Plant-Specific Cation/H\(^+\) Exchanger 17 and Its Homologs Are Endomembrane K\(^+\) Transporters with Roles in Protein Sorting*\(^{1,2,3,§, ‡, †}

Received for publication, May 5, 2011, and in revised form, July 8, 2011. Published, JBC Papers in Press, July 27, 2011, DOI 10.1074/jbc.M111.252650

Salil Chanroj\(^{3}\), Yongxiong Lu (路永憲)\(^{4,1}\), Senthilkumar Padmanaban\(^{4}\), Kei Nanatani (七谷圭)\(^{3}\), Nobuyuki Uozumi (魚住信之)\(^{5}\), Rajini Rao\(^{6}\), and Heven Sze (斯海文)\(^{2,7}\)

From the \(^{1}\)Department of Cell Biology and Molecular Genetics and the Maryland Agricultural Experiment Station, University of Maryland, College Park, Maryland 20742, the \(^{2}\)Department of Physiology, Johns Hopkins University School of Medicine, Baltimore, Maryland 21205, and the \(^{3}\)Department of Biomolecular Engineering, Graduate School of Engineering, Tohoku University, Sendai 980-8579, Japan

The complexity of intracellular compartments in eukaryotic cells evolved to provide distinct environments to regulate processes necessary for cell proliferation and survival. A large family of predicted cation/proton exchangers (CHX), represented by 28 genes in Arabidopsis thaliana, are associated with diverse endomembrane compartments and tissues in plants, although their roles are poorly understood. We expressed a phylogenetically related cluster of CHX genes, encoded by CHX15–CHX20, in yeast and bacterial cells engineered to lack multiple cation-handling mechanisms. Of these, CHX16–CHX20 were implicated in pH homeostasis because their expression rescued the alkaline pH-sensitive growth phenotype of the host yeast strain. A smaller subset, CHX17–CHX19, also conferred tolerance to hygromycin B. Further differences were observed in K\(^+\)-and low pH-dependent growth phenotypes. Although CHX17 did not alter cytoplasmic or vacuolar pH in yeast, CHX20 elicited acidification and alkalization of the cytosol and vacuole, respectively. Using heterologous expression in Escherichia coli strains lacking K\(^+\) uptake systems, we provide evidence for K\(^+\) (\(^{86}\)Rb) transport mediated by CHX17 and CHX20. Finally, we show that CHX17 and CHX20 affected protein sorting as measured by carboxypeptidase Y secretion in yeast mutants grown at alkaline pH. In plant cells, CHX20-RFP co-localized with an endoplasmic reticulum marker, whereas RFP-tagged CHX17–CHX19 co-localized with prevacuolar compartment and endosome markers. Together, these results suggest that in response to environmental cues, multiple CHX transporters differentially modulate K\(^+\) and pH homeostasis of distinct intracellular compartments, which alter membrane trafficking events likely to be critical for adaptation and survival.

The dynamic endomembrane system of eukaryotic cells is emerging as a critical and central coordinator in cell development, growth, signaling, and adaptation to stress (1, 2). Recent studies have illustrated the increasing complexity of intracellular compartments that sort proteins and membranes in the anterograde or retrograde direction through the biosynthetic and/or the endocytic pathway. Such compartments probably evolved to provide distinct environments in a temporal manner for specific biochemical reactions and protein-protein interactions necessary for cell proliferation and survival (3), yet the mechanisms that regulate the internal and external environment of these endomembrane compartments in plants are not well understood. In plants, the dynamic endomembrane affects cell polarity, cytokinesis, cell wall formation and stress tolerance (1, 4).

Endomembranes of eukaryotic cells, including those from yeast, mammals, and plants, share several transporters in common, indicating that the mechanisms controlling the physico-chemical environment of diverse intracellular compartments are conserved in general. The most prominent is the H\(^+-\)pumping vacuolar ATPase, which acidifies intracellular compartments, including yeast (5) and plant vacuoles (6), trans-Golgi network (7), clathrin-coated vesicles, lysosomes, and endosomes of diverse animal cells (8). Because the V-ATPase is an electrogenic H\(^+\)-pump, acidification of membrane vesicles occurs only if counter ions, such as anions, enter to dissipate the electrical potential (9). Several alkali-cation/proton exchangers belonging to the cation-proton antiporter 1 (CPA1) family have been found in endomembranes of eukaryotes (2, 10, 11). Examples include vacuolar ScNHX1 of yeast, six Na\(^+/\)H\(^+\) exchanger (NHX)\(^{3}\) genes in Arabidopsis, and NHE6 to -9 in humans. Alkali-cation/proton exchangers provide a leak pathway for H\(^+\) and thus modulate luminal pH and cation homeostasis within the endomembrane system, where they have profound effects on vesicle biogenesis and trafficking in cells (2).

In plants, NHX1 represents the best characterized member of the CPA1 family. NHX1 was initially characterized as a vac-

* This work was supported, in whole or in part, by National Institutes of Health Grant R01DK054214 (to R.R.). This work was also supported by National Science Foundation Grant IBN0209788 and United States Department of Energy Grant DEFG0207ER15883 (to H.S.). Japan Society for the Promotion of Science Grants-in-Aid for Scientific Research 22020002 and 22380056 (to N.U.), and a Royal Thai Government Fellowship (to S.C.).

† Present address: Carnegie Inst. for Science, Dept. of Plant Biology, 260 Panama St., Stanford, CA 94305.

‡ To whom correspondence should be addressed: 0120 Bioscience Research Bldg. (413), Dept. of Cell Biology and Molecular Genetics, University of Maryland, College Park, MD 20742. Tel.: 301-405-1645; Fax: 301-314-1248; E-mail: hsze@umd.edu.

§ This work was supported, in whole or in part, by National Institutes of Health Grant R01DK054214 (to R.R.). This work was also supported by National Science Foundation Grant IBN0209788 and United States Department of Energy Grant DEFG0207ER15883 (to H.S.). Japan Society for the Promotion of Science Grants-in-Aid for Scientific Research 22020002 and 22380056 (to N.U.), and a Royal Thai Government Fellowship (to S.C.).

