F), expression of *OsENT2* proved lethal. These results showed that *OsENT2* mediated adenosine and uridine transport in the yeast system.

Transport Properties of Yeast Cells Expressing *OsENT2*

The properties of *OsENT2*-mediated adenosine and uridine transport were examined using the *ade2* and Δ FUI1 mutants, respectively. In initial time course experiments with [³H]adenosine and [³H]uridine, the uptake rate of both radiochemicals was practically constant for the first 10 min (data not shown). Therefore, subsequent quantifications of uptake kinetics were based on a 2-min period. Figure 4 shows the Michaelis-Menten kinetics of adenosine and uridine uptake mediated by *OsENT2*. The deduced Eadie-Hofstee plots (Fig. 4, insets) indicate K_m values for adenosine and uridine of 3.0 and 0.7 μ M, respectively. The V_{max} values were 3.9 μ mol (10⁷ cells)⁻¹ h⁻¹ for adenosine, and 1.6 μ mol (10⁷ cells)⁻¹ h⁻¹ for uridine. Thus, *OsENT2* exhibited higher affinity for uridine than for adenosine, whereas the V_{max} was lower. In competition experiments, [³H]adenosine uptake via *OsENT2* was significantly and consistently reduced by a series of purine and pyrimidine nucleosides and 2'-deoxynucleosides (Table I), suggesting that *OsENT2* possesses broad substrate specificity for nucleosides. In addition, nucleotides slightly inhibited [³H]adenosine uptake, while none of the nucleobases tested exerted any significant effects (Table I).

Figure 4. Characterization of [^3H]adenosine and [^3H]uridine uptake by yeast cells expressing OsENT2. [^3H]adenosine uptake was examined in BY4735 *ade2* cells expressing OsENT2 (A), whereas [^3H]uridine uptake was tested in the yeast mutant ΔFU11 expressing OsENT2 (B). Cells were incubated for 2 min at the indicated substrate concentrations at pH 6.0. Background uptake rates (empty vector pYES2) were subtracted. Affinity constants were calculated on the basis of Eadie-Hofstee plots (insets). Data shown are means \pm SE ($n = 3$).



The sensitivity of OsENT2-mediated uptake of [^3H]adenosine uptake to typical inhibitors of mammalian ENT proteins, such as NBMPR, dilazep, dipyrindamole, and the protonophore, carbonyl cyanide *m*-chlorophenyl-hydrazone (CCCP), was tested. The effector concentrations and duration of the treatment in our experiments were almost identical to those in previous studies on AtENTs (Möhlmann et al., 2001; Li et al., 2003; Wormit et al., 2004). All three inhibitors of mammalian ENTs tested did not affect the transport activity of OsENT2 (Table II). On the other hand, CCCP reduced [^3H]adenosine uptake by about 30% (Table II). To characterize the pH dependency of the transport in more detail, we examined OsENT2-mediated

[^3H]adenosine transport at different extracellular pH values. The maximum transport activity was observed at pH 5.0 (Fig. 5), but 40% of the activity still was recorded at pH 8.0. These characteristics of OsENT2 transport, namely, low sensitivity to CCCP and slight dependency on pH, resemble those of AtENT3 (Li et al., 2003; Wormit et al., 2004).

OsENT2 Transports Nucleoside-Type CKs in Yeast

Recently, the Arabidopsis purine transporters AtPUP1 and AtPUP2 have been shown to translocate adenine and its various derivatives, such as caffeine, and as well as CK nucleobases (Gillissen et al., 2000; Bürkle et al., 2003). In this context, we speculated that

Table I. Inhibition of *OsENT2*-mediated [^3H]adenosine uptake in BY4735 *ade2* yeast cells by nucleosides, nucleobases, and nucleotides

[^3H]Adenosine uptake was examined at a substrate concentration of $2.0\ \mu\text{M}$ for 2 min at pH 6.0. The concentrations of various additives were $20\ \mu\text{M}$. Mean \pm SE ($n = 3$).

Additive	[^3H]Adenosine Uptake (% of Control)
None	100
Nucleosides:	
Adenosine	23.8 \pm 2.6
Cytidine	35.1 \pm 4.0
Guanosine	20.4 \pm 3.5
Uridine	13.3 \pm 2.2
Inosine	16.6 \pm 2.4
2'-Deoxyadenosine	37.5 \pm 5.6
2'-Deoxycytidine	25.3 \pm 3.8
2'-Deoxyguanosine	29.7 \pm 3.3
2'-Deoxythymidine	6.7 \pm 1.6
2'-Deoxyinosine	24.4 \pm 3.2
Nucleobases:	
Adenine	100.5 \pm 0.7
Cytosine	94.6 \pm 0.9
Guanine	98.8 \pm 2.4
Thymine	89.7 \pm 2.6
Uracil	85.7 \pm 2.8
Hypoxanthine	79.7 \pm 2.1
Nucleotides:	
dATP	77.2 \pm 2.8
ATP	73.5 \pm 1.5
ADP	71.9 \pm 0.3
AMP	72.3 \pm 2.7
CTP	69.4 \pm 2.8
GTP	62.9 \pm 0.1
UTP	64.4 \pm 1.0

