Functional Characterization of Betaine/Proline Transporters in Betaine-accumulating Mangrove*

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Betaine is an important osmoprotectant in many plants, but its transport activity has only been demonstrated using a proline transporter from tomato, a betaine-nonaccumulating plant. In this study, two full-length and one partial transporter genes were isolated from betaine-accumulating mangrove Avicennia marina. Their homologies to betaine transporters from bacteria and betaine/4-aminobutyrate transporters from mammalian cells were low but were high to proline transporters from Arabidopsis and tomato. Two full-length transporters could complement the Na+-sensitive phenotype of the Escherichia coli mutant deficient in betT, putPA, proP, and proU. Both transporters could efficiently take up betaine and proline with similar affinities (Km, 0.32–0.43 mM) and maximum velocities (1.9–3.6 nmol/min/mg of protein). The uptakes of betaine and proline were significantly inhibited by mono- and di-methylglycine but only partially inhibited by betaine aldehyde, choline, and 4-aminobutyrate. Sodium and potassium chloride markedly enhanced betaine uptake rates with optimum concentrations at 0.5 mM, whereas sucrose showed only modest activation. The change of amino acids Thr290-Thr-Ser292 in a putative periplasmic loop to Arg290-Gly-Arg292 yielded the active transporter independent of salts, suggesting the positive charge induced a conformational change to the active form. These data clearly indicate that the betaine-accumulating mangrove contains betaine/proline transporters whose properties are distinct from betaine transporters of bacteria and mammalian cells.

The accumulation of osmoprotectants is an important process for the adaptation to adverse environmental conditions (1, 2). The increase of osmoprotectants is achieved either by altering metabolism (increasing biosynthesis and/or decreasing degradation) or by transport (increased uptake and/or decreased export). Glycine betaine (betaine) is a major osmoprotectant in bacteria, algae, plants, and animals (1, 2). In plants, betaine is synthesized in chloroplasts from choline via two-step oxidations (3) and must be transported for a long distance upon the environmental stresses (4, 5). However, very little is known about the betaine transport in plants, and the betaine transport activity has only been demonstrated using a proline transporter from tomato (LeProT1) (6).

Recent molecular cloning of several amino acid transporters by functional complementation in yeast revealed that plant amino acid transporters are classified into two superfamilies; the amino acid, polyamine, and choline transporter superfamily (ATF superfamily (7, 8). ProT is a member of the ATF superfamily together with the broad substrate specificity amino acid permease and putative indole acetic acid transporter. But few studies have been carried out on plant ProTs. ProT genes were isolated from Arabidopsis (AtProT1 and -2) (9), tomato (LeProT1–3) (6), and rice (OsProT) (10). Among three tomato LeProTs, only one clone, LeProT1, could complement functional deficiencies in proline transport in yeast (6). LeProT1 was specifically expressed in pollen, whereas the mRNA for LeProT2 and LeProT3 could not be detected in any organs (6). It was shown that LeProT1 could take up proline, betaine, and 4-aminobutyrate (GABA) (6), and AtProT2 could take up GABA as well as proline (9, 11), suggesting that in plants, proline, betaine, and GABA are transported with the same transporter (6, 11).

Properties of the above ProTs from plants are different from those in bacteria and mammalian cells. In Escherichia coli, betaine and proline betaine were essentially taken up by the ATP-binding cassette transporter ProU (12) and secondary transporter ProP (13), whereas proline was taken up by the proline-specific secondary transporter PutP (14). The affinities of ProU and ProP for betaine and proline betaine were very high, in the micromolar range, whereas the affinity for proline was low (15). In mammalian cells, betaine was taken up by a betaine/GABA or proline/GABA transporter (16).

Hitherto, a betaine-specific transporter has not been reported in plants. Transport activity of betaine has only been demonstrated using a proline transporter from tomato, a beta-

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The nucleotide sequence(s) reported in this paper has been submitted to the DDBJ/GenBankTM/EMBL Data Bank with accession numbers AB075992, AB075993, and AB075994.