Supplemental Material can be found at: http://www.jbc.org/content/suppl/2011/07/27/M111.252650.DC1.html

The on-line version of this article (available at http://www.jbc.org) contains supplemental Tables S1–S10, Figs. S1–S10, and additional methods and references.

© 2011 by The American Society for Biochemistry and Molecular Biology, Inc. Printed in the U.S.A.
uolar Na+/H+ exchanger, although later findings broadened cation selectivity to include K+ (11–15). In addition to vacuole and prevacuolar endosomes, plant NHX isoforms are found in other endomembranes. Tomato LeNHX2, a homolog of AtNHX5/6, is a K+-selective H+ exchanger localized to the Golgi and endosomes (15). Recently, AtNHX5 and AtNHX6 were localized to Golgi or the trans-Golgi network and shown to play a role in cell expansion because double mutants were dwarfed (16). In addition, NHX7/SOS1, a plasma membrane member, is important for conferring salt tolerance probably by controlling long distance Na+ transport (17). These findings show that members of the plant NHX family modulate Na+ and K+ homeostasis at various endomembrane compartments as well as the plasma membrane.

Given the multiplicity of plant NHX genes, it was surprising to discover a large family of putative cation/H+ exchangers (CHXs) (18) in a distinct CPA2 superfamily, sharing sequence similarity to bacteria NhaA and KefB (10). Intriguingly, most of the eukaryotic genes in CPA2 come from plants and fungi (10, 18). Metazoan genomes each carry only two CPA2 members, the eukaryotic genes in CPA2 come from plants and fungi (10, 18). Yeast, Bacterium, and Plant—Saccharomyces cerevisiae (Saccharomyces cerevisiae yeast) (19). In striking contrast, Arabidopsis thaliana (Arabidopsis thaliana) plants were grown on Miracle-Gro/ H9004 strain LB2003 (22, 25) was grown in YPAD medium. Five ml of serial dilution was spotted onto YNB or SDAP medium with 2% glucose at varied pH and K+ concentration was monitored at 30 °C for 3–5 days. Drop test media had 20 mM MES, and pH was adjusted to 7.5 with arginine or to acidic pH values with glutamic acid. K+ concentration was adjusted with KCl.

pH对外部和pHvac Determination—To monitor cytosolic pH, we used a pH-sensitive GFP variant, superecliptic pHiIuorin (GenBank accession number A533296) from Dr. Miesenbock (Yale University). A destination vector (pDYpH) harboring pHiIuorin was generated with or without a CHX gene by PCR-directed in vivo homologous recombination in yeast (28) (supplemental Fig. S2A) using primers shown in supplemental Table S8. To measure cytosolic pH, an aliquot of normalized cells was mixed with YNB medium supplemented with 2% glucose at varied pH and K+ concentration. The plate was immediately placed in a multimode plate reader (BMG FLUOstar Optima model, BMG Labtechnologies) set to 30 °C. Emission at 460 nm (F460) and 510 nm (F510) was read using excitation at 400 nm. F510/F460 was converted to pH units using a pH calibration curve as described by Brett et al. (29).

To estimate vacuolar pH (30), cells were loaded with 50 μM 2',7'-bis(2-carboxyethyl)-5(6)-carboxyfluorescein acetoxymethyl ester (BCECF) (B8806, Sigma) and mixed with YNB medium supplemented with 2% glucose at varied pH and K+ concentration. The ratio of fluorescence at 530 nm was determined at F490/F530 and converted to pH units using a pH calibration curve (see supplemental Methods).

Measuring Carboxypeptidase Y Secretion—Twenty μl of normalized cells was added to a white opaque ELISA plate (15042, Pierce) containing 180 μl of YNB-galactose medium, pH 5.5, supplemented with 4 mM K2SO4 or 8 mM KOH (pH 7.3). Plates

Endomembrane K+ Transporters and Membrane Trafficking

MATERIALS AND METHODS

Yeast, Bacterium, and Plant—Saccharomyces cerevisiae strains used here are shown in supplemental Table S1. Untransformed strains were grown in YPAD medium. Escherichia coli strain LB2003 (trkAΔ, klp1Δ, kdpABCDEΔ) (22, 25) was grown in YTMK medium at pH 7.3 (supplemental Table S7B). Arabidopsis thaliana (Col-0) plants were grown on Miracle-Gro® potting mix with continuous light at 50 microeinsteins m−2 s−1, 21 °C, and 65% humidity.

cDNA Cloning and Plasmid Preparation—CHX15 to CHX19 ORFs were PCR-amplified (supplemental Table S2) from cDNA obtained by reverse transcription of seedling RNA and cloned into Gateway pDONR221 as described for CHX20 (21). CHX17 was amplified from a cDNA clone in pSMCHX17. DNA was recombined into entry and destination vectors for expression in yeast (supplemental Tables S3 and S4) using Gateway LR Clonase II Enzyme Mix (supplemental Table S5). For empty vector, a 90-bp sequence (supplemental Table S3) was inserted into Gateway pENTR/D-TOPO. Plasmid was amplified in E. coli strain DH5α (18265-017, Invitrogen) and purified with a miniprep kit (27106, Qiagen). For expression in E. coli, CHX17 and CHX20 were amplified from entry vectors, pECHX17 and pECHX20 (supplemental Table S3), using 1-proof DNA polymerase (172-5331, Bio-Rad) and suitable primers (supplemental Table S6) and subcloned in plasmid pPAB404 (26). Plasmids were amplified in E. coli NEB10B (C3019H, New England Biolabs) and purified as above.

