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Article

Overexpression of Gene Encoding Tonoplast Intrinsic Aquaporin Promotes Urea Transport in *Arabidopsis*

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Complementation assay of the urea uptake-defective yeast mutants led to the identification of the Arabidopsis *AtTIP4;1* gene encoding the aquaporin. However, its physiological functions still remain elusive. In the present study, histochemical and genetic analyses were performed to understand the physiological roles of *AtTIP4;1* in urea uptake. The *AtTIP4;1* product was detectible in the roots, but not in the leaves, the stem, and the flower. Its promoter allowed the expression of the β-glucuronidase reporter gene in the roots and the apical meristem in *Arabidopsis*. The *AtTIP4;1* products were induced under nitrogen-deficient conditions. To investigate the role of the tonoplast intrinsic protein in urea transport and developments, *Arabidopsis* with the loss- and the gain-of-function mutations by T-DNA insertion in *AtTIP4;1* and 35S promoter-mediated overexpression of *AtTIP4;1* were identified, respectively. The transfer DNA insertion and the *AtTIP4;1*-overexpressed plants showed normal growth and development under normal or abiotic stress growth conditions. The urea-uptake studies using ^{14}C-labeled urea revealed higher accumulation of urea in the *AtTIP4;1*-overexpressed plants. These results provide evidence that overexpression of *AtTIP4;1* leads to the increase in the urea-uptake rate in plants without detectable defects to the growth and development.

Key words: *aquaporins, T-DNA insertion mutant, transgenic plants, tonoplast intrinsic proteins (TIP), urea transport*

Nitrogen fertilizers are heavily applied on a global scale to increase the crop yield and achieve high-quality crop. Although nitrogen fertilizers are significantly important contributors to the increase in the world agricultural productivity, high inputs of nitrogen fertilizers have a negative effect on the environment, including biodiversity, air and water quality, and the global climate. In spite of these opposing characteristics, urea fertilizers possess the advantages of high availability to plants and retardation of the microbial degradation into nitrate.

It has long been believed that urea is easily taken into the cells via passive diffusion due to its low molecular weight and neutral charge [Galluci *et al.*, 1971], up until Wilson *et al.* [1988] reported for the first on the protein-mediated urea uptake via a short-term influx experiment of ^{14}C-labeled urea in the algal cells. They demonstrated that the influx of ^{14}C-urea in the algal cells was dependent on the ATPase inhibitors, thereby indicating the presence of a urea transport system, which uses the proton gradient across the plasma membrane. Because the concentration-dependent uptake followed bi- or even multiphase kinetics, the authors suggested the combined actions of a high- and a low-affinity urea transport systems in the

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Abbreviations: BLAST, basic local alignment and search tool; BSA, bovine serum albumin; GUS, β-glucuronidase; KLH, key-hole limpet hemocyanin; NIP, NOD26-like intrinsic protein; PCR, polymerase chain reaction; PIP, plasma membrane intrinsic protein; RT, room temperature; SDS-PAGE, sodium dodesyl sulfate - polyacrylamide gel electrophoresis; SSS, sodium-solute symporter; T-DNA, transfer DNA; TIP, tonoplast intrinsic aquaporin.

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algal cells. Through short-term uptake experiments using ^{14}C-labeled urea, Cooper and Sumrada [1975] revealed two major pathways for urea uptake in the yeast cells: one being an active transport system with a rather low K_m of 14 M, and the other a passive transport system that operates at concentrations above 0.5 mM. Active transport is the predominant route of the urea entry at the extracellular concentrations below 0.25 mM, making it possible to isolate the yeast mutant cells from the yeast carrying a defect in the urea-active transport system [Sumrada *et al*., 1976]. The *ScDUR3* gene responsible for the urea-active transport was identified by transformation of this mutant using the yeast genomic library [ElBerry *et al*., 1993]. ScDUR3 is a member of the SSS gene family, which is widespread among microorganisms, animals, and plants. Members of the SSS family have been found to transport sugars, amino acids, nucleotides, inositols, vitamins, anions, and urea [Reizer *et al*., 1994; Turk and Wright, 1997; Saier, 2000]. Proteins of the SSS family possess between 400 and 700 amino acid residues and are predicted to form 12 to 15 transmembrane-spanning domains. A number of the plant ESTs from Arabidopsis, maize, rice, soybean, and barley exhibited significant homologies with the *ScDUR3* gene. Heterologous expression of the *Arabidopsis* cDNA gene, *AtDUR3*, similar to *ScDUR3* was revealed to confer the urea uptake-defective yeast growth in 2 mM urea [Liu *et al*., 2003]. The *AtDUR3* is predicted to encode a protein with 14 transmembrane-spanning domains belonging to the SSS superfamily. The ubiquitous presence of the genes homologous to *ScDUR3* in plants and their functional complementation suggest that the urea uptake system is essential for the support of a proper growth in plants.

In addition to the energy-dependent pathway in the plant roots, urea uptake is also mediated through facilitative urea transporters. Two aquaporins from tobacco, NtPIP1 and NtTIPa, stimulated the ^{14}C-urea fluxes when expressed in *Xenopus* oocytes [Eckert *et al*., 1999; Gerbeau *et al*., 1999]. In the yeast complementation, which used an expression library from zucchini, an aquaporin belonging to the NIP subfamily was identified [Klebl *et al*., 2003]. The heterologous complementation of a urea uptake-defective yeast mutant made it possible to isolate four genes encoding the TIP aquaporin from an Arabidopsis cDNA library [Liu *et al*., 2003].

