Role of Positively Charged Amino Acids in the M2 D Transmembrane Helix of Ktr/Trk/HKT Type Cation Transporters

Naoki Kato1,†
Masaro Akai1,†
Lalu Zulkifli1
Nobuyuki Matsuda1
Yasuhiro Kato1
Shinobu Goshima1
Akihito Hazama2
Mutsumi Yamagami3
H. Robert Guy4
Nobuyuki Uozumi1,5,*

1Bioscience and Biotechnology Center, Nagoya University, Nagoya, Japan
2Department of Physiology, School of Medicine, Fukushima Medical University, Fukushima, Japan
3Institute for Environmental Sciences, Aomori, Japan
4Laboratory of Cell Biology, Center for Cancer Research, National Cancer Institute; National Institutes of Health, Bethesda, Maryland USA
5Department of Biomolecular Engineering, Graduate School of Engineering, Tohoku University, Sendai, Japan

†Both authors contributed equally to this work.
*Correspondence to: Nobuyuki Uozumi; Department of Biomolecular Engineering, Graduate School of Engineering, Tohoku University, Aobayama 6-6-07, Sendai 980 8579 Japan; Tel.: 81.22.795.7280; Fax: 81.22.795.7293; Email: uozumi@biophy.ch.chem.tohoku.ac.jp

INTRODUCTION

Membrane transport proteins can be divided into three groups; pumps, transporters and channels, based on their structures and functions. Ion channels allow ions to diffuse passively down their electrochemical gradients, but transporters and pumps use energy to transport ions actively against these gradients. The transport rates of ions and water channels are much greater than those of transporters. Typically, a channel molecule can transport more than $10^6$ ions per second, but the rates of ion transporters are usually in the range of $10^{-2} – 10^4$ ions sec$^{-1}$. Crystal structures of ion channels such as bacterial KcsA have been solved, and the structure-function relationship of these proteins has been extensively studied. The K'/Na$^+$ transporter family, encompassing prokaryotic Krs and Trks, fungal TRKs, and plant HKTs, referred to as the Ktr/Trk/HKT-type transporter family, has been suggested to belong to a large superfamily of K$^+$ channels based on structural similarity. K$^+$ channels such as KcsA form an ion-channel pore by the homo-tetramerization, and each subunit is composed of two transmembrane helices (M1 and M2) that flank a reentrant loop called the P segment (MPM motif) (Fig. 1B). K$^+$ selectivity is conferred by GYG motifs within the P segments at the outer pore of the channel. Interestingly, Ktr/Trk/HKT-type transporters have several sequential MPM motifs within a single polypeptide (M1PM2$_A$M1PM2$_B$M1PM2$_C$M1PM2$_D$), and plant HKTs exhibit a K$^+$ channel-like selectivity filter in which the first Gly of the GYG motif determines K$^+$/Na$^+$ selectivity, supporting the idea of an evolutionary relationship between K$^+$ channels and transporters. A structural model of the MPM motifs of Ktr/Trk/HKT-type transporters has been proposed based on the structure of KcsA, and structural differences between the transporters and channels likely determine their different activities. In particular, positively charged amino acids are found in the M2 D

KEY WORDS

positive charge, transporter, Ktr, HKT, salt bridge, channel

ACKNOWLEDGEMENTS

This work was supported by a grants in aid for scientific research (19380058 and 17078005) to Nobuyuki Uozumi from MEXT and JSPS.
helices of Ktr/Trk/HKT-type transporters, unlike the M2 segments of most K+ channel and transporters (Fig. 1C). Among the positive residues in the M2$_D$ segment, an Arg residue, corresponding to R415 in *Synechocystis* KtrB, is highly conserved in the transporters of this family with a few exceptions; e.g., Lys, Gln, and Leu occur at this position in some KtrB sequences and a Lys substitutes for Arg in *Saccharomyces cerevisiae* TRK1.28 We assumed that the local distribution of positively charged amino acids in M2$_D$ helices would be associated with the characteristics of this transporter family because the presence of an unpaired positively charged residue within a cation transporter would be an electrostatic barrier to the passage of cations. Some models of ion transporters indicate that a barrier is required to prevent free diffusion of ions down their electrochemical gradient, and it is possible that the positively charged residues within the transporter pore could help regulate its activity.

In this study, we substituted positively charged residues in M2$_D$ of three different types of Ktr/Trk/HKT-type transporters (Fig. 1): Na$^+$ uniporter AtHKT1, K$^+$ uniporter KtrB, and Na$^+$/K$^+$ symporter TaHKT1. TaHKT1 enables yeast cells lacking K$^+$-uptake systems (*TRK1, TRK2*) to grow at low K$^+$ concentrations,17 and it functions as a K$^+$/Na$^+$ symporter when expressed in *Xenopus* oocytes.29 At low external Na$^+$ concentrations, TaHKT1 mediates K$^+$/Na$^+$ symport, but only Na$^+$ transport when the external concentration of Na$^+$ is high.30 The *Arabidopsis* ortholog, AtHKT1, is highly specific for Na$^+$ transport; inward Na$^+$, but no K$,^+$ current was observed in oocytes expressing this protein.11,18,27 The cyanobacteria *Synechocystis* transporter KtrB exhibits K$^+$ transport activity in a Na$^+$-dependent manner.6 The results reported here support the existence of salt bridge formed by the conserved positive amino acids in the M2$_D$ helix.

