

Full Length Research Paper

# AtCCX1 transports Na<sup>+</sup> and K<sup>+</sup> in *Pitch pastoris*

Zhiyuan Chen<sup>1</sup>, Yongjun Wu<sup>2</sup>, Lijun Di<sup>1</sup>, Yufang Shen<sup>3</sup> and Guodong Wang<sup>1\*</sup>

<sup>1</sup>College of Sciences, Northwest A&F University, Yangling, 712100 Shaanxi, China.

<sup>2</sup>College of Life Sciences, Northwest A&F University, Yangling, 712100 Shaanxi, China.

<sup>3</sup>Institute of Soil and Water Conservation, Chinese Academy of Sciences and Ministry of Water Resources, Yangling 712100 Shaanxi, China.

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**Compartmentation of Na<sup>+</sup> into vacuoles is an important way for cells to avoid the toxic effects of salt. Here, *Arabidopsis* AtCCX1 was studied in *Pitch pastoris* GS115. Yeast expressing AtCCX1 grew better in high H<sup>+</sup> concentration medium and high salt medium than original strain and increased Na<sup>+</sup> accumulation and decreased K<sup>+</sup> accumulation. AtCCX1 was located in tonoplast in yeast. Transport essays were indicated by fluorescence SBFI and PBFI, respectively. Results show that AtCCX1 simultaneously participate in transports both of Na<sup>+</sup> and K<sup>+</sup> and the process was inhibited by H<sup>+</sup>-ATPase inhibitor vanadate. In conclusion, we suggest that AtCCX1 is an H<sup>+</sup>-dependent Na<sup>+</sup>/K<sup>+</sup> exchanger.**

**Key words:** AtCCX1, SBFI, PBFI, Na<sup>+</sup> accumulation, K<sup>+</sup> accumulation.

## INTRODUCTION

Ion ratios in plants are altered by the influx of Na<sup>+</sup> through K<sup>+</sup> pathways (Blumwald, 2000; Santa-Cruz et al., 1999; Rees et al., 1992). The similarity of the hydrated ionic radii of Na<sup>+</sup> and K<sup>+</sup> makes it difficult to discriminate between them and this is the basis of Na<sup>+</sup> toxicity. For plant cell to deal with this deleterious ionic effect of salinity, it has to perform two tasks: to exclude or sequester Na<sup>+</sup> and Cl<sup>-</sup> to avoid their toxicity in the cytoplasm and to maintain appropriate cellular levels of K<sup>+</sup> and Ca<sup>2+</sup> necessary for metabolic activities (Mansour et al., 2003).

CAX7 to CAX11 were recently renamed as CCX1 to CCX5 (Cai and Lytton, 2004; Shigaki et al., 2006) because they display limited primary amino-acid sequence homology with other CAX, but have a striking sequence similarity to the mammalian K<sup>+</sup>-dependent exchanger family is one of the five families of the CaCA.

superfamily. CAXs are a group of proteins that export cations from the cytosol to maintain optimal ionic concentrations in the cell. CAXs are energized by the pH gradient established by proton pumps such as H<sup>+</sup>-ATPase and H<sup>+</sup>-pyrophosphatase (Kamiya and Maeshima, 2004). Several plant CAXs have been characterized as vacuole-localized transporters, which function in H<sup>+</sup>-coupled antiport of Ca<sup>2+</sup>, Mg<sup>2+</sup> and Mn<sup>2+</sup>, resulting in the accumulation of these cations in vacuoles (Hirschi et al., 1996; Hirschi, 1999; Pittman et al., 2004; Mei et al., 2007). However, most members of CCXs have not yet to be identified and their transport properties are needed to complete urgently.

The gene of AtCCX1 had been cloned and subcloned into *Pitch pastoris* GS115. In this study, static and dynamic transport properties of AtCCX1 in *P. pastoris* GS115 were investigated.

## MATERIALS AND METHODS

Yeast was grown overnight in YPD medium at 28°C. After measuring the OD600, equal numbers of cells were grown in 50 ml of yeast peptone dextrose (YPD) +supplements.

AtCCX1 was amplified from *Arabidopsis thaliana* genomic DNA using PCR. Two oligonucleotide primers that are complementary to the 5' and 3' ends of the predicted AtCCX1 gene were generated: AtCCX1 forward primer, 5'-GGGGGTACCCCT CATTATTGT-TCCAATTCATACCC3' and AtCCX1 reverse primer, 5'-GGGCCGCGGCCTCTCGATAGTAATTCAACTATGCACA3'. Kpn

\*Corresponding author. E-mail: [guowang211@yahoo.com.cn](mailto:guowang211@yahoo.com.cn).  
Fax: (+86) 29 87092262.