Even though a number of reports have revealed the urea uptake activities of various aquaporins belonging to different subgroups, the yeast complementation experiment exclusively identified the genes encoding the TIP aquaporin in Arabidopsis. This could be explained, at least partially, by the high abundance of TIP cDNAs in the library used for the yeast complementation experiments. This possibility is also supported by reports showing high levels of *AtTIP1;1, AtTIP1;2*, and *AtTIP2;1* transcripts [Quigley *et al*., 2002; Liu *et al*., 2003]. On the other hand, the expression of *AtTIP4;1* transcript was very low. These findings could reflect the higher potential of *AtTIP4;1* products for urea transport in Arabidopsis. Therefore, histochemical and genetic analyses were performed to further understand the physiological roles of the aquaporins in urea uptake in the plant, and the results showed that the *AtTIP4;1* products are specifically present in the root and the apical meristem. In addition, the *AtTIP4;1* products were up-regulated under the N nutritional status in Arabidopsis. The knock-out mutant was also characterized using T-DNA in *AtTIP4;1* gene and the transgenic Arabidopsis with the overexpression of *AtTIP4;1* driven by the 35S promoter.

Materials and Methods

Plant growth. All experiments were performed using *A. thaliana* ecotype Columbia. The *Arabidopsis* seeds were germinated and grown in agar plates containing the nutrient solution described by Liu *et al*. [2003]. Ten-day-old seedlings were transferred onto the agar plates containing the same nutrient solution mentioned above except without NH_4NO_3 and were subjected to nitrogen deficiency for different days as described in the text.

Molecular cloning of full-length *AtTIP4;1* gene and plant transformation. Total RNA was obtained from the 10-day-old seedlings. Poly (A^+) RNA was isolated from the total RNA using the Poly (A^+) Track Kit (Promega, Madison, WI) following the instruction of the manufacturer. cDNA was synthesized from 0.5 μg mRNA using oilgo$(dT)_{12-18}$ as a primer and Moloney murine leukemia virus reverse transcriptase (Invitrogen, Carlsbad, CA). A full-length *AtTIP4;1* cDNA was obtained by PCR reaction using the Pfu polymerase (Stratagene, La Jolla, CA) and the gene-specific primers to the 5'- and 3'-noncoding regions of the *AtTIP4;1* gene (TIP41-up, 5' GCTCTAGAATGAAGAAGATCGAGTTAGG-3'; and TIP41-dn, 5' GCGAGCACTAATTCAACAATGGTTGC TCG-3'). The *AtTIP4;1* PCR fragment was subcloned into the pGEM T-easy vector (Promega) and sequenced. These gene-specific primers were designed to create *Xba*I and *Sac*I sites at the 5' and 3' ends of the amplified fragment. The *AtTIP4;1* cDNA was subcloned into the *Xba*I and *Sac*I sites of the plant binary vector pBI121 with the cauliflower mosaic virus 35S promoter. Agrobacterium-mediated transformation was used to introduce the *AtTIP4;1* gene driven by the CaMV 35S promoter into the wild type *Arabidopsis* (Col-O) using the flower-dipping method of Clough and Bent [1998].

Promoter identification and plant transformation. Two promoter-specific primers, PT41up (5'-GCAAGCT TGTGGAGATTGTGGCTATGTAAG-3') and PT41dn (5'-GCGGATCCAAAGTTCTAATCTAACGAAATTTTG-3'), were designed using the Arabidopsis genomic DNA sequence. A PCR reaction was performed using the GeneAmp PCR system 9700 (PE Applied Biosystems, Foster, CA). *Hind*III and *Bam*HI recognition sites were incorporated at the 5' ends of PT41up and PT41dn, respectively, for cloning into the plasmid vector. The DNA fragment resulting from the PCR reaction was cloned into the *Hind*III and *Bam*HI sites of the pGEM vector (Promega) and sequenced. The resulting plasmid and the plant binary vector pBI121 were digested with *Hind*III and *Bam*HI, and the fragment containing the *AtTIP4;1* promoter and the binary vector containing the GUS gene were gel-purified. The DNA fragment containing the *AtTIP4;1* promoter was ligated into the pBI121 vector. The resultant plant transformation vector was used to transform the Arabidopsis plants.

T-DNA mutant identification by PCR analysis. A BLAST search of the SALK (SIGnAL T-DNA Express, SALK institute Genomic Analysis Laboratory, La Jolla, CA) database using the *AtTIP4;1* genomic sequence (At2g25810) resulted in the identification of a line (SALK_050663) with a T-DNA insertion in the first intron. The genomic DNAs from the leaves of each individual line were extracted to determine the integration site of the T-DNA by PCR amplification using two gene-specific primers (primer 1, 5'-CCATGTGCTCCTTAGTC CTTACC-3' and primer 3, 5'-GTGAACCGGAGTTCCC TAATCAC-3') and the T-DNA left border primer (primer 2, 5'-CAGGTGCCCACGGAATAGT-3') (Fig 4). The gene-specific primers flanking the T-DNA were used for the first PCR analysis to verify the presence of a wild-type allele. The second PCR employed a gene-specific primer and the T-DNA left border primer to confirm the presence of the mutant allele. The PCR products were separated on a 0.8% agarose gel. The plants heterozygous for the T-DNA insertion gave rise to the *AtTIP4;1* gene fragments with two gene-specific primers as well as DNAs containing the T-DNA left border with a gene-specific primer and the T-DNA left border primer without the gene-specificity, whereas no band corresponding to the *AtTIP4;1* locus was amplified from the homozygous plants. Position of the T-DNA insertion was confirmed by the DNA sequence analysis of the PCR product.

