Isolation and Functional Characterization of Ca\(^{2+}/H^+\) Antiporters from Cyanobacteria*

Received for publication, September 16, 2003, and in revised form, October 13, 2003
Published, JBC Papers in Press, October 14, 2003, DOI 10.1074/jbc.M310282200

Rungaroon Waditee\(^{\ddagger}\), Gazi Sakir Hossain\(\ddagger\), Yoshito Tanaka\(\ddagger\), Tatsunosuke Nakamura\(\ddagger\), Masamitsu Shikata\(\ddagger\), Jun Takano\(\ddagger\), Tetsuko Takabe\(\ddagger\), and Teruhiro Takabe\(\ddagger\)

From the \(\ddagger\)Research Institute, and \(\ddagger\)Graduate School of Environmental and Human Sciences, Meijo University, Nagoya, 468-8502, \(\ddagger\)Faculty of Pharmacy, Niigata University of Pharmacy and Applied Life Science, Niigata, 950-3081, \(\ddagger\)Shimadzu Co. Ltd, Nakagou-ku, Kyoto, 604-8511, and \(\ddagger\)Graduate School of Agricultural Science, Nagoya University, Chikusa-ku, Nagoya, 464-8601, Japan.

Genome sequences of cyanobacteria, Synechocystis sp. PCC 6803, Anabaena sp. PCC 7120, and Thermosynechococcus elongatus BP-1 revealed the presence of a single Ca\(^{2+}/H^+\) antiporter in these organisms. Here, we isolated the putative Ca\(^{2+}/H^+\) antiporter gene from Synechocystis sp. PCC 6803 (synCAX) as well as a homologous gene from a halotolerant cyanobacterium Aphanothece halophytica (apCAX). In contrast to plant vacuolar CAXs, the full-length apCAX and synCAX genes complemented the Ca\(^{2+}\)-sensitive phenotype of an Escherichia coli mutant. ApCAX and SynCAX proteins catalyzed specifically the Ca\(^{2+}/H^+\) exchange reaction at alkaline pH. Immunological analysis suggested their localization in plasma membranes. The Synechocystis sp. PCC 6803 cells disrupted of synCAX exhibited lower Ca\(^{2+}\) efflux activity and a salt-sensitive phenotype. Overexpression of ApCAX and SynCAX enhanced the salt tolerance of Synechococcus sp. PCC 7942 cells. Mutagenesis analyses indicated the importance of two conserved acidic amino acid residues, Glu-74 and Glu-324, in the transmembrane segments for the exchange activity. These results clearly indicate that cyanobacteria contain a Ca\(^{2+}/H^+\) antiporter in their plasma membranes, which plays an important role for salt tolerance.

Modulation of cytosolic Ca\(^{2+}\) levels is essential for adapted physiological responses and is determined by two opposite fluxes, Ca\(^{2+}\) influx via channels and Ca\(^{2+}\) efflux via active transporters (1–3). For Ca\(^{2+}\) efflux, the primary pump Ca\(^{2+}\)-ATPase and secondary transporter Ca\(^{2+}\) exchanger are believed to play important roles. When compared with other Ca\(^{2+}\) transporters, few studies have been focused on the molecular mechanisms of H\(^-\)–coupled Ca\(^{2+}\) antiporter (4).

Ca\(^{2+}/H^+\) antiporters (CAXs)\(^3\) have been cloned from bacteria, fungi, and plants, most of which are vacuolar CAXs (5–7). CAXs have in general 10–14 transmembrane (TM)-spanning domain with about 400 amino acid residues (2–7). CAXs contain a central hydrophilic motif rich in acidic amino acid residues that bisect the polypeptide into two approximately equal segments (5–7). Information on the molecular properties of CAX has been emerging from the studies on plant CAXs, especially from Arabidopsis (4, 7–15). Four Arabidopsis CAXs (AtCAX1–4) were identified by their ability to sequester Ca\(^{2+}\) into yeast vacuoles in Saccharomyces cerevisiae mutants deleted of the vacuolar Ca\(^{2+}\)-ATPase and Ca\(^{2+}/H^+\) antiporter (ScV CX) (7, 9, 12, 14). It was shown that AtCAX1, AtCAX3, and AtCAX4 specifically transport Ca\(^{2+}\), whereas AtCAX2 transports Ca\(^{2+}\), Mn\(^{2+}\), and Cd\(^{2+}\) (7, 9, 14). In these vacuolar type CAXs, the presence of an N-terminal autoinhibition domain and a 9-amino-acid region required for Ca\(^{2+}\) transport (Ca\(^{2+}\) domain) has been reported (9–14). By contrast, little is known about H\(^-\)–coupled Ca\(^{2+}\) efflux antiporters. Hitherto, a plasma membrane Ca\(^{2+}/H^+\) antiporter gene (chaA) has only been isolated from Escherichia coli (5). In ChaA, neither an N-terminal autoinhibition domain nor a 9-amino-acid region was reported. ChaA has been shown to catalyze both Na\(^+/H^+\) and Ca\(^{2+}/H^+\) exchange reactions at alkaline pH (16). Essentially nothing is known about molecular properties of Ca\(^{2+}/H^+\) antiporters from other organisms, especially those on plasma membranes.