**Materials and Methods**

Plasmid construction. Site-directed mutagenesis was conducted on the 565-bp of *BsiW* I-Sph I, and 160-bp of *Xba I-Sph I fragments from *Arabidopsis* HKT1 (AtHKT1), and wheat HKT1 (TaHKT1), respectively, using overlap extension PCR. cDNA encoding TaHKT1 was kindly provided by J. Schroeder (University of California, San Diego). Plasmids carrying the AtHKT1 and TaHKT1 cDNAs on pYES2, which was modified to insert a poly(A) termination sequence downstream of the multiple cloning site for expression in *Xenopus* oocytes,18 were used as templates, and the corresponding regions of the plasmids were replaced by mutated fragments. Construction of the mutated ktrB genes was also carried out with overlap PCR. The amplified fragments, corresponding to 471-bp Spe I-Pst I fragment, were cloned back to KtrB on pPA404.6 All PCR-derived DNA fragments were verified by sequencing.

Bacterial and yeast strains and growth conditions. *E. coli* strain LB2003 (F', thi, lacZ, gal, rha, ΔkdpFABC5, trkD1, ΔtrkA), lacking K$^+$-uptake systems, was used as a host strain for experiments to evaluate KtrB-mediated K$^+$ transport.31 *E. coli* transformants carrying ktrABE were grown at 30˚C in the minimum medium containing appropriate antibiotics, and KCl was added to give the final concentrations indicated in the text. *S. cerevisiae* strain CY162 (MATa, Δatrk1.2::pCK64, his3, leu2, ura3, trp1, ade2), lacking a K$^+$-uptake system, was used as a host strain for complementation assays to evaluate K$^+$ uptake activities of plant HKT variants.17,18,32 Yeast transformation was carried out as described previously.33 The transformants carrying the plant HKT constructs on pYES2 were selected on standard SD medium lacking uracil and supplemented with 100 mM KCl. *URA* transformants obtained were precultured in SD medium containing 100 mM KCl at 30˚C for 24 h. The cells were collected by centrifugation, resuspended with arginine-based medium34 without KCl, and a dilution series (OD$_{600}$ 1 to 10$^{-6}$) of the cell suspension was prepared. Five µl of each dilution was inoculated onto the arginine-based medium supplemented with 1 mM KCl, and incubated at 30˚C for three days.

**Measurement of ion uptake in E. coli.** K$^+$ uptake by *Synechocystis* KtrABE in *E. coli* was measured as described previously.11,31,35 Briefly, plasmid-containing *E. coli* LB2003 cells were cultured at 30˚C for 16 h in the minimum medium supplemented with 30 mM KCl, IPTG, thiamine, and appropriate antibiotics. The cells were collected by centrifugation, resuspended in 120 mM Tris-HCl (pH 8.0) and 1 mM EDTA, and incubated at room temperature for
A positively charged residue in the M2_d helix is essential for K^+ transport activity of cyanobacterial KtrB. Unlike the other transporters studied here, Synechocystis KtrB has only one positively charged residue, R415, in its M2_d segment (Fig. 1C). We introduced point mutations to replace R415 with Ala, Asn, Asp, Gln, Glu, Gly, Met, Leu and Lys. The transport activities of the mutated KtrBs were examined using a heterologous expression system in E. coli.38,61,63 KtrB can complement the K^+ uptake deficiency of E. coli strain LB2003 (ΔkdpFABC5, trkD1, ΔtrkA) with two additional subunits, KtrA and KtrE(13,31,39 (Fig. 1A). The mutated ktrB on pPAB404, also carrying ktrE, was introduced into bacteria with ktrA on pSTV28. Transformants expressing the wild-type KtrB with KtrA and KtrE grew well on the medium containing 7.5 mM KCl, and the control cells transformed with vector alone were not able to grow at K^+ concentrations below 7.5 mM (Fig. 2A). Interestingly, KtrB_R415K allowed for marked growth in the presence of 7.5 mM K^+, conditions incompatible with growth for all other KtrB variants. We also examined the effects of substitution for R415 on overall K^+ transport in E. coli LB2003 by measuring the net uptake of K^+ using flame photometry (Fig. 2B). Rapid K^+ uptake was observed when wild-type KtrB was expressed in cells, and KtrB_R415K exhibited a comparable activity to the wild-type transporter. The other variants had no or very small K^+ uptake activity (data not shown), confirming the results of the complementation assay (Fig. 2A). These results suggest that a positively charged residue at position 415 in the M2_d segment is required for the K^+ uptake activity of Synechocystis KtrB, and could not be replaced by negatively charged, uncharged polar or hydrophobic amino acids.

The conserved Arg residues in the M2_d helix of plant HKTs are not replaceable with Ala or Glu. The positive residues corresponding to R415 in Synechocystis KtrB are present in TaHKT1 and AtHKT1 (Fig. 1C). To demonstrate that plant HKTs also require the conserved Arg for their activities, we generated HKT variants in which R519 of TaHKT1 and R487 of AtHKT1 were substituted with Ala (A), Gln (Q), Glu (E), and Lys (K). The transport activities of these variants were then examined using S. cerevisiae and Xenopus oocytes expression systems.

