Identification of a Crucial Histidine Involved in Metal Transport Activity in the Arabidopsis Cation/H+ Exchanger CAX1*

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In plants, yeast, and bacteria, cation/H+ exchangers (CAXs) have been shown to translocate Ca2+ and other metal ions utilizing the H+ gradient. The best characterized of these related transporters is the plant vacuolar localized CAX1. We have used site-directed mutagenesis to assess the impact of altering the seven histidine residues to alanine within Arabidopsis CAX1. The mutants were expressed in a Saccharomyces cerevisiae strain that is sensitive to Ca2+ and other metals. By utilizing a yeast growth assay, the H338A mutant was the only mutation that appeared to alter Ca2+ transport activity. The CAX1 His338 residue is conserved among various CAX transporters and may be located within a filter for cation selection. We proceeded to mutate His338 to every other amino acid residue and utilized yeast growth assays to estimate the transport properties of the 19 CAX mutants. Expression of 16 of these His338 mutants could not rescue any of the metal sensitivities. However, expression of H338N, H338Q, and H338K allowed for some growth on media containing Ca2+. Most interestingly, H338N exhibited increased tolerance to Cd2+ and Zn2+.

Endomembrane fractions from yeast cells were used to measure directly the transport of H338N. Although the H338N mutant demonstrated 25% of the wild type Ca2+/H+ transport, it showed an increase in transport for both Cd2+ and Zn2+ reflected in a decrease in the Kᵣ for these substrates. This study provides insights into the CAX cation filter and novel mechanisms by which metals may be partitioned across membranes.

Cation exchanger (CAX)1-like cation/proton antiporters are a group of proteins that export cations out of the cytosol to maintain ion homeostasis across biological membranes (1). They are energized by the pH gradient established by proton pumps such as the H+-ATPase or H+-pyrophosphatase (2). CAX-like exchangers have been classified as one of the five families of exchangers that make up the cation/H+ exchanger superfamily (3). The plethora of genomic sequencing has identified ~130 CAX-like transporters from bacteria, fungi, and plants.2

The term CAX was first used to describe the cation exchangers CAX1 and CAX2 in Arabidopsis thaliana (5). These transporters were identified by function. Namely, N-terminal truncations of these proteins (sCAX1 and sCAX2) suppressed the Ca2+ sensitivity of a yeast mutant defective in vacuolar Ca2+ transport. Cation specificities of CAXs may vary, and there is evidence that specificities may be determined by local primary structures. By using yeast assays, a sequence of 9 amino acids from CAX1 can confer strong calcium transport ability to its homologue CAX3 (6). A 3-amino acid-long manganese specificity determinant in TM4 of CAX2 has also been identified (7).

Substrate selectivity of the rice cation/H+ exchanger OsCAX1a appears to be dependent on residues in internal repeat regions (termed c-1 and c-2) located between TM3 and TM4 and between TM8 and TM9 (8). The ability to manipulate the expression levels and substrate specificity of these transporters may positively affect plant properties through increasing the nutrition quality of foods or through the removal of toxic metals from soils (9, 10).

Other transporters have also been engineered with altered transport properties. For example, histidyl residues in many transporters have been shown to play critical roles in transporter function (11). This is particularly the case for membrane proteins involved in H+ translocation or those that exhibit H+ sensing. Histidine residues may also function in substrate recognition, interaction of helices, and for coupling conformational changes between helices (12). All the characterized CAX transporters are about 400 amino acids long and are predicted to have 11 membrane-spanning domains with several conserved histidine residues.2 However, no methodical analysis has been undertaken on the functional role of these amino acids in CAX transporters.

Identification of novel substrates of CAXs, and determining structural requirements for ion specificity, will provide important information for constructing “designer” proteins to utilize for plant improvement. With this long range goal in mind, this immediate study demonstrates the effects of point mutations in the histidine residues of CAX1. Although a series of mutations in six of the seven histidines did not appear to alter transporter function, we show that a histidine to alanine mutation at

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† The abbreviations used are: CAX, cation exchanger; sCAX, N-terminal truncated CAX; TM, transmembrane domain; MES, 4-morpholineethanesulfonic acid.