Recent genome sequences of cyanobacteria, Synechocystis sp. PCC 6803, Anabaena sp. PCC 7120, and Thermosynechococcus elongatus BP-1 suggest the presence of a single putative Ca\(^{2+}/H^+\) antiporter gene (17–19). Cyanobacteria are oxygen-evolving photosynthetic prokaryotes that can acclimate to a wide range of environmental changes (20, 21). Although the role of Ca\(^{2+}\) for stress responses in prokaryotic cells has not been clearly demonstrated, direct evidence of Ca\(^{2+}\) signaling in cyanobacteria has become available recently (22). Therefore, it was interesting to characterize the putative Ca\(^{2+}/H^+\) antiporter of cyanobacteria. Here, we isolated the CAX genes from Synechocystis sp. PCC 6803 (synCAX) and from a halotolerant cyanobacterium Aphanothece halophytica (apCAX). It is shown that SynCAX and ApCAX are localized on plasma membranes and catalyze the efflux of Ca\(^{2+}\), but not of Na\(^+\). The exchange activity between Ca\(^{2+}\) and H\(^-\) is essential for salt tolerance at alkaline pH in which the acidic amino acid residues in TM segments are involved.

**MATERIALS AND METHODS**

**Strains and Culture Conditions—** A. halophytica cells were grown photoautotrophically in BG11 liquid medium plus 18 mM NaNO\(_3\) and Turk Island salt solution at 28 °C as described previously (23, 24). Synechocystis sp. PCC 6803 and Synechococcus sp. PCC 7942 cells were grown...
Ca^{2+}/H^{+} Antiporter from Cyanobacteria

| Primers | 5’-3’ | Base pairs |
|---------|-------|-----------|
| ApCa/H-F1 | TTAATGGTTAATAAATACGATCCTTTT | 28 mer |
| ApCa/H-R1 | CTTGACCACCCATTTTGCAAGAACCA | 27 mer |
| SynCa/H-F1 | AACGCTGCCATGAACTGTTTCA | 25 mer |
| SynCa/H-R1 | ACGGATCCACGCTGTTGGAAGAA | 27 mer |
| pACYCmcl-F | ATGGATCCCAAAAGTAAGATTTTTCTTG | 28 mer |
| pACYCmcl-R | GTGGATCCATCCTGGCGATCGCCAT | 27 mer |
| E74DQH-R | E74DQH-F | 27 mer |
| E74DQH-F | GTGAGCTGACGCGCCSMGTTATTCGGT | 29 mer |
| E74DQH-R | GCTTGGGCTAGATGACGGCGCGG | 29 mer |
| ApCa/H-R1 | AATCTTCTTATTTAGTGCAAGACGGCA | 28 mer |
| ApCa/HproR | CGATCTGCTCACTAATKAANAGGATT | 28 mer |
| ApCa/HproF | AACATGGCAGCCTTTGTTGGTTAAGT | 28 mer |
| ApCa/HproR | GCCATGATGACGCGCCSMGTTATTCGGT | 28 mer |
| SynCa/HproF | TGGATGCTCAGCTTGGTGAAGGCTACCAATAG | 30 mer |
| SynCa/HproR | GGTATCCATCTGGGCTAGGGCGCAT | 25 mer |
| HisBamHI | GGTATCCATCTGGGCTAGGGCGCAT | 25 mer |
| HisBamHI | GGTATCCATCTGGGCTAGGGCGCAT | 25 mer |

grown at 30 °C under continuous fluorescent white light (40 microeinsteins m⁻² s⁻¹) in BG11 liquid medium supplemented with 10 mM HEPES-KOH and buffered with 3% CO₂. The cells with the disrupted synCAX gene were grown in the same conditions as the wild type except for supplementation with chloramphenicol (150 μg/ml). E. coli TO114 cells were grown at 37 °C in LBK medium or Tris-E medium (25). Ampicillin, erythromycin, kanamycin, and chloramphenicol were used at final concentrations of 50, 160, 30, and 30 μg ml⁻¹, respectively.