**Abbreviations:** [K<sup>+</sup>], K<sup>+</sup> Concentration; [K<sup>+</sup>]m, K<sup>+</sup> concentration in medium; [K<sup>+</sup>]c, K<sup>+</sup> content in yeast cells; [Na<sup>+</sup>], Na<sup>+</sup> concentration; [Na<sup>+</sup>]m, Na<sup>+</sup> concentration in medium; [Na<sup>+</sup>]c, Na<sup>+</sup> content in yeast cells; **AtCCX1**, the *Pitch pastoris* GS115 expressing AtCCX1; **T[Control]**, the *Pitch pastoris* GS115; **[H<sup>+</sup>]m**, H<sup>+</sup> concentration in culture media

I and Sac II sites (underlined) were introduced for sub-cloning. Genomic DNA was isolated using the E.Z.N.A. TM Plant DNA Maxi Kit (Omega, USA), according to the manufacturer's instructions. AtCCX1 genomic DNA was subcloned into the yeast expression vector pGAPZ B (Invitrogen, USA).

EGFP (enhanced green fluorescent protein) sequence was cloned from vector pIRES2-EGFP (Invitrogen, USA) using PCR by two oligonucleotide primers. The forward primer was GCGCAAGCTTGTATGGGATCTGATCTGGGGCCTCG and the reverse primer was GCGCAAGCTTTGTTTCAGGTTTCAGGGGGAGGTGTG. Hind III sites (underlined) were introduced for sub-cloning. Then, EGFP sequence was fused to C-terminal of AtCCX1 in vector pGAPZ B. The primers CGTTGCTTGAGACACTAGGGTTCTT and ACCTCTACAAATGTGGTATGGCTGAT were used to test the correct reading order of EGFP by PCR. Yeast vacuolar protein P51 was used as a marker protein and fused to EGFP as described previously (Morris et al., 2008; Carter et al., 2004).

Inductively coupled plasma mass spectrometry (ICP-MS) analysis was done as described previously with a little modification (Eide et al., 2005). Yeast cells were collected from the 50 ml yeast culture suspension by centrifugation at  $3,000 \times g$  for 10 min and washed with 50 ml of distilled, deionized H<sub>2</sub>O. To ensure remove unbound elements, this process was repeated for three times. The cells were then placed in a 50 ml conical flask with stopper and 5 ml 30% HNO<sub>3</sub> was added. The samples were digested overnight in a 65°C water bath. Afterward, 5 ml of distilled, deionized H<sub>2</sub>O was added, the samples were vortexed briefly. Before ICP-MS analysis, the cell digests were diluted 100 times or even more. ICP-MS analysis was performed with a Varian 820-MS ICP mass spectrometer (made in USA) with a three-channel peristaltic pump.

Yeast membrane vesicles were prepared as described previously with a few modifications (Nakanishi et al., 2001). Yeast cells were precultured at 30°C for 2 days in YPD medium that contained supplements. The cell culture was diluted 64-fold and then grown for 12 h to reach an exponential phase. After being washed with 0.1 M Tris-HCl, pH 9.4, 50 mM 2-mercaptoethanol and 0.1 M glucose at 30°C for 10 min, cells were treated with a zymolyase medium at 30°C for 1 h with gentle agitation. The medium contained 0.05% zymolyase 20 T, 0.9 M sorbitol, 0.1 M glucose, 50 mM Tris-Mes, pH 7.6, 5 mM DTT, 0.043% yeast nitrogen base without amino acids and ammonium sulfate and 0.25× dropout solution composed of all amino acids and adenines. Spheroplasts were collected from the suspension by centrifugation at  $3,000 \times g$  for 10 min and washed with 1 M sorbitol.

The spheroplasts were resuspended in 50 mM Tris-ascorbate, pH 7.6, 1.1 M glycerol, 1.5% polyvinylpyrrolidone (Mr 40,000), 5 mM EGTA-Tris, 1 mM DTT, 0.2% bovine serum albumin, 1 mM PMSF and 1 mg/l leupeptin and then homogenized with a motor-driven Teflon homogenizer. After centrifugation at  $2,000 \times g$  for 10 min, the precipitate was suspended in the same buffer and centrifuged again. All of the supernatant fractions were pooled and centrifuged at  $120,000 \times g$  for 30 min. The precipitate was suspended in 15% (w/w) sucrose and layered on a 35% (w/w) sucrose solution. Both sucrose solutions contained 10 mM Tris-Mes, pH 7.6, 1 mM EGTA-Tris, 2 mM DTT, 25 mM KCl, 1.1 M glycerol, 0.2% bovine serum albumin, 1 mM PMSF and 1 mg/l leupeptin. After centrifugation at  $150,000 \times g$  for 30 min, the interface portion was collected and diluted with 5 mM Tris-Mes, pH 7.6, 0.3 M sorbitol, 1 mM DTT, 1 mM EGTA-Tris, 0.1 M KCl, 1 mM PMSF, 1 mg/l leupeptin and 5 mM MgCl<sub>2</sub>. The precipitate after centrifugation at  $150,000 \times g$  for 30 min was resuspended in 5 mM Tris-Mes, pH 7.6, 0.3 M sorbitol, 1 mM DTT, 1 mM EGTA-Tris, 1 mM PMSF, 1 mg/l leupeptin and 1.5 mM MgCl<sub>2</sub>. The suspension was stored at -80°C until use.