OsENT2 might play a role as a CK nucleoside transporter. Competition experiments using various CKs showed that the transport of [^3H]adenosine mediated by *OsENT2* was partially inhibited by isopentenyladenine riboside (iPR; Fig. 6A). The main characteristics of iPR and trans-zeatin riboside (tZR) transport through *OsENT2* were examined using the yeast *ade2* mutant. Since [^3H]iPR and [^3H]tZR were chemically unstable (data not shown), we prepared them just prior to the transport experiments as described previously (Takei et al., 2003). In initial time course experiments, the uptake rate of both radiochemicals was found to be practically constant for the first 10 min (data not shown). Consequently, uptake periods of 5 min formed the basis of kinetic analyses in subsequent experiments. Eadie-Hofstee plots (Fig. 6, inset) deduced from the Michaelis-Menten kinetics of *OsENT2*-mediated uptake of iPR and tZR (Fig. 6) indicated K_m values for iPR and tZR of 32 and 660 μM , respectively. The V_{max} values for uptake were $1.3\ \mu\text{mol}\ (10^7\ \text{cells})^{-1}\ \text{h}^{-1}$ for iPR and $6.2\ \mu\text{mol}\ (10^7\ \text{cells})^{-1}\ \text{h}^{-1}$ for tZR. We concluded that *OsENT2* transported CK nucleosides and had a higher affinity for iPR than for tZR.

Table II. Inhibition of *OsENT2*-mediated [^3H]adenosine uptake in BY4735 *ade2* yeast cells

[^3H]Adenosine uptake was examined at a substrate concentration of $2.0\ \mu\text{M}$ for 2 min at pH 6.0. The concentration of the inhibitor applied is shown in parenthesis. Mean \pm SE ($n = 3$).

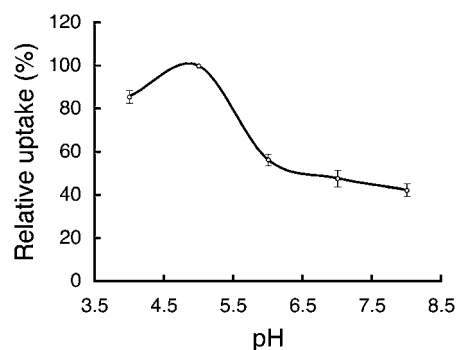
Additive	[^3H]Adenosine Uptake (% of Control)
None	100
NBMPR (20 nM)	97.2 \pm 3.7
NBMPR (20 μM)	86.6 \pm 1.1
Dilazep (20 nM)	98.2 \pm 0.2
Dilazep (20 μM)	95.9 \pm 3.9
Dipyridamole (20 nM)	96.9 \pm 4.1
Dipyridamole (20 μM)	91.4 \pm 4.0
CCCP (5 μM)	70.7 \pm 3.7

OsENT2 Is Predominantly Expressed in the Vasculature and during Germination

To study the tissue specificity of *OsENT2* expression throughout plant development, the promoter region was isolated and fused to the *Escherichia coli* β -glucuronidase (GUS) gene. Twenty-five independent T1 transgenic lines were analyzed for reporter gene activity. All lines analyzed showed a very similar pattern of expression, varying only in the intensity of GUS staining. Four representative lines were used to determine the expression pattern in more detail.

In 1-d-old seedlings, a strong signal for *OsENT2* promoter activity was detected predominantly in the root primordium and in the scutellum (Fig. 7A). Intense staining was also observed in the root and in the coleoptile tip of 2-d-old seedlings (Fig. 7B). *OsENT2* mRNA accumulation was induced within 6 h after the initiation of germination by imbibition of the dry seeds, indicating that *OsENT2* gene expression is up-regulated at the earliest stages of germination (Fig. 7C). The GUS staining in the scutellum was observed for at least 2 weeks after the start of imbibition (data not shown).

In adult plants, the *OsENT2* promoter was active in the vasculature throughout the whole plant (roots, stems, leaves, and grains; Fig. 8, A–F). In roots, GUS

**Figure 5.** pH dependency of [^3H]adenosine uptake into BY4735 *ade2* yeast cells expressing *OsENT2*. [^3H]adenosine uptake was tested at a substrate concentration of $2.0\ \mu\text{M}$ for 2 min at various pH. Data shown are means \pm SE ($n = 3$).