Construction of Expression Vectors for ApCAX and SynCAX—The apCAX gene was amplified from genomic DNA of A. halophytica by the primer set, ApCa/H-F1 and ApCa/H-R1. The sequences of all the primers are shown in Table I. The synCAX (slr1336) was amplified from genomic DNA of Synechocystis sp. PCC 6803 by the primer set, SynCa/H-F1 and SynCa/H-R1. PCR products for apCAX and synCAX were ligated into the EcoRV restriction site of pBSK⁺ (Stratagene, La Jolla, CA) and sequenced. The resulting plasmids were designated as pApCAX-SK, pSynCAX-SK, respectively. The plasmid pApCAX-SK was digested with NcoI and BamHI, whereas plasmid pSynCAX-SK was digested with BamHI. The resulting fragments were ligated into the E. coli vector pUC184 (New England Biolabs, Beverly, MA) as a template. The PCR product was subcloned into the EcoRV restriction site of pBSK⁺ and then digested with HindIII and Smal of pBSK⁺. The blunt-ended fragment of mlw⁺ was ligated into the NcoI site at position 543 bp of synCAX in pSynCAX-SK⁺, which was prepared by partial digestion with NcoI and blunting. Insertion of the cmr cassette into the correct site was confirmed by DNA sequencing. The cmr-containing synCAX was transferred to Synechocystis sp. PCC6803 by electroporation (500 V, 45 ohms, and 125 microfarads) using an Electrocell Manipulator (model 600 M, BTX). The disrupted mutants were selected on BG11 medium containing 0.5% agar supplemented with chloramphenicol at a final concentration of 150 μg/ml.

Detection of Ca^{2+} Efflux and Membrane Potential—To prepare the Ca^{2+}-loading cells, both the wild type and disrupted cells were incubated for 1 h with 50 mM Ca^{2+} in buffer A (20 mM HEPES and 140 mM KCl) at various pH values (30, 31). Ca^{2+}-loaded cells were diluted 10-fold in buffer A and then transferred to the assay medium containing Ca^{2+} indicator (2 mM) (20 mM HEPES, pH 7.2, 3 mM MgSO₄, 27 mM arsenazo III) (21). Ca^{2+} efflux from the SynCAX cells was measured by excitation at 620 nm and emission at 670 nm. The membrane potential of Synechocystis cells was assayed with the potential-sensitive cyanide dye diS-C₅(5) (31). The assay medium (1 ml) had the same composition as that used for the detection of Ca^{2+} efflux, but it was supplemented with 1 μM diS-C₅(5). The fluorescence was thereby quenched. Upon the addition of salt, fluorescence increased due to the excretion of H⁺ by the antiporters and consequent leakage of the dye. The changes in fluorescence (ΔF) were measured by excitation at 650±70 nm. The fluorescence was measured by excitation at 620 nm and emission at 670 nm with a Shimadzu RF-5300PC spectrofluorophotometer.

Other Methods—The nucleotide sequences were determined using an ABI310 genetic analyzer (Applied Biosystems, Foster City, CA). Cellular ions were determined by Shimadzu Personal Ion Analyzer PIA-1000. SDS-PAGE and Western blotting analysis were carried out as described previously (27, 28). An antibody raised against His₆-His₆ (His₆-His tag) was obtained from R&D systems (Minneapolis, MN). Protein was determined by Lowry’s method as described (27). Chlorophyll a was extracted by 90% methanol in dim light and calculated from the absorbance at 665 nm (21, 32). For the preparation of phycobiliproteins, Synechocystis sp. PCC6803 cells were sonicated, streptomycin sulfate (1%) was added, and homogenate was centrifuged (32). The resulting supernatant, the phycobiliprotein content was determined spectrophotometrically. Plasma membranes were prepared by a discontinuous sucrose density gradient centrifugation method (33).

Computer Analysis—The hydrophobic profile of proteins was predicted by the computer-assisted procedure according to the method of Kyte and Doolittle (34). The possible TM structure of ApCAX was predicted by the computer program TopPredII (35).

RESULTS
Cloning of Putative Ca^{2+}/H^{+} Antiporter Genes from A. halophytica and Synechocystis Cells—From the shotgun clones of A. halophytica, one open reading frame homologous to the synCAX was isolated. Its gene was isolated by a PCR method and sequenced as described under “Materials and Methods.” The predicted gene product ApCAX consists of 373 amino acids with a molecular mass of 39,710 Da (Fig. 1A). The ClustalW analysis of seven kinds of CAXs (Fig. 1B) showed highest homology to the CAX from Synechocystis sp. PCC6803 (SynCAX) (69%) and then to vacuolar antiporters from Saccharomyces cerevisiae (ScVCX1) (43%), Neurospora crassa (NeCAX) (40%), Vigna ra-