RNA hybridization analysis. Total RNA (10 μg) was extracted from the 10-day-old seedlings, separated by electrophoresis on a 1.5% agarose gel containing formaldehyde, and transferred to a nylon membrane. The DNA probe was obtained from a 252-bp DNA fragment containing the 5'- UTR (untranslated region) and the N-terminal region of *AtTIP4;1* from the genomic DNA, which displayed the lowest similarity with the genes encoding other TIP proteins. The probe was radiolabeled using a Nick Translation Kit (Amersham Bioscience, Piscataway, NJ). The hybridization was carried out overnight at 65°C. The membrane was then washed in 0.1 ×SSC, 0.1% SDS at 24°C for 15 min, followed by two 10-min washes in 0.1×SSC, 0.1% SDS at 42°C [Kwon *et al.*, 2007].

Generation of AtTIP4;1-specific antibodies and Western analysis. A specific anti-AtTIP4;1 antibody was produced using a synthetic peptide for the first 12 amino acids of AtTIP4;1 (MKKIELGHSEA) as an antigen. The antibody was raised in rabbits by injection of 500 μg KLH (keyhole limpet hemocyanin)-conjugated peptide emulsified in the complete Freund's adjuvant (Peptron, Daejon, Korea). Two booster doses of 500 μg antigen emulsified in the incomplete Freund's adjuvant at 21-day intervals were injected into the rabbits. The rabbits were then bled 8 days after the second boost.

Total protein from seedlings was extracted in the SDS sample buffer [60 mM Tris-HCl, pH 8.0, 60 mM dithiothretol, 2.0% (w/v) SDS, 15% (w/v) sucrose, 5 mM ε-amino-*N*-caproic acid, 1 mM benzamidine] using a homogenizer. The protein concentration was determined through a Coomassie Blue dye-binding assay with BSA as the standard [Ghosh *et al.*, 1988]. All protein samples were also examined by SDS-PAGE and Coomassie Blue staining, and the protein concentrations were found to be consistent with the results of the protein assay [Hong and Vierling, 2000].

Standard methods were used for the SDS-PAGE separation of the protein samples on 15% polyacrylamide gel. For Western analysis, the proteins were blotted on to the nitrocellulose. After washing, the secondary antibodies labeled with the alkaline phosphatase were added for 2 h. Subsequently, the filters were washed, and the immuno-reacting proteins were detected using the alkaline phosphatase conjugate substrate kit (Bio-Rad, Hercules, CA). The primary and the secondary antisera were used at 1/1,000 and 1/3,000 dilutions, respectively.

Urea uptake studies in Arabidopsis. Leaves from 3-week-old plants were detached and then transferred to 1/2 MS medium containing 10% (v/v) ^{14}C-labelled urea (specific activity of 57.0 mCi/mol; American Radiolabelled Chemicals Inc., St. Louis, MO). At the end of 0.5 and 1 h incubations, the leaves were removed from the 1/2 MS medium containing the ^{14}C-labeled urea and washed five times with the 1/2 MS medium containing 10 mM cold urea to remove the ^{14}C-labeled urea. They were then homogenized in 200 μL of the homogenization buffer (10

mM HEPES, 0.3 mM EDTA; pH 7.5). Uptake of the ^{14}C-labeled urea was determined by obtaining the extracts and counting them in a liquid scintillation counter. Fresh weight of the leaves was determined to normalize the urea uptake.

Results

Molecular cloning of AtTIP4;1, a member of tonoplast intrinsic protein family in Arabidopsis. The full-length *AtTIP4;1* cDNA obtained from RT-PCR was 766 bp with an open reading frame of 750 bp, encoding 249-amino-acid protein. AtTIP4;1 has a predicted molecular mass of 26.1 kD and a calculated pI value of 6.2. Analysis of the deduced amino acid sequences using TRUST, an internet-based program (http://zeus.cs.vu.nl/programs/trustwww/) for determination of the internal repeats showed the head-to-head arrangement of two halves, which is a characteristic feature of the aquaporin proteins [Jung *et al.*, 1994; Heymann and Engel 1999; Fujiyoshi *et al.*, 2002]. The transmembrane regions were also analyzed with the TMpred program (http://www.ch.embnet.org/software/TMPRED_form.html) and are presented by dotted lines in Fig. 1. The N-terminal half (residues 14 through 102, 89 amino acids) is related to the C-terminal domain (residues 132 through 215, 84 amino acids) by a quasi-twofold axis that lies in the mid-membrane plane. Each one-half of the aquaporin consists of two transmembrane helices, followed by a conserved loop with the amino acid motif NPA and ended with a third transmembrane helix, as shown in many aquaporin proteins [Johanson *et al.*, 2001]. AtTIP4;1 exhibited the highest and the lowest sequence identities at the amino acid level with AtTIP1;1 and AtTIP5;1 (50.6 and 34.9%, respectively). The similarities in the sequences were also observed immediately after the NPA motif in the two