Mutated TaHKT1 constructs were introduced into the yeast K^+-uptake deficient strain CY162,32 and the ability of transformants to grow on synthetic medium supplemented with 1 mM KCl were examined. Yeast transformants containing wild-type TaHKT1 and all the mutated TaHKT1s were able to grow on medium containing 1 mM K^+, whereas no growth was observed for cells transformed with the empty vector (Fig. 3A). Among the four TaHKT1 variants, only cells expressing TaHKT1_R415K showed a normal growth like the wild type. Yeast expressing the other variants grew slower than wild type, though growth of cells carrying TaHKT1_R519Q was rather better than those of TaHKT1_R519A and TaHKT1_R519E.
Positively charged residues in M2D helix of K⁺/Na⁺ transporters

Ion uptake currents of the HKT-variants were measured using the two-electrode voltage clamp technique in Xenopus oocytes (Fig. 3B). Na⁺ and K⁺ current amplitudes mediated by TaHKT1R519A were comparable to those of wild type. The current amplitudes associated with TaHKT1R519Q were smaller than those seen in wild type and TaHKT1R519K expressing cells. In contrast, oocytes expressing TaHKT1R519A and TaHKT1R519E gave only minimal currents.

Overall, the abilities of mutant TaHKT1 constructs to produce currents in the presence of high concentrations of Na⁺ or K⁺ correlated with their abilities to support yeast growth under the K⁺-limited conditions.

Similar experiments were performed with AtHKT1-variants. Since AtHKT1 is a Na⁺-selective transporter unlike TaHKT1, a Na⁺ current only was observed in oocytes expressing wild-type AtHKT1 (Fig. 4). AtHKT1R487Q exhibited a smaller activity than that of the wild type. Currents associated with AtHKT1R487K were observed in response to increases in Na⁺ concentration, while little or no current was detected in oocytes expressing AtHKT1R487A and AtHKT1R487E. Consequently, replacement of the conserved Arg with Ala or Glu, but not Lys, severely impaired the ion transport activities of TaHKT1 and AtHKT1 as well as Synechocystis KtrB (Fig. 2), indicating that positively charged residues at the middle of M2D helices play an important role in the ion transport.

Positively charged residues in M2D helices are involved in ion transport of plant HKTs. Replacement of conserved Arg residues with Glu had a smaller effect on Na⁺ and K⁺ transport activities of plant HKTs, whereas the same mutation in Synechocystis KtrB abolished its K⁺ transport activity. Structural differences between Synechocystis KtrB and plant HKTs (TaHKT1 and AtHKT1) may explain this difference. In addition to the conserved Arg (R519 in TaHKT1 and R487 in AtHKT1), several positively charged residues are located in the M2D helices of plant HKTs (Fig. 1C); three...
residues (K508, K521, and K529) in TaHKT1 and four residues (K476, K489, K494, and R497) in AtHKT1 could also participate in the cation transport of plant HKTs. To investigate this possibility, we replaced each of these residues in the M2D helix with Gln and examined their ion uptake activities using Xenopus oocytes and/or yeast expression system(s).

Cells expressing TaHKT1K508Q, TaHKT1R519Q, or TaHKT1K521Q exhibited a slightly lower growth compared to cells containing wild type or TaHKT1K529Q (Fig. 5A). Differences in ion transport activities for these variants were clearer when their electrophysiological characteristics were tested using Xenopus oocytes; ion transport activity of TaHKT1K508Q and TaHKT1R519Q were reduced compared to that for the other proteins (Fig. 5B).

**Figure 5.** Contribution of positive residues surrounding the conserved Arg in M2D of TaHKT1. (A) Complementation of the K+ uptake-deficient yeast mutant strain CY162 with wild-type TaHKT1 (WT) and Gln-substituted variants at amino acid position K508, R519, K521 and K529. The cells were precultured in SD medium containing 0.1 M KCl at 30°C for 24 h. Cells were collected and 10-fold serial dilutions were prepared (OD600 1 to 10⁻⁴). Cells suspensions were incubated at 30°C for three days on arginine-based medium containing 1 mM KCl. TaHKT1R519Q shown in Figure 3A was used in this experiment. (B) Representative Na+ current traces elicited by TaHKT1 variants in Xenopus oocytes (upper) and the current-voltage curves for Na+ K+ and Tris at the end of the pulses (lower). Xenopus oocytes were injected with cRNAs of wild-type TaHKT1 and TaHKT1 variants, and ion uptake currents were measured with the two electrode voltage clamp method one or two days after injection. Currents at 100 mM Na+ (open circles), 100 mM K+ and 1 mM Na+ (closed circles), and 100 mM Tris (squares) were recorded. TaHKT1R519Q shown in Figure 3B was used in this experiment.

