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Characterization of an *AtCCX5* gene from *Arabidopsis thaliana* that involves in high-affinity K^+ uptake and Na^+ transport in yeast

Xinxin Zhang [a], Min Zhang [a], Tetsuo Takano [b], Shenkui Liu [a,*]

[a] Key Laboratory of Saline-alkali Vegetation Ecology Restoration in Oil Field (SAVER), Ministry of Education, Alkali Soil Natural Environmental Science Center (ASNESC), Northeast Forestry University, Harbin 150040, China
[b] Asian Natural Environment Science Center (ANESC), The University of Tokyo, Midori Cho 1-1-1, Nishitokyo City, Tokyo 188-0002, Japan

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ABSTRACT

The gene for a putative cation calcium exchanger (CCX) from *Arabidopsis thaliana*, *AtCCX5*, was cloned and its function was analyzed in yeast. Green fluorescent protein-tagged AtCCX5 expressed in yeast was localized in the plasma membrane and nuclear periphery. The yeast transformants expressing *AtCCX5* were created and their growth in the presence of various cations (K^+, Na^+, Ca^{2+}, Mg^{2+}, Fe^{2+}, Cu^{2+}, Co^{2+}, Cd^{2+}, Mn^{2+}, Ba^{2+}, Ni^{2+}, Zn^{2+}, and Li^+) were analyzed. *AtCCX5* expression was found to affect the response to K^+ and Na^+ in yeast. The AtCCX5 transformant also showed a little better growth to Zn^{2+}. The yeast mutant 9.3 expressing *AtCCX5* restored growth of the mutant on medium with low K^+ (0.5 mM), and also suppressed its Na^+ sensitivity. Ion uptake experiments showed that AtCCX5 mediated relatively high-affinity K^+ uptake and was also involved in Na^+ transport in yeast. Taken together, these findings suggest that the AtCCX5 is a novel transport protein involves in mediating high-affinity K^+ uptake and Na^+ transport in yeast.

1. Introduction

Ca^{2+}/cation antiporter (CaCA) superfamily proteins are integral membrane proteins that transport cytosolic Ca^{2+} or other cations across membranes against their electrochemical gradient by utilizing the downhill gradients of H^+, Na^+ or K^+ generated by primary transporters [1]. The CaCA superfamily is composed of five families: K^+-independent Na^+/Ca^{2+} exchangers (NCXs), K^+-dependent Na^+/Ca^{2+} exchangers (NCKXs), cation/Ca^{2+} exchangers (CCXs), YBRG transporters and cation exchangers (CAXs). Members of this big superfamily have been identified in animals, plants, protozoa, fungi, archaea and bacteria [2]. Phylogenetic and alignment studies indicate that one of the mammalian NCKXs, NCKX6 [3], and its related proteins form a unique group, designated cation/Ca^{2+} exchangers (CCXs) [2].

The *Arabidopsis thaliana* genome has five putative exchangers in CCXs group, originally named CAX7 to CAX11 [4]. Each has an amino acid sequence similar to that of NCKX6 [3]. In addition, like NCKX6 and other Na^+/Ca^{2+} exchangers, they have two internally homologous domains known as the α-1 and α-2 domains, with consensus sequences GNG (A/S) PD in α-1 and (G/S) (N/D) SxGD in α-2 [2,3,5]. Therefore, based on the sequence homology and characteristic α-repeats, the genes *CAX7* to *CAX11* were renamed as *CCX1* to *CCX5* [5]. Their functions are also different from those of the other six CAX genes in *Arabidopsis* [5]. Three other *Arabidopsis* CAXs have been characterized and shown to have different transport specificities. CAX1 is a specialized Ca^{2+} transporter [6,7], whereas, CAX2 can transport multiple cations such as Ca^{2+}, Cd^{2+} and Mn^{2+} into the vacuole [8–10]. CAX4 can not transport Ca^{2+} into the vacuole, but can partially rescue yeast mutant Cd^{2+} sensitivity [11]. CAX4 is thought to play an important role in root growth under heavy metal stress conditions [12]. Among *Arabidopsis* CCX1 to CCX5, AtCCX3 was found to be an endomembrane H^+-dependent K^+ transporter [13]. However, whether the other *Arabidopsis* CCXs transport K^+ has not been addressed. K^+ plays an important role in plant growth and development, nutrition, membrane potential, enzyme function, and the homeostasis of many other ions [14,15]. The extraction of K^+ from soil and its distribution within the plant require the presence of membrane transport proteins. A larger numbers of transporters have now been identified at the molecular level, such as HAK transporters, CHX transporters, HKT transporters, demonstrating the complex nature of K^+ transport. The physiological roles of these proteins in K^+ or Na^+ transport within the plant have been partially characterized [15,16], while many other putative K^+ transporters are currently under investigation or not studied.