Table I

| Primers | 5’-3’ | Base pairs |
|---------|-------|-----------|
| ApCa/H-F1 | TTAATGGTTAATAAATACGATCCTTTT | 28 mer |
| ApCa/H-R1 | CTTGACCACCCATTTTGCAAGAACCA | 27 mer |
| SynCa/H-F1 | AACGCTGCCATGAACTGTTTCA | 25 mer |
| SynCa/H-R1 | ACGGATCCACGCTGTTGGAAGAA | 27 mer |
| pACYCmcl-F | ATGGATCCCAAAAGTAAGATTTTTCTTG | 28 mer |
| pACYCmcl-R | GTGGATCCATCCTGGCGATCGCCAT | 27 mer |
| E74DQH-R | E74DQH-F | 27 mer |
| E74DQH-F | GTGAGCTGACGCGCCSMGTTATTCGGT | 29 mer |
| E74DQH-R | GCTTGGGCTAGATGACGGCGCGG | 29 mer |
| ApCa/H-R1 | AATCTTCTTATTTAGTGCAAGACGGCA | 28 mer |
| ApCa/HproR | CGATCTGCTCACTAATKAANAGGATT | 28 mer |
| ApCa/HproF | AACATGGCAGCCTTTGTTGGTTAAGT | 28 mer |
| ApCa/HproR | GCCATGATGACGCGCCSMGTTATTCGGT | 28 mer |
| SynCa/HproF | TGGATGCTCAGCTTGGGCTAGGGCGCAT | 30 mer |
| SynCa/HproR | GGTATCCATCTGGGCTAGGGCGCAT | 25 mer |
| HisBamHI | GGTATCCATCTGGGCTAGGGCGCAT | 25 mer |
| HisBamHI | GGTATCCATCTGGGCTAGGGCGCAT | 25 mer |
FIG. 1. Comparison of the deduced amino acid sequences of Ca\(^{2+}\)/H\(^+\) antiporters. A, alignment of the deduced amino acid sequences of Ca\(^{2+}\)/H\(^+\) antiporters from seven organisms. The sequences were aligned by the program ClustalW. The amino acid residues conserved in all sequences are shown by a star, and conservative substitutions are shown by a dot. Predicted membrane-spanning regions are marked above the sequences.

B) Phylogenetic tree
from N. crassa could not grow if the medium contained 100 mM CaCl$_2$ (Fig. 1A). The growth rates of the cells transformed with the control cells and SynNhaP1-expressing cells could not grow in the absence of the vacuolar CAX. It would be noted that ApCAX showed lower homology to E. coli ChaA than it showed to vacuolar CAXs. It is also evident that vacuolar CAXs have longer N-terminal sequences than those of prokaryotic CAXs (Fig. 1A). Analysis of the hydropathy plot and the TM prediction program predicted 11 putative TM-spanning segments in these CAXs (Fig. 1A, A and C). All CAXs contain a central hydrophilic motif, rich in acidic amino acid residues (Fig. 1, A and C).

Construction of Expression Vectors for apcAX and synCAX, and Their Expression in E. coli—Expression vectors for ApCAX and SynCAX were constructed as described under "Materials and Methods" (Fig. 2A). Western blotting analysis indicated that ApCAX and SynCAX could be expressed in E. coli with reasonable size and similar expression levels, which would provide the basis for functional comparison of these CAXs (Fig. 2B).

Complementation Test in E. coli TO114—It has been reported that plant vacuolar CAXs such as AtCAX1–4 could not complement the Ca$^{2+}$-sensitive phenotype of a yeast vacuolar mutant deleted of the vacuolar Ca$^{2+}$-ATPase and Ca$^{2+}$/H$^+$ antipporter (ScVCX1) when expressed at full length (7, 9, 12, 14) but did complement the yeast mutant when expressed with an N-terminal deletion (7, 9, 12–14). In AtCAX1, the autoinhibition domain contains 36 amino acid residues. Therefore, we examined whether ApCAX and SynCAX could complement the salt-sensitive phenotype of E. coli mutant TO114 cells. Due to the absence of the nhaA, nhaB, and chaA genes, TO114 cells could not grow if the medium contained 100 mM CaCl$_2$ (Fig. 3A), 200 mM NaCl (Fig. 3B), or 4 mM LiCl (Fig. 3C) (16, 26, 27). However, the TO114 cells expressing ApCAX and SynCAX could grow on solid medium containing 100 mM CaCl$_2$, whereas the control cells and SynNhaP1-expressing cells could not grow (Fig. 3A). The growth rates of the cells transformed with ApCAX, SynCAX, and empty vector in the liquid medium were in this order (Fig. 3D). These results indicate that ApCAX and SynCAX complemented the Ca$^{2+}$-sensitive phenotype of E. coli mutant.

Fig. 3B shows that the cells expressing ApCAX and SynCAX could not grow in the presence of 200 mM NaCl, whereas the cells expressing SynNhaP1 could grow under the same conditions, which is consistent with the results of a previous report (27). Essentially similar results were obtained in the case of LiCl (Fig. 3C). These data indicate that ApCAX and SynCAX catalyze Ca$^{2+}$/H$^+$ exchange but not Na$^+$/H$^+$ or Li$^+$/H$^+$ exchange reactions. Ca$^{2+}$/H$^+$ Antipporter Activity—Next, as shown in Fig. 4, we examined exchange activities using everted membrane vesicles of TO114 (26, 27). Exchange activity between Ca$^{2+}$ and H$^+$ was observed in the cells expressing ApCAX and SynCAX with ApCAX exhibiting higher exchange activity than SynCAX. The pH optimum of exchange activity for ApCAX was 8.8, whereas that of SynCAX was 8.0. These results are consistent with the complementation tests (Fig. 3). When choline was replaced with KCl in the reaction buffer, the exchange activity increased, especially at alkaline pHs, suggesting an effect of K$^+$ on Ca$^{2+}$ binding and/or transport. Exchange activity between