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position 338 can effectively abolish the transport function of sCAX1. A systematic analysis of sCAX1 variants in which His338 has been altered to 19 other amino acids affords new insights into the involvement of this region in substrate specificity. Most interestingly, specific sCAX1 variants increase metal transport while dramatically decreasing Ca2+ transport. These mutants now offer novel tools by which nutrients and toxic metals can be redistributed across plant membranes.

**Experimental Procedures**

**Yeast Strains, Vectors, and DNA Manipulations**—The yeast strain K667 (enb1::LEU2 pnc1::TRP1 cex1) was used to express the wild type CAX1 and mutant constructs. The clones were propagated in either pBluescript (Stratagene, La Jolla, CA) or pCRII-TOPO (Invitrogen), and inserts were transferred to the shuttle vector pHGpdl (13) for their expression in yeast. The plasmids were introduced into yeast by lithium acetate/polyethylene glycol transformation (14), and standard techniques were used to manipulate DNA (15).

**Site-directed Mutagenesis**—CAX1 mutants were produced by class IIS restriction enzyme-mediated site-directed mutagenesis (16), using the primers shown in Table I.

**Construction and Detection of c-myc-tagged CAX1 and Its Derivatives**—The c-myc epitope was amplified by PCR and ligated in-frame at the C terminus of CAX1 or the CAX1 mutants. Western analysis was performed as described previously (6, 7).

**Ca2+ and Metal Transport Assays**—Yeast vacuole membrane vesicles were prepared as described previously (7, 17), and protein content was determined by the method of Bradford (18). For the measurement of 45Ca2+ uptake, membrane vesicles were incubated in buffer containing 0.3 mM sorbitol, 5 mM Tris-MES (pH 7.6), 25 mM KCl, 0.1 mM sodium azide, and 0.2 mM sodium orthovanadate. H+ translocation by the vacuolar H+-ATPase was initiated by the addition of 1 mM MgSO4 and 1 mM ATP. The vesicles were allowed to reach steady state with respect to pH gradient for 5 min at 25 °C before the addition of CaCl2 (6 mM, with the final concentration of 0.1 mM). The Vesicle transport activity was measured by coupling metal exchange was measured in yeast vacuole vesicles (30 μg of protein) by monitoring the metal-dependent recovery of quinacrine (6-chloro-9-[(4-diethylamino)-1-methylbutylamino]-2-methoxyacridine dihydrochloride) fluorescence following the formation of an inside acid pH gradient across the vesicles generated by activation of the vacuolar H+-ATPase, as described previously (19). Fluorescence was monitored in a thermostatted cell at 25 °C using a fluorescence spectrometer (model LS-50, PerkinElmer Life Sciences) at excitation and emission wavelengths of 427 and 495 nm, respectively, both with a slit width of 5 nm. As shown by Bennett and Sphanswick (20), the rate of fluorescence recovery is directly proportional to proton flux. Thus initial rates of fluorescence recovery represent initial rates of proton-dependent metal transport.