In this study, to investigate the function of AtCCX5, we cloned *AtCCX5* and expressed it in two yeast strains to evaluate the growth

* Corresponding author. Address: Hexing Road No. 26, Xiangfang District, Harbin City, Heilongjiang Province 150040, China. Fax: +86 0451 82191394.
E-mail address: shenkuiliu@nefu.edu.cn (S. Liu).

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of the yeast on media containing various cations. We then analyzed the intracellular localization of AtCCX5 in the yeast cells and measured their uptake of K^+ or Rb^+ and Na^+.

2. Materials and methods

2.1. DNA manipulations

The cDNA sequence for the *AtCCX5* (At1g08960) coding region was amplified by PCR using gene-specific primers from the plasmid pUN151-*AtCCX5* provided by the Arabidopsis Biological Resource Center (ABRC). The plasmid contains the full-length ORF of the *AtCCX5* gene. Primers are forward primer 5′-ATGGAATTGATTTCTTCTTC-3′ and reverse primer 5′-TCAGGTTGAAACGCTGGCA-3′. For construction of a yeast expression plasmid, the ORF of *AtCCX5* cDNA was ligated to the pAUR123 vector (TaKaRa) digested with *Sal*I and *Hpa*I to construct the plasmid pAUR123-*AtCCX5*. To construct GFP fusion proteins in yeast, the *AtCCX5* gene without the stop codon was amplified with the primers 5′-AAGCTTG ATGGAATTGATTT-3′ and 5′-CCCGGGTGGTTGAAACGCTG-3′, and was ligated to the pYES2-*GFP* plasmid digested with HindIII and SmaI to obtain the plasmid of pyes2-*AtCCX5-GFP*.

2.2. Yeast strains, media, and growth conditions

The plasmids pAUR123-*AtCCX5* and pAUR123 empty vector were introduced into yeast strain K667 (*cnb1::LEU2 pmc1::TRP1 vcx1*Δ), which lacks vacuolar Ca^{2+}-ATPase (PMC1) and vacuolar Ca^{2+}/H^+ antiporter (VCX1) [17]. These two plasmids were also introduced into yeast strain 9.3 (*MATa, trk1Δ, trk2Δ, ena1Δ::HIS3::ena4Δ, leu2, ura3, trp1, ade2*) (ATCC, USA, No. 20140), in which the original potassium transporters (TRK1, 2) and P-type ATPase involved in Na^+ extrusion (ENA1-4) were deleted. Transformed yeast strains were grown in synthetic defined (SD) medium minus the appropriate amino acids for selective growth for the expression plasmids. For metal tolerance assays, K667 yeast overnight cultures were adjusted to an OD_{600} of 0.3. Ten-fold serial dilutions of K667 yeast were prepared, and 5-μl aliquots of each dilution were spotted on solid YPD medium containing a range of metal salts including $CaCl_2$, $MgCl_2$, $FeCl_2$, $CuCl_2$, $CoCl_2$, $CdCl_2$, $MnCl_2$, $BaCl_2$, $NiSO_4$, $ZnCl_2$, and LiCl. For determination of 9.3 yeast growth responding to K^+ and Na^+, 9.3 yeast overnight cultures were prepared as described as above, and 5-μl aliquots of each dilution were spotted on solid arginine phosphate (AP) medium and SD medium with or without various concentrations of KCl or NaCl as indicated. All of the plates were incubated at 30 °C for 3–7 d.