alignment. The central hydrophilic motif rich in acidic amino acid residues in AtCAX1 is boxed. The N-terminal autoinhibition domain in AtCAX1 is underlined. B, phylogenetic analysis of seven Ca$^{2+}$/H$^+$ antipporters. Multiple sequence alignment and generation of a phylogenetic tree were performed with ClustalW and TreeView software, respectively. C, hydropathy plots of ApCAX, SynCAX, AtCAX1, and ChaA. Hydropathy was computed, and the central hydrophilic motif rich in acidic residues is underlined. The accession numbers for CAXs are GenBank$^{25}$ D90912 from Synochystris sp. PCC 6803 (SynCAX), U826603 from S. cerevisiae vacuolar antipporter (SeVCX1), AB012932 from V. radiata (VrCAX), AF053229 from N. crassa (NcCAX), AF461691 from A. thaliana (AtCAX1), and L28709 from E. coli (ChaA).
Disruption of synCAX Caused the Rapid Degradation of Pigments at Alkaline pH—To study the physiological function of CAX, the synCAX gene of Synechocystis sp. PCC 6803 was disrupted as described under “Materials and Methods.” Complete segregation of synCAX was checked by PCR (data not shown). When the wild type and synCAX-disrupted cells were grown in BG11 medium at pH 7.5, the growth rates and their phenotypes were similar (see Fig. 5, B–G). However, clear evidence of the difference in phenotypes was observed after long term incubation at alkaline pH. Cell cultures with the disrupted gene became pale green after 14 days of incubation, whereas the wild type cells retained their green color (Fig. 5A).

Under these conditions, the cell number and total soluble proteins in the wild type and disrupted cells were similar (Fig. 5, B and C). However, the levels of chlorophyll, phycocyanin, and phycobiliprotein in the disrupted cells were only about 30, 20, and 10% of the wild type cells, respectively (Fig. 5, D and E). These results indicate that SynCAX plays an important role for stabilization in pigments at alkaline pH.

Disruption of synCAX Caused Salt-sensitive Phenotype at Alkaline pH—Next, we examined the stress tolerance of synCAX-disrupted Synechocystis sp. PCC 6803 cells. Fig. 6 shows that both the wild type and disrupted cells could grow at pH 7.5 with similar rates when the concentration of NaCl in BG11 medium was as high as 0.3 M. However, when the concentration of NaCl was increased to 0.5 M, only the wild type cells could survive, although their growth rate was reduced.
exhibit the Ca\textsuperscript{2+}/H\textsuperscript{+} antiporter activity at alkaline pH. At pH 8.8, the gene-disrupted cells could not grow in BG11 medium containing 0.3 and 0.5 M NaCl, whereas the wild type cells could grow under these conditions (Fig. 6B). These results clearly indicate that the disruption of synCAX caused a salt-sensitive phenotype at alkaline pH.

**Effects of synCAX Disruption on the Efflux Activity**—Next, we examined whether the SynCAX could catalyze the efflux of Ca\textsuperscript{2+} from *Synechocystis* sp. PCC 6803 cells. After Ca\textsuperscript{2+} loading, both the wild type and gene-disrupted cells were transferred to a Ca\textsuperscript{2+}-free assay medium containing the Ca\textsuperscript{2+} indicator arsenazo III as described under "Materials and Methods." Fig. 7A shows that the effluxed Ca\textsuperscript{2+} by the wild type cells was higher than that by the gene-disrupted cells. The differences between wild type and disrupted cells were larger at alkaline pH, which is consistent with the results of exchange activities (Fig. 4). We also examined the effects of synCAX disruption on the membrane potential because the electrogenic properties of a plant vacuolar Ca\textsuperscript{2+}/H\textsuperscript{+} antiporter with H\textsuperscript{+}:Ca\textsuperscript{2+} stoichiometry of 3 has been reported (36). Membrane potentials of wild type and synCAX-disrupted cells were measured by using the potential-sensitive probe diS-C\textsubscript{3}(5) (31). Fig. 7B shows that membrane potentials of the gene-disrupted cells were more negative than those of wild type cells, which is consistent with the electrogenic properties of SynCAX. These data support the view point that SynCAX catalyzes the efflux of Ca\textsuperscript{2+} in exchange for H\textsuperscript{+} with H\textsuperscript{+}:Ca\textsuperscript{2+} stoichiometry higher than 2 and diminishes the membrane potentials.