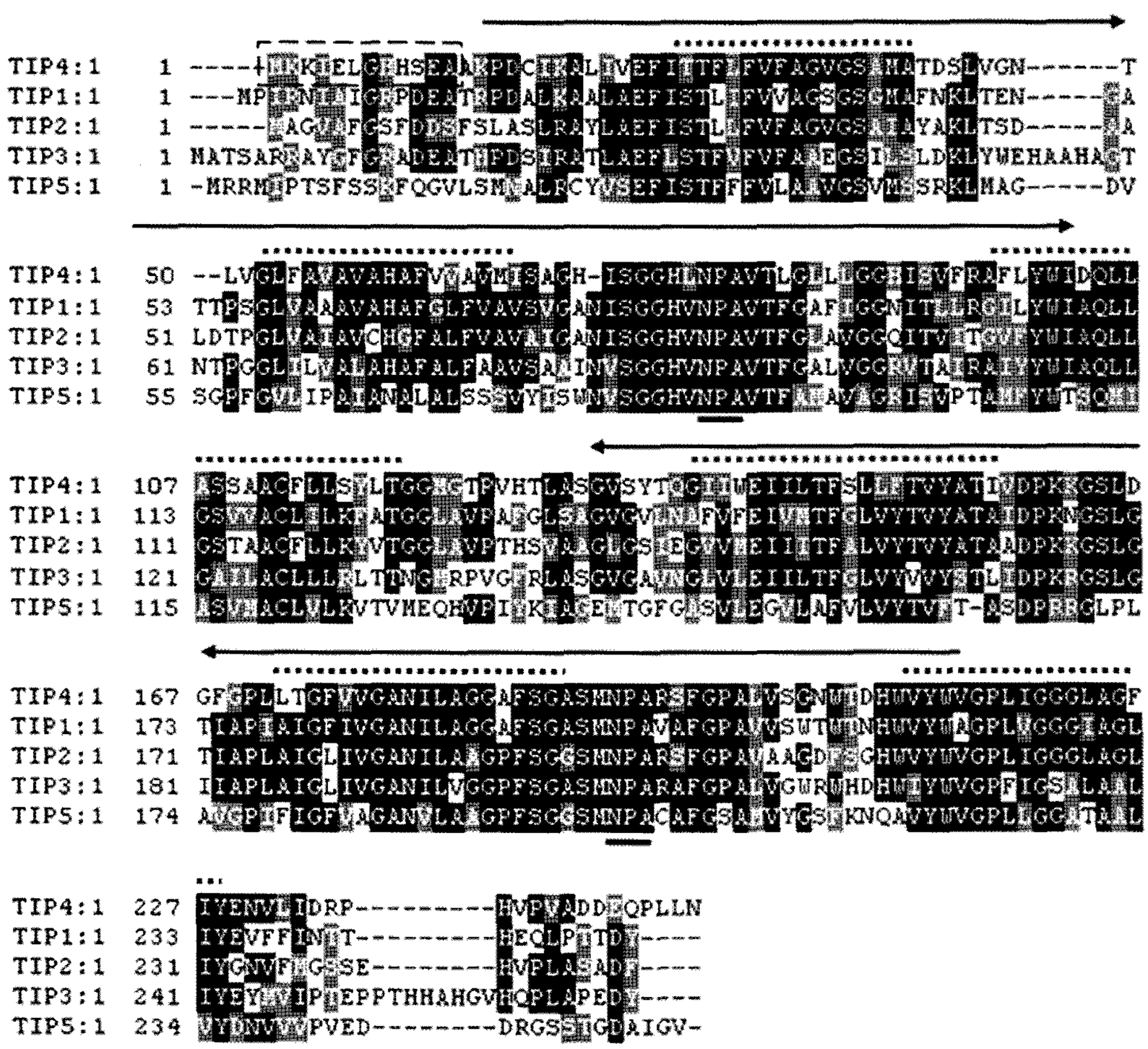

Fig. 1. Amino acid alignments of the members of tonoplast intrinsic protein (TIP) aquaporin family of *Arabidopsis*. Amino acids are given single-letter designations, and dashes indicate gaps. Residues are shown in white letters on black if three or more sequences have identical residues at the aligned positions. Gray areas indicate sequence homologies. An antibody was raised against a synthetic peptide that corresponded to the first 12 amino acids of the full-length AtTIP4;1, boxed by dotted lines. The transmembrane domains are presented by dotted lines. The lines below the sequences indicate the MIP family signature [Schaffner, 1998]. Two symmetric halves oriented 180° with respect to the membrane are indicated by arrows.