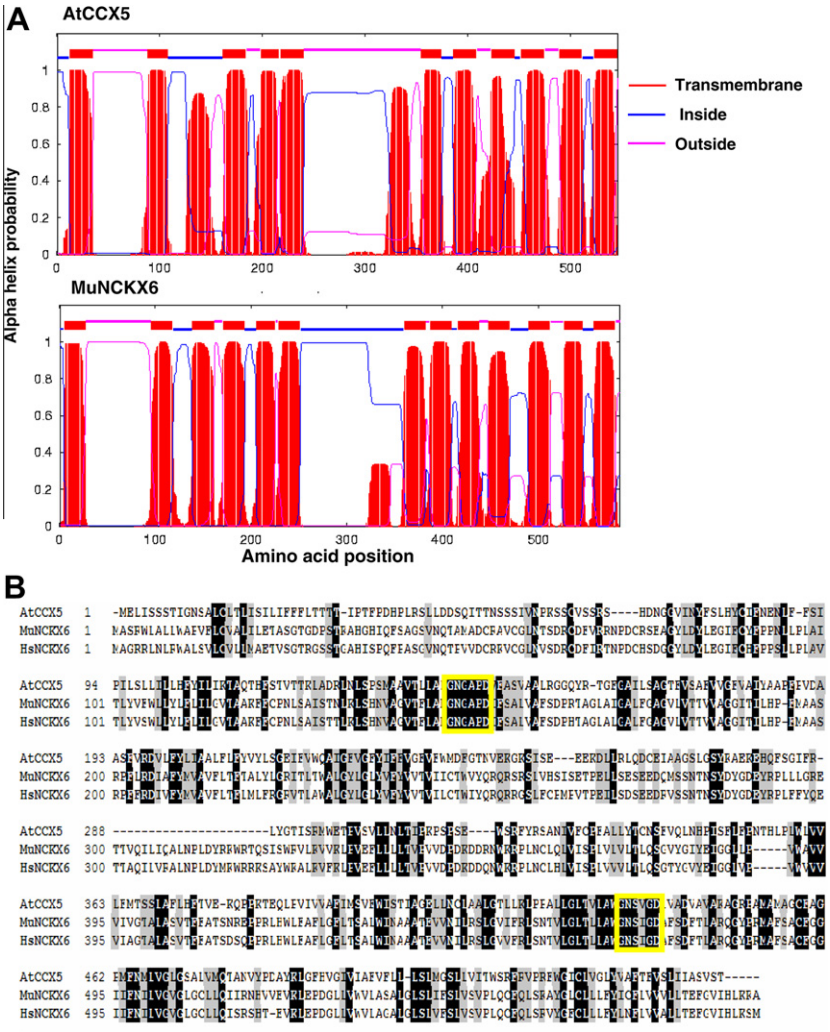

Fig. 1. Comparison of deduced amino acid sequences of AtCCX5 and other K^+-dependent Na^+/Ca^{2+} exchangers (NCKX). (A) Transmembrane helices in AtCCX5 (GenBank No. AE28373) and MuNCKX6 (GenBank No. NP_573484) were predicted by the TMHMM algorithm (http://www.cbs.dtu.dk/services/TMHMM/). (B) Alignment of AtCCX5, MuNCKX6 and HsNCKX6 (GenBank No. Q6J4K2). The yellow boxes mark the highly conserved α-1 and α-2 domains. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

2.3. Subcellular localization of AtCCX5-GFP in yeast

The plasmids pYES2-*AtCCX5-GFP* and pYES2-*GFP* were introduced into yeast strain INVSc1, respectively. The yeast transformants were pre-cultured in liquid YPD (1% yeast extract + 2% peptone + 2% glucose) medium overnight at 30 °C and washed three times. The cells were cultured in liquid YPG (1% yeast extract + 2% peptone + 2% galactose) medium for 8 h at 30 °C to induce expression of GFP and AtCCX5-GFP. GFP fluorescence was detected using a laser-scanning confocal imaging system (Olympus Fluoview, FV500).

2.4. Ion uptake in transformed yeast

Yeast 9.3 cells transformed with pAUR123 and pAUR123-*AtCCX5* were grown in SD medium and suspended in uptake buffers [2.0% glucose, 10 mM MES, and pH 6.0 adjusted with $Ca(OH)_2$] containing various concentrations of KCl or RbCl and NaCl. After 3 h, cells were rinsed twice in distilled water, and were acid-extracted overnight in 0.1 M HCl. Cell samples were collected by centrifuge, and the K^+, Rb^+ and Na^+ concentrations in the supernatant were determined with an atomic absorption spectrophotometer (AA800, Perkin Elmer, USA).