Yeast Transformation and Functional Assays—Yeast was transformed with the lithium acetate method (27) and selected on YNB medium containing adenine and required amino acids at pH 5.8. Growth was synchronized in YNB without a carbon source unless galactose was added to induce gene expression. Cells were normalized in water or K+-free YNB to A600 of 0.2. Five μl of each serial dilution was spotted onto YNB or SDAP medium with 2% glucose or 2% galactose (supplemental Table S7A) and incubated at 30 °C for 3–5 days. Drop test media had 20 mM MES, and pH was adjusted to 7.5 with arginine or to acidic pH values with glutamic acid. K+ concentration was adjusted with KCl.

pH对外部和pHvac Determination—To monitor cytosolic pH, we used a pH-sensitive GFP variant, superecliptic pHiIuorin (GenBank accession number A533296) from Dr. Miesenbock (Yale University). A destination vector (pDYpH) harboring pHiIuorin was generated with or without a CHX gene by PCR-directed in vivo homologous recombination in yeast (28) (supplemental Fig. S2A) using primers shown in supplemental Table S8. To measure cytosolic pH, an aliquot of normalized cells was mixed with YNB medium supplemented with 2% glucose at varied pH and K+ concentration. The plate was immediately placed in a multimode plate reader (BMG FLUOstar Optima model, BMG Labtechnologies) set to 30 °C. Emission at 460 nm (F460) and 510 nm (F510) was read using excitation at 400 nm. F510/F460 was converted to pH units using a pH calibration curve as described by Brett et al. (29).

To estimate vacuolar pH (30), cells were loaded with 50 μM 2’,7’-bis(2-carboxyethyl)-5(6)-carboxyfluorescein acetoxymethyl ester (BCECF) (B8806, Sigma) and mixed with YNB medium supplemented with 2% glucose at varied pH and K+ concentration. The ratio of fluorescence at 530 nm was determined at F490/F530 and converted to pH units using a pH calibration curve (see supplemental Methods).
were incubated at 30 °C to early log phase. Cells were removed, and CPY attached to plates were determined using mouse anti-CPY (1:1000; A-6428, Invitrogen), followed by anti-mouse IgG conjugated with horseradish peroxidase (31430, Thermo Scientific). Chemiluminescence from 10 cycles was pooled. Relative luminescence units were normalized to cell density at A600 (see supplemental Methods).

E. coli Growth and 86Rb(K⁺) Transport—E. coli strain LB2003 was transformed with pPAB404 (KATJ) (31), CHX17, or CHX (20) by heat shock. Transformants were selected on YTMK or synthetic-glycerol-mannitol (SGM)-KN medium (supplemental Table S7B) with 50 μg/ml ampicillin (32). Cell growth at A600 was tested on medium with 50 μg/ml ampicillin, 0.5 mM IPTG, and varied cations or pH (see supplemental Methods). 86Rb (K⁺) transport was determined by filtration using a 0.45-μm nitrocellulose membrane (Protran BA85, Whatman). Freshly transformed cells were K⁺-depleted for 2 h and normalized. A typical reaction of 750 μl contained cells at A600 0.4 (~2.5 × 10⁸ cells/ml), 0.5 mM isopropyl β-D-thiogalactopyranoside, 0.6 mM RbCl, 86Rb (0.5 μCi/ml) in a 0.25X basal SGM with 80 mM glycerol, 10 mM MES, and 10 mM MOPS, pH 6.2. Cells and 86Rb were added to start the reaction, and an aliquot was counted to convert dpm to nmol.

Transfection of Plant Protoplast—Cells without cell walls were isolated from 3-week-old leaves and transfected by the PEG-Ca method (33, 34). Plasmids containing GFP or RFP fusion constructs are shown in supplemental Table S9. Transfected cells were incubated in the dark at 22 °C for 18–24 h. Efficiency of transformation with a single gene is ~50% and with two genes is less due to differential signals and gene expression (see supplemental Methods).

Microscopy—Fluorescent proteins in cells were examined with a Zeiss LSM510 confocal microscope (Carl-Zeiss, Germany) and C-Apochromat ×63 numerical aperture/1.2 water immersion lens. Five μl was dropped onto a slide using two 0.5-mm parafilm strips as spacers between slide and coverglass. To enhance cell density, protoplasts were concentrated >10-fold by centrifugation. Filter sets for excitation and emission are as follows: 488 nm/BP505–530 nm for GFP; 543 nm/BP560–615 nm for RFP; 543 nm/LP650 nm for chlorophyll, and 633 nm for bright field. Signals were captured in multichannel mode. Images were analyzed and processed in a Zeiss LSM image browser (Carl-Zeiss). Yeasts expressing GFP were grown overnight in YNB medium, pelleted, and incubated in YPAD for 4 h at 30 °C. Before microscopy, cells were incubated for 10 min in 10 μM FM4-64 (T-3166, Invitrogen), washed with 2% sucrose, and suspended in 0.05% agarose. The filter set for FM4-64 is 488 nm (excitation)/LP650 nm (emission).

RESULTS

Phylogenetic Relationship of AtCHX, ScKHA1, and NHXI—According to cDNA sequences, the deduced proteins CHX15–CHX20 have a hydrophobic domain with 12 transmembrane spans at the N-terminal half (427–433 residues) and a hydrophilic C tail of 372–409 residues (supplemental Fig. S1) (18). Phylogenetic analysis has revealed that CHX15–CHX20 are tightly clustered away from yeast KHA1 within the CPA2 family (Fig. 1); however, the CHX cluster and ScKHA1 are both clearly separated from the CPA1 family represented by yeast and Arabidopsis NHXs (10). Although ScKHA1 has only 38–41% overall similarity to CHX15–CHX20, the transmembrane domain shared 60% homology (supplemental Fig. S1 and Table S10). Thus, ScKHA1 is considered orthologous to AtCHX15–AtCHX20.

AtCHX16–AtCHX20 Confer Yeast Growth at Alkaline pH—We tested CHX15–CHX20 function in a panel of yeast mutants variously defective in Na⁺ extrusion and K⁺ (Na⁺)/H⁺ antiport (Fig. 2A). Cation/proton antiporters maintain cellular pH homeostasis and growth in response to large perturbations in external pH. Thus, yeast strain KTA40-2 proliferated at pH 5.6 but failed to grow at pH 7.5. We showed that KTA40-2 expressing CHX16, -17, -18, or -19 recovered tolerance to alkaline pH, similar to the AXT3 strain that expresses the endogenous ScKHA1 (Fig. 2B, right). Similar results were obtained when CHX17 or CHX20 was expressed in the single kha1Δ mutant at low K⁺ (Fig. 2E). Alterations in membrane potential and membrane trafficking significantly influence sensitivity to the cationic drug hygromycin B (35, 36). KTA40-2 was shown to be sensitive to HygB (100 μg/ml) at pH 5.6 (Fig. 2B, middle). Interestingly, only CHX17, -18, or -19 conferred resistance to the drug (Fig. 2B), similar to ScKHA1, whereas CHX15, CHX16, and CHX20 did not. CHX15 was unable to restore growth under any of the conditions tested. These results suggest that multiple CHX genes share a role in pH homeostasis, although they differ in the ability to confer hygromycin B resistance.