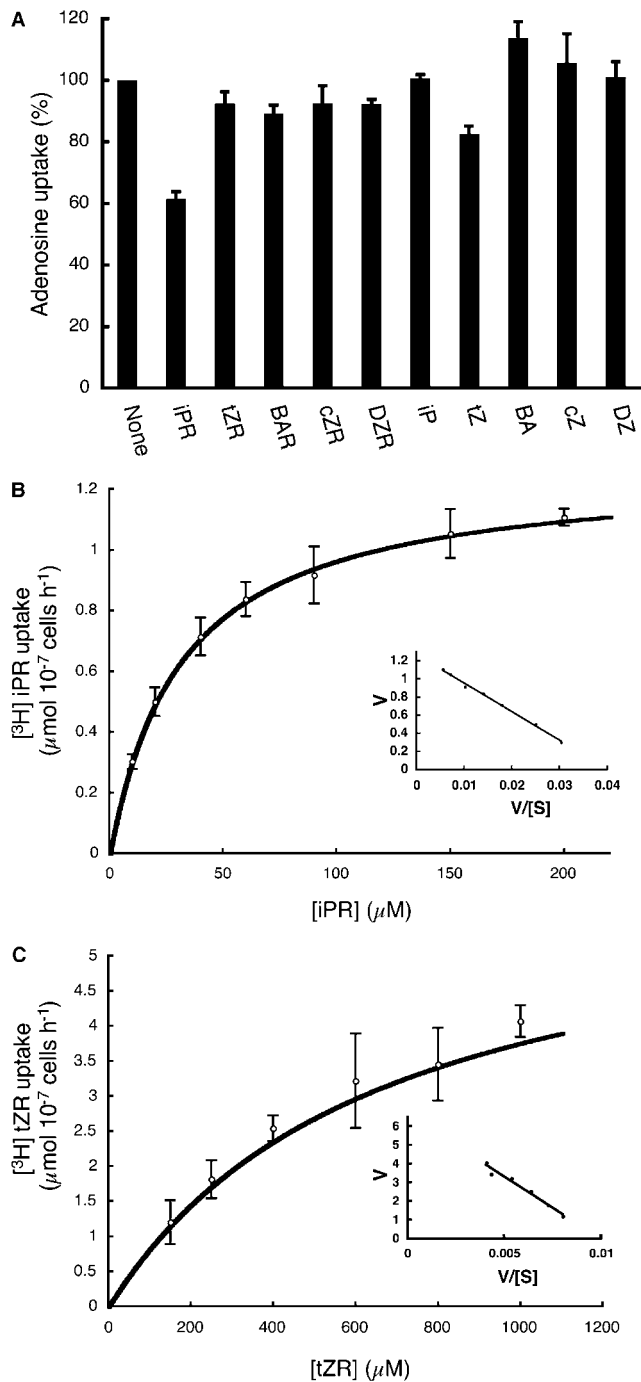


Figure 6. Uptake of nucleoside-type CKs by yeast cells expressing *OsENT2*. A, Inhibition of *OsENT2*-mediated [³H]adenosine uptake in BY4735 *ade2* yeast cells by various CKs. [³H]adenosine uptake was tested at a substrate concentration of 2.0 μM for 2 min at pH 6.0. The concentrations of the various additives were 20 μM. [³H]iPR (B) and [³H]tZR (C) uptake was examined in BY4735 *ade2* cells expressing *OsENT2*. Cells were incubated for 5 min at the substrate concentrations indicated at pH 6.0. Background uptake rates (empty vector pYES2) were subtracted. Affinity constants were calculated on the basis of an Eadie-Hofstee plot (inset). Data shown are means ± SE (n = 3). BAR, benzyladenine riboside; cZR, cis-zeatin riboside; DZR, dihydrozeatin riboside; BA, benzyladenine; cZ, cis-zeatin; DZ, dihydrozeatin.

staining derived from the *OsENT2* promoter activity was observed within the stele and lateral roots, but not in epidermal, exodermal, or cortical cells (Fig. 8, A and B). In leaf sheaths, GUS activity was detected exclusively in the phloem (Fig. 8D). In 10-DPA-old grains, the GUS signal was found in the vascular bundle on the dorsal side and in the inner integument (Fig. 8F). Taken together, these results suggested that *OsENT2* participates in the long-distance transport of nucleosides.