**Assay for Yeast Suppression**—The assay for metal tolerance on solid agar was as described previously (6, 7).

**Results**

The aim of this study was to characterize the role of histidine residues in the translocation of metal ions in the Arabidopsis CAX1 transporter. Like other CAXs from a variety of different organisms, analysis of the 463-amino acid sequence suggests that the protein has 11 TMs (Fig. 1A). CAX1 has an acidic motif in the cytosolic loop between TM6 and TM7 and repetitive domains between TM3 and TM4 and between TM5 and TM9 of 36 residues, 14 of which are identical (repeats a-1 and a-2 (8)). There are seven histidine residues in the CAX1 polypeptide at positions 35, 210, 259, 338, 412, 443, and 446. We initially generated a H35A variant of CAX1 to determine whether this would disrupt the autoinhibitory domain. Previously, we showed that the N-terminal domain (the first 36 amino acids) of CAX1 inhibits the ion transport activity in yeast assays (17). We cloned the H35A CAX1 open reading frame into a high copy yeast expression plasmid and expressed this plasmid in a yeast strain deficient in vacuolar Ca2+-transporters and lacking functional calcineurin (5, 21). This strain (K667) lacks the endogenous vacuolar Ca2+-ATPase PMC1 and vacuolar H+/Ca2+ antiporter VCX1 and thus is defective in vacuolar Ca2+ transport, making it unable to grow on high Ca2+ media such as 200 mM CaCl2 (21). The H35A mutant of CAX1 remained autoinhibited when expressed in yeast (unlable to grow on the Ca2+-containing media; data not shown). For the remainder of our studies, we used a truncated CAX1 (sCAX1) that removed the first 36 residues from the full-length cDNA (CAX1). High level expression of sCAX1 suppresses the Ca2+-sensitive phenotype of K667 cells. When the six sCAX1 mutants (H210A, H259A, H338A, H412A, H443A, and H446A) were similarly expressed in K667 cells on Ca2+-containing media, only the H338A mutant showed an altered phenotype compared with sCAX1. In H338A-expressing K667 cells, the Ca2+-sensitive phenotype was not suppressed, highlighting the importance of this residue in the Ca2+ transport of CAX1 (Fig. 1C). To demonstrate that the changes in transport were not due to alterations in protein expression or stability, we generated a c-myc tagged version of H338A and several of the other histidine mutants, and the tags were expressed in K667 cells. These tagged transporters retained the same growth phenotypes as the original sCAX1 variants. Immunoblotting of the crude membrane fractions prepared from the various CAX1 mutants expressing yeast cells demonstrated approximately equal accumulation of the tagged protein (Fig. 1D; data not shown).

We confirmed the differences in calcium sensitivity of the mutants by measuring 45Ca2+ uptake activity using isolated membrane vesicles. In this system, the pH gradient across yeast vacuolar membrane vesicles was generated by activation of the vacuolar H+-ATPase. The vesicles of sCAX1 expressing cells took up 45Ca2+ from the medium in a pH- and time-dependent manner for up to 10 min (Fig. 2, sCAX1). The accumulated 45Ca2+ was released after the addition of the Ca2+ ionophore A23187 (Fig. 2, 12 min). The absence of ATP or in the presence of gramicidin, a protonophore that dissipates the pH gradient (6), no such activity was detected. Membrane vesicles of yeast cells expressing an empty vector or H338A had negligible activity (Fig. 2). These results corroborate that sCAX1 but not H338A could function as a Ca2+/H+ exchanger in yeast.

The His338 residue of CAX1 is part of the c-2 repeat that contains the consensus sequence GNAAEHX3AAMDXLGXGSX3QX3FX found in various CAX transporters (Fig. 1B). This region has been proposed to function as a cation selectivity filter (8). In order to determine further the importance of His338, this residue was changed to all possible amino acids by site-directed mutagenesis (Table I) (16), and yeast cells expressing these variants of sCAX1 were tested on a variety of media containing different metals. Yeast cells expressing 3 of the 19 His338 variants and the wild type sCAX1 demonstrated tolerance to 50 mM CaCl2. The cells expressing the wild type sCAX1 were the most tolerant to Ca2+ in the media, but H338N > H338Q > H338K were all able to grow at low levels of Ca2+ (Table II). To confirm the yeast growth phenotypes on Ca2+-containing media, we measured 45Ca2+ transport activity directly. Membranes isolated from yeast expressing the H338N mutant demonstrated ~25% of the Ca2+ transport activity when compared with His338-expressing cells (Fig. 2). Because of the limited sensitivity of the transport assay, we were unable to reproducibly detect Ca2+ transport activity in the H338Q- and H338K-expressing cells.