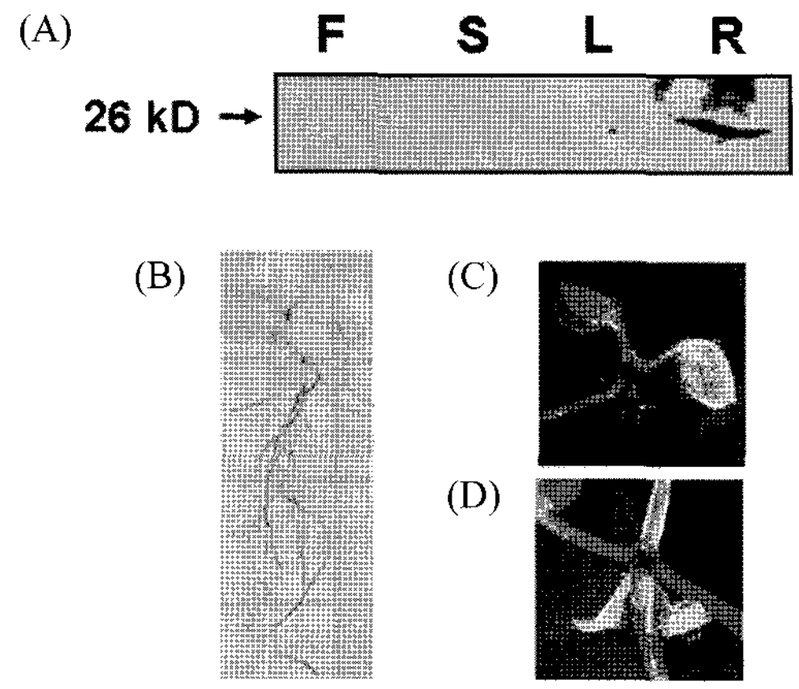

Fig. 2. *AtTIP4;1* expression pattern. (A) AtTIP4;1 protein expressions in different tissues. Each 10 μg of the total protein from leaves (L), stem (S), flower (F), and root (R) were separated by SDS-PAGE and subjected to Western blotting using AtTIP4;1 antiserum. (B-D) GUS expressions in the *Arabidopsis* plants transformed with the *AtTIP4;1* promoter-GUS construct: B, 2-week-old seedlings; C and D, magnification of 5-day-old seedlings and 2-week-old seedlings showing staining of the apical meristem.

halves, which are known to form a pore that allows the substrate to circumvent the biological membrane.

AtTIP4;1 is expressed in root and apical meristem. The expression patterns of *AtTIP4;1* in different organs were determined to understand the physiological function of *AtTIP4;1*. Northern blot analysis was performed using a 252-bp DNA fragment containing the 5'-UTR (untranslated region) and the N-terminal region of *AtTIP4;1* from the genomic DNA as a probe. However, the *AtTIP4;1* transcript was not detected in all of the organs tested. Thus, two different approaches were employed to establish its expression pattern: Western blot analysis with an antibody against the synthetic peptide of the first 12 amino acids of AtTIP4;1 and examination of the transgenic plants harboring the GUS gene driven by the *AtTIP4;1* promoter. Sequence alignments among several different TIP proteins revealed that the amino-terminal 12 amino acids of AtTIP4;1 are unique. Therefore, N-terminal region was chosen for the generation of the antibody. The AtTIP4;1 antibody recognized a protein of about 26 kD, consistent with the deduced molecular mass of the protein encoded by *AtTIP4;1*, which was detected only in the root (Fig. 2A).

The spatial expression pattern of *AtTIP4;1* was examined using the transgenic plants that harbored the *GUS* gene under the control of the *AtTIP4;1* promoter. This *pAtTIP4;1::GUS* construct contained a 1537-bp DNA fragment upstream of the start codon of *AtTIP4;1* fused to the GUS gene. The seedlings grown for 14 days on 1/2 MS plates were histologically stained to reveal the

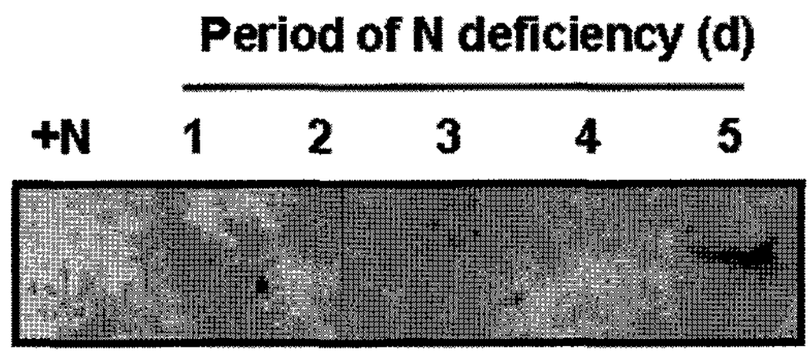

Fig. 3. *AtTIP4;1* product is induced under the nitrogen-deficient conditions. Western-blot analysis performed with the seedlings grown for 10 days in 2 mM NH_4NO_3 and then starved of nitrogen for 1, 2, 3, 4 or 5 days. Equal amounts of the total protein (10 μg) of the leaves were separated on a 12% polyacrylamide SDS gel, and the protein blot was probed with the anti-AtTIP4;1 as indicated in the Materials and Methods section.

GUS expression pattern. The GUS expression was restricted to the root and the apical meristem (Fig. 2B, C, and D). When the seedlings were exposed to a low temperature, dehydration or salt stress, no detectable changes were observed in the GUS activity. Thus,, the GUS activities of the fully developed *AtTIP4;1-GUS* transgenic plants were studied. Under the control conditions, the GUS activity was detected in the root, but not in the leaves, the inflorescence, and the flower.

Nitrogen-deficiency up-regulates AtTIP4;1 expression. To understand the possible roles of AtTIP4;1 in the plant nitrogen nutrition, an expression analysis was performed in *Arabidopsis* grown on the agar plates containing the nutrient solution described by Liu *et al.* [2003]. Before harvest, 10-d-old *Arabidopsis* seedlings were subjected to N deficiency for 1, 2, 3, 4, or 5 days as described in the Materials and Methods section. The transcript level of *AtTIP4;1* was nearly undetectable under both normal and nitrogen-deficient conditions (data not shown), possibly due to the extremely low expression of the *AtTIP4;1* gene. Therefore, Western blot analysis was conducted to determine the change in the AtTIP4;1 protein level in response to the nitrogen-deficient conditions. The AtTIP4;1 protein showed a weak but clear induction after N-starvation for 5 d (Fig. 3). This result suggested that AtTIP4;1 might be involved in the transport process for N nutrition or N metabolism.