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ine-nonaccumulating plant. Therefore, it was of interest to characterize ProTs in betaine-accumulating plants. In a previous study (17), we constructed a cDNA library from betaine-accumulating mangrove Avicennia marina and showed that A. marina has unique betaine aldehyde dehydrogenase genes. Here we isolated betaine transporter genes from A. marina and characterized the molecular properties of their gene products. We show that A. marina contains betaine/proline transporters with substrate specificity and salt sensitivity distinct from previously reported proline transporters from plants. Moreover, one of these transporters showed a novel regulation of activity by introducing the additional positive charges on the periplasmic loop of the transporter.

Experimental Procedures

Culture Conditions—E. coli DH5α cells were grown at 37°C in Luria-Bertani medium. E. coli MKH13 cells deficient in betT, putPA, proP, and proU genes (12) were grown at 37°C in minimal medium A containing 0.2% glucose and ampicillin (50 µg/ml). Radiolabeled [1-14C]betaine (55 mCi/mmol) and [U-14C]proline (50 µCi/mmol) were purchased from Amersham Biosciences, respectively. Isolation of A. marina Betaine and/or Proline Transporter—The cDNA library from A. marina was constructed using the ZAP cDNA synthesis kit (Stratagene, La Jolla, CA) (17). For the isolation of betaine transporter genes, the mixed oligonucleotides were synthesized based on the known sequences of ProTs. The sequences of all the primers and probes are shown in Table I. The forward primer ProT-F corresponds to the 175–195 bases from the translation start codon of AtProT1 cDNA (18, 19). The reverse primer ProT-R corresponds to the 718–738 bases of AtProT1 cDNA. The cDNA library was divided into 96 fractions so that each well contained about 104 plaque-forming units. The betaine/proline transporter clones were further diluted 100-fold and amplified. After sequencing more than 20 clones, three kinds of gene fragments were fused in-frame to six histidines. The expression plasmids were transferred first into E. coli DH5α cells and then to MKH13 (12) cells in which betT, putPA, proP, and proU genes were deleted. Construction of AmT1 Mutant Transporter—A site-directed mutant in which the tripeptide Thr192-Thr-Ser206 in AmT1 was replaced with Arg206-Gly-Arg207 was constructed following the fragment covered by the restriction sites, BamHI site at 859 in pAMT1 and EcoRI site in pTrCHsi2C, was amplified by the PCR technique using pAMT1 as a template. The forward primer AmT1-BamHI contains the BamHI site and AGGGGAAG encoding Arg206-Gly-Arg207. The amplified fragment was ligated into the BamHI- and EcoRI-digested pAMT1, which generated the plasmid pAMT1m.

Complementation Test—For the complementation test on agar plates, E. coli MKH13 cells transformed with pTrCHsi2C, pAMT1, and pAMT2 were grown overnight at 37°C in minimal medium A (pH 6.7) containing 0.2% glucose and ampicillin (50 µg/ml) and were inoculated into the same fresh medium with an absorbance at 620 nm (A620) of 0.05. IPTG (1 mM) was added to the mid log-phase cells (A620 between 0.5 and 0.8). After a 5-h incubation, cells were harvested, washed twice, and suspended to an A620 of 1.0 in the same medium. Subsequently the cells were incubated with shaking for 5 min at 37°C, and transport was initiated by the addition of 1 µl of [1-14C]betaine or [L-14C]proline. For Ks and Vmax determinations, the concentrations of betaine or proline were varied from 0.01 to 5 mM. Glucose was added to a final concentration of 5 mM to energize the cells, and where indicated, salt (NaCl or KCl) or sucrose was added to the indicated concentrations. Cells were collected on 0.2-µm-pore-size cellulose nitrate filters (Advantec MFS, Chiba, Japan). Filters were washed with 3 ml of buffer (same salinity as the assay buffer), and the radioactivity trapped in the cells was measured with a liquid scintillation counter (model 3200C, Aloka Instruments Co., Tokyo, Japan). Quantification for betaine uptake and proline uptake were performed in the presence of a 100-fold molar excess (10 µM) of competitors.