Yeast cells expressing the same four constructs (sCAX1, H338N, H338Q, and H338K) also conferred tolerance to Cd2+ (Fig. 3 and data not shown). Vector controls and strains expressing other CAX transporters will not grow well in media containing 2 or 3 μM Cd2+ (data not shown). Furthermore, ion competition studies suggest that sCAX1 is capable of transporting both...
Ca\(^{2+}\) and Cd\(^{2+}\) (6), discounting the possibility that other metal ions transported by CAX1 produce this metal tolerance. To investigate further the difference in metal tolerance of H338N-expressing cells as compared with sCAX1, we studied metal-dependent H\(^{+}\)-transport in isolated membrane vesicles from yeast vacuoles. The ability of H338N-expressing yeast cells to confer higher Cd\(^{2+}\) and Zn\(^{2+}\) tolerance suggested a greater transport capability of this mutant for these metals. Quinacrine fluorescence recovery was used to monitor the dissipation of a preset transmembrane pH gradient (generated by activation of the vacuolar H\(^{+}\)-ATPase), following the addition of different metal ions. For both Zn\(^{2+}\) and Cd\(^{2+}\) ions, H338N demonstrated a higher metal/H\(^{+}\)-dependent transport activity than that measured in sCAX1 (Fig. 4, A and B). Other metal ions tested, including Ni\(^{2+}\), Co\(^{2+}\), and Mn\(^{2+}\), showed no detectable H\(^{+}\)-dependent transport activity in either sCAX1 or the H338N-expressing cells, confirming the growth phenotype observed on these metals (Fig. 5; data not shown). Furthermore, no Zn\(^{2+}\)- nor Cd\(^{2+}\)-dependent H\(^{+}\) transport was measured in the K667 vector control strain confirming that metal transport required the presence of sCAX1 (data not shown).

Michaelis-Menten kinetic analysis of the data showed that increased metal transport in the H338N-expressing yeast cells was a result of a decrease in the \(K_m\) value for the metals with little or no change in the \(V_{\text{max}}\) values. The \(K_m\) values for Cd\(^{2+}\) decreased from a value of 230 \(\mu\)M in sCAX1 to a value of 187 \(\mu\)M in the H338N mutant, whereas the \(V_{\text{max}}\) values increased slightly from 150% \(V_{\text{min}}\) per mg protein to 164% \(V_{\text{min}}\) per mg protein (Fig. 6A). In the case of Zn\(^{2+}\), the \(K_m\) values decreased from a value of 225 \(\mu\)M in sCAX1 to a value of 182 \(\mu\)M in H338N, whereas the \(V_{\text{max}}\) values showed little change; from 144% \(V_{\text{min}}\) per mg protein in sCAX1 to 149% \(V_{\text{min}}\) per mg protein in H338N (Fig. 6B).

**DISCUSSION**

We used site-directed mutagenesis to modify the histidine residues in CAX1. Histidine residues have been shown to be important functional determinants in various membrane pro-
protons, including those involved in H⁺ translocation and H⁺ sensing (12). Our data presented here strongly implicate the His338 as being vital for the kinetic properties and ion selectivity of this transporter.

We performed histidine-scanning mutagenesis throughout the CAX1 transporter. Only His338 appears to be required for sCAX1 Ca²⁺ transport, as mutations at His35, His210, His259, His412, His443, and His446 did not alter transport as measured by the yeast suppression assay (Fig. 1). His338 is closely associated with predicted transmembrane domain 8 and is part of the c-2 domain (8). Although previous studies have reported on the importance of this region, our studies firmly establish that this motif is involved in the ionic filter.

Previously, work with the rice cation/H⁺ exchanger (OsCAX1a) has shown that replacing His330 (in the equivalent position of His338 in CAX1) with arginine or alanine (H330R or H330A) decreased the transport activity of the transporter (9). Our findings support the idea that His338 is critical for the function of CAX1.

**FIG. 2.** Time course of [⁴⁵]Ca²⁺ uptake into vacuolar vesicles prepared from the yeast strain K667 expressing sCAX1 and mutants. Square, pH-dependent [⁴⁵]Ca²⁺ uptake; circle, uptake in the presence of the protonophore gramicidin. The Ca²⁺ ionophore, A23187 (5 μM), was added at 12 min. The data represent means of three replications, and the bars indicate S.E.