The fluorescence quenching of SBFI and PBF1 were used to monitor the dynamic of Na<sup>+</sup> and K<sup>+</sup> concentration inside membrane vesicles. Membrane vesicle suspensions were recollected by centrifugation at  $150,000 \times g$  for 30 min and the precipitate was kept. The precipitate was then loaded with SBFI-AM (or PBF1-AM) by

adding 50 μl of 10 μmol/l SBFI-AM (or PBF1-AM) with 0.04% pluronic F-127. After 100 min, excess SBFI-AM (or PBF1-AM) was removed by washing with buffer. While the indicator is SBFI, the buffer is 5 mM Tris-Mes, pH 6.0, 0.3 M sorbitol, 1 mM DTT, 1 mM EGTA-Tris, 0.1 M KCl, 1 mM PMSF, 1 mg/l leupeptin. While the indicator is PBF1, the buffer is 5 mM Tris-Mes, pH 6.0, 0.3 M sorbitol, 1 mM DTT, 1 mM EGTA-Tris, 0.1 M NaCl, 1 mM PMSF, 1 mg/l leupeptin. Excitation ratios of fluorescence at 344 and 400 nm measured at emission 500 nm were used to estimate the content of Na<sup>+</sup> or K<sup>+</sup> inner vesicle. The time-dependent fluorescence changes were monitored on a Hitachi F4500 fluorescence spectrophotometer. Results were corrected for background fluorescence and presented as percentage of untreated control.