DISCUSSION

In contrast to the *Arabidopsis* genome, which harbors eight ENT genes, the rice genome contains only four (Fig. 1A). *OsENT1*, *OsENT2*, and *OsENT3* possessed the 11 putative TMs that are typical of ENTs. On

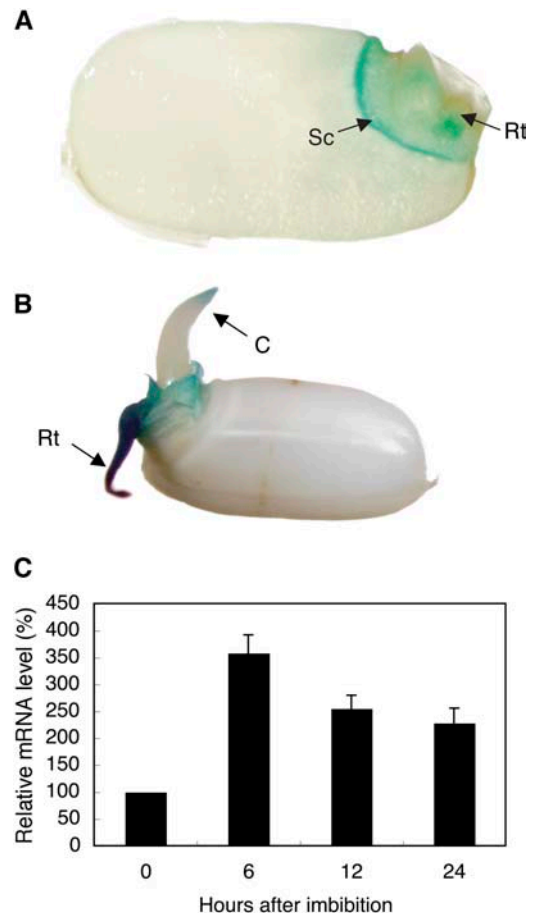


Figure 7. Expression of the *OsENT2* gene during germination. Histochemical localization of *OsENT2*-promoter-controlled GUS activity in seedlings at 1 d (A) and 2 d (B) after the start of imbibition. A longitudinal section of a representative grain is shown in A. C, Coleoptile; Rt, root; Sc, scutellum. Relative amount of *OsENT2* mRNA during germination is shown in C. The *OsENT2* mRNA level was normalized with respect to the corresponding *actin* transcript level. The data are given as relative values with respect to the value determined at 0 h. Data shown are means ± SE (n = 3).

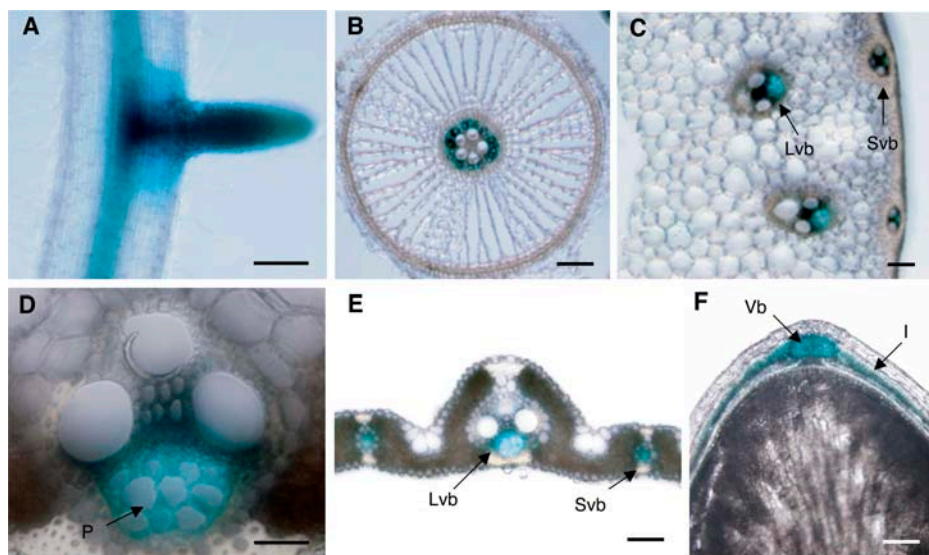


Figure 8. Tissue specificity of the activity of the *OsENT2* promoter. Histochemical localization of *OsENT2* promoter-controlled GUS activity in a representative root (A), a root cross-section (B), a stem cross-section (C), a vascular bundle in a leaf sheath (D), a leaf blade cross-section (E), and a longitudinal section of a grain (F). I, Inner integument; Lvb, large vascular bundle; P, phloem; Sv, small vascular bundle; Vb, vascular bundle on the dorsal side of grain. Bar = 500 μm for A; 100 μm for B, C, E and F; 50 μm for D.

the other hand, *OsENT4* appeared to be carboxy-terminally truncated. In this context, it is interesting that the human *ENT2* gene (*hENT2*) is alternatively spliced to produce a number of variants, including a hydrophobic nucleolar family of proteins (Sankar et al., 2002). An amino-terminally truncated form of *hENT2* failed to stimulate uridine transport in transiently transfected cultured cells (Crawford et al., 1998). Additional studies are required to determine whether *OsENT4* actually is translated as a truncated ENT in rice and whether it has a role in nucleoside transport.