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**Table I**

The names of the primers denote the change that they create with suffix F and R indicating forward and reverse primer, respectively. The underlined sequence is the BsmBI site that is required for the class IIS restriction enzyme-mediated site-directed mutagenesis, and the boldface and underlined sequence is the mutation incorporated into the primer. The letters M and N indicate “A or C” and “any nucleotide,” respectively.

| Primers used to change the histidine residues to alanine |  |
|--------------------------------------------------------|----------------------------------|
| H35A-F 5'-GAATTCGTCCTCCGCCTGCCAATGTCTTCCTCCTTTTGAGG-3' |  |
| H35A-R 5'-GAATTCGTCCTCCGCGTTCCTCTAATGTTGGGGATAC-3' |  |
| H35A-F 5'-GAATTCGTCCTCCGGCACACAAGAAACCCAGTAGAAGTAAAAAGAAGTTCACATC-3' |  |
| H210A-R 5'-GAATTCGTCCTCCGGCACACAAGAAACCCAGTAGAAGTAAAAAGAAGTTCACATC-3' |  |
| H259A-F 5'-GAATTCGTCCTCCGGCACACAAGAAACCCAGTAGAAGTAAAAAGAAGTTCACATC-3' |  |
| H259A-R 5'-GAATTCGTCCTCCGGCACACAAGAAACCCAGTAGAAGTAAAAAGAAGTTCACATC-3' |  |
| H338A-F 5'-GAATTCGTCCTCCGGCACACAAGAAACCCAGTAGAAGTAAAAAGAAGTTCACATC-3' |  |
| H338A-R 5'-GAATTCGTCCTCCGGCACACAAGAAACCCAGTAGAAGTAAAAAGAAGTTCACATC-3' |  |
| H412A-F 5'-GAATTCGTCCTCCTACATGAAGGGACTAGTCCTCTTTGC-3' |  |
| H412A-R 5'-GAATTCGTCCTCCTACATGAAGGGACTAGTCCTCTTTGC-3' |  |
| H443A-F 5'-GAATTCGTCCTCCTCCTCCTCAAGCGATGAACAATGTCGTC-3' |  |
| H443A-R 5'-GAATTCGTCCTCCTCCTCCTCAAGCGATGAACAATGTCGTC-3' |  |
| H446A-F 5'-GAATTCGTCCTCCTCCTCCTCAAGCGATGAACAATGTCGTC-3' |  |
| H446A-R 5'-GAATTCGTCCTCCTCCTCCTCAAGCGATGAACAATGTCGTC-3' |  |

Primers used to change His338 to other amino acids

| Primers used to change His338 to other amino acids |  |
|----------------------------------------------------|----------------------------------|
| H338A-F 5'-GAATTCGTCCTCCTCCTCCTCAAGCGATGAACAATGTCGTC-3' |  |
| H338A-R 5'-GAATTCGTCCTCCTCCTCCTCAAGCGATGAACAATGTCGTC-3' |  |

Histidine Mutants of Arabidopsis CAX1
Suppression of metal sensitivity of the yeast strain K667 expressing various sCAX1 mutants

Saturated liquid cultures of pmc1vcc1cnb yeast strain (K677) expressing sCAX1 with mutations at the amino acid position 338 were spotted onto medium containing the indicated metals. The growth was recorded after incubating for 2 (50 nM CaCl₂) or 3 days (5 μM CdCl₂) at 30 °C. The values were summed up for each mutant and tolerance is expressed by +++++ for the highest tolerance exhibited by the variants tested (designated 100%), +++++ (−80% tolerance), +++++ (−60% tolerance), + + (−40% tolerance), + (−20% tolerance), and − (no tolerance). WT indicates wild type.

| Amino acids at position 338 | Growth on 50 mM Ca²⁺ | Growth on 5 μM Cd²⁺ |
|-----------------------------|---------------------|---------------------|
| Ala                         | −                   | −                   |
| Arg                         | −                   | −                   |
| Asn                         | +++++               | +++++               |
| Asp                         | −                   | −                   |
| Cys                         | −                   | −                   |
| Glu                         | −                   | −                   |
| Gln                         | +++++               | −                   |
| Gly                         | −                   | −                   |
| His (WT)                    | +++++               | ++                  |
| Ile                         | −                   | −                   |
| Leu                         | −                   | −                   |
| Lys                         | ++                  | +                   |
| Met                         | −                   | −                   |
| Phe                         | −                   | −                   |
| Pro                         | −                   | −                   |
| Ser                         | −                   | −                   |
| Thr                         | −                   | −                   |
| Trp                         | −                   | −                   |
| Tyr                         | −                   | −                   |
| Val                         | −                   | −                   |