Quantification of mRNA for AmT1–3 by a Real Time Quantitative Reverse Transcription (RT)-PCR—Quantification of AmT1–3 mRNA was carried out using a TaqMan fluorescent chemical analysis method (17, 21, 22). Total RNA was extracted by using the SDS-phenol method. RT-PCR and DNA amplification were carried out using TaqMan Reverse Transcriptase and DNA amplification reagents and TaqMan Universal PCR Master Mix, respectively (Applied Biosystems). The clone-specific primers and TaqMan fluorescent probes, shown in Table I, were used for amplification. A computer algorithm was used for comparison of the amount of reporter dye emission with the quenching dye emission during the PCR amplification, generating a ∆Rn value as follows: ∆Rn = (∆Rn+) − (∆Rn−), where ∆Rn+ is (emission intensity of reporter)/emission intensity of quencher at any given time in the reaction tube), and ∆Rn− is (emission intensity of reporter)/emission intensity of quencher before PCR amplification in the reaction tube). The ∆Rn value was used for the construction of amplification plots.

Computer Analysis and Other Methods—The hydrophy profile of the deduced amino acid sequence was predicted according to the method of Kyte and Doolittle (23). The possible transmembrane (TM) segments of the AmT1 sequence were deduced by a computer program TopPredII (24). SDS-PAGE and immunoblotting were carried out as described previously (17). Protein concentration was determined by the method of Lowry et al. (17). An antibody raised against a six-histidine (His6) tag was obtained from R&D Systems, Inc. (25).

Results

Cloning of Betaine/Proline Transporter from A. marina—Using the mixed oligonucleotides, three kinds of gene fragments were amplified from the cDNA library of A. marina. Nucleotide sequencing of isolated pure clones showed that clones 1, 2, and 3 contain the 1830–1794, and 1432-1428 bp DNA sequences, respectively, and poly(A) sequences in their 3’-terminal regions (data not shown). The homology search revealed that clones 1 and 2 encode the full length of transporters, whereas clone 3 encodes the N-terminal-missing transporter. Several attempts to isolate the full length of clone 3 were unsuccessful. The predicted gene products for clone 1 (AmT1) and clone 2 (AmT2)
consist of 446 amino acids with a molecular mass of 48,742 Da and 447 amino acids with a molecular mass of 48,645 Da, respectively (Fig. 1A). The gene product for clone 3 (AmT3) consists of 440 amino acids (Fig. 1A). It was found that AmT1–3 are highly homologous to each other and also to other ProTs from plants as shown in Fig. 1A (6, 9, 10). In contrast, AmT1–3 showed low homology to amino acid permease AAP1 (18, 19) (Fig. 1B). AmT1 and AmT2 Could be Expressed in E. coli Membranes—Hitherto, the complementation of a salt-sensitive betaine-deficient mutant by plant transporters has not been reported. To test this, E. coli MKH13 cells were transformed with pTrcHis2C, pAmT1, and pAmT2. Western blotting analysis of

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**Fig. 1.** A, alignment of the deduced amino acid sequences of *A. marina* transporters from six organisms. The sequences were aligned by the program ClustalW. The amino acid residues conserved in all sequences are designated by stars, and conservative substitutions are shown by dots. Predicted membrane-spanning regions are marked above the alignment (I-XI). B, phylogenetic analysis of six transporters. Multiple sequence alignment and the generation of the phylogenetic tree were performed with ClustalW and TreeView software, respectively.
the membrane fractions revealed that the MKH13 cells transformed with pAmT1 and pAmT2 exhibited a single cross-reaction band corresponding to ∼48 kDa when IPTG was included in the culture medium, whereas no band could be detected without IPTG (Fig. 2A). The levels of AmT1 and AmT2 increased with increasing concentrations of IPTG (from 0.2 to 1.0 mM) and were almost saturated at 0.5 mM IPTG. As a positive control of membrane proteins, the outer membrane protein A of E. coli (OmpA), which was stained with Coomassie Brilliant Blue R250, is shown. A, Western blotting of AmT1 and -2. The AmT1- and AmT2-expressing MKH13 cells were grown at 37 °C in minimal medium A (pH 6.7) containing 0.2% glucose and ampicillin (50 μg/ml) until the absorbance at 620 nm reached 0.6, and then the indicated concentrations of IPTG were added. After 5-h incubations, the cells were harvested and sonicated, and membrane fractions were used for Western blotting.