Growth analysis of yeast cells expressing *OsENTs* and competition tests indicated that *OsENT2* can transport both purine and pyrimidine nucleosides, while *OsENT1*, *OsENT3*, and *OsENT4* did not exhibit any transport competence (Fig. 3; Table I). Not all ENTs may be functionally expressed in this yeast system: For instance, *AtENT8*, an Arabidopsis ENT, also did not exhibit transport activity when it was expressed in *ade2* and *fui1* mutants (Wormit et al., 2004). Further studies will establish whether the lack of transport activity we observed in *OsENT1* and *OsENT3* was due to ineffective plasma membrane targeting in the heterologous expression system and whether these two *OsENTs* transport substrates other than nucleosides.

OsENT2 resembles *AtENT1*, *AtENT3*, *AtENT4*, *AtENT6*, and *AtENT7* (Möhlmann et al., 2001; Li et al., 2003; Wormit et al., 2004) in its low sensitivity to NBMPR, dilazep, and dipyridamole (Table II). Amino acids at positions 33 and 154 have been identified as the residues responsible for the difference in the sensitivity to vasodilator drugs that is observed between *hENT1* and *hENT2* (Hyde et al., 2001; Visser et al., 2002, 2005). Comparing the sequences of *AtENTs* with that of *hENT1*, Li et al. (2003) found that Met-33 of *hENT1* is replaced by Ile or Leu and that Gly-154 of *hENT1* is exchanged for Asp in all *AtENTs*. The authors specu-

lated that these two exchanges are responsible for the low sensitivity of *AtENTs* to vasodilator drugs. In *OsENT2*, Leu and Asp occupied the positions corresponding to residues 33 and 154 of *hENT1*, respectively (Fig. 1A). Since *OsENT2* is also resistant to vasodilator drugs, as demonstrated in this investigation, these two residues appear likely to be involved in the resistance to vasodilators of all plant ENTs tested so far.

Our analysis of cloned *OsENT2* implicates a role of the ENT as a CK nucleoside transporter. At first sight, the affinity constant for the CK nucleosides, especially that of tZR, may seem high (Fig. 6) compared to the predicted concentration of CKs in plant tissues in the nanomolar range. In some enzymes of the purine salvage pathway and of CK metabolism, the affinity constants and specificity constants have been determined. As for cloned Arabidopsis adenosine kinases, K_m values for iPR and adenosine were 3 to 5 μM and 0.3 to 0.5 μM , respectively, while the V_{max}/K_m for iPR was 100-fold lower than for adenosine (Moffatt et al., 2000). The K_m value of cloned APT2 for isopentenyladenine (iP) was 110 μM and that of APT3 for trans-zeatin (tZ) was 76 μM . In both cases, the V_{max}/K_m was significantly lower than for adenine (Allen et al., 2002). K_m values of cloned maize (*Zea mays*) *O*-glucosyltransferases for cis-zeatin were between 46 and 95 μM (Veitch et al., 2003), and the K_m values of cloned Arabidopsis *N*-glucosyltransferases for iP and tZ ranged from 70 to 240 μM (Hou et al., 2004). In cloned maize CK oxidase/dehydrogenase, a CK-degrading enzyme, the K_m values for iP and tZ ranged from 1.5 to 14 μM (Bilyeu et al., 2001). Thus, the substrate affinities of enzymes involved in CK metabolism and that of *OsENT2* seem to be in a compatible range (at least for iPR). Moreover, the K_m value of a CK nucleobase transporter, *AtPUP1*, for tZ was calculated to be around 40 μM (Bürkle et al., 2003). In this context, we speculated that *OsENT2* functions as an iPR transporter in planta.

Analysis of *OsENT2 promoter:GUS* transgenic rice enabled us to deduce the physiological function of *OsENT2* (Figs. 7 and 8). *OsENT2* was predominantly expressed in the scutellum of the germinating seed (Fig. 7A). The epithelium layer in the dorsal portion of the scutellum elongates and develops during germination and functions as the absorptive tissue of storage reserves from the endosperm (Hoshikawa, 1989). Significant levels of nucleosides such as adenosine and guanosine were released into the incubation medium when halved endosperm of castor bean seeds was incubated in buffer (Kombrink and Beevers, 1983). Active nucleotide biosynthesis is needed to provide the embryos with sufficient purine and pyrimidine nucleotides to support nucleic acid synthesis (Deltour, 1985). In this context, we assumed that *OsENT2* might be involved in the uptake of nucleosides derived from the endosperm, which are subsequently metabolized to nucleotides in developing embryos. Strong GUS staining was detected in the roots of 2-d-old seedlings (Fig. 7B) and in lateral roots of mature plants (Fig. 8A), reflecting the high demand for nucleotides in young sink organs. Real-time PCR analysis also indicated that *OsENT2* generally is at high levels in roots of mature plants (Fig. 2B).