**Table II**

Histidine Mutants of Arabidopsis CAX1

Effect of Histidine Mutants on Transport Activity of CAX1

- H330A abolished activity in yeast (8).
- His338 of OsCAX1a, like His338 of CAX1, is located within a repetitive motif designated c-2 that contains a conserved GNXXEH motif, and similar residues are found in animal Na⁺/Ca²⁺ exchangers (8).
- Mutation of the glutamic acid residue of OsCAX1a (E329D) severely reduced the ability of mutant yeast to grow in high Ca²⁺ medium (8). This evidence has been used to suggest that this region of the CAX transporters may form an ion filter within the protein (8). Like OsCAX1a H330R, the CAX1 H338A mutant, and 15 other His338 mutants, lost Ca²⁺ transport activity (Table II).
- There are several possible mechanisms that could contribute to the reduced transport activity of a particular mutant. For example, the mutation may cause the transporter to misfold and consequentially be targeted for degradation (22). Alternatively, the altered protein may not be correctly targeted to the tonoplast. However, the epitope-tagged version of H338A is highly expressed in crude membrane fractions, and several mutants at position 338 are active in Ca²⁺ transport (Fig. 1D; see below), implying proper expression and localization of CAX1 variants that have replaced His338 with another amino acid.
- Furthermore, the H338N-expressing cells demonstrate increased transport of Cd²⁺ and Zn²⁺ when compared with wild type. This implies that there is not a decrease in tonoplast-located CAX1 protein among these variants.

- In the topology of CAX1, the c-2 repeat is thought to form a re-entrant loop and act as a region involved in cation translocation (8). The c-2 region contains several hydrophilic residues that may act as a solvent-accessible region that forms an ion filter (8). Yeast cells expressing several of the His338 variants (H338K, H338N, and H228Q) are only able to weakly suppress the Ca²⁺-hypersensitive phenotype, but some confer robust growth on media containing several different metals (Table II; Fig. 3).

**Fig. 3. Suppression of metal sensitivity of the yeast strain K667.** Saturated liquid cultures of pmc1vcc1cnb yeast strains containing the indicated plasmid were spotted onto medium permissive for growth (synthetic complete medium without histidine (−His)) or medium that selects for the presence of plasmid-borne vacuolar CAX mutant transporter capable of Cd²⁺ or Zn²⁺ transport (yeast extract/peptone/dextrose containing 7 μM CdCl₂ or 5 mM ZnCl₂). The plates were incubated for 3 days.

**Fig. 4. Metal-dependent proton transport in isolated vacuolar membrane vesicles from yeast cells expressing sCAX1 or H338N.** A preset, steady-state pH gradient (inside acidic) was generated by activation of the H⁺-ATPase, as described under “Experimental Procedures.” The recovery of quinacrine fluorescence, indicative of metal/H⁺ exchange, was measured upon addition of 500 μM CdCl₂ (A) or 200 μM ZnCl₂ (B) as indicated by the arrows. The results are original traces from one experiment representative of a total of four.

**Fig. 5. Specificity of metal-dependent proton transport in isolated vacuolar membrane vesicles from yeast cells expressing H338N.** A preset, steady-state pH gradient (inside acidic) was generated by activation of the H⁺-ATPase, as described under “Experimental Procedures.” The recovery of quinacrine fluorescence, indicative of metal/H⁺ exchange, was measured upon addition of 500 μM CdCl₂, ZnCl₂, MnCl₂, NiCl₂, or CoCl₂. The results are original traces from one experiment representative of a total of four.
measurements from membranes prepared from H338N-expressing cells agreed with the growth phenotypes, i.e., the tolerant mutants had higher transport rates for Zn$^{2+}$/Cd$^{2+}$ reflected in lower $K_m$ values but reduced Ca$^{2+}$ transport (Figs. 2 and 4). These experimental observations suggest that rather than destroying the filter, these mutants have altered ion selectivity. Our hypothesis is that the H338N variants have an increased $K_m$ value for Ca$^{2+}$ transport; however, the negligible Ca$^{2+}$ transport measured in H338N-expressing cells makes precise measurements of changes in Ca$^{2+}$ kinetic parameters difficult.