**Figure 6.** Effects of combined substitution of positive residues in M2D of TaHKT1 on K+ uptake activity. (A) Yeast strains CY162 expressing TaHKT1 variants containing two or more positively charged residues replaced with Gln in the M2D helix. Cells were precultured in SD medium containing 0.1 M KCl at 30°C for 24 h. Cells were collected and 10-fold serial dilutions were prepared (OD600 1 to 10⁻⁴). Cells suspensions were incubated at 30°C for three days on the arginine-based medium containing 1 mM KCl. The loss of the function of TaHKT1K508Q/R519Q was confirmed by the electrophysiological measurement. (B) Multiple substitutions in the M2D helix of TaHKT1. TaHKT1 variants containing multiple substitutions exhibited decreased cation transport; K508Q/K521Q, R519Q/K521Q, and K508Q/R519Q double mutants, triple mutant, and quadruple mutant, in order. Opened circles indicate positively charged residues in the M2D helices, and closed circles indicate the substituted residues.

**Figure 6A**

**Figure 6B**

**Figure 6 Effects of combined substitution of positive residues in M2D of TaHKT1 on K+ uptake activity.** (A) Yeast strains CY162 expressing TaHKT1 variants containing two or more positively charged residues replaced with Gln in the M2D helix. Cells were precultured in SD medium containing 0.1 M KCl at 30°C for 24 h. Cells were collected and 10-fold serial dilutions were prepared (OD600 1 to 10⁻⁴). Cells suspensions were incubated at 30°C for three days on the arginine-based medium containing 1 mM KCl. The loss of the function of TaHKT1K508Q/R519Q was confirmed by the electrophysiological measurement. (B) Multiple substitutions in the M2D helix of TaHKT1. TaHKT1 variants containing multiple substitutions exhibited decreased cation transport; K508Q/K521Q, R519Q/K521Q, and K508Q/R519Q double mutants, triple mutant, and quadruple mutant, in order. Opened circles indicate positively charged residues in the M2D helices, and closed circles indicate the substituted residues.
TaHKT1_{K508Q/R519Q/K521Q} and quadruple, TaHKT1_{K508Q/R519Q/K521Q/K529Q} variants were not able to complement the yeast K\(^+\)-transport deficiency. These data indicate that at least residues K508, R519 and K521 are necessary for the formation of the K\(^+\) conduction pore in TaHKT1. To further define the residues needed for K\(^+\) transport activities, double mutants were tested (Fig. 6A). The double mutants had decreased growth compared to the single mutant TaHKT1_{R519Q}. In particular, TaHKT1_{K508Q/R519Q} exhibited severely impaired K\(^+\) transport activity. This is consistent with the inactivity of KtrB_{R415Q} as KtrB does not have positively charged residues in positions analogous to 508, 521 and 529 of TaHKT1. Replacing two or more positively charged residues with Gln caused a considerable loss of activity in TaHKT1 (Fig. 6B). Conversely, yeast K\(^+\) uptake efficiency was partially rescued by TaHKT1_{K508Q/K521Q} and TaHKT1_{K508Q/R519Q/K521Q}. These results indicate that the contributions of K508 and R519 for K\(^+\) transport are higher than that of K521. The loss of the function of TaHKT1_{K508Q/R519Q} was confirmed by electrophysiological measurements (data not shown and Table 1).

### DISCUSSION

In K\(^+\) channels, electrostatic interactions occur primarily between K\(^+\) ions and the backbone oxygen atoms of the polypeptide selectivity filter. On the other hand, few, if any, charged residues are present in the putative selectivity filter regions of the K\(^+\) transporters studied here. Positively charged residues do exist, however, within the M2\(_D\) segment of Ktr/Trk/HKT-type transporters. In this study, we have evaluated the contribution of these positively charged residues for three different Ktr/Trk/HKT-type transporters; AtHKT1-mediated Na\(^+\) uniport, *Synechocystis* KtrB-mediated K\(^+\) uniport, and TaHKT1-mediated K\(^+\) and Na\(^+\) symport.

The atomic-scale model developed by Durell and Guy\(^{24}\) indicates that a conserved Arg within the M2\(_D\) segment, corresponding to R415 in *Synechocystis* KtrB, is placed nearby the ion conduction pore. It has been predicted that this best-conserved positive residue in the middle of M2\(_D\), which is absent in K\(^+\) channels, may be one of the determinants for the nature of the transporters. However, there was a lack of information on the necessity of the positive charge at this site. As shown in Figure 2 and Table 1, removal of the positive charge within the M2\(_D\) segment of *Synechocystis* KtrB severely disrupted its K\(^+\) transport activity. For TaHKT1 and AtHKT1, replacement of the corresponding residue with Ala or Glu impaired cation transport, whereas replacement with Gln did not severely affect cation transport activity. This difference may be due to the presence of four or five other positive residues in the M2\(_D\) segment of the plant HKTs. Replacement of the TaHKT1 M2\(_D\) positive residues both individually and in combination indicated that K508 and R519 have a greater contribution towards K\(^+\) and Na\(^+\) transport than K521 and K529 (Fig. 6). Interestingly, K508 and R519, but not K521 and K529, lie on the same face of the M2\(_D\) helix (Fig. 7A and B). To deduce the spatial disposition of these residues in TaHKT1, we used the atomic-scale structural model that was developed based on crystal structure data for KcsA channels.\(^{24,25}\) This model suggests that the spatial region mostly highly conserved among prokaryote Ktr, fungal TRK, and plant HKT transporters involves the P\(_3\) and P\(_4\) segments, and residues in M2\(_D\), with which these segments interact (Fig. 7). Positively charged side chains near the beginning of M2\(_D\) (K508 in