Identification of loss-of-function and gain-of-function mutations in AtTIP4;1. Even though increasing evidence indicated the possible role of TIPs in the transport process for N nutrition in plants, their physiological functions still remain elusive. Therefore, the reverse genetic approaches were employed to study the potential physiological role of *AtTIP4;1* in plants. The T-DNA insertion plants of *AtTIP4;1* and the transgenic *A. thaliana* expressing the *ATTIP4;1* cDNA under the control of the CaMV35S promoter were analyzed to

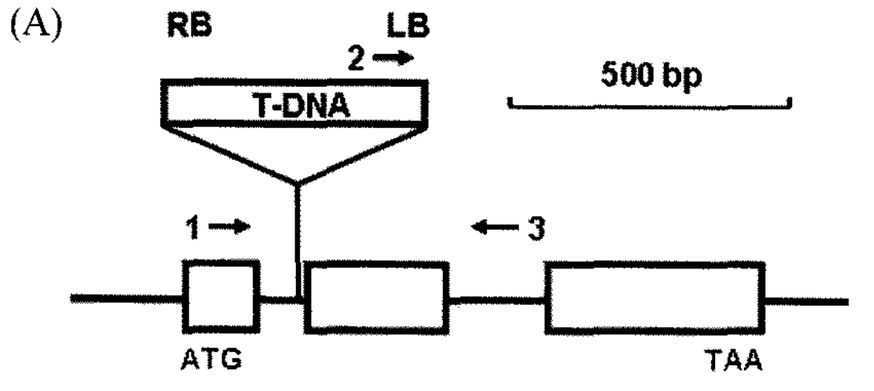

Fig. 4. Schematic representation of the *AtTIP4;1* genomic DNA with T-DNA insertion, and the construct of *P35S-AtTIP4;1-Tnos*. (A) Diagram of *AtTIP4;1* genomic structure and site of T-DNA insertion. Positions of exons (box) and introns (solid line) are shown. Arrows indicate positions of the primers used for screening to confirm the T-DNA insertion. (B) PCR fragments obtained by amplification of the genomic DNA from the wild-type (Col-0) plants and those homozygous for T-DNA insertion (TIP4;1KO:HM). DNA fragments shown in lane 1 and 3 were obtained with primers 1 and 2 (A), whereas fragments in lanes 2 and 4 were obtained with primers 1 and 3. (C) The binary vector pBI121 was used to introduce *AtTIP4;1* gene driven by the 35S CaMV promoter. 35S-P, a cauliflower mosaic virus 35S promoter, NOS-T, nopaline synthase terminator.

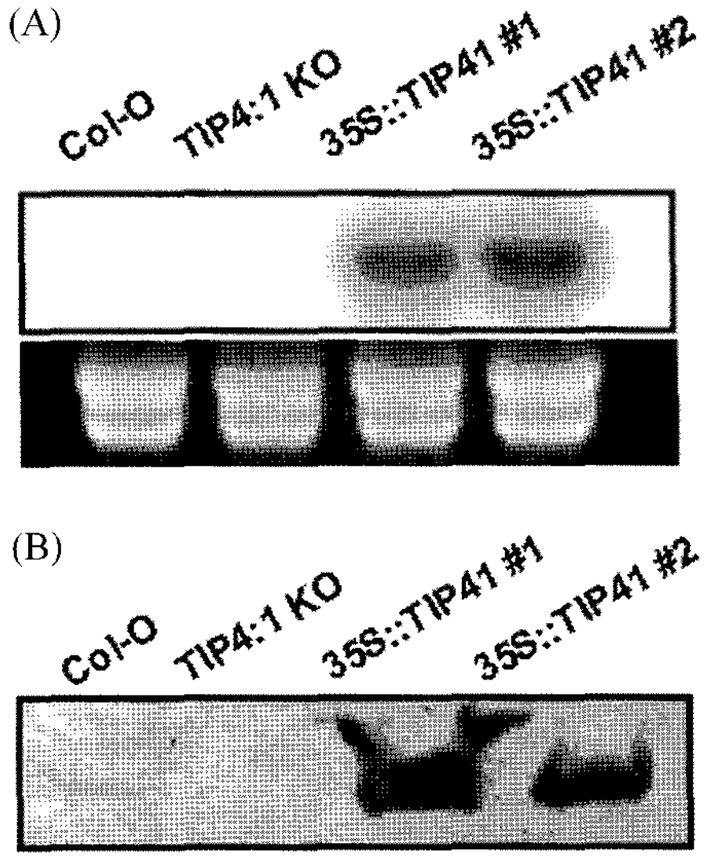

Fig. 5. Confirmation of *AtTIP4;1* products in 10-d-old seedlings of wild-type, T-DNA insertion, and *AtTIP4;1*-overexpressed plants. (A) *AtTIP4;1* transcript levels of the seedlings. RNA gel-blot was hybridized with ^{32}P-labled DNA fragment containing the 5'- UTR and N-terminal region of *AtTIP4;1* and visualized by autoradiography. The rRNA was used as a loading control. (B) AtTIP4;1 levels of the seedlings. Ten micrograms each of the total proteins of the wild-type, the T-DNA insertion, and the *AtTIP4;1*-overexpressed plants were separated on 12% polyacrylamide SDS gel, transferred on to the nitrocellulose membrane, and sequentially probed with the anti-AtTIP4;1 antiserum.

obtain the loss-of-function and the gain-of-function mutations in *AtTIP4;1*, respectively.