AtCHX17 and AtNHXI Have Distinct yet Overlapping Functions—Yeast strains with endogenous ScNHX1 but lacking ScKHA1 (LMB01) grew poorly on alkaline medium, compared with strains expressing either AtCHX17 (in KTA40-2) or ScKHA1 (AXT3) (Fig. 2C, right). Furthermore, alkaline pH sensitivity of KTA40-2 could not be rescued by Arabidopsis NHXI.
or NHX2 (Fig. 2D). Thus, CPA2 transporters (AtCHX17 and ScKHA1) were more effective than CPA1 transporters (ScNHX1, AtNHX1, and AtNHX2) in supporting yeast growth at alkaline pH. LMB01, expressing endogenous ScNHX1, clearly showed stronger resistance against HygB (Fig. 2C, middle) compared with cells expressing AtCHX17 or ScKHA1. Interestingly, cells expressing both CHX17 and endogenous ScNHX1 showed enhanced HygB resistance, suggesting an additive or cooperative effect by these two activities. These results indicate that ScKHA1 (CPA2) and ScNHX1 (CPA1) are involved in separate functions in yeast and that AtCHX17 is a functional ortholog of ScKHA1.

Growth Rescue by AtCHX16–AtCHX20 of K⁺/H⁺ Uptake-deficient Mutant Depends on pHext—LMM04, a K⁺/H⁺ uptake-deficient mutant, showed little or no growth on 8 mM KCl (Fig. 3A), although growth of TRK1/2- and TOK1-positive strains (KTA40-2 and LMB01) was vigorous (Fig. 3C), suggesting a
crucial role of $K^+$ uptake in restoring growth that was optimal at pH 5.6. Intriguingly, mutants expressing CHX17, CHX18, or CHX19 showed enhanced growth, especially at pH 7.5 (Fig. 3A). Furthermore, CHX16 expression improved mutant growth partially at 8 mM $[K^+]_{ext}$ at acidic or alkaline pH values, but growth was fully restored at pH 4.3 at 58 mM $K^+$ (Fig. 3B). However, CHX19 rescued mutant growth at pH 7.5 under 8 mM $K^+$ and also at pH 4.3 when $K^+$ was 58 mM, but it was ineffective at pH 5.6. In contrast, CHX20 enhanced growth at pH 4.3 (58 mM $K^+$) and 5.6 (8 mM $K^+$) but not at pH 7.5 (Fig. 3A). The differential effectiveness in supporting growth suggests that CHX17, -18, or -19 facilitated $K^+$ homeostasis under alkaline conditions, whereas CHX16 or CHX20 was needed under an acidic environment.

**CHX17 and CHX20 Exert Differential Effects on Cytosolic and Vacuolar pH in Yeast**—A pH calibration curve shows that the responsive range of pHluorin (37) is between pH 6.5 and 8.0 (Fig. 4A), which agrees with the apparent $pK_a$ of 7.6 for the superniclereptic pHluorin (38). Furthermore, yeast mutants co-expressing pHluorin and CHX17 or CHX20 retained their activity as shown by their ability to restore growth at alkaline pH (supplemental Fig. S2B).

We tested whether acidic pH$_{ext}$ and $[K^+]_{ext}$ might alter pH$_{cyt}$. Basal pH$_{cyt}$ of yeast strain AXT3 carrying the wild-type KHA1 or KTA40-2 strain disrupted in kha1 was estimated to be pH 7.2 after a 30-min incubation at pH 5.6 (Fig. 4B). Expression of CHX17 in KTA40-2 did not significantly change pH$_{cyt}$. However, pH$_{cyt}$ in the mutant expressing CHX20 was lowered to pH 7.06, suggesting acidification resulting from activity of CHX20. When the $K^+$ level was lowered to 0.1 or 0.8, basal pH$_{cyt}$ of kha1 mutant dropped to about pH 6.9 and 7.1, respectively (supplemental Fig. S3, A and C). Expression of either KHA1, CHX17, NHX1 (LMB01 strain), or both NHX1 and CHX17 did not change pH$_{cyt}$ significantly.

After cells were exposed to alkaline pH$_{ext}$ (Fig. 4C), the pH$_{ext}$ of all strains initially became basic (pH ~7.6); however, within 30 min, the pH$_{ext}$ of most strains reached a steady pH of 7.16 – 7.18. Similar to the acid pH shift, the mutant expressing CHX20 showed a slightly lower pH$_{ext}$ of pH 7.11, which dropped to pH 7.01 after 50 min (Fig. 4C). However, at $[K^+]_{ext}$ of 0.1 or 0.8 mM, the pH$_{ext}$ dropped from ~7.4 to 7.05 and 7.15, respectively, in all strains (supplemental Fig. S3, B and D) except in mutants expressing CHX20, which had a pH$_{ext}$ of 6.88 at 50 min (supplemental Fig. S3B). These results indicate that (i) KHA1, CHX17, or NHX1 did not affect pH$_{cyt}$ significantly at the external pH conditions tested; (ii) CHX20-expressing cells consistently acidified their pH$_{ext}$ by up to 0.2 units, especially when $[K^+]_{ext}$ was limiting; and (iii) adequate levels (8 mM) of $K^+$ seemed critical for maintaining a steady pH$_{cyt}$ homeostasis.