### Table 1

**Summary of ion uptake activities of KtrB and HKT variants**

|            | KtrB   | TaHKT1 | AtHKT1 |
|------------|--------|--------|--------|
|            | **K\(^+\)** (E. coli) | **Na\(^+\)/K\(^+\)** (oocytes) | **K\(^+\)** (yeast) | **Na\(^+\)** (oocytes) |
| Single mutants |       |        |        |        |
| R415 A      |      |        |        |        |
| E           |      |        |        |        |
| Q           |      |        |        |        |
| K           | ++    |        |        | ++    |
| G, D, N, M, L | ++ | ++     | ++     | ++    |
| Multiple mutants |       |        |        |        |
| (double)    | K508Q/R519Q |        |        |        |
| (triple)    | K508Q/R519Q/K521Q |        |        |        |

**KtrB** and **TaHKT1** variants were not able to complement the yeast K\(^+\)-uptake deficiency. These data indicate that at least residues K508, R519 and K521 are necessary for the formation of the K\(^+\) conduction pore in TaHKT1. To further define the residues needed for K\(^+\) transport activities, double mutants were tested (Fig. 6A). The double mutants had decreased growth compared to the single mutant TaHKT1_{R519Q}. In particular, TaHKT1_{K508Q/R519Q} exhibited severely impaired K\(^+\) transport activity. This is consistent with the inactivity of KtrB_{R415Q} as KtrB does not have positively charged residues in positions analogous to 508, 521 and 529 of TaHKT1. Replacing two or more positively charged residues with Gln caused a considerable loss of activity in TaHKT1 (Fig. 6B). Conversely, yeast K\(^+\) uptake efficiency was partially rescued by TaHKT1_{K508Q/K521Q} and TaHKT1_{K508Q/R519Q/K521Q}. These results indicate that the contributions of K508 and R519 for K\(^+\) transport are higher than that of K521. The loss of the function of TaHKT1_{K508Q/R519Q} was confirmed by electrophysiological measurements (data not shown and Table 1).
TaHKT1 and K476 in AtHKT1 bind to an Asp (D78 and D55, respectively) near the beginning of the P$_A$ helix, and other positively charged side chains 11 positions further downstream (R415 in KtrB, R519 in TaHKT1, and R487 in AtHKT1) form salt bridges with a Glu (E381 in KtrB, E464 in TaHKT1, and E433 in AtHKT1) in the P$_A$ helix (Figs. 7 and 8A). The membrane topology and the residues responsible for cation selectivity in Ktr/Trk/HKT-type transporters have been experimentally evaluated, and the results showed good agreement with the atomic-scale structural model.

Amino acid sequence alignment of Ktr/Trk/HKT-type transporters whose activities have been demonstrated so far shows that charged residues corresponding to D78, K508 and R519 in TaHKT1 are highly conserved in this protein family (Fig. 8A), whereas the negative charged residue corresponding to E464 is well-conserved among prokaryote Ktrs and eukaryote Trks/HKTs, but not in prokaryote Trks. Table 2 shows the percentage of conservation of the charged residues in M2$_A$, M2$_D$, P$_A$, and P$_D$ among 307 Ktr/Trk/HKT-type transporters which have been registered in the public databases. The positive residue corresponding to R519 of TaHKT1 is significantly conserved with score of 96% in Ktr/Trk/HKT-type transporters (93% in eukaryote Trks/HKTs and prokaryote Ktrs, and 100% in prokaryote Ktrs). The negative charged residue corresponding to E464 of TaHKT1 is also conserved with score of 96%. Accordingly, the pair of positive and negative residues corresponding to R519-E464 in TaHKT1 is found in 90% of the proteins belonging to eukaryote Trks/HKTs and prokaryote Ktr groups. Likewise, the other pair corresponding to K508-D78 in TaHKT1 is moderately conserved (64%). Taking into consideration the experimental results on positive residues in M2$_D$ in this study, high degree of the conservation in the family, and the possible assignment of the residues in structural model of Ktr/Trk/HKT-type transporters, we propose that an electrostatic interaction exists between K508-D78 and R519-E464 in TaHKT1 (Fig. 7B–D).

Prokaryote Trks have other highly conserved charged residues in P$_A$, M2$_A$, and M2$_D$ (E104, R131 and E468 in E. coli TrkH) (Fig. 8A and Table 2). The tertiary structural model shows that the Glu in M2$_D$ (99%) is located nearby the conserved Arg in M2$_D$ (100%). Moreover, the Arg in M2$_A$ (100%) and the Glu in P$_A$ (98%) are also close to each other. Most (98%) of prokaryote Trks have both pairs of R-E in M2$_D$ and R-M2$_A$-E in P$_A$. These pairs may be stabilized by...
electrostatic interaction (Fig. 8A). Phylogenetic analysis of Ktr/Trk/ HKT-type transporters revealed that the cluster of prokaryote Trk proteins was rather distant from those of eukaryote Trks/HKTs and prokaryote Ktrs (Fig. 8B). The presence and absence of conserved charged residues involving electrostatic interaction may be closely related to evolutionary classification proposed by Durell et al. 25 Interestingly, many K+ channels have Lys or Arg near the beginning of M2 or S6 helices, corresponding to position 508 in the M2D helix of TaHKT1 (e.g., R89 in the M2 in KcsA shown in Fig. 7A), but few have the positively charged residue at the position corresponding to R519 of TaHKT1. This supports the idea that the pair of positive and negative residues (R519 and E464 in TaHKT1) would play an important role in the Ktr/Trk/HKT-type transporter function of this family.