A BLAST search of the SALK database was performed to find *Arabidopsis* with T-DNA insertion in the *AtTIP4;1* gene (Fig. 4A). The individual plants of the T-DNA insertion line (SALK_050663) from the Arabidopsis Biological Resource Center were analyzed by PCR to confirm the presence of the T-DNA insertion. Two gene-specific PCR primers flanking the T-DNA insertion were used in combination with the T-DNA left border primer. The presence of a wild-type *AtTIP4;1* genomic DNA was confirmed by the PCR product amplified with the upstream and downstream gene-specific primers, while amplification of a smaller fragment using the downstream gene-specific primer and T-DNA left border primer indicated a T-DNA insertion. The lines homozygous for T-DNA insertion in *AtTIP4;1* were identified by PCR analysis. DNA from the homozygous lines exhibited the PCR product with the downstream gene-specific primer and the T-DNA left border primer, but not with the two gene-specific primers (Fig. 4B). Sequencing of the PCR products, which flanked the T-DNA insert by the T-DNA left border primer, located the insertion of T-DNA into the first intron of the *AtTIP4;1* gene at 69 bp downstream of the ATG initiation start codon (Fig. 4A). The insertion was also accompanied by a 15-bp deletion of the *AtTIP4;1* sequence at the integration site. Three successive backcrosses and the subsequent identification of the plants homozygous for the T-DNA were performed to eliminate additional possible mutations in the T-DNA insertion plants.

AtTIP4;1 was placed under a strong plant promoter, the cauliflower mosaic virus 35S promoter (Fig. 4C), and introduced into *Arabidopsis* by *Agrobacterium*-mediated transformation. For this process, 36 independent kanamycin-resistant lines were selected on the 1/2 MS medium containing kanamycin, and their segregation ratios of kanamycin resistance in T1 and T2 generations were examined. To determine the effect of overexpression, two independent lines showing the segregation ratio of 3:1 (kanamycin-resistant:kanamycin-sensitive) were selected among these independent transgenic plants.

Northern blot analysis showed a significant induction of the *AtTIP4;1* transcripts in the *35S::AtTIP4;1*

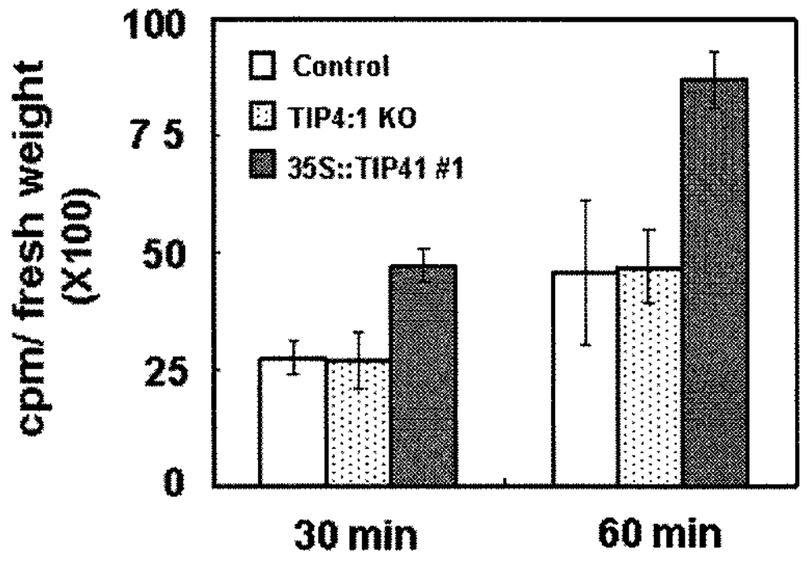

Fig. 6. Uptake of urea by T-DNA insertion and *AtTIP4;1*-overexpressed plants. Three-week-old leaves of the control, the T-DNA insertion, and the *AtTIP4;1*-overexpressed plants were exposed to 1/2 MS solution containing ^{14}C-labeled urea for 30 or 60 min. The transgenic plants introduced with an empty vector served as controls. Uptake of ^{14}C-labeled urea was determined by obtaining extracts from the seedlings and counting them in a liquid scintillation counter. The fresh weight of the leaves was determined to normalize the amount of ^{14}C-labeled urea. Mean and standard deviation were derived from three independent measurements.

transformants, with no detectable signals in the wild-type and the T-DNA insertion plants (Fig. 5A). Thus, Western blot analysis was performed by using the T-DNA insertion to further confirm the absence of the *AtTIP4;1* products. A weak signal was observed in the wild-type plants; however, the signal corresponding to the *AtTIP4;1* products was hardly detected in the T-DNA-insertion plants (Fig. 5B). This finding indicated that the T-DNA insertion in the first intron of *AtTIP4;1* resulted in a knock-out mutation and that the AtTIP4;1 antibody had a specific binding activity toward AtTIP4;1. Consistent with the Northern blot analysis, significant inductions were also detected in the *35S::AtTIP4;1* transformants (Fig. 5B). The effects of the absence or the overproduction of the AtTIP4;1 antibody in response to the abiotic stresses and plant developments were examined. However, no detectable differences were observed in the growth and the development of all organs including roots, cotyledons, leaves, inflorescence, and flowers among the T-DNA-insertion, the overexpressed, and the wild-type plants. The T-DNA-insertion and the overexpressed plants also exhibited the same response of the wild-type plants to the abiotic stresses including drought, salinity, and osmosis (data not shown). The absence of the obvious phenotypic changes in the loss-of-function and the gain-of-function plants might be due to the overlapping functions of the TIP aquaporin subfamily members and/or to the non-essential function of the *AtTIp4;1* gene.