Vacuolar pH was estimated using BCECF, which accumulates within yeast endosomes and vacuoles (30, 39). BCECF fluorescence in H$^+$-permeabilized cells showed an apparent $pK_a$ between pH 6.5 and 7.0 (Fig. 4D), consistent with a previous report in yeast (30). At acidic pH$_{ext}$, the resting pH$_{vac}$ of strains KTA40-2 and AXT3, except for CHX20-expressing cells, had reached a steady pH of 5.9 after 30 min (Fig. 4E). Expression of CHX17 or KHA1 did not alter yeast pH$_{vac}$. However, expression of NHX1 (LMB01 strain) significantly raised pH$_{vac}$ to 6.1 (Fig. 4E) as reported previously (39). Surprisingly, expression of CHX20 also elevated pH$_{vac}$ to 6.13 at 50 min. Unlike cytosolic pH, vacuolar pH was slightly altered by external $K^+$. When $K^+$ was 0.1 mM, basal pH$_{vac}$ of kha1 mutant remained at pH ~5.9 (supplemental Fig. S3E). Expression of KHA1 or CHX17 did not significantly change pH$_{vac}$. Remarkably, expression of CHX20...
FIGURE 5. CHX17 or CHX20 mediated K⁺ uptake into E. coli. A, cell density at varied K⁺ concentration. E. coli strain LB2003 harboring pPAB404 vector (EV) or vector with CHX17, CHX20, or KAT1 was starved for K⁺ in basal SGM medium at pH 6.6 for 2 h. Cells were added to SGM-MES-MOPS medium at pH 6.6 with 1–8 mM K⁺ and incubated 14 h at 30 °C. B, pH, on cell growth at 18 h; same as A, except medium contained 2 mM K⁺ at varied pH values. Error bars, S.E. (n = 4). C, time course of Rb⁺ uptake. Isopropyl β-D-thiogalactopyranoside was added to medium during K⁺ starvation. Reaction mixture consisted of 2 mM RbCl at pH 6.2 and cells expressing vector (EV; ×) or vector harboring CHX17 (X17; ●), CHX20 (X20; △), or KAT1 (○). An aliquot was filtered and washed at the indicated times. Data are from two independent experiments. D, Rb⁺ uptake at pH 6.2 or 6.6. The reaction was similar to that in C. Uptake at 30 min is shown. Error bars, S.E. (n = 2). E, cations reduced CHX17-dependent Rb⁺ uptake. Cells expressing CHX17 were prepared as in C and normalized. The reaction mixture consisted of 0.6 mM RbCl (0.5 μCi/ml 86Rb) and 2.5 × 10⁵ cells/ml in SGM-MES-MOPS at pH 6.2 plus one cation chloride (lithium (Li), sodium (Na), potassium (K), cesium (Cs), rubidium (Rb), or NH₄) near 0, 1, 3, 10, or 30 mM. 86Rb uptake was measured at 30 min. Uptake without added cation (100%) was 0.8 nmol of Rb⁺/10⁶ cells. F, cations on CHX20-dependent uptake. The method was the same as in E. Uptake without added cation (100%) was 2.4 nmol of Rb⁺/10⁶ cells. Data are from two independent experiments. Error bars, S.E. (n = 2). G, CCCP on Rb⁺ uptake at pH 6.2 versus pH 7.2. Assays were similar to C, except cells were incubated with CCCP at 0–30 μM for 30 min. Data are from two independent experiments. Data of KAT1 are representative of three independent experiments. Bars, S.E. (n = 2). H, Ba⁺⁺ decreased E. coli growth supported by CHX17 or KAT1. Cells expressing vector only or CHX17, CHX20, or KAT1 were incubated in medium containing 5 mM K⁺ at pH 6.6 plus 5 mM choline chloride, 1.25 mM BaCl₂, or 5 mM CsCl for 18 h. Error bars, S.E. (n = 4). I, rescue of mutations in three K⁺ uptake transport systems, Trk, Kup, and Kdp, in E. coli LB2003 by exogenous Arabidopsis CHX transporters.

Further alkalinized pHvac to 6.28 (supplemental Fig. S3E), but this alkalinization was reduced in strain LB301, as observed by pHvac 6.03 at limiting K⁺ concentration.

In alkaline medium, BCECF signal reflects the average intracellular pH (pHin) because the dye was localized at both cytosol and vacuole. The pHin of cells expressing CHX17 or KHA1 was pH 7.1 initially but dropped to 6.6 after 1 h. However, expression of NHX1 resulted in a pHin of 0.2 units higher. Because these strains maintained a similar pHcyt (Fig. 4C and supplemental Fig. S3, B and D), the results imply that only NHX1 alkalinized pHin. The role of CHX20 in yeast is less certain because it altered both pHcyt and pHvac. These results showed that CHX17 or KHA1 had no effect on regulating bulk vacuolar pH when pHext was either acidic or alkaline.

Together, the ability of CHX17 or KHA1 to confer yeast growth tolerance on alkaline medium was not accompanied by alteration of bulk cytosolic pH or vacuolar pH. NHX1 did alkalinize vacuolar pH, consistent with prior reports (29, 39). CHX20, however, differed because cells expressing this gene consistently showed a more acidic pHcyt and a more basic pHvac.

CHX17-mediated K⁺ Uptake in E. coli—We were unable to detect significant changes in K⁺ (86Rb⁺) fluxes resulting from expression of CHX17 or CHX20 in yeast LMM04 cells at either pH 7.5 or pH 4.5 (supplemental Fig. S4); therefore, we expressed CHX17 and CHX20 in E. coli strain LB2003 that is defective in three K⁺ uptake systems (25). In this system, heterologously expressed membrane protein would be inserted in the plasma membrane (40). Both CHX17 and CHX20 restored bacterial growth on YTMM medium containing 1–4 mM K⁺ at pH 6.6 (supplemental Fig. S5A). Increasing concentrations of K⁺ and Rb⁺, but not Na⁺, enhanced growth of cells harboring CHX17, CHX20, or KAT1 at pH 5.5 (supplemental Fig. S5B), indicating that Rb⁺ can partially substitute for K⁺. KAT1 is an inward rectifying K⁺ channel from Arabidopsis (41) and serves as a positive control.
Using SGM medium to better control K⁺ levels, we showed that CHX17 restored growth of LB2003 strain at 2–4 mM K⁺, similar to KAT1. CHX20 was more effective because it increased bacterial growth at 1 mM K⁺ (Fig. 5A). By 8 mM K⁺, cells harboring empty vector grew as fast as others, indicating that K⁺ is taken up via nonspecific pathways. CHX20 rescued bacteria growth at all pH values tested (Fig. 5B), although CHX17- or KAT1-enhanced growth was nearly optimal at pH 7.0. These results indicate that growth rescue by CHX17 and CHX20 is influenced differently by external K⁺ and pH.