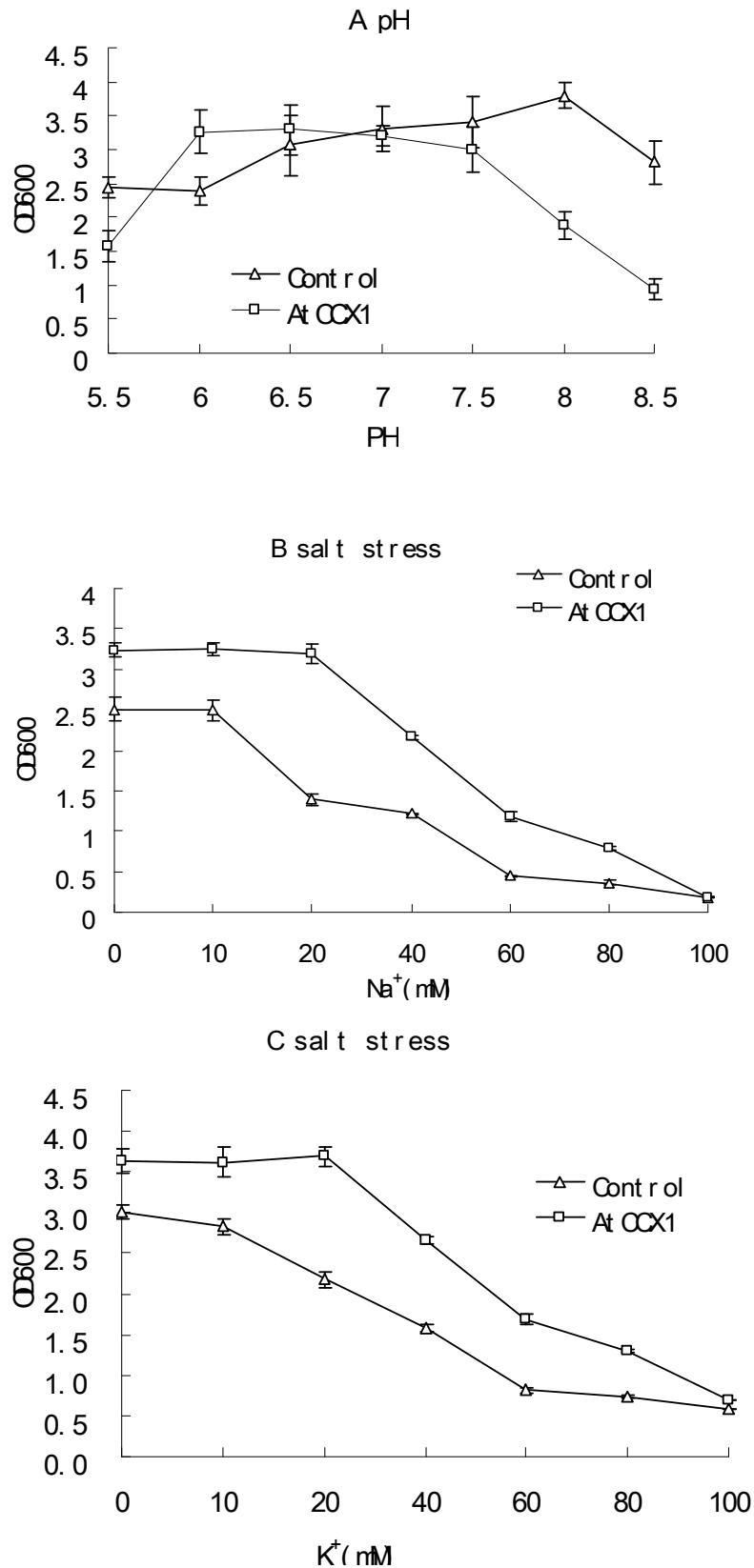
## RESULTS

AtCCX1 was introduced into *P. pastoris* GS115. To test the properties of AtCCX1 in yeast, yeast growth density was tested in OD600 by ultraviolet spectrophotometer. After expressing ATCCX1, yeast showed the different performance in [H<sup>+</sup>] gradient medium. The variant expressing AtCCX1, unlike original strain, preferred to growing best at [H<sup>+</sup>]m 0.01 μM (pH = 6) rather than at [H<sup>+</sup>]m 1 μM (pH = 8). Figure 1A shows that GAtCCX1 grew more better in high [H<sup>+</sup>]m than T[control]. And the most suitable fittest [H<sup>+</sup>]m shifted from 0.01 to 1 μM. The growth of yeast under salt stress was also tested. The variant expressing AtCCX1 significantly grew better in both NaCl and KCl medium than control (Figure 1B,C). The results indicate that expression of AtCCX1 might be helpful for yeast against salt stress.

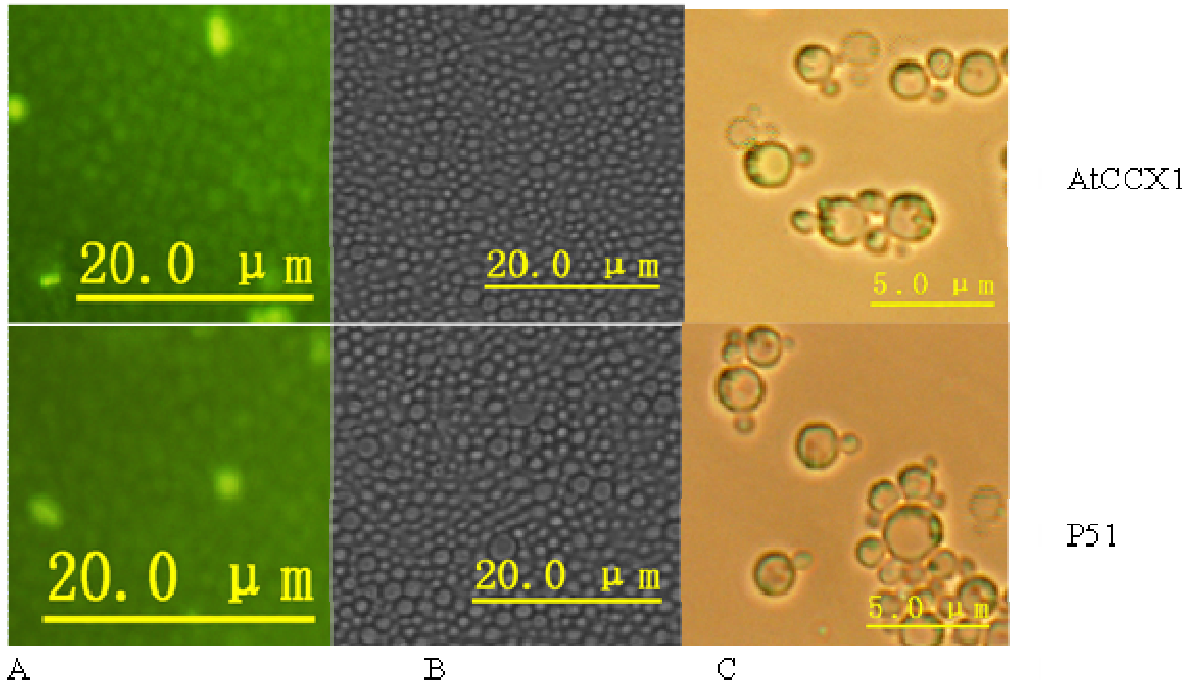
To identify localization of AtCCX1 in yeast cell, C-terminal of AtCCX1 was fused to EGFP (enhanced green fluorescent protein). Vacuolar protein P51 was also fused to EGFP as a marker protein. Fluorescent pictures were obtained by Olympus fluorescence microscope. Figure 2 shows that AtCCX1 was located in tonoplast apparently, consistent with P51.