Predominant expression of *OsENT2* in vascular tissues throughout the whole plant (Fig. 8, A–F), and particularly in the phloem of leaf sheaths (Fig. 8D), suggests an involvement of *OsENT2* in the long-distance transport of nucleosides by loading and unloading into and from the phloem, respectively. *OsENT2* was expressed in the vascular tissues of grains during the period of seed loading, pointing to a role of *OsENT2* in providing grains with nucleosides, possibly derived from senescing tissues where nucleosides are liberated by the nucleolytic breakdown of RNA and DNA. Long-distance translocation of nucleoside-type CKs is believed to occur in the xylem and phloem (Latham, 1994). The highest concentrations of CKs were found in the developing rice seed (Yang et al., 2001, 2002). It has been suggested that cell number and cell division activity in rice endosperm are regulated by CK levels in this tissue (Yang et al., 2002). *OsENT2* might contribute to the retrieval of CKs transported through the vascular systems in developing grains.

Distinct distributions of iPR and tZR in phloem and xylem, respectively, were reported in *Sinapis alba* (Lejeune et al., 1994). We found similarly distinct distributions of CK nucleosides in rice as well (N. Hirose and H. Sakakibara, unpublished data). Considering that *OsENT2* has different affinities for iPR and tZR, vascular-localized *OsENT2* might play an important role in the selective transport of nucleoside-type CKs. The sites of CK biosynthesis recently have been revealed in *Arabidopsis* (Miyawaki et al., 2004; Takei et al., 2004), but not yet in rice. To more precisely deduce the possible roles of *OsENT2* in CK transport, future studies will have to focus on the identification of the sites of CK biosynthesis in rice. Furthermore, detailed analyses of knockout or RNA interference

mutants will enable us to further elucidate the physiological roles of *OsENT2* in the transport of nucleosides, including nucleoside-type CKs.

MATERIALS AND METHODS

Plant Materials and Growth Conditions

For the determination of organ distribution of *OsENT* mRNA and histochemical analysis, rice (*Oryza sativa*) L. cv Nipponbare plants were hydroponically grown in an environment-controlled greenhouse at a 12-h-light (30°C)/12-h-dark (25°C) photoperiod as described by Kamachi et al. (1991). For the propagation of transgenic rice plants, single plants were grown in 0.3-L pots with 150 g of synthetic culture soil (Mitsui-Toatsu No. 3; Mitsui-Toatsu, Tokyo) in a greenhouse under the conditions described above. For germination experiments, rice grains were surface sterilized and incubated on wet filter paper at 30°C in the dark.

Cloning of *OsENT* cDNAs and Comparative Analysis of Amino Acid Sequence of *OsENTs*

The *OsENT* genes were identified by BLAST queries in GenBank (www.ncbi.nlm.nih.gov/BLAST), The Institute for Genomic Research (TIGR rice genome project; <http://www.tigr.org/tdb/e2k1/osa1/index.shtml>), the DNA Data Bank of Japan (DDBJ; <http://www.ddbj.nig.ac.jp/search/blast-j.html>), and the rice cDNA database at KOME (<http://cdna01.dna.affrc.go.jp/cDNA>) using the amino acid sequence of *AtENT1* as a search sequence. To amplify cDNAs of *OsENT1* and *OsENT3* using reverse transcription-PCR, the following primers were used: 5'-AAGATCCCCACCCAAATCCACCTC-3' and 5'-AGCATGGCACTATGATACACTGAC-3' for *OsENT1*; 5'-GGGTCACTCTAGITGACTACAAAC-3' and 5'-TGGGAAAAAGACATGTAAATGCAACTATG-3' for *OsENT3*. Total RNA was prepared from 3-week-old seedlings with the RNeasy plant mini kit (Qiagen, Valencia, CA) with RNase-free DNase I (Qiagen). cDNA was synthesized by SuperScriptII reverse transcriptase (Invitrogen, Carlsbad, CA) with oligo(dT)₁₂₋₁₈ primers. The amplified fragment was cloned into pCR-Blunt-TOPO (Invitrogen) and sequenced to confirm that no substitution had occurred during PCR. Analysis of cDNA and amino acid sequences was carried out using GENETYX-MAC version 11 (Software Development, Tokyo). To investigate phylogenetic relationships among *OsENTs* and *AtENTs*, the amino acid sequences were aligned using the ClustalW program (Thompson et al., 1994) at the DDBJ Web site with default settings; phylogenetic trees were developed with Tree View (Page, 1996). The locations of transmembrane helices in *OsENTs* were predicted by the program TMHMM 2.0 (<http://www.cbs.dtu.dk/services/TMHMM>) with manual adjustment aided by the comparison of the amino acid sequences of *OsENTs* with that of *hENT1* for which structural information is currently available (Sundaram et al., 2001).