Urea uptake rate is increased by overexpression of AtTIP4;1. To investigate the effects of the loss-of-function and the gain-of-function mutations of *AtTIP4;1* on the urea uptake in plants, the rosette leaves were removed from the T-DNA-insertion, the overexpressed, and the wild-type plants, and incubated for 30 and 60 min in 1/2 MS medium containing the ^{14}C-labeled urea. The transgenic plants introduced with empty vectors served as the control for the urea uptake experiments. The urea uptake in the leaves of the T-DNA-insertion plants showed no difference to those of the control leaves, whereas the overexpression of *AtTIP4;1* resulted in 71.6 and 90.2% increases in the urea uptake after incubation for 30 and 60 min, respectively (Fig. 6). No reduction in the urea uptake of the loss-of-function mutants may suggest a functional redundancy in the TIP aquaporins in *Arabidopsis*. Taken together, these results suggest that the overexpression of *AtTIP4;1* can facilitate the urea transport in plants without causing detectable defects in the growth and the development under normal and abiotic stress conditions.

Discussion

In plants several passive and active transport pathways are available for the transportation of urea across the cellular membranes [Kojima *et al.*, 2006]. A number of biochemical studies have shown that the plant aquaporins contribute to the urea import as well as the water transport in a dose-dependent manner. This transport kinetics makes aquaporins to become more important in the urea application to soils and leaves as a liquid fertilizer. Although biochemical studies combined with the heterologous expression approaches have shown that many aquaporins mediate the facilitation of urea uptake into the cells, little is known of their physiological roles in the growth and the development of plants. Expanding the knowledge of their biological functions and regulations in plants will not only allow a better understanding of the urea transport system, but also result in an improvement of their utilization as a nitrogen fertilizer for soil and foliar application in the crop production.

Reverse genetic approaches were undertaken to study the biological function of *AtTIP4;1* in urea uptake, as well as the growth and the development in plants. The present study showed that the expression of the *AtTIP4;1* products was restricted to the root and the apical meristem (Fig. 2A-D). Western blot analysis also revealed an induction following the nitrogen starvation for 5 d, suggesting possible physiological roles of AtTIP4;1 in the plant nitrogen nutrition (Fig. 3). In addition, the increase

in the rate of the urea uptake could be achieved by the overexpression of *AtTIP4;1* without causing any detectable defects in the growth and the development of *Arabidopsis* (Fig. 6). These results suggested that the *AtTIP4;1* products might play a role in the urea accumulation in the vacuole for the transient storage of the nitrogen source. The present study also showed the possible way to improve the utilization of urea as a nitrogen fertilizer through the overexpression of genes encoding the aquaporins in plants. However, these findings raised several questions on their function and regulation in plants.

Aquaporins have been shown to function as urea transport systems only under relatively high concentration of urea. However, the urease secreted by microorganisms in nature rapidly degrades urea into ammonium and CO_2 [Watson *et al.*, 1994]. This biological activity keeps urea concentration at very low concentrations, ranging between 0.1-0.3 μM [Mitamura *et al.*, 2000]. If so, then urea transport systems must exist to mediate the active transport. Heterologous complementation study of the urea uptake-defective yeast showed that the *AtDUR3* products conferred the yeast growth in 2 mM urea and mediated the proton-stimulated urea transport [Liu *et al.*, 2003]. This active transport system might mediate the high accumulation of urea in the cytoplasm from the environments with low concentrations of urea. One can thus speculate that the *AtTIP4;1* products would contribute to a high accumulation of urea in the vacuole through facilitation of a passive transport using the potential gradient generated by the active transport systems.

Even though the urea uptake was increased by the overexpression of the *AtTIP4;1* products (Fig. 6), it cannot be ruled out that urea might not be an endogenous substrate for the *AtTIP4;1* products under normal conditions. Northern and Western blot analyses showed very low expression levels of the *AtTIP4;1* products under normal and nitrogen-deficient conditions. These low expression levels might be adverse for the rapid transport of urea under the nitrogen-deficient conditions, which suggest the transport was possible due to the structural similarity between the uncharged organic molecule and urea. This broad spectrum of the substrates might allow the increase in the urea-uptake rate in the transgenic plants overexpressing *AtTIP4;1*. Recent reports have shown that the plant aquaporins promote the membrane transport of small substances such as CO_2, hydrogen peroxide, boron, and silicon [Uehlein *et al.*, 2003; Ma *et al.*, 2006; Bienert *et al.*, 2007]. Resolving their substrate specificity and the connected physiological function would give an opportunity for a better understanding of the plant physiology and response to the environmental changes. Biochemical studies using the *AtTIP4;1* knock-out and the overexpressed plants would provide essential information on their endogenous substrates in Arabidopsis.

Although the high induction of the *AtTIP4;1* transcript and the protein by a strong 35S promoter was confirmed by both Northern and Western blot analyses, the *AtTIP4;1*-overexpressed plants exhibited 90.2% increase in the urea-uptake rate when compared to that of the wild-type plants, which suggests that the post-translational modification could be important in the regulation of the activity of *AtTIP4;1*. Accumulating evidence has shown that a number of factors affecting the aquaporin activity could involve phosphorylation, glycosylation, heteromerization, pH, Ca^{2+}, and solute gradient beyond the regulation through alteration of the transcript level according to the cell type, the developmental stage, and the environmental conditions in plants [Vera-Estrella *et al.*, 2004; Chaumont *et al.*, 2005]. Therefore, the *35S::AtTIP4;1* transgenic plant could be a potential source for elucidating these mechanisms, which contribute to the further understanding of the aquaporin regulation. A better understanding of the physiological function and regulation of AtTIP4;1 would provide considerably more information on the urea transport systems and improve the quality of the nitrogen fertilizer used in the crop production.

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