**Site-directed Mutagenesis of Glu-74 and Glu-324 in ApCAX**—The analyses of hydropathy plot and TM prediction program suggest the presence of several conserved amino acid residues in TM segments (Fig. 1A). A topological model of ApCAX is shown in Fig. 8, which indicates that Glu-74 and Glu-324 are the only conserved charged amino acid residues in TM segments. The function of acidic amino acid residues in TM has never been reported in any CAXs. Therefore, we examined the mutation of Glu-74 and Glu-324 to Asn, Gln, and His. It was found that the E74D and E74H mutants did not exhibit the Ca\textsuperscript{2+}/H\textsuperscript{+} antiporter activity at all pHs tested (Fig. 9A) and could not complement the Ca\textsuperscript{2+}-sensitive phenotype of TO114 (data not shown), whereas the E74Q mutant exhibited partial activity, 50% of the wild type. The mutants E342D, E324H, and E324Q did not exhibit Ca\textsuperscript{2+}/H\textsuperscript{+} antiporter activity at all pHs (Fig. 9B) and did not complement the Ca\textsuperscript{2+}-sensitive phenotype of TO114 (data not shown). These results indicate that not only the negative charges on Glu-74 and Glu-324, but also their side chains, are crucial for the Ca\textsuperscript{2+}/H\textsuperscript{+} exchange activity.
We isolated putative Ca\textsuperscript{2+}/H\textsuperscript{+} antiporter genes homologous to plant vacuolar ones from a halotolerant cyanobacterium *A. halophytica* (*apCAX*) and *Synechocystis* sp. PCC 6803 (*synCAX*). Based on the findings that the antiporter-deficient *E. coli* TO114 mutant cells became Ca\textsuperscript{2+}-tolerant by transformation with the *apCAX* and *synCAX* genes (Fig. 3), by the direct observation of Ca\textsuperscript{2+}/H\textsuperscript{+} antiporter activity in the membrane vesicles of transformants (Fig. 4), by the reduced Ca\textsuperscript{2+} efflux activity of *synCAX*-disrupted cells (Fig. 7), and by immunoblotting analysis (Fig. 10), it was concluded that the putative genes, *apCAX* and *synCAX*, encode the plasma membrane Ca\textsuperscript{2+}/H\textsuperscript{+} antiporters. The most striking physiological function of ApCAX and SynCAX is in their roles for salt tolerance (Figs. 6 and 10).

Hydropathy profiles, polypeptide size (about 400 amino acid residues), and hydrophilic acidic motif in the central region are conserved among CAXs from plant, yeast, cyanobacteria, and *E. coli* (Fig. 1, A and C), suggesting that the transport mechanisms of these CAXs might be similar. The facts that ApCAX and SynCAX are more homologous to the plant CAXs than to *E. coli* ChaA (Fig. 1B) suggest that plant CAXs were evolved from cyanobacteria CAX and that the N-terminal regulatory domain and the Ca\textsuperscript{2+} domain were evolved later.

The results of Figs. 3 and 4 indicate that both full-length ApCAX and full-length SynCAX could complement the Ca\textsuperscript{2+}-sensitive phenotype of TO114 and exhibited the Ca\textsuperscript{2+}/H\textsuperscript{+} exchange activity, which is different from that of plant CAXs. Since a stop codon appeared just before the first Met of ApCAX, it is unlikely that the Ca\textsuperscript{2+}/H\textsuperscript{+} antiporter from *A. halophytica* encodes a longer polypeptide (data not shown). These facts indicate that ApCAX and SynCAX are both active, at least when expressed in *E. coli*. Results on the plant vacuolar AtCAX1 indicated that the exchange activity of AtCAX1 is regulated by protein factors (14). From the present data, the regulatory mechanisms of cyanobacterial CAXs are unknown. One possibility is that of regulation by non-protein factors, such as membrane potentials, since the Ca\textsuperscript{2+} efflux by SynCAX affected the membrane potential (Fig. 7). Unlike *Arabidopsis* with its 11 putative CAX genes (2), cyanobacteria have only a single CAX gene (17–19), which might be more suitable for studying the physiological function of CAX. The role of H\textsuperscript{+}-coupled Ca\textsuperscript{2+} efflux by CAX in Ca\textsuperscript{2+} homeostasis in cyanobacteria is an important subject to be clarified.
hitherto, the N-terminal regulatory domain (1–36 amino acid residues) and the Ca\(^{2+}\) domain (89–95 amino acid residues in the loop connecting TM5 and TM6) have been reported in AtCAXs (9–14). However, these domains seem to be absent in ApCAX and SynCAX, and no information was available previously on the specific amino acid residues in the TM region needed for exchange activity. Here, we have shown that two acidic amino acid residues, Glu-74 and Glu-324, are involved in cation binding and/or transport of the cations, Ca\(^{2+}\) and H\(^{+}\). Putative TM segments of Glu-74 and Glu-324 are TM3 and TM10, respectively, and are distributed symmetrically around the central TM segments, TM6 and TM7 (Fig. 8). Recently, it has been proposed that the Na\(^{+}/\text{Ca}^{2+}\) exchanger has a two-domain structure, each with five TM segments with opposite membrane topologies (37). How these acidic amino acid residues, Glu-74 and Glu-324, are involved in cation binding and/or transport is an interesting question to be tested.