Quantitative Real-Time PCR

Total RNA was prepared from the roots, stems, leaves, and young grains (10 DPA) of ripening rice plants with the RNeasy plant mini kit (Qiagen) and from mature grains by the method of Shirzadegan et al. (1991). Total RNA extracted was treated with RNase-free DNase I (Qiagen). cDNA was synthesized using SuperScriptII reverse transcriptase (Invitrogen) with oligo(dT)₁₂₋₁₈ primers. Accumulation levels of the transcripts of interest were analyzed by a real-time PCR method, with the ABI Prism 7000 sequence detection system (PE-Applied Biosystems, Foster City, CA). The primers for PCR were designed using Primer Expression software (PE-Applied Biosystems) and checked for the specific product formation by polyacrylamide gel analysis. The sequences of the primers used for PCR were 5'-GCTCAGAAGGAGGAGGAAGATG-3' and 5'-CAACAATGCTCCACAAGGTTGA-3' for *OsENT1*; 5'-CATCGTGAAGCACTACCGATCA-3' and 5'-CCTGCAGCAGCAAGATCACTT-3' for *OsENT2*; 5'-AGTGAAAAACTGGATCAACA-3' and 5'-ATACGTTATA-GCTCGCGATCAAGA-3' for *OsENT3*; 5'-TGTCATCGGTTGCAATAACC-3' and 5'-GGCCATCTTGGAGTTCTCAAA-3' for *OsENT4*; 5'-CGAGGCG-CAGTCCAAGAG-3' and 5'-CCCAGTTGCTGACGATACCA-3' for *actin1* (ACT1; McElroy et al., 1990). In each case, plasmid DNA containing the corresponding cDNAs was used as a template to generate a calibration curve.

Expression of *OsENTs* in the Yeast Mutant

The coding regions of *OsENT* cDNAs were amplified by PCR using the primers 5'-TGGATCCAAGCGAAAGGCTCAAGAAAGC-3' and 5'-GTCTA-GATCAAATGACCCAAAACCAAGC-3' for *OsENT1*; 5'-GAAGCTTATCAT-GAGCTCGAGGTCGA-3' and 5'-GTCTAGATCACCAGCCTTTTCCTATC-3' for *OsENT2*; 5'-AGGTACCATGGCATATGGTAAAGGAG-3' and 5'-GTCTA-GATCACCACCTTTACCTATC-3' for *OsENT3*; 5'-AGGTACCATGGCAG-CCTTCTCC-3' and 5'-ACTCGAGCTATCTGTTGCTTAATCG-3' for *OsENT4*. The amplified fragments were cloned into pCR-Blunt-TOPO (Invitrogen) and sequenced. The *OsENT1* cDNA was cut out as a *Bam*HI-*Xba*I fragment and was cloned into *Bam*HI-*Xba*I sites of pYES2 (Invitrogen). The *OsENT2* cDNA was cut out as a *Hind*III-*Xba*I fragment and was ligated into *Hind*III-*Xba*I sites of pYES2. The *OsENT3* cDNA was cut out as a *Kpn*I-*Xba*I fragment and was cloned into *Kpn*I-*Xba*I sites of pYES2. The *OsENT4* cDNA was cut out as a *Kpn*I-*Xho*I fragment and was ligated into *Kpn*I-*Xho*I sites of pYES2. The resulting plasmids were transformed into a yeast (*Saccharomyces cerevisiae*) *ade2* strain (BY4735, Mat α , *ade2 Δ :hisG*, *his3 Δ 200*, *leu2 Δ* , *met15 Δ* , *trp1 Δ 63*, *ura3 Δ* , purchased from America Type Culture Collection, Manassas, VA) or the yeast mutant Δ FU11 (BY4742, Mat α , *his3 Δ 1*, *leu2 Δ* , *lys2 Δ* , *ura3 Δ* , *YBL042C::KanMX4*, purchased from Open Biosystems, Huntsville, AL) by the lithium acetate method (Gietz et al., 1992), and the transformants were selected on synthetic minimal medium lacking uracil (BD Biosciences Clontech, Palo Alto, CA) as described in Yeast Protocols Handbook (Clontech). For plate assays, the cells were grown on three types of medium: minimal medium lacking uracil but containing Gal (20 g L⁻¹), minimal medium lacking both uracil and adenine but containing adenosine (150 μ M) and Gal, and minimal medium lacking uracil but containing fluorouridine (1 mM) and Gal.