CHX20 mediated 86Rb⁺ uptake at a faster rate than that facilitated by CHX17 or KAT1 at pH 6.2 and 6.6 (Fig. 5, C and D). We chose pH 6.2 for further experiments to minimize nonspecific Rb influx. These results demonstrated that CHX17 and CHX20 mediated K⁺ uptake in a pH-dependent manner. K⁺ or Rb⁺ reduced CHX17-dependent 86Rb uptake with an IC₅₀ (half-maximal inhibitory concentration) of about 2.6 mM, relative to an IC₅₀ of 17–36 mM by Li⁺ or Na⁺ (Fig. 5, E and F, and supplemental Fig. S10). The concentration of Cs⁺, Rb⁺, and K⁺ required to inhibit CHX20-dependent Rb⁺ flux (IC₅₀) was 1.5, 2.5, and 3.8 mM, respectively, indicating that CHX20 preferred Cs⁺ > Rb⁺ > K⁺. KAT1 also showed a slight preference of K⁺ over Rb⁺ (supplemental Fig. S10) based on the IC₅₀ of 2.1 and 3.2 mM, respectively, consistent with the study by Uozumi et al. (42). Thus, CHX17 and CHX20 are monovalent cation transporters with an apparent Kₘ, for K⁺ in the low millimolar range.

C CCCP decreased Rb⁺ uptake mediated by CHX17, CHX20, and KAT1 (Fig. 5G) at pH 6.2. Curiously, an increase in Rb⁺ uptake in cells harboring CHX20 was observed at pH 7.2 in the presence of CCCP. However, CCCP caused no change in Rb uptake mediated by CHX17 or KAT1. This result would suggest that CHX20-mediated K⁺ transport is coupled to a pH gradient, whereas CHX17-dependent K⁺ transport is not. Ba²⁺ at 1.25 mM reduced E. coli growth dependent on CHX17 or KAT1 by 50% relative to the control but reduced growth of cells harboring CHX20 by only 20% (Fig. 5H). Moreover, Cs⁺ at 5 mM inhibited CHX17- or KAT1-dependent growth more than that of cells harboring CHX20. Thus, the mode of K⁺ transport mediated by CHX17 might differ from that of CHX20.

Localization of CHXs to Endomembrane in Yeast and in Plant Cells—All CHXs tagged at the carboxyl end to GFP were shown to restore growth of KTA40-2 mutants on alkaline medium (supplemental Fig. S6). Furthermore, resistance to HygB was also retained in cells expressing GFP-tagged CHX17, CHX18, or CHX19. Cells expressing CHX16-GFP or CHX20-GFP were hypersensitive to HygB (supplemental Fig. S6, middle), as shown before for the untagged protein (Fig. 2B). These results demonstrate that CHX16–CHX20 tagged at the C-terminal end with GFP retained their native activity.

In wild-type yeast (FY833), CHX17-GFP and CHX20-GFP fluorescence appeared on tubular and reticulate structures (Fig. 6A). The GFP signal co-localized poorly with that of FM4-64 (Fig. 6A), indicating that CHX17 or CHX20 proteins were not associated with the PM, endosomes, or vacuole. These patterns suggested that CHX17 and CHX20 were localized to distinct endomembranes. In contrast, AtNHX1-GFP labeled intracellular membranes that were mainly reticulate and partially overlapped with FM4-64.

In plant cells, CHX17-GFP alone showed a punctate pattern (supplemental Fig. S7). CHX17 did not co-localize with sialyltransferase or syntaxin 41, suggesting that CHX17 was not associated with the Golgi or the trans-Golgi network. However,
CHX17 co-localized very well with syntaxin 21 and partially with Rab2b or Ara7 (Fig. 6B), suggesting that CHX17 was in prevacuolar compartments (PVC), a subset of endosomes. Moreover, CHX18 and CHX19 co-localized with CHX17, suggesting that these two proteins were also at the PVC (Fig. 6C). In contrast, CHX20-RFP gave a reticulate pattern that co-localized with an ER marker (GFP-HDEL) but not with syntaxin 22 (Fig. 6C). In addition, CHX16-GFP showed a reticulate pattern (supplemental Fig. S7), implying that CHX16 and CHX20 were associated with ER.

**CHX17 or CHX20 Altered Efficiency of CPY Sorting Depending on pHext**—We tested if CHX17 or CHX20 had a role in protein sorting. Carboxypeptidase Y (CPY) is normally trafficked from Golgi to the vacuole lumen via the prevacuolar/late endosomal compartment in yeast. Cells with the same density were cultured in medium at pH 5.7 or 7.3, and growth was monitored continuously using $A_{600}$ of 0.2, and added to K$^{+}$-free YNB with 2% galactose and 8 mM K$^{+}$ at pH 5.5. CPY on plate was detected at early log phase (18–20 h). Signals were normalized to cell density ($A_{600}$) and converted to ng of CPY/$A_{600}$ cells. Data are from seven replicates of two independent lines. Error bars, S.E. (n = 14).

To test if CHX17 or CHX20 had a role in protein sorting, we compared CPY secretion from yeast strains expressing CHX17 or CHX20 with those expressing NHX1 (in LMB01 cells) in a pH-dependent manner. Curiously, cells expressing CHX20 did not show a similar reduction in CPY secretion levels at alkaline pH$^\text{ext}$, suggesting that it may influence protein sorting in a differential manner.