Figs. 6 and 10 indicate that cyanobacteria CAXs are involved in salt tolerance at alkaline pH. This conclusion was obtained from the facts that disruption of synCAX caused a salt-sensitive phenotype at alkaline pH (Fig. 6) and overexpression of ApCAX and SynCAX enhanced the salt tolerance (Fig. 10). A simplistic explanation for these facts is that circulation of Ca\(^{2+}\) via Ca\(^{2+}\) efflux is important. Its inhibition causes the salt-sensitive phenotype. In addition to the efflux of Ca\(^{2+}\), maintaining a neutral pH inside cells would be important for salt tolerance at alkaline pH. Due to the electronegative properties of the Ca\(^{2+}/\text{H}^{+}\) antiporter, overexpression of the Ca\(^{2+}/\text{H}^{+}\) antiporter would confer the salt tolerance to cyanobacteria.

The data of Fig. 3 show that the complementability of ApCAX is more effective than that of SynCAX. This finding would suggest an interesting application of ApCAX for the genetic engineering of salt-tolerant plants. Previously, we showed that the DnaK (38) and NhaP Na\(^{+}/\text{H}^{+}\) antiporter (26) from A. halophytica exhibited unique properties absent in the homologous genes from freshwater cyanobacteria. Transfer of these genes to plants and a cyanobacterium significantly improved their stress tolerance (21, 39). Enhanced salt tolerance by introducing the vacuolar Na\(^{+}/\text{H}^{+}\) antiporter into plants has also been reported (40). Further studies on transgenic plants using ApCAX and NhaP Na\(^{+}/\text{H}^{+}\) antiporter from A. halophytica would enable us to obtain improved salt-tolerant plants.

Acknowledgments—We thank Dr. Andre T. Jagendorf for critical reading of manuscript. We thank Eiko Tsunekawa for expert technical assistance.

REFERENCES

1. Sze, H., Liang, F., Hwang, I., Curran, A. C., and Harper, J. F. (2000) Annu. Rev. Plant Physiol. Plant Mol. Biol. 51, 433–4621.

2. Maser, P., Thomine, S., Schroeder, J. I., Ward, J. M., Hirschi, K., Sze, H., Tazkia, I. N., Amstmann, A., Maathuis, F. J., Sanders, D., Harper, J. F., Tschui, J., Griswold, M., Persans, M. W., Salt, D. E., Kim, S. A., and Gueriniot, M. L. (2000) Plant Physiol. 126, 1646–1667.

3. Gasiora, R. A., Fink, G. R., and Hirschi, K. D. (2002) Plant Physiol. 129, 967–973.

4. Shiokaki, T., Pittman, J. K., Cheng, N. H., and Hirschi, K. D. (2001) J. Biol. Chem. 276, 43152–43159.

5. Ivey, D. M., Guffanti, A. A., Zemskys, J., Carpen, E., Padan, E., Schubert, S., and Kruwich, A. J. (1995) J. Biol. Chem. 268, 11286–11303.

6. Cunningham, K. W., and Fink, G. R. (1996) Mol. Cell. Biol. 16, 2226–2237.

7. Hirschi, K. D., Zhou, E. G., Cunningham, K. W., Rea, P. A., and Fink, G. R. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 8782–8786.

8. Ueoka-Nakanishi, H., Nakanishi, Y., Tanaka, Y., and Maeshima, M. (1999) Eur. J. Biochem. 262, 417–425.

9. Hirschi, K. D., Korenkev, Y. D., Wilanowski, N. L., and Wagner, G. J. (2000) Plant Physiol. 124, 125–1338.

10. Pittman, J. K., and Hirschi, K. D. (2001) Plant Physiol. 127, 1020–1029.

11. Pittman, J. K., Shiokaki, T., and Cheng, N. H., and Hirschi, K. D. (2002) J. Biol. Chem. 277, 26452–26459.

12. Cheng, N. H., and Hirschi, K. D. (2000) J. Biol. Chem. 275, 12452–12524.

13. Pittman, J. K., Sreeviya, C. S., Shiokaki, T., Ueoka-Nakanishi, H., and Hirschi, K. D. (2002) Plant Physiol. 130, 1054–1062.