**Fig. 2.** Expression and complementation of AmT1 and -2 in salt-sensitive *E. coli* MKH13 cells. A, Western blotting of AmT1 and -2. The AmT1- and AmT2-expressing MKH13 cells were grown at 37 °C in minimal medium A (pH 6.7) containing 0.2% glucose and ampicillin (50 μg/ml) until the absorbance at 620 nm reached 0.6, and then the indicated concentrations of IPTG were added. After 5-h incubations, the cells were harvested and sonicated, and membrane fractions were used for Western blotting. As a control, the outer membrane protein A of *E. coli* (OmpA), which was stained with Coomassie Brilliant Blue R250, is shown. B, complementation test of betaine-deficient MKH13 mutant cells by AmT1 and -2. AmT1- and AmT2-expressing cells were grown for 5 days on agar plates containing 0.7 M NaCl and 1 mM betaine or proline as described under “Experimental Procedures,” and then photographs were taken. The control represents MKH13 cells transformed with pTrcHis2C.

**Fig. 3.** Kinetics of betaine and proline uptakes. A and B, time courses of betaine (A) and proline (B) uptakes by AmT1- and AmT2-expressing MKH13 cells. The control represents MKH13 cells transformed with pTrcHis2C. C and D, double-reciprocal plots of betaine (C) and proline (D) transport kinetics by AmT1- and AmT2-expressing MKH13 cells. Reaction mixtures contained minimal medium A (pH 6.7) and 0.1 mM [1-14C]betaine or L-[U-14C]proline. Each value shows the average of three independent measurements.
AmT1 and AmT2 Complement Salt-sensitive E. coli mutant MKH13 cells by Betaine Uptake or Proline Uptake—The E. coli MKH13 cells are unable to grow in high osmolality medium containing betaine due to the lack of a betaine transport system as well as betaine synthesis genes (12, 26, 27). Fig. 2B shows that the MKH13 cells transformed with vector alone (pTrcHis2C) could not grow on agar plates containing minimal medium A, 0.7 mM NaCl, and 1 mM betaine either in the presence or absence of IPTG. Neither pAmT1 nor pAmT2 could complement the E. coli MKH13 cells under the same conditions in the absence of IPTG (data not shown). However, the pAmT1 and pAmT2 could complement the MKH13 cells if IPTG, at a concentration higher than 0.1 mM, was included in the agar plate (Fig. 2B). The inclusion of betaine was indispensable for the complementation (data not shown). The complementation by pAmT2 was slightly less effective than that by pAmT1 (Fig. 2B). These data indicate that both AmT1 and AmT2 are involved in the uptake of betaine by the MKH13 cells, thus allowing their growth under high salinity.

Since the MKH13 cells do not contain the proline transporters ProP and ProU (2, 12, 15), we therefore tested whether the MKH13 cells could grow under high salinity in the presence of proline. Fig. 2B shows that the E. coli MKH13 cells transformed with pAmT1 and pAmT2 could grow on the agar plates containing minimal medium A, 0.7 mM NaCl, 1 mM proline, and IPTG (>0.2 mM). In this case, the complementation by pAmT1 was less effective than that by pAmT2 (Fig. 2B). Both proline and IPTG were indispensable for the complementation (data not shown). These data indicate that the uptake of proline by AmT1 and AmT2 enables the growth of the MKH13 cells under high salinity.