The electrostatic balances described above may explain why the presence of positively charged residues near the putative transport pathway does not prevent passage of ions. Moreover, the additional salt bridge of K508-D78 in TaHKT1 and K476-D55 in AtHKT1, which is absent in KtrB (Q404 and V38), may stabilize HKT transporters (Fig. 7). The Lys in M2D and Asp in P_A are

| Charged residues | M2_D-P_D (R519) | M2_D-P_A (K508) | M2_A-M2_D (R466) | M2_A-P_A (R131) | M2_A-P_A (K455) |
|------------------|----------------|----------------|------------------|----------------|----------------|
| K                | 12 [7]         | 113 [66]       | 135 [99]         | 133 [98]       | 118 [87]       |
| E                | 163 [95]       | 7 [4]          | 12 [7]           | 2 [1]          | 4 [3]          |
| D                | 2 [1]          | 121 [71]       | 2 [1]            | 43 [68]        |                |
| Q                | 9 [5]          | 28 [16]        | 8 [5]            | 1 [1]          | 4 [3]          |
| N                | 2 [1]          |                | 1 [1]            | 22 [16]        |                |
| L                | 3 [2]          | 2 [1]          | 9 [5]            | 8 [6]          |                |
| T                | 4 [2]          | 1 [1]          | 10 [6]           | 5 [4]          | 1 [1]          |
| V                | 2 [1]          | 8 [5]          | 2 [1]            | 4 [3]          | 2 [1]          |
| G                |                |                | 1 [1]            | 64 [17]        |                |
| H                | 1 [1]          |                | 2 [1]            | 1 [1]          |                |
| A                |                |                | 1 [1]            | 1 [1]          |                |
| P                | 2 [1]          |                | 4 [3]            |                |                |
| F                | 1 [1]          |                | 1 [1]            |                |                |
| Y                | 1 [1]          |                | 1 [1]            |                |                |

Amino acid combination

| (+)/[‑] | R/E | 141 [82] | 135 [99] | 133 [98] |
|         | R/D | 2 [1]    | 6 [4]    | 2 [1]    |
|         | K/E | 12 [7]   |          |          |
|         | K/D | 103 [60] | 87 [64]  |          |
| Subtotal|     | 155 [90] | 109 [64] | 135 [99] |

Others

| K/N | 1 [1]    | 1 [1] |
| Q/E | 7 [4]    | 11 [6] |
| R/T | 4 [2]    | 3 [2] |
| T/D | 1 [1]    | 5 [4] |
| L/D | 4 [2]    | 1 [1] |
| K/V | 4 [2]    | 1 [1] |
| K/S |          | 4 [3] |
| L/E | 3 [2]    | 1 [1] |
| Q/Q | 4 [2]    | 4 [2] |
| Q/V |          | 4 [2] |
| others | 2 [1] | 29 [17] | 1 [1] | 1 [1] | 15 [11] |

1Residues of TaHKT1 represents in parenthesis; 2Residues of EcTrkH represents in parenthesis; 3No corresponding residue could be identified to D78 in TaHKT1 from the amino acid alignment

Table 2  Conserved charged residues positioned in M2A, M2D, PA and PD, and possible combinations forming salt bridges

| Charged residues | M2_D-P_D (R519) | M2_D-P_A (K508) | M2_A-M2_D (R466) | M2_A-P_A (R131) | M2_A-P_A (K455) |
|------------------|----------------|----------------|------------------|----------------|----------------|
| K                | 12 [7]         | 113 [66]       | 135 [99]         | 133 [98]       | 118 [87]       |
| E                | 163 [95]       | 7 [4]          | 12 [7]           | 2 [1]          | 4 [3]          |
| D                | 2 [1]          | 121 [71]       | 2 [1]            | 43 [68]        |                |
| Q                | 9 [5]          | 28 [16]        | 8 [5]            | 1 [1]          | 4 [3]          |
| N                | 2 [1]          |                | 1 [1]            | 22 [16]        |                |
| L                | 3 [2]          | 2 [1]          | 9 [5]            | 8 [6]          |                |
| T                | 4 [2]          | 1 [1]          | 10 [6]           | 5 [4]          | 1 [1]          |
| V                | 2 [1]          | 8 [5]          | 2 [1]            | 4 [3]          | 2 [1]          |
| G                |                |                | 1 [1]            | 64 [17]        |                |
| H                | 1 [1]          |                | 2 [1]            | 1 [1]          |                |
| A                |                |                | 1 [1]            | 1 [1]          |                |
| P                | 2 [1]          |                | 4 [3]            |                |                |
| F                | 1 [1]          |                | 1 [1]            |                |                |
| Y                | 1 [1]          |                | 1 [1]            |                |                |

Amino acid combination

| (+)/[‑] | R/E | 141 [82] | 135 [99] | 133 [98] |
|         | R/D | 2 [1]    | 6 [4]    | 2 [1]    |
|         | K/E | 12 [7]   |          |          |
|         | K/D | 103 [60] | 87 [64]  |          |
| Subtotal|     | 155 [90] | 109 [64] | 135 [99] |