3. Results and discussion

3.1. AtCCX5 isolation and predicted protein

Sequencing of plasmid pUN151-*AtCCX5* revealed an *AtCCX5* ORF consisting of 1641 nucleotides. The ORF encoded 546 amino acids corresponding to a putative protein of 59.8 kDa. The deduced amino acid sequence contains a short N-terminal region, followed by five transmembrane domains, a 115-residue hydrophilic region, another six transmembrane domains and a short C-terminal region (Fig. 1A). Phylogenetic analyses clearly indicates that CCXs are more closely related to the mouse K^+-dependent Na^+/Ca^{2+} antiporter MuNCKX6 than to any of the CAXs [5,13]. AtCCX5 and MuNCKX6 share similar membrane topologies (Fig. 1A). Especially, AtCCX5 shares the characteristic α-repeats GNG (A/S) PD in α-1 and (G/S) (N/D) SxGD in α-2 with MuNCKX6 and HsNCKX6 (Fig. 1B) [2,3,5]; these are highly conserved Ca^{2+} and Na^+ domains [18,19], which may help to maintain the proper chemical microenvironment for ion binding [3]. These phylogenetic relationships suggest that the biochemical and regulatory properties of CCX transporters are different from those of CAXs.

3.2. Subcellular localization of AtCCX5-GFP in yeast

To determine the subcellular localization of AtCCX5, a fusion of AtCCX5 with GFP at the C-terminus (AtCCX5-GFP) was constructed. In yeast, AtCCX5-GFP was predominantly localized peripherally, consistent with plasma membrane localization (Fig. 2A). This result is similar to the localization of AtCHX13 that is a plasma membrane K^+ transporter in *Arabidopsis* [20]. According to the computer program PSORT, the intracellular localization of AtCCX5 with the highest certainty was also the plasma membrane (0.640). In addition, AtCCX5-GFP fluorescence was also observed around the nuclear periphery (Fig. 2A). Therefore, the precise localization of AtCCX5 is necessary to be further investigated in plant cell. On the other hand, in control cells carrying a construct that encoded only GFP, green fluorescence was almost evenly distributed throughout the cytoplasm (Fig. 2B).

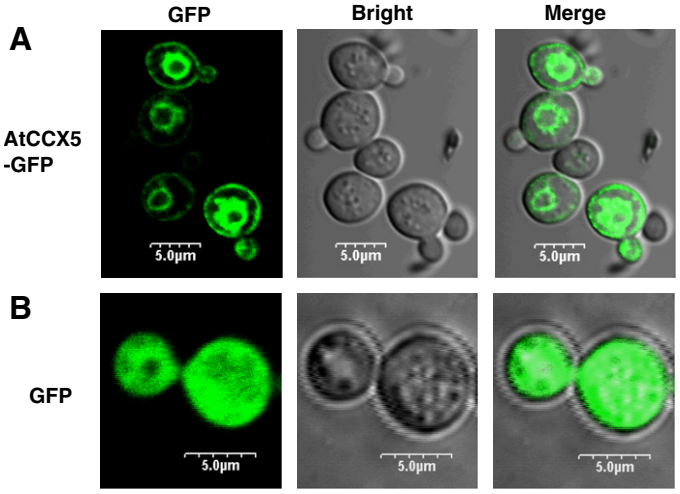

Fig. 2. Subcellular localization of AtCCX5 in yeast. The localization was observed with a confocal microscope. Left, middle and right images are fluorescent, bright-field and merged images, respectively. (A) Expression of the AtCCX5-GFP protein in yeast; (B) expression of the GFP protein in yeast. Bars = 5 μm.

3.3. AtCCX5-overexpressing cells respond to various metal cations

Growth of yeast mutant K667 expressing *AtCCX5* was the same as that of the vector transformant on media containing Ca^{2+}, Fe^{2+}, Mn^{2+}, Mg^{2+}, Cu^{2+}, Ba^{2+} or Ni^{2+} (Fig. 3A). Ca^{2+} uptake by yeast cells expressing *AtCCX5* was similar to that of vector controls (data not shown). In the presence of Co^{2+}, Cd^{2+}, or Li^+, the growth of the *AtCCX5* transformant was weaker than that of the vector transformant (Fig. 3A), this result is interesting and future study need to done to determine whether the mechanism is mediated directly by AtCCX5 or not. However, the AtCCX5 transformant grew a little better than the vector transformant in the presence of 2.5 mM Zn^{2+} (Fig. 3A). Morris et al. reported that various yeast mutant strains expressing *AtCCX3* were not distinctly different from cells with vector controls in the presence of Al^{3+}, Cd^{2+}, Cu^{2+}, Ni^{2+}, Mg^{2+}