Kinetic Properties of AmT1 and AmT2—To examine directly the transporter activity of AmT1 and AmT2, we measured the kinetic parameters for these transporters. Hitherto, the kinetic parameters for betaine uptake by plant transporters have only been reported for LeProT1 (6). No measurable uptake of [1-14C]betaine or of L-[U-14C]proline was observed for the MKH13 cells transformed with pTrcHis2C (Fig. 3, A and B). Consistent with the results of the complementation test, the MKH13 cells transformed with pAmT1 and pAmT2 could take up betaine and proline (Fig. 3, A and B). The $K_m$ and $V_m$ values for betaine uptake by AmT1 at pH 6.7 and 0.3 M NaCl were 0.34 mM and 3.3 nmol of betaine min$^{-1}$ (mg of protein)$^{-1}$, respectively (Fig. 3C). The corresponding $K_m$ and $V_m$ values by AmT2 were 0.36 mM and 1.9 nmol of betaine min$^{-1}$ (mg of protein)$^{-1}$, respectively (Fig. 3C). Similar values were obtained for proline uptake (Fig. 3D). The $K_m$ and $V_m$ values for proline uptake by AmT1 were 0.43 mM and 3.6 nmol of proline min$^{-1}$ (mg of protein)$^{-1}$, and those by AmT2 were 0.32 mM and 2.7 nmol of proline min$^{-1}$ (mg of protein)$^{-1}$. Results in Fig. 3, A and C, are compatible with the results in Fig. 2B showing slightly more effective complementation of betaine-deficient MKH13 cells by AmT1 than that by AmT2, whereas the results in Fig. 3, B and D, are apparently incompatible with the results of complementation of proline-deficient MKH13 cells in Fig. 2B. Nonetheless, all the above data indicate that both AmT1 and AmT2 transport betaine and proline with similar kinetic properties.

Competitions for Betaine and Proline Uptake Mediated by AmT1 and AmT2—To obtain the information on the substrate specificity, we performed the competition experiments. Consistent with the betaine and proline uptake experiments (Fig. 3), the betaine uptake by AmT1 was significantly inhibited by both L- and D-proline (Fig. 4A). However, it was only partially inhibited by GABA (Fig. 4A), which is in contrast to that of LeProT1 (6). In LeProT1, the betaine uptake was significantly inhibited by betaine, proline, and GABA (6). Fig. 4A shows that other amino acids such as alanine, arginine, glycine, glutamic acid, and histidine did not inhibit the betaine uptake by AmT1. It was also observed that proline betaine, betaine aldehyde, and choline only partially inhibited the betaine uptake by AmT1. In contrast, mono- and dimethylglycine strongly inhibited betaine uptake by AmT1. Essentially similar competition patterns were obtained for the betaine uptake by AmT2 (Fig. 4B). These results strongly suggest that the AmT1 and AmT2 are a betaine and proline transporter but not a GABA transporter.

Next we examined the competition for proline uptake medi-
ated by AmT1 and AmT2. For AmT1, the proline uptake was insensitive to histidine but significantly inhibited by betaine (Fig. 4C). The precursors of betaine, namely betaine aldehyde and choline, showed less inhibition of proline uptake than did betaine. Dimethylglycine, monomethylglycine, and GABA also showed less competition than did betaine for proline uptake mediated by AmT1. Essentially similar competition patterns for proline uptake were obtained for AmT2 (Fig. 4C). Since proline uptake by LeProT1 was significantly inhibited by beta-aldehyde, choline, and GABA (6), the competition patterns for the proline uptake mediated by AmT1 and AmT2 were also different from that of LeProT1.

Effects of Salts and pH on the Rates of Betaine Uptake by AmT1 and AmT2—Recently it has been demonstrated that *E. coli* ProP (28), *Corynebacterium glutamicum* BetP (26, 29), and *Lactococcus lactis* (30) are osmosensor and osmotically activated transporters. However, no information is available for plant transporters. To determine whether AmT1 and AmT2 could be osmotically activated, we examined the effects of salts on their betaine transport activities. The rates of betaine uptake by AmT1 increased with increasing concentrations of NaCl (Fig. 5A) and KCl (Fig. 5B) with maximum uptake at 0.5 M. Sucrose also activated betaine uptake, although its extent was much smaller than that by NaCl and KCl (Fig. 5C). Similar effects of three osmotica on betaine uptake were obtained for AmT2. In these experiments, the MKH13 cells were initially energized with 50 mM glucose. Therefore, the increase of transport rates upon the increase of salts was the consequence of activation of AmT1 and -2 by salts rather than the differences in membrane potential energy.