To investigate substrates of AtCCX1, ICP-MS analysis was performed in H<sup>+</sup> gradient medium. 8 elements concentration in different [H<sup>+</sup>] concentration medium were tested with ICP-MS and relative concentration variation percentages of 8 elements were calculated with  $\Delta\text{cation} = (\text{C}[\text{AtCCX1}]\text{cation} - \text{C}[\text{control}]\text{cation}) / \text{C}[\text{control}]\text{cation}$  (Figure 3). The results show that  $\Delta\text{Na}^+$  and  $\Delta\text{K}^+$  were the highest, especially while [H<sup>+</sup>]m=1 μM, respectively to be 160.5% and -49.1%.

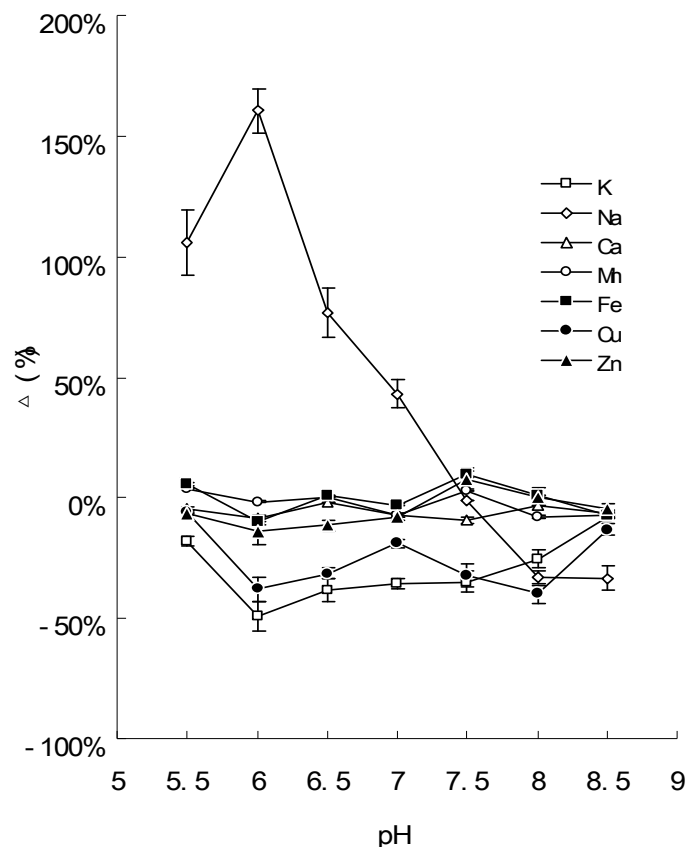
ICP-MS analysis was performed in K<sup>+</sup> gradient medium to further investigate accumulation of Na<sup>+</sup> and K<sup>+</sup>. Both GAtCCX1 and T[control] were grown in [K<sup>+</sup>] gradient culture medium for 14 h and [Na<sup>+</sup>]c and [K<sup>+</sup>]c were tested with ICP-MS. Accumulation of K<sup>+</sup> in original strains raised up significantly accompanying with the increasing of [K<sup>+</sup>]m, but accumulation of K<sup>+</sup> in variants stayed at a steady level in all [K<sup>+</sup>] gradient culture medium (Figure 4). Accumulation of Na<sup>+</sup> in original strains raised up significantly with the increasing of [K<sup>+</sup>]m, but accumulation of Na<sup>+</sup> in variants was strengthened more than that of original strains (Figure 5).