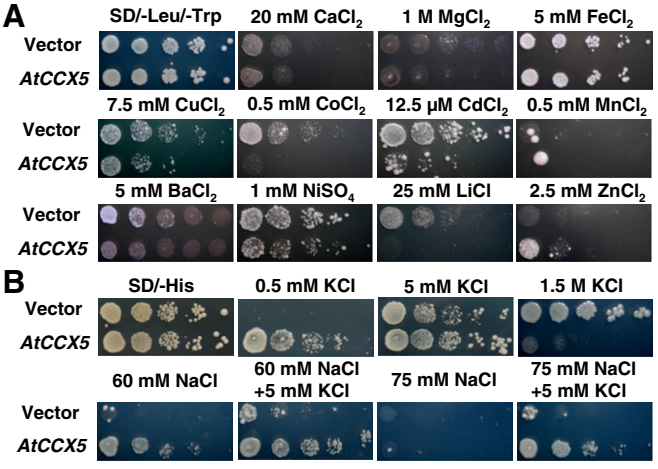

Fig. 3. (A) Growth of *AtCCX5*-overexpressing cells in the presence of various metal cations. Ten-fold dilutions of K667 yeast cells containing pAUR123-*AtCCX5* and pAUR123 vector were spotted onto solid SD/-Leu/-Trp medium or YPD media supplemented with the indicated cations. (B) Growth of yeast cells expressing the *AtCCX5* gene in the presence of low K^+, high K^+ and Na^+. Ten-fold dilutions of 9.3 yeast cells containing pAUR123-*AtCCX5* and pAUR123 vector were spotted onto solid SD/-His medium, AP media plus 0.5 or 5 mM KCl, and SD/-His media supplemented with or without additional KCl and NaCl. In (A) and (B), cells were grown at 30 °C, each panel shows growth after 3–7 d.

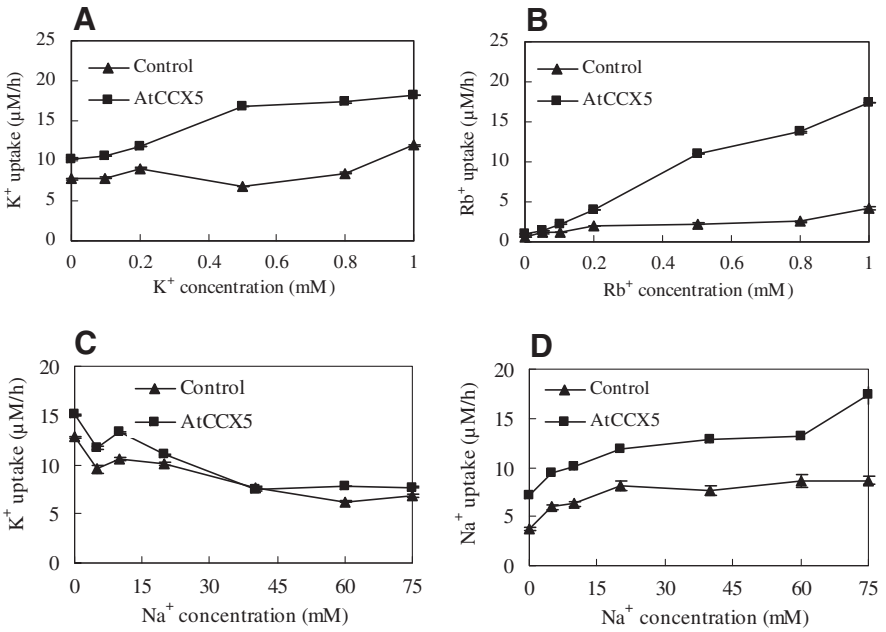

Fig. 4. (A and B) Concentration dependence of K^+ (A) or Rb^+ (B) uptake in yeasts expressing *AtCCX5*. Strain 9.3 yeast cells were transformed with empty vector pAUR123 (closed triangles) or with the vector containing *AtCCX5* (closed squares). (C and D) Concentration dependence of K^+ uptake (C) or Na^+ influx (D) in yeast expressing *AtCCX5* in the presence of 5, 10, 20, 40, 60 and 75 mM NaCl and 5 mM KCl. Yeast strains and symbols are indicated as in (A) and (B). Results are expressed as means ± SE (n = 3).

or Zn^{2+} [13]. Also, yeast cells expressing the *AtCCX3* or *AtCCX4* were unable to suppress the Ca^{2+} sensitivity of yeast strains deficient in vacuolar transport. Collectively, AtCCX5, AtCCX3 and AtCCX4 and did not show the same transport properties as the CAXs.