The proline uptake rate by LeProT1 has been shown to increase with decreasing pH (6.7-fold higher at pH 4.5 than at pH 5.5), whereas the proline uptake rate was inhibited by 2,4-dinitrophenol (6). From these data, it was concluded that LeProT1 is a proton/proline symporter. To determine whether this is also the case for mangrove transporter, we examined effects of pH on betaine uptake by AmT1 and AmT2. As shown in Fig. 5D, the rates of betaine uptake by AmT1 increased with increasing pH, reaching a maximum at pH 6.5, and then decreased. A similar pH-dependent uptake pattern was obtained for AmT2, although, in this case, the pH optimum was 6.0, and a more rapid decrease was observed at higher pH. Betaine uptakes by AmT1 and -2 were completely inhibited by 0.1 mM 2,4-dinitrophenol and also by 20 J/m carbonyl cyanide m-chlorophenylhydrazone. The data of both pH dependence and inhibition by the protonophore suggest that AmT1 and -2 might function as sodium/betaine symporters.

Analysis of AmT1 Mutant—Until now, no information was available on the substrate binding site in plant proline transporter. In some bacteria betaine transporters, i.e. OpuD (31), BetP (32), and BetL (33), many basic amino acids as well as the tripeptide Arg-Gly-Arg are found on the predicted cytoplasmic loop connecting to the eighth TM segment that were speculated to play an important role for transport (30). From the data of Fig. 5, we speculated that the binding of cations to the periplasmic loop of AmTs would activate the betaine transporter. To test this possibility, we introduced the positive charges on the periplasmic loop of AmTs. Analysis of the hydropathy plot (23) and the TM prediction program (24) predicted 11 putative TM segments for AmT1 as shown in Fig 6A. We constructed an AmT1 mutant (AmT1m) in which Thr 290-Thr-Ser292 in AmT1 was changed to Arg 290-Gly-Arg292 (Fig. 6B).

Western blotting analysis revealed that the AmT1m could be expressed in *E. coli* MKH13 cells and assembled as in the case of AmT1 (data not shown). It was also found that AmT1m could complement the salt-sensitive *E. coli* MKH13 mutant by both betaine uptake and proline uptake (Fig. 7A). The kinetic experiments revealed that AmT1m has slightly lower $K_m$ and $V_m$. 

**FIG. 5.** Effects of salts, sucrose, and pH. A and B, effects of NaCl (A) and KCl (B) on betaine uptake by AmT1- and AmT2-expressing MKH13 cells. C, effect of sucrose on betaine uptake by AmT1- and AmT2-expressing cells. D, effect of pH on betaine uptake by AmT1- and AmT2-expressing cells. Basic reaction mixtures were the same as those in Fig. 3. In D, the value at pH 6.0 was used as 100%, and pH was adjusted by solid MES. Each value shows the average of three independent measurements.
values than AmT1 for both betaine uptake and proline uptake (Fig. 7B). The competition experiments for the betaine uptake by AmT1m showed a pattern similar to that by AmT1 with the exception that less inhibition of betaine uptake by AmT1m was observed with GABA, monomethylglycine, and dimethylglycine (Figs. 4A and 7C).

Interestingly we found that the AmT1m has a very high betaine uptake activity even without salts as shown in Fig. 8, A and B. The betaine uptake rates were almost constant when the concentrations of salts were between 0.0 and 0.3 M. At higher concentrations of salts, the uptake rates decreased. Addition of sucrose significantly inactivated AmT1m (Fig. 8C). These data suggest that the positive charges on the periplasmic loop mimicked the salt-induced activation of the transporter.

**DISCUSSION**

Using the mixed oligonucleotides, we isolated two full-length and one N-terminal-deleted cDNA clones. Based on the following three independent experiments, we could conclude that betaine-accumulating *A. marina* contains at least two betaine/proline transporters. First, the gene products of two clones, AmT1 and -2, could complement the salt-sensitive betaine and proline-deficient *E. coli* mutant (Fig. 2). Second, AmT1 and -2 could transport both betaine and proline at similar rates (Fig. 3). Third, the transport activities of betaine and proline by AmT1 and -2 were significantly inhibited by proline and betaine but only partially inhibited by GABA (Fig. 4). To our knowledge, this is the first report demonstrating betaine transport activity in betaine-accumulating plants.