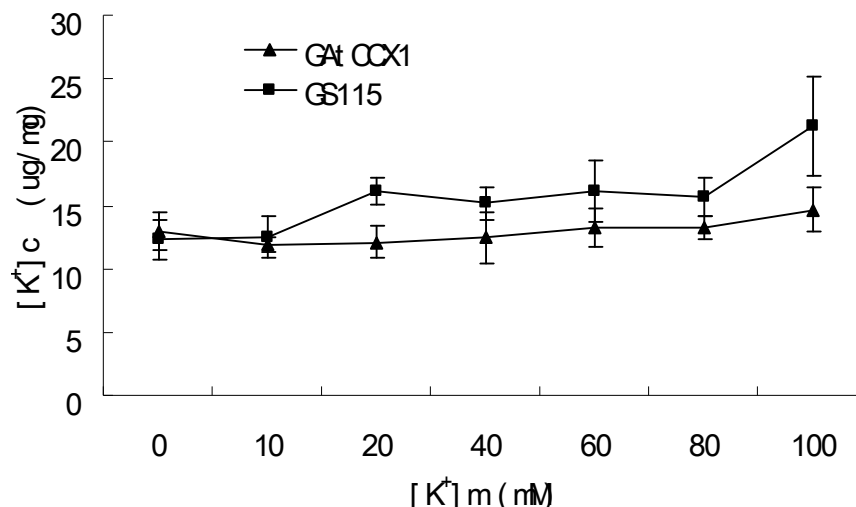
**Figure 1.** Growth of AtCCX1-expressing yeast strains (A) growth of yeast in various [H<sup>+</sup>] gradient medium; (B and C) growth of yeast in various [Na<sup>+</sup>] and [K<sup>+</sup>] gradient medium.



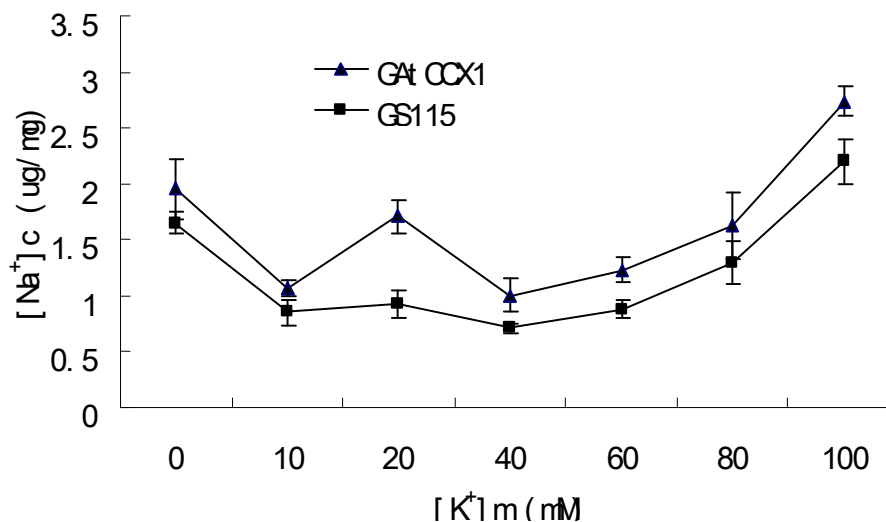
**Figure 2.** Localization of AtCCX1 in yeast (A) excited at 488 nm, filtered between 500 and 510 nm; (B) bright field; (C) excited at 488 nm, observed at full wavelength cation content in yeast.



**Figure 3.** ICP-MS analysis from dry weight of unit volume yeast culture.



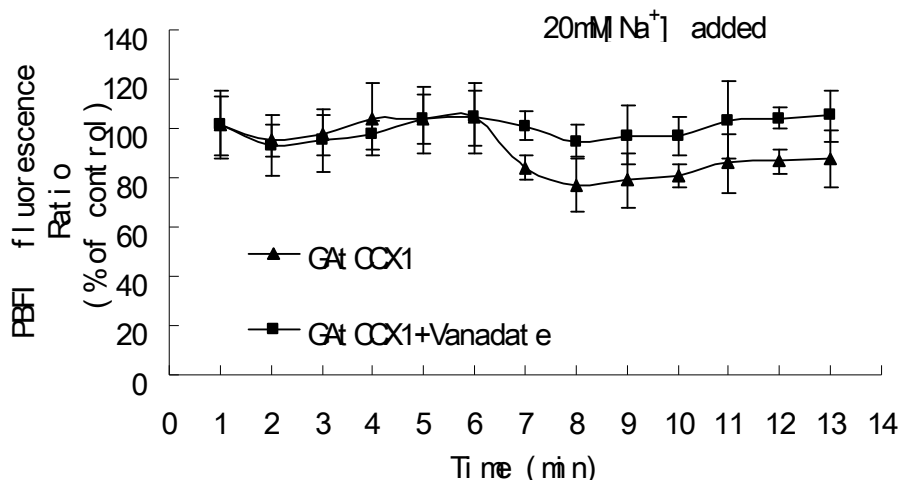
**Figure 4.** Accumulation of K<sup>+</sup> in yeast strains growing in [K<sup>+</sup>] gradient medium.