**DISCUSSION**

**Tolerance to Alkaline pH: Diversity among Five CHXs**—For years, it had been challenging to determine CHX gene functions because they failed to restore growth in various yeast mutants. A striking phenotype of AtCHX16–AtCHX20 expressed in KTA40-2 yeast mutant is their ability to restore growth at alkaline pH. This breakthrough was made initially using AtCHX20 (21) and AtCHX17 (24), although the mechanistic basis was unknown. Without any obvious working hypothesis, we have first compared properties of five CHXs with NHX1. Here we show that Arabidopsis CHX17, -18, or -19 is most effective in conferring tolerance to alkaline pH of KTA40-2 mutants, similar to yeast KHA1. By contrast, CHX20 or CHX16 is less effective in conferring alkaline tolerance. Furthermore, cells expressing CHX17, -18, and -19 were resistant to hygromycin B, whereas cells expressing CHX16 or CHX20 were sensitive or hypersensitive. Importantly, the properties of CHX17 differed from those of ScNHX1 or AtNHX1. ScNHX1 was unable to restore growth of KTA40-2 mutant at alkaline pH and was more effective in conferring HygB resistance than CHX17, as seen between ScNHX1 and ScKHA1 (45). The variations observed among five AtCHXs cannot be attributed to unequal expression of protein levels because (i) the qualitative phenotypes are consistently observed in independent transformants, (ii) GFP-tagged CHX16 through CHX20 at the C terminus are active in conferring alkaline tolerance and are localized to intracellular membranes in yeast, and (iii) CHX17-GFP conferred HygB resistance, whereas CHX20-GFP or CHX16-GFP did not. Thus, all five CHX proteins (CHX16–CHX20) appear to function in pH homeostasis in yeast in a manner distinct from NHX1. Moreover, CHX17, CHX18, and CHX19 diverge in activity from CHX16 and CHX20.
Importance of $K^+$ in pH Homeostasis—Several observations pointed to a role of $[K^+]_{\text{ext}}$ in pH homeostasis and in growth of yeast mutants expressing CHX: (i) CHX17- or CHX20-enhanced growth of single $\text{kha1}$ mutant at alkaline pH was evident at 0.6–10 mM $[K^+]$ but not at 100 mM (Fig. 2E); (ii) CHX17–CHX19 restored growth of $K^+$ uptake-deficient mutant LMM04 at pH 7.5, similar to yeast haboring $K^+$ uptake systems, TrkJ1/2 and Tok1j (Fig. 3, A and C); and (iii) CHX16, CHX19, and CHX20 restored growth at pH 4.3 (Fig. 3B). The increased growth of $K^+$ uptake-deficient mutants by CHX17, -18, or -19 at alkaline pH would suggest that intracellular $K^+$ rather are important in cation uptake, $K^+$ flux at pH 7.5 or pH 4.5 into $K^+$ uptake-deficient yeast (LMM04) expressing CHX17 (supplemental Fig. S4, A and B). Curiously, KTA40-2 strains expressing CHX17 or CHX20 took up less $K^+$ than in mutants with vector only, and cells grown at pH 7.5 took up 3-fold more $K^+$ than at pH 4.5 (supplemental Fig. S4, C and D). Because both CHX17 and CHX20 were localized to endomembranes in yeast, these results suggest that both CHX proteins do not enhance net $K^+$ uptake into cells but rather are important in cation uptake, $K^+$ sorting and/or cycling through certain endomembrane compartments and the cell. This interpretation could also apply to yeast $\text{kha1}$, which has been localized to Golgi (24, 46).

CHX17- and CHX20-mediated $K^+$ Transport—Here we provide the first functional characterization of CHX17- and CHX20-mediated $K^+$ transport using a $K^+$ uptake-deficient $E. coli$ strain (Fig. 5). CHX20 enhanced $E. coli$ LB2003 growth at acidic pH (pH 5.8–6.2), whereas cells expressing CHX17 or KAT1 showed optimal growth at pH 6.6–7. Cation competition studies showed that CHX17 preferred $K^+$ ($^{86}\text{Rb}^+$) flux at pH 7.5 or pH 4.5 into $K^+$ uptake-deficient yeast (LMM04) expressing CHX17 (supplemental Fig. S4, A and B). These results would suggest that tolerance to alkaline pH conferred by either CHX17 or CHX20 was not due largely to changes in $\text{pH}_{\text{cyt}}$ or $\text{pH}_{\text{vac}}$. Instead, altered pH in cells expressing CHX20 could be related to $\text{H^+}/\text{K^+}$ symporter (see above).

Several observations suggested that CHX20 mediated $H^+$-coupled $K^+$ transport: (i) $K^+$ influx into $E. coli$ is optimal when $\text{pH}_{\text{ext}}$ is acidic, and (ii) CCCP, a protonophore, inhibited $K^+$ uptake at pH 6.2 but not at $\text{pH}_{\text{ext}}$ 7.2, when there is little or no pH gradient across the PM. However, at pH 7.2, increasing CCCP concentration enhanced CHX20-dependent $\text{Rb}^+$ uptake into cells. This finding is surprising, unless the protein has switched its mode of transport.

Transport properties of CHX17 mimicked that of KAT1, an inwardly rectifying $K^+$ channel. They both showed optimal transport near neutral pH, and $K^+$ influx was insensitive to the protonophore CCCP at neutral pH. Bacterial growth stimulated by both CHX17 and KAT1 is inhibited $\approx 50\%$ by 1.25 mM Ba$^{2+}$, a $K^+$ channel blocker (41, 48), whereas CHX20-supported growth is less sensitive. It is possible that CHX20 behaves like a $H^+$-coupled $K^+$ symporter and CHX17 has channel-like properties, although this idea will need to be rigorously verified. Interestingly, examples are emerging where channel properties exist in transporters (49). For example, KefC (a homolog of AtKEA1/2) has channel-like properties and cation/proton antiport activity when co-expressed with a peripheral protein, KefF (50). Despite the ambiguity, we postulate that $K^+$ transport mediated by CHX is probably coupled either directly or indirectly to a $H^+$ flux, which would change compartment pH or localized pH at an endomembrane.