The competition experiments for betaine (Fig. 4, A and B) and proline (Fig. 4C) uptake suggest that AmT1 and -2 transport specifically betaine and proline. Other amino acids such as neutral (Ala), basic (Arg), and acidic (Glu) residues were ineffective for the inhibition of betaine uptake. Interestingly betaine aldehyde and choline were relatively ineffective, which is quite different from the results of competition studies for proline uptake by tomato LeProT1 (6). The proline uptake by LeProT1 was significantly inhibited by these compounds. Since tomato and *Arabidopsis* are betaine-nonaccumulating plants, the physiological function of the putative proline/betaine/
GABA transporter (LeProT1 and AtProT) in these plants is probably to transport proline and GABA.

The ineffectiveness of proline betaine for the inhibition of betaine uptake by AmT1 and -2 seems to be different from bacteria transporters ProP (13) and ProU (12), which transport both betaine and proline betaine. ProU and ProP have high affinities for betaine and proline betaine and low affinities for proline. These facts suggest that bacteria betaine transporters ProP and ProU can discriminate between the substrates betaine and proline but not between betaine and proline betaine, whereas the betaine-accumulating plant transporters AmT1 and -2 can discriminate between betaine and proline betaine but not between betaine and proline. The mechanism underlying substrate specificities among different transporters remains to be clarified. For further insight into substrate specificity, it might be interesting to isolate the proline betaine transporter from proline betaine-accumulating plants such as *Lamium* (1) and compare the substrate specificity among proline betaine, beta-

![A) Complementation test for mutant AmT1](image)

**Fig. 7.** Complementation, kinetic parameters, and competition for the AmT1m-expressing MKH13 cells. A, complementation test of betaine- and proline-deficient MKH13 mutant cells by AmT1 and AmT1m. Experimental conditions were the same as in Fig. 2B. B, kinetics of betaine and proline uptake by AmT1- and AmT1m-expressing MKH13 cells. Experimental conditions were the same as in Fig. 3A and D. C, competition of betaine uptake by the AmT1-expressing MKH13 cells. Experimental conditions were the same as in Fig. 4. Each value in B and C shows the average of three independent measurements. Pro-Bet, proline betaine; Bet-ald, betaine aldehyde; Cho, choline; DM-Gly, dimethylglycine; MM-Gly, monomethylglycine.

ine, and proline. Since AmT1 and -2 could be expressed in functional forms in *E. coli*, structural analysis obtained for the NhaA Na’/H’ antiporter (34) might be another useful approach to unveil the complicated and important substrate specificity of betaine transporters.

Monomethylglycine and dimethylglycine are effective competitors for betaine uptake by AmT1 and -2, whereas they are less efficient competitors for proline uptake (Fig. 4). These facts suggest that the methyl group attached to glycine is important for betaine uptake, whereas it plays a minor role for proline uptake. AmT1 and -2, showing that betaine aldehyde and choline were rather inefficient to inhibit betaine uptake by AmT1 and -2, suggesting the importance of zwitterionic form for efficient binding and/or transport. The importance of zwitterionic form for GABA transport by AtProT2 has also been reported (11). However, of the two zwitterionic compounds, proline and proline betaine, only the former significantly inhibited the betaine uptake by AmT1 and -2 (Fig. 4, A and B). Hence the zwitterionic form of the compound cannot totally account for its uptake by betaine/proline transporter. Altogether it seems that AmT1 and -2 can bind betaine and proline at the same site, and the core structures of betaine and proline are key determinants for binding to the transporter, i.e. neither the methyl group nor the zwitterionic form can solely direct the binding of the compound to the transporter.

Considering the relative expression levels of AmT1 and AmT2, the complementation of betaine-deficient MKH13 mutant cells by AmT1 seems to be more effective than that by AmT2, whereas the reverse was observed for the complementation of proline-deficient MKH13 mutant cells (Fig. 2). Comparisons between the kinetic parameters (Figs. 3, C and D, and 7B) and the effects on complementation (Figs. 2B and 7A) suggest that the effective complementation was achieved with MKH13 cells showing high affinity for betaine or proline but not with cells showing high maximal velocity.