3.4. Ion uptake in transformed yeast

In order to determine whether AtCCX5 mediates K^+ or Na^+ transport, we examined whether expression of *AtCCX5* affected the growth of yeast mutant 9.3 (Fig. 3B). In the presence of low $[K^+]_{Ext}$ (0.5 mM), the *AtCCX5* transformant grew very well while the vector transformant could not grow (Fig. 3B). When $[K^+]_{Ext}$ was increased to 5 mM, the growth rates of the *AtCCX5* and vector transformants were similar (Fig. 3B). When $[K^+]_{Ext}$ was raised to 1.5 M, the *AtCCX5* transformant could not grow, while the vector transformant grew well. Because K^+ is required to sustain growth for all cells, the better growth of *AtCCX5* transformant at low K^+ level suggests that AtCCX5 has a role in acquiring K^+ and maintaining suitable cellular homeostasis for cell growth. This idea is supported by the very similar growth rates of the vector and *AtCCX5* transformants in the presence of 5 mM K^+. When $[K^+]_{Ext}$ was increased to 1.5 M, the *AtCCX5* transformant acquired a toxic level of K^+. Therefore, the *AtCCX5* transformant was more sensitive to 1.5 M KCl than the vector transformant. The *AtCCX5* transformant could grow on medium containing 60 mM NaCl (Fig. 3B). Furthermore, the suppression of growth of the *AtCCX5* transformant by 75 mM NaCl was rescued by the addition of 5 mM K^+ (Fig. 3B). These results suggest that AtCCX5 improves the tolerance to Na^+ stress, probably through its ability to take up K^+.

The yeast expressing *AtCCX5* showed a high K^+ uptake ability in the K^+ (Rb^+) uptake experiment (Fig. 4A and B). AtCCX5 functioned in the micromolar range of K^+ (Rb^+), indicating that AtCCX5 has a high-affinity for K^+. This result is supported by the better growth of the *AtCCX5* transformant at low K^+ as shown in Fig. 3B. In contrast, AtCCX3 appeared to behave as a low affinity K^+/proton exchanger in a manner similar to AtNHX1 [12]. In the presence of NaCl, the K^+ uptake ability of AtCCX5 rapidly decreased with increasing NaCl concentration (Fig. 4C). Furthermore, AtCCX5 showed not only K^+ uptake but also Na^+ permeability. Na^+ influx was greater in AtCCX5 transformant than in the control expressing pAUR123 empty vector at each Na^+ concentration (Fig. 4D). At low $[K^+]_{Ext}$, AtCCX5 mediates high-affinity K^+ uptake (Figs. 3B and 4A and B). When $[Na^+]_{ext}$ is at toxic millimolar levels and greater than $[K^+]_{Ext}$, AtCCX5 mediates Na^+ uptake while the K^+ uptake is blocked (Fig. 4C and D). These data show that AtCCX5 is highly selective for K^+ and Na^+ under various ionic conditions, which supports the idea that AtCCX5 activity accumulates K^+ and Na^+ into the cell. The *TaHKT2;1* gene from wheat (*Triticum aestivum*) (previously named *HKT1* (high-affinity K^+ transporter 1)), was originally characterized as the K^+–H^+ symporter that mediated high-affinity uptake system in wheat roots [21], but it was later found that it acted as a Na^+–K^+-symporter when expressed in yeast cells or *Xenopus* oocytes [22,23]. The *PutHKT2;1* gene from *Puccinellia tenuiflora* expressed in yeast was able to take up K^+ in low K^+ concentration medium or in the presence of NaCl and was also permeable to Na^+, which suggested that *PutHKT2;1* has a high-affinity K^+–Na^+ symport function in yeast [24]. The function of AtCCX5 is similar to that of TaHKT2;1 and PutHKT2;1, however, the mechanism of competitive transport of K^+ and Na^+ in a working model of AtCCX5 is necessary to be further investigated in plants.

In summary, we have characterized the function of the *AtCCX5* gene in yeast. We demonstrate that AtCCX5 localizes to the plasma membrane and nuclear periphery, and has a role in mediating high-affinity K^+ uptake and Na^+ transport in yeast distinct from previously characterized plant CAX transporters, and that it might be useful in transforming other plants to promote their K^+ uptake (when K^+ is limiting in the environment) and Na^+ tolerance.

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