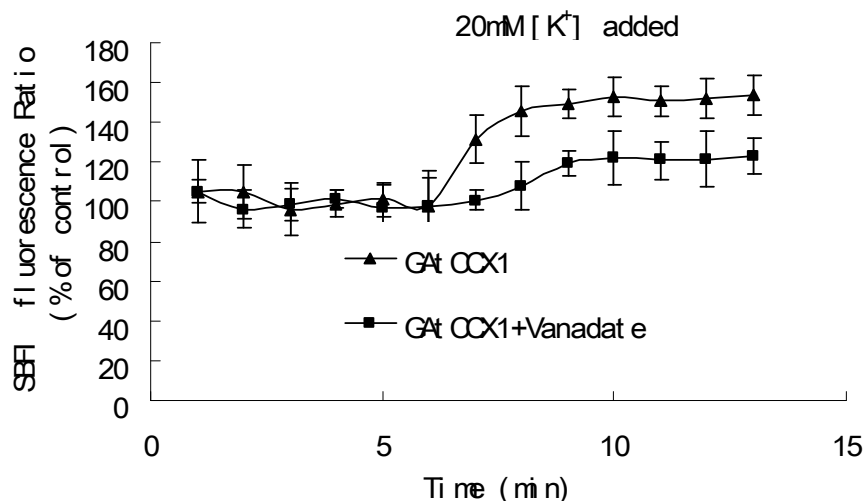
**Figure 5.** Accumulation of Na<sup>+</sup> in yeast strains growing in [K<sup>+</sup>] gradient medium.

It was reasonable to infer that AtCCX1 took part in transports of Na<sup>+</sup> and K<sup>+</sup>, according to the results of ICP-MS analysis. However, a piece of evidence that AtCCX1 dynamically transported Na<sup>+</sup> and K<sup>+</sup> are still needed. In this section, dynamic of Na<sup>+</sup>/K<sup>+</sup> transport essay by AtCCX1 was described. SBFI and PBF1, which were fluorescent indicators for sodium and potassium, respectively, were used in this experiment. Membrane vesicle of the variants which had been treated with AM-PBF1 was preloaded with a K<sup>+</sup> buffer (5 mM Tris-Mes, pH 6.0, 0.3 M sorbitol, 1 mM DTT, 1 mM EGTA-Tris, 0.1 M KCl, 1 mM PMSF, 1 mg/l leupeptin) (Figure 6). After detecting for 6 min, 20 mM [Na<sup>+</sup>] was added into the buffer. Without [Na<sup>+</sup>] in the solution, a steady-state PBF1 fluorescence ratio level was observed. While 20 mM [Na<sup>+</sup>

was added into the solution, PBF1 fluorescence ratio began to reduce sharply. However, if vanadate, a H<sup>+</sup>-ATPase inhibitor, was contained in the solutions, PBF1 fluorescence ratio was kept in a steady level all the time, no matter whether [Na<sup>+</sup>] was added. Compared with PBF1 fluorescence ratio, similar performance was observed in SBFI fluorescence ratio, but a slightly difference. Membrane vesicle of variants that had been treated with AM-SBF1 was preloaded with a Na<sup>+</sup> buffer (5 mM Tris-Mes, pH 6.0, 0.3 M sorbitol, 1 mM DTT, 1 mM EGTA-Tris, 0.1 M NaCl, 1 mM PMSF, 1 mg/l leupeptin) (Figure 7). After detecting for 6 min, 20 mM [K<sup>+</sup>] was added into the buffer. Without [K<sup>+</sup>] in the solution, a steady-state SBFI fluorescence ratio level was observed while 20 mM [K<sup>+</sup>] was added into the solution, SBFI fluorescence ratio



**Figure 6.** Fluorescent image of membrane vesicles loaded with PBF1. 20 mM  $[Na^+]$  was added after the beginning of testing for 6 min in form of NaCl.



**Figure 7.** Fluorescent image of membrane vesicles loaded with SBFI. 20 mM  $[K^+]$  was added after the beginning of testing for 6 min in form of KCl.

raised up dramatically. However, if vanadate was contained in the solution, PBF1 fluorescence ratio will be kept in a steady level all the time, no matter whether  $[K^+]$  was added.