14. Cheng, N. H., and Hirschi, K. D. (2000) J. Biol. Chem. 278, 6503–6509.

15. Cheng, N. H., and Hirschi, K. D. (2000) Plant Physiol. 15, 347–364.

16. Oyama, T., Igarashi, K., and Kobayashi, H. (1995) J. Bacteriol. 176, 4311–4315.

17. Kaneko, T., Sato, S., Kato, H., Tanaka, A., Amasimuzu, K., Nakanuma, Y., Miyajima, N., Hirose, M., Sugiura, M., Sasamoto, S., Kimura, T., and Hiraishi, K. D.
18. Kaneko, T., Nakamura, Y., Wolk, C. P., Kuritz, T., Sasamoto, S., Watanabe, A., Iriguchi, M., Kawashima, K., Kimura, T., Kishida, Y., Kohara, M., Matsumoto, M., Matsuno, A., Nakazaki, N., Shimpo, S., Sugimoto, M., Takeuchi, C., Yamada, M., Yasuda, M., and Tabata, S. (2001) DNA Res. 31, 205–213

19. Nakamura, Y., Kaneko, T., Sato, S., Ikeuchi, M., Katoh, H., Sasamoto, S., Watanabe, A., Iriguchi, M., Kawashima, K., Kimura, T., Kishida, Y., Kiyokawa, C., Kohara, M., Matsumoto, M., Matsuno, A., Nakazaki, N., Shimpo, S., Sugimoto, M., Takeuchi, C., Yamada, M., and Tabata, S. (2002) DNA Res. 31, 123–130

20. Marin, K., Suzuki, I., Yamaguchi, K., Ribbeck, K., Yamamoto, H., Kanesaki, Y., Hagemann, M., and Murata, N. (2003) Proc. Natl. Acad. Sci. U. S. A. 100, 9061–9066

21. Waditee, R., Hibino, T., Nakamura, T., Incharoenakdi, A., and Takabe, T. (2002) Proc. Natl. Acad. Sci. U. S. A. 99, 4109–4114

22. Torrecilla, I., Leganes, F., Bonilla, I., and Fernandez-Pinas, F. (2000) Plant Physiol. 123, 161–176

23. Ishitani, M., Takabe, T., Kojima, K., and Takabe, T. (1993) Aust. J. Plant Physiol. 20, 693–703

24. Hibino, T., Kaku, N., Yoshikawa, H., Takabe, T., and Takabe, T. (1999) Plant Mol. Biol. 40, 409–418

25. Brockman, R. W., and Heppel, L. A. (1968) Biochemistry 7, 2554–2562

26. Waditee, R., Hibino, T., Tanaka, Y., Nakamura, T., Incharoenakdi, A., and Takabe, T. (2001) J. Biol. Chem. 276, 36931–36938

27. Hamada, A., Hibino, T., Nakamura, T., and Takabe, T. (2001) Plant Physiol. 125, 437–446

28. Waditee, R., Hibino, T., Tanaka, Y., Nakamura, T., Incharoenakdi, A., Hayakawa, S., Futsuhara, Y., Kawamitsu, Y., Takabe, T., and Takabe, T. (2002) J. Biol. Chem. 277, 18373–18382

29. Nakamura, T., Komano, Y., and Unemoto, T. (1995) Biochim. Biophys. Acta 1230, 170–176

30. Tsujibo, H., and Rosen, B. P. (1983) J. Biol. Chem. 154, 854–858

31. Nazarenko, L. V., Andreev, I. M., Lyukevich, A. A., Pisareva, T. V., and Los, D. A. (2003) Microbiology (Read.) 149, 1147–1153

32. Tandeau de Marsac, N., and Houmard, J. (1988) Methods Enzymol. 167, 318–328

33. Murata, N., and Omata, T. (1988) Methods Enzymol. 167, 245–251

34. Kyte, J., and Doolittle, R. F. (1982) J. Mol. Biol. 157, 105–132

35. Hofmann, K., and Stoffel, W. (1992) Comput. Appl. Biosci. 8, 331–337

36. Blackford, S., Rea, P. A., and Sanders, D. (1990) J. Biol. Chem. 265, 9617–9620

37. Saaf, A., Baars, L., and von Heijne, G. (2001) J. Biol. Chem. 276, 18905–18907

38. Lee, B. H., Hibino, T., Je, J., Viale, A. M., and Takabe, T. (1997) Plant Mol. Biol. 35, 763–775

39. Ono, K., Hibino, T., Kishinata, T., Suzuki, S., Tanaka, Y., Nakamura, T., Takabe, T., and Takabe, T. (2001) Plant Sci. (Limerick) 160, 455–461

40. Zhang, H.-X., and Blumwald, E. (2001) Nat. Biotechnol. 98, 12832–12836
Isolation and Functional Characterization of Ca\(^{2+}/H^+\) Antiporters from Cyanobacteria

Rungaroon Waditee, Gazi Sakir Hossain, Yoshito Tanaka, Tatsunosuke Nakamura, Masamitsu Shikata, Jun Takano, Tetsuko Takabe and Teruhiro Takabe

*J. Biol. Chem.* 2004, 279:4330-4338.
doi: 10.1074/jbc.M310282200 originally published online October 14, 2003

Access the most updated version of this article at doi: 10.1074/jbc.M310282200

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 38 references, 21 of which can be accessed free at http://www.jbc.org/content/279/6/4330.full.html#ref-list-1