Fig. 5, A and B, shows that the AmT1 and -2 were activated by salts such as NaCl and KCl, which is similar to the results of the bacteria betaine transporters BetP and BetL (26, 28, 29). These transporters contain C- and/or N-terminal extension(s). The importance of these extensions for the osmolality-regulated activity was clearly demonstrated in the case of BetP from *C. glutamicum* (26, 29). However, the topological model of AmT1 showed the absence of an N- or C-terminal extension (Fig. 6A), indicating its non-involvement in the salt-induced activation. Sucrose slightly activated AmT1- and AmT2-mediated betaine transport (Fig. 5C), which was in contrast to a much greater activation by sorbitol observed for the bacterial betaine transporter BetP (26). At equivalent osmolality, sucrose slightly activated AmT1- and AmT2-mediated betaine transport as compared with that by NaCl or KCl. This suggests that both osmolality and ionic effects contribute to the activation of betaine transport in AmT1 and -2. More importantly, we observed that the introduction of positive charges on the periplasmic loop of AmT1 would perturb its interaction with the membrane, thereby making it independent of salt-induced activation. It would be of interest to find out
whether AmT1m could alter interactions between the transporter and the membrane under varying osmolality. This would probably explain how sucrose inactivated AmT1m (Fig. 8C).

Fig. 9 shows that the three genes encoding AmT1, -2, and -3 are all expressed in *A. marina*, different from the genes encoding LeProT2 and -3, which show no detectable transcript even after salt stress (6). The accumulation levels of mRNA for AmT1, -2, and -3 increased with increasing concentration of NaCl (0.4 M), especially in leaves. Since betaine synthesis occurs in chloroplasts upon the salt stress (3), it is reasonable that the synthesis of betaine transporters is also induced upon the salt stress because the synthesized betaine must be transported to the other plant organs where betaine synthesis activity is low (1, 5). Therefore, under high salinity conditions, the betaine/proline transporters (AmT1, -2, and -3) appear to be involved in the accumulation of betaine by increasing the mRNA levels (Fig. 9) as well as post-translational activation (Fig. 8) in betaine-accumulating *A. marina*. In contrast, the mRNA for LeProT1 was significantly accumulated in pollen. It can be envisaged that the physiological function of LeProT1 might be different from the physiological function of AmT1, -2, and -3, i.e. general supply of proline for the former as opposed to the supply of a compatible solute, betaine, for the latter. The data showing different levels of mRNA for AmT1, -2, and -3 under salt stress indicate that AmT1 of mangrove plays a major role for the transport of betaine when mangrove encounters osmotic stress. In addition, since AmT1 appeared to have favorable kinetic properties especially with respect to betaine (Fig. 3, A and C), it is clearly advantageous for mangrove to thrive well in changing osmotic conditions by virtue of its preferential activation of the gene encoding AmT1. Our study here provides strong evidence for the existence of at least three betaine/proline transporter proteins. Both AmT1 and AmT2 do not appear to transport GABA, which is distinct from other proline transporters from plants. The study on this important trans-

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**Fig. 8.** Effects of salts and sucrose on betaine uptake by AmT1m- and AmT1-expressing cells. Experimental conditions were the same as in Fig. 5. Each value shows the average of three independent measurements.

**Fig. 9.** Changes of mRNA levels for AmT1, -2, and -3 upon salt stress by NaCl. The levels of mRNA for AmT1, -2, and -3 in leaf and root of mangrove were measured. The mangrove *A. marina* was grown with 0.4 M NaCl or without NaCl, and the total RNAs were extracted. Quantification of AmT1–3 mRNAs was carried out using a TaqMan fluorescent analysis method as described under "Experimental Procedures." The levels of mRNA for AmT3 in non-stressed leaf is set to have the value of 1.0. Data are shown as mean ± S.E. of three different measurements.
porter remains an active area of research for the understanding of the mechanism for adaptation of plants to osmotic stress.

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