Others

| K/N | 1 [1]    | 1 [1] |
| Q/E | 7 [4]    | 11 [6] |
| R/T | 4 [2]    | 3 [2] |
| T/D | 1 [1]    | 5 [4] |
| L/D | 4 [2]    | 1 [1] |
| K/V | 4 [2]    | 1 [1] |
| K/S |          | 4 [3] |
| L/E | 3 [2]    | 1 [1] |
| Q/Q | 4 [2]    | 4 [2] |
| Q/V |          | 4 [2] |
| others | 2 [1] | 29 [17] | 1 [1] | 1 [1] | 15 [11] |

1Residues of TaHKT1 represents in parenthesis; 2Residues of EcTrkH represents in parenthesis; 3No corresponding residue could be identified to D78 in TaHKT1 from the amino acid alignment
Positively Charged Residues in M2\textsubscript{D} Helix of K\textsuperscript{+}/Na\textsuperscript{+} Transporters

Figure 8. Possible electrostatic interactions between conserved charged residues in Ktr/Trk/HKT transporters. (A) Multiple alignment of the amino acid sequences of P\textsubscript{A}, P\textsubscript{D} and M2\textsubscript{D} segments of Ktr/Trk/HKT transporters. Sequences used here are the ones of transporters so far reported their cation transport activities: Bacillus subtilis KtrB (BsKtrB, NP_390988), Vibrio alginolyticus KtrB (VaKtrB, BAA32063), Enterococcus hirae NtpJ (EhNtpJ, P43440), Synechocystis KtrB (SyKtrB), B. subtilis KtrD (BsKtrD, NP_389233), S. cerevisiae TRK1 and TRK2 (ScTrk1, ScTrk2, NP_012406, NP_012976), Debaryomyces occidentalis TRK1 (SoTRK1, CAB91046), Neurospora crassa TRK1 (NcTRK1, CAA08813), Schizosaccharomyces pombe TRK1 (SpTRK1, CAA93300), Eucalyptus camaldulensis HKT1 and HKT2 (EcHKT1, EcHKT2, AAF97728, AAD53890), AtHKT1, Mesembryanthemum crystallinum HKT1 (MyHKT1, AAK52962), Oryza sativa HKT1 and HKT2 (OsHKT1, OsHKT2, AAG37278, BAB61791), TaHKT1, Hordeum vulgare subsp. vulgare HKT1 (HvHKT1, CAJ01327), E. coli TrkH (EcTrkH, NP_756655), Halomonas elongate TrkH (HeTrkH, AAR191792), V. alginolyticus TrkH (VaTrkH, BAA31229), H. elongate Trk1 (HeTrk1, AAR91793), E. coli TrkG (EcTrkG, CAA40103). Positively charged amino acids and negatively charged residues are shaded by blue and red, respectively. The well-conserved Gly residue in M2\textsubscript{D} segment is indicated by yellow. Possible electrostatic interactions are indicated by dot lines. (B) Phylogenetic tree of Ktr/Trk/HKT-type transporters. The three major branches are prokaryote Trk, prokaryote Ktr and eukaryote Trk/HKT, as depicted by the sketches on their membrane topology. The scale bar corresponds to a genetic distance of 0.1 substitutions per position.
found in all reported transporters, except for Synechocystis KtrB (Gln-Val) and HeTrkI (Lys-Asn) (Fig. 8A). Transitions between open and closed states involve a large conformational change in a mecanosensitive channel, 40 K+ channels, 41 acethylcholine receptor, 42 and Ca2+ pump. 43-45 The region involving the salt bridge in Ktr/Trk/HKT-type transporters may undergo conformational changes during the transport cycle. Among Ktr/Trk/HKT family members, 46 a Gly residue (G414 in KtrB, G518 in TaHKT1, G486 in AtHKT1) immediately preceding the Arg in M2D is also completely conserved (Fig. 8A). The analogous residue in K+ channels like KcsA is also usually a Gly (Fig. 7A), which serves as a hinge region where M2 (or S6 in 6TM K+ channels) bends when the channel opens. 2 If the C-terminal region of M2D moved away from the central axis of the ion conducting pore during the open state, the salt bridge of R519 with E464 in TaHKT1 may constrain the transporters despite similar membrane topology.