The uptake of tritiated adenosine, iPR, and tZR was evaluated by using the BY4735 *ade2* yeast strain, while that of [³H]uridine was assessed in the yeast mutant Δ FU11. Gal (20 g L⁻¹) was applied to culture media as a sole carbon source to activate the *GAL1* promoter on pYES2. Cells were cultured to an *A*₆₀₀ of 0.8 to 1.2. They were harvested by centrifugation (4,000g, 10 min), and washed twice with 25 mM phosphate buffer, pH 6.0. Washed cells were resuspended in the same buffer to an *A*₆₀₀ of 5. For uptake experiments at different pH, cells were first suspended in distilled water, distributed, and resuspended in 25 mM phosphate buffer of the given pH. The [³H]nucleoside uptake was measured by the method of Smith et al. (1995), with minor modification (Kataoka et al., 2004). The uptake was terminated by centrifuging (10,000g, 1 min) 100 μ L of the yeast cell suspension through 150 μ L of 1:1 silicone oil (SH-150):dinonylphthalate into 14 μ L of 60% perchloric acid (Nakalai Tesque, Kyoto) placed at the bottom of 250- μ L microcentrifuge tubes. After centrifugation, the tips of these tubes were cut off and the contents were transferred into 2 mL of Ultima Gold scintillation cocktail (Perkin-Elmer, Boston) to determine the radioactivity with a liquid scintillation counter (Aloka, Tokyo). For investigating the sensitivity of *OsENT2*-mediated transport to inhibitors, vasodilator drugs (diazepam or dipyrindamole; Sigma, St. Louis), a nucleoside analog (NBMPR; Research Biochemicals, Natick, MA), or a protonophore (CCCP; Sigma) were added to the yeast cell suspensions 30 min before the addition of [³H]adenosine (Visser et al., 2002).

Transformation of Rice

Genomic sequences containing putative promoter regions of *OsENT2* (-2,012 to -1 bp from the translational initiation codon) were amplified by PCR with genomic DNA. Primers were forward, 5'-GAAGCTTCTTCTTTGATTTTTATGGTCATC-3' and reverse, 5'-AGTCGACGATAAAGTTCACCTGCGCCTAATC-3'. The resulting PCR product was cloned into pCR-Blunt-TOPO (Invitrogen) and verified by complete sequencing. The verified fragments were introduced into the upstream region of the open reading frame of the GUS gene that is inserted into the *Sma*I-*Spe*I site of the pCAMBIA1390 vector (CAMBIA, Canberra, Australia).

Agrobacterium tumefaciens (EHA101) carrying the above construct was used to transform rice following the method of Hiei et al. (1994). Hygromycin B-resistant plants from callus, defined as transgenic plants of the T0 generation, were transplanted into soil and grown in a greenhouse. The primary transformants were self-pollinated and the resulting seeds (T1) were collected.

Histochemical Analysis

Histochemical analysis of GUS activity was performed by the method of Jefferson (1987), modified by Kosugi et al. (1990). The tissues of transgenic

plants were infiltrated with GUS reaction buffer: 1 mM 5-bromo-5-chloro-3-indolyl- β -D-glucuronide, 0.5% Triton X-100, 50 mM sodium phosphate buffer, pH 7.0, and 20% (v/v) methanol. For fresh sections, the tissues of transgenic plants were cut with a scalpel into approximately 0.5-cm sections. These sections were embedded in 5% agar and then cut into 80- to 130- μ m sections using a DTK-100 microslicer (Dosaka EM, Kyoto). The sections were incubated at 37°C for between 30 min and overnight in GUS reaction buffer. After staining, the sections were washed in 70% ethanol overnight to remove chlorophyll and stored in 70% ethanol until further analysis. GUS staining was observed using an Olympus BX100 microscope (Olympus, Tokyo) following the manufacturer's instructions.

Chemicals

iP, tZ, benzyladenine, dihydrozeatin, iPR, tZR, and dihydrozeatin riboside were purchased from Sigma, cis-zeatin from ICN Biomedicals (Irvine, CA), benzyladenine riboside from Fluka (Buchs, Switzerland), and [2-³H]adenosine (TRK423; 814 GBq/mmol) and [5-³H]uridine (TRK178; 1.04 TBq/mmol) from Amersham Biosciences (Uppsala). The preparations and quality checks of [³H]iPR and [³H]tZR were performed as described previously (Takei et al., 2003).

Sequence data from this article have been deposited with the EMBL/GenBank data libraries under accession numbers AP005125, AK059439, AK102045, AK058524, AK101098, AK065096, and AB201113.

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