The four amino acids at residue 338 that can confer calcium tolerance are similar in hydropathy (Fig. 7). This suggests hydropathy is an important determining factor for cation selectivity within the c-2 region. Despite the similar hydropathy of Asp and Glu to His, Lys, Asn, and Gln residues, these negatively charged amino acids at position 338 cannot confer calcium tolerance in yeast (Table II). Given that there is another Glu residue adjacent to His$^{338}$, we hypothesize that the H338D and H338E variants are nonfunctional because of an excess of negative charge within the filter domain (Fig. 1B). The selectivity of H338N for Cd$^{2+}$/Zn$^{2+}$ (Figs. 3–5) suggests that the putative filter domain of H338N appears to be able to discriminate between these similar ions (ionic radius for Cd$^{2+}$ is 97 Å whereas that of Ca$^{2+}$ is 99 Å). However, extensive alterations in this region appear to inhibit completely the Ca$^{2+}$ activity of the transporter (see above). The fact that H338N also has increased affinity for Zn$^{2+}$ is intriguing (ionic radius of 74 Å) and confirms that alterations in His$^{338}$ affect the uptake of other metals, as indicated by our preliminary findings using the yeast growth assay and transport experiments (data not shown and Fig. 5). The observation that changes in hydrophilic residues within the transporter filter can change substrate specificity has been detailed with K$^+$ channels and other transporters (23, 24). For example, a Q103A mutation in the plant metal transporter IRT1 apparently eliminated zinc transport but did not alter the transport of other metals (25). Similarly, a T305A mutant of OsCAX1 may increase Mn$^{2+}$/Ca$^{2+}$ transport without altering Ca$^{2+}$ transport (8). However, the IRT1 and OsCAX1 studies utilized yeast growth assays to infer transport function, and thus the kinetic changes in these transporters can only be inferred.

The role of histidines can vary for different transporters and can also depend on the position of the residue within the protein sequence. For some channel proteins such as the guard cell inward-rectifying K$^+$ channel, KST1, and the cardiac pacemaker channel NCN2, the histidine residues appear to be involved in a pH-sensing mechanism (26, 27). In the sucrose/H$^+$ cotransporter, SUC1, a histidine residue is implicated in substrate recognition and directly in the transport reaction (28). In human H$^+$/peptide cotransporters, two different histidines play two different roles; one conserved histidyl residue appears to be important for translocation of protons (29), whereas the second seems to be responsible for the recognition of transportable peptide substrates (11). Although these are

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**Fig. 6.** Michaelis-Menten kinetic analysis of the initial rate of metal/H$^+$ exchange. A preset, steady-state pH gradient (inside acidic) was generated in isolated vacuolar membrane vesicles from yeast cells expressing sCAX1 or H338N by activation of the H$^+$-ATPase. Initial rates of H$^+$-dependent metal recovery of quinacrine fluorescence were calculated over a range of metal concentrations from 10 to 1000 μM (Cd$^{2+}$ in A or Zn$^{2+}$ in B). All data represent means ± S.E. of experiments performed using three independent membrane preparations.

**Fig. 7.** Hydropathy values of biological amino acids according to Kyte and Doolittle (4). The four amino acids, when inserted at the position 338 of AtCAX1, that suppressed the Ca$^{2+}$ sensitivity of the pmc1 vcx1 cnb yeast strain are indicated in boldface letters.
only some examples, such a diverse role of histidine residues is not surprising; histidine is capable of forming intra- and intermolecular hydrogen bonds. These bonds can have a variety of effects on the protein, from inducing conformational changes and changes in pore size to influencing activity and transport of H\(^+\) and other ions (12).