19. Fairbairn DJ, Liu W, Schachteman DP, Gomez-Gallego S, Day SR, Teasdale RD. Characterisation of two distinct HKT1-like potassium transporters from Eucalyptus camaldulensis. Plant Mol Biol 2000; 43:515-25.
20. Horie T, Yoshioka K, Nakayama H, Yamada K, Oki S, Shinmyo A. Two types of HKT transporters with different properties of Na+ and K+ transport in Oryza sativa. Plant J 2001; 27:129-38.
21. Su H, Balderas E, Vera-Estrella R, Goldack D, Quigley F, Zhao C, Pantotto O, Bohmer HJ. Expression of the cation transporter McHKT1 in a halophyte. Plant Mol Biol 2003; 52:967-80.
22. Gasciodelias B, Senn ME, Banuelos MA, Rodriguez-Navaro A. Sodium transport and HKT transporters: The rice model. Plant J 2003; 34:788-801.
23. Haro R, Banuelos MA, Senn ME, Barretto-Gil J, Rodriguez-Navaro A. HKT1 mediates sodium uptake in roots: Pitfalls in the expression of HKT1 in yeast. Plant Physiol 2005; 139:1495-506.
24. Durell SR, Guy HR. Structural models of the KtrB, TrkH, and TrkL2 symporters based on the structure of the KcsA K+ channel. Biophys J 1999; 77:889-907.
25. Durell SR, Hao Y, Nakamura T, Bakker EP, Guy HR. Evolutionary relationship between K+ channels and symporters. Biophys J 1999; 77:775-88.
26. Kato Y, Sakaguchi M, Mori Y, Sato K, Nakano T, Bakker EP, Sato Y, Goshima S, Uozumi N. Evidence in support of a four transmembrane-pore-transporter topology model for the Arabidopsis thaliana Na+/K+ co-transporting ATPase, a member of the superfamily of K+ transporters. Proc Natl Acad Sci USA 2001; 98:6488-93.
27. Maerz P, Hosoo Y, Goshima S, Horie T, Eckelman B, Yamada K, Yoshioka K, Bakker EP, Shinmyo A, Oki S, Schroeder JL, Uozumi N. Glycine residues in potassium channel-like selectivity filters determine potassium selectivity in four-loop-per-subunit HKT transporters from plants. Proc Natl Acad Sci USA 2002a; 99:6628-33.
28. Haro R, Rodriguez-Navaro A. Functional analysis of the M2D helix of the TRK1 potassium transporter of Saccharomyces cerevisiae. Biochim Biophys Acta 2003; 1613:1-6.
29. Rubio E, Gassmann W, Schroeder JL. Sodium-driven potassium uptake by the plant potassium transporter HKT1 and mutations conferring salt tolerance. Science 1995; 270:1660-3.
30. Gassmann W, Rubio E, Schroeder JL. Alkali cation selectivity of the wheat root high-affinity potassium transporter HKT1. Plant J 1996; 10:869-52.
31. Stumpe S, Schlösser A, Schleyer M, Bakker EP, K+ circulation across the prokaryotic cell membrane: K+ uptake systems. In: Koning WN, Kaback HR, Lolkema JS, eds. Handbook of Biological Physics. Amsterdam: Elsevier Science B V, 1996:2:473-99.
32. Anderson JA, Huprikar SS, Kochian LV, Lucas WJ, Gaber RF. Functional expression of a probable Arabidopsis thaliana sodium-channel potassium transporter in Saccharomyces cerevisiae. Proc Natl Acad Sci USA 1992; 89:3736-40.
33. Dohmen RJ, Strasser AW, Honer CB, Hollenberg CP. An efficient transformation procedure enabling long-term storage of competent cells of various yeast genera. Yeast 1991; 7:691-2.
34. Rodriguez-Navaro A, Ramos J. Dual system for potassium transport in Saccharomyces cerevisiae. J Bacteriol 1984; 159:945-50.
35. Tholema N, Bakker EP, Suzuki A, Nakamura T. Change to alanine of one out of four selectivity filter glycines in Krb causes a two orders of magnitude decrease in the affinities for both K+ and Na+ of the Na+ dependent K+ uptake system KtrAb from Vibrio alginolyticus. FEBS Lett 1999; 450:217-20.
36. Bakker EP, Mangerich WE. Interconversion of components of the bacterial proton motive force by electrogenic potassium transport. J Bacteriol 1981; 147:820-6.
37. Thompson JD, Higgins DG, Gibson TJ. CLUSTAL W: Improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res 1994; 22:4673-80.
38. Saitou N, Nei M. The neighbor-joining method: A new method for constructing phylogenetic trees. Mol Biol Evol 1987; 4:406-25.
39. Uozumi N. Escherichia coli as an expression system for K+ transporters from plants. Am J Physiol 2001; 281:C733-C9.
Positively Charged Residues in M2 Helix of K+/Na+ Transporters

40. Sukharev S, Betanzos M, Chiang CS, Guy HR. The gating mechanism of the large mechanosensitive channel MscL. Nature 2001; 409:720-4.

41. Jiang Y, Lee A, Chen J, Ruta V, Cadene M, Chait BT, MacKinnon R. X-ray structure of a voltage-dependent K+ channel. Nature 2003; 423:33-41.

42. Miyazawa A, Fujiyoshi Y, Unwin N. Structure and gating mechanism of the acetylcholine receptor pore. Nature 2003; 423:949-55.

43. Toyoshima C, Nakasako M, Nomura H, Ogawa H. Crystal structure of the calcium pump of sarcoplasmic reticulum at 2.6 A resolution. Nature 2000; 405:647-55.

44. Toyoshima C, Mizutani T. Crystal structure of the calcium pump with a bound ATP analogue. Nature 2004; 430:529-35.

45. Toyoshima C, Nomura H, Tsuda T. Lumenal gating mechanism revealed in calcium pump crystal structures with phosphate analogues. Nature 2004; 432:361-8.

46. Gambale F, Uozumi N. Properties of Shaker-type potassium channels in higher plants. J Membrane Biol 2006; 210:1-19.