## DISCUSSION

To avoid the toxic effects of salt, cell sequesters  $Na^+$  into vacuoles (Ye et al., 2009). In *Arabidopsis*, the AtNHX family of  $Na^+/H^+$  antiporters functions in  $Na^+$  compartmentation (Blumwald, 2000; Hanana et al., 2009; Xu et al., 2009). AtNHX1 and AtNHX2 are localized in the tonoplast membrane and their transcript levels are upregulated by ABA or osmotic stress (Yokoi et al., 2002). However, recently, CCXs in *Arabidopsis* were also

found to be related to  $Na^+$  compartmentation. Heterologous expression of AtCCX3 in yeast increased accumulation of  $Na^+$  in yeast (Morris et al., 2008), similar to NHX1 to promote  $Na^+$  uptake (Kinclova et al., 2003; Darley et al., 2000; Fukuda et al., 2011). When compared with NHX1, AtCCX3 simultaneously transported  $K^+$  and  $Mn^{2+}$  apart from  $Na^+$ . In this study, we found that  $Na^+$  and  $K^+$  were the main substrates of AtCCX1. AtCCX1 was located in tonoplast (Figure 2) and increased accumulation of  $Na^+$  and decreased accumulation of  $K^+$  and  $Cu^{2+}$  (Figures 3 to 5). Dynamic of  $Na^+/K^+$  transport assay indicated that AtCCX1 facilitated  $Na^+$  influx and  $K^+$  efflux depending on  $H^+$  gradient. These results suggest that AtCCX1 be an  $H^+$  dependent  $Na^+/K^+$  exchanger. Actually, the results are consistent with AtCCX3 and AtCCX4 (Morris et al., 2008). Vacuolar sequestration of

Na<sup>+</sup> not only lowers Na<sup>+</sup> concentration in the cytoplasm, but also contributes to osmotic adjustment to maintain water uptake from saline solutions. Na<sup>+</sup> compartmentation is an economical means of preventing Na<sup>+</sup> toxicity in the cytosol because the Na<sup>+</sup> can be used as an osmolyte in the vacuole to help to achieve osmotic homeostasis (Li et al., 2010; Zhu, 2001).

A wealth of evidence indicates that most of the transporters in plant are regulated by pH (Sze, 1993; Duan et al., 2007; Kuchitsu et al., 1992). Here, we analyzed the effect of pH on AtCCX1. After expressing AtCCX1, the fittest growth pH in culture medium shifted from 8 to 6. While pH in the medium is identical to 6, relative changes of Na<sup>+</sup> and K<sup>+</sup> of GAtCCX1, compared with wild type, were the highest. Dynamic of Na<sup>+</sup>/K<sup>+</sup> transport assay showed that exchange between Na<sup>+</sup> and K<sup>+</sup> caused by AtCCX1 could be prevented by vanadate, an H<sup>+</sup>-ATPase inhibitor. These results suggest that [H<sup>+</sup>] gradient regulated ion transport of AtCCX1. Similar results were found in other transporters. Structure-function studies have proved that CAX1, CAX2 and CAX3 share a similar sequence domain participating in pH regulation (Pittman et al., 2005). In *Escherichia coli* NhaA, it was found that His-226 is part of the pH sensor (Gerchman et al., 1993; Rimon et al., 1995).

A high cytosolic K<sup>+</sup>/Na<sup>+</sup> ratio is important for maintaining cellular metabolism (Zhu, 2003; Hauser and Horie, 2010; Leidi et al., 2010). Various reports have indicated that increasing cytosolic K<sup>+</sup> levels relative to Na<sup>+</sup>, thus, increasing the K<sup>+</sup>/Na<sup>+</sup> ratio, is crucial for Na<sup>+</sup> tolerance in plants and maintaining high K<sup>+</sup>/Na<sup>+</sup> ratio in shoots is highly correlated with salinity tolerance in glycophytes (Dubcovsky et al., 1996; Ren et al., 2005; Sunarpi et al., 2005; Zhu et al., 1998; Mason et al., 2010). The *Arabidopsis* AtHKT1 protein, a Na<sup>+</sup>-K<sup>+</sup> co-transporter, mediates Na<sup>+</sup> influx when expressed in heterologous systems such as *Xenopus oocytes*, yeast and wheat (Mason et al., 2010; Uozumi et al., 2000; Laurie et al., 2002; Baek et al., 2011; Plett et al., 2010). The novel CCXs family is also possibly correlated with maintenance of Na<sup>+</sup>/K<sup>+</sup> ratio in plant. In this study, the results indicate that AtCCX1 was a Na<sup>+</sup>/K<sup>+</sup> exchanger and is regulated by [H<sup>+</sup>] gradient. It was proposed that AtCCX1 contribute to lower cytosolic Na<sup>+</sup> concentration by K<sup>+</sup> reverse flow.

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