Differential Effects of CHX17 and CHX20 on Intracellular pH—The inability to detect changes in cytosolic or vacuolar pH in yeast expressing CHX17 or $\text{ScKHA1}$ could be due to the relatively weak or limited signal from small compartments. In contrast, pH changes in vacuoles were detected because cells expressing $\text{NHX1}$ had a slightly basic pH in vacuoles (pH 6.1) relative to pH 5.9 of vacuoles in KTA40-2 ($\text{nhx1\Delta}$) (Fig. 4E). Our results are consistent with the model that NHX1 alkalinizes yeast pH (29).

Furthermore, cells expressing CHX20 alkalinized the vacuole and acidified the cytosol by about 0.2 units, indicating that the roles of CHX20 differed from that of CHX17. At limiting $K^+$ (0.1 mM; supplemental Fig. S3E) $\text{pH}_{\text{vac}}$ increased from 5.92 to 6.28 (0.4 units) in cells expressing CHX20, whereas $\text{NHX1}$ alkalinized $\text{pH}_{\text{vac}}$ by about 0.1 unit to pH 6.03. This result suggested that CHX20 and NHX1 show differential modes of transport. It was shown before that vacular $K^+$ concentration changed according to $K^+$ availability in the tissue (51). If so, $K^+$ transport into the vacuole via NHX1-mediated electroneutral $K^+/H^+$ exchange would be reduced at low $K^+$; however, the increase in vacuolar alkalinization and cytosolic acidification seen in cells with CHX20 would favor a model of a $H^+/K^+$ symporter (see above).

Second, yeast mutant KTA40-2 lacking several endomembrane cation/ $H^+$ exchanger secreted vacuolar CPY, however, mutants expressing CHX17 or CHX20 secreted less CPY at pH 7.3 (37–39%) relative to that seen at pH 5.5 (Fig. 7, B and C).
Like CHX17, ScKHA1 expression also reduced CPY secretion (52–60%) relative to KTA40-2 mutant alone at alkaline pH. In contrast, ScNHX1 reduced CPY secretion in LMB01 yeast at both acidic and alkaline pH. Our results are consistent with previous work demonstrating that pH modulation of endosome/PVC by ScNHX1 (29) is important for trafficking of vacuolar sorting receptor, Vps10, which normally binds and sorts CPY from Golgi to vacuole (39, 44) (Fig. 8). Because CHX17 (Fig. 6) and ScKHA1 (24, 46) were localized to endomembranes, we propose a model where modulation of cation and pH homeostasis in ER, Golgi, and/or related compartments is critical for proper protein sorting and membrane trafficking. CHX17 could serve a similar model in endosomes/PVC of plant cells.

Third, the ability of CHX17 and ScNHX1 to confer hygromycin B resistance in yeast could be related to vesicle trafficking. The cellular basis of aminoglycoside resistance in yeast is still debatable. Toxicity has been attributed to the extent of hygromycin B transport into the cytosol, where it binds the 30 S ribosome and blocks protein translation (54). Yeasts grown under K\textsuperscript+ starved condition are sensitive, suggesting an increase in potential-driven uptake of HygB\textsuperscript+ (35), yet kha1 or nhx1 mutants showed no change in membrane potential (55, 56). One study showed that gentamicin, another aminoglycoside, enters pig kidney cells (LLC-PK1) via endocytosis and is then delivered by retrograde traffic to the lysosome and ER/Golgi, where its leakage into the cytosol poisons the cell (57). Furthermore, several gentamicin-sensitive yeast mutants are defective in genes associated with the Golgi-associated retrograde pathway or homotypic fusion and vacuolar protein sorting (58). Thus, gentamicin sensitivity in yeast is related to altered intracellular membrane trafficking. We found that CHX17, KHA1, or NHX1 conferred both HygB and gentamicin resistance (supplemental Fig. S9). Some mutants of vma, vps, or pep genes are also sensitive to HygB. Because Vps44 or NHX1 alkalizes the vacuole (29) and confers tolerance to HygB or gentamicin, we propose that NHX1 could influence sorting of the antibiotic into a compartment (e.g. vacuole) for degradation or inactivation. CHX17 confers an additive effect of HygB resistance on cells harboring wild-type NHX1 (Fig. 2B), suggesting that CHX17 also modulated pH or cation balance of compartments that collaborate to facilitate retrograde transport, leading to degradation or inactivation of the antibiotic. Furthermore, the inability of CHX20 to confer HygB resistance could be attributed to its different transport properties, its association with distinct endomembranes, or both, which would interfere with antibiotic sorting and inactivation.

**Summary**—This study has revealed distinct properties of two *Arabidopsis* K\textsuperscript+ transporters, CHX17 and CHX20, after expression in yeast and *E. coli*. Our findings point to a common role of CHXs in modulating cation and pH homeostasis of diverse endosomal compartments. Apart from the role of CHX20 in guard cell movement, phenotypic analyses of single or high order mutants of CHX16 through CHX19 have been remarkable so far. Mutant chx17 plants grown under K\textsuperscript+ starvation showed reduced K\textsuperscript+ content, supporting a role in K\textsuperscript+ homeostasis (23). Increased expression of CHX17 transcripts by K\textsuperscript+ starvation, abscisic acid, or high salt (23) would suggest additional roles in stress tolerance. Our finding that endosome-associated CHX17 alters vacuolar CPY sorting in yeast supports a role in membrane trafficking. Whether it influences the anterograde or retrograde trafficking among ER, PVC, and endosomes has yet to be established. It is clear that NHX1 and CHX17 functions can partially overlap yet are distinct in yeast and possibly in plants. Together, our studies provide the first indication that members of the CHX family are monovalent cation transporters with distinct and critical roles in membrane trafficking that would impact stress tolerance, development, and growth of plants.

**Acknowledgments**—We thank Drs. Chunxin Wang, Alex Bao, Quan-sheng Qui, and Dorothy Belle Poli (University of Maryland) for conducting preliminary studies on the CHX gene family and Lalu Zulkiﬁ (Tohoku University) for initial studies using *E. coli*. Jose Pardo (Instituto de Recursos Naturales y Agrobiologia-Consejo Superior de Investigaciones Científicas) provided AtNHX1-GFP plasmid.

**REFERENCES**