This site-directed mutagenesis study will help future crystallography studies to define the quaternary structure of CAX1 and other H\(^+\)/cation exchangers. The ability to manipulate the metal transport activity and selectivity of CAX transporters through simple mutations, as has been demonstrated in this study, holds promise that plants can be custom-engineered to become metal accumulators, enhancing characteristics needed when selecting plants to be employed as phytoremediators.

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REFERENCES

1. Pittman, J. K., and Hirschi, K. (2003) Curr. Opin. Plant Biol. 6, 257–262
2. Gaxiola, R., Fink, G., and Hirschi, K. (2002) Plant Physiol. 129, 967–973
3. Cai, X., and Lytton, J. (2004) Mol. Biol. Evol. 21, 1692–1703
4. Kyte, J., and Doolittle, R. F. (1982) J. Mol. Biol. 157, 105–132
5. Hirschi, K., Zhen, R., Cunningham, K. W., Rea, P. A., and Fink, G. R. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 8782–8786
6. Shigaki, T., Cheng, N., Pittman, J. K., and Hirschi, K. (2001) J. Biol. Chem. 276, 43152–43159
7. Shigaki, T., Pittman, J. K., and Hirschi, K. (2003) J. Biol. Chem. 278, 6610–6617
8. Kamiya, T., and Maeshima, M. (2004) J. Biol. Chem. 279, 812–819
9. Park, S., Kim, C., Pike, L., Smith, R., and Hirschi, K. (2004) Mol. Breed. 14, 275–282
10. Hirschi, K. (2004) Plant Physiol. 136, 2338–2342
11. Chen, X. Z., Steel, A., and Hediger, M. A. (2000) Biochem. Biophys. Res. Commun. 272, 726–730
12. Wiebe, C. A., Dibattista, E. R., and Fliegel, L. (2001) Biochem. J. 357, 1–10
13. Nathan, D. F., Vos, M. H., and Lindquist, S. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 1409–1414
14. Geitz, R. D., Schiestl, R. H., Willems, A., and Woods, R. A. (1995) Yeast 11, 355–360
15. Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A., and Struhl, K. (eds) (1998) Current Protocols in Molecular Biology, Greene Publishing Associates/Wiley Interscience, New York
16. Shigaki, T., and Hirschi, K. (2001) Anal. Biochem. 298, 118–120
17. Pittman, J. K., and Hirschi, K. (2001) Plant Physiol. 127, 1020–1029
18. Bradford, M. (1976) Anal. Biochem. 72, 248–254
19. Barkla, B. J., Vera-Estrella, R., Maldonado-Gamma, M., and Pantoja, O. (1999) Plant Physiol. 120, 811–819
20. Bennett, A., and Spanswick, R. (1983) J. Membr. Biol. 71, 95–107
21. Cunningham, K. W., and Fink, G. R. (1996) Mol. Cell. Biol. 16, 2236–2237
22. Zhou, F., Pan, Z., Ma, J., and You, G. (2004) Biochem. J. 384, 87–92
23. Gaber, R., Dreyer, I., Horeau, C., Lemailliet, G., Zimmermann, S., Bush, D., Rodriguez-Navarro, A., Schachtman, D., Spalding, E., and Sentenac, H. (1999) J. Exp. Bot. 50, 1073–1087
24. Uozumi, N., Nakamura, T., Schroeder, J. I., and Muto, S. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 9773–9778
25. Rogers, E. E., Edle, D. J., and Guerinot, M. L. (2000) Proc. Natl. Acad. Sci. U. S. A. 97, 12356–12360
26. Hoth, S., Dreyer, I., Dietrich, P., Becker, D., Muller-Rober, B., and Hedrich, R. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 4806–4810
27. Zeng, X., Steiber, J., Ludwig, A., Hofmann, P., and Biel, M. (2001) J. Biol. Chem. 276, 6313–6319
28. Lu, J. M.-Y., and Bush, D. R. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 9025–9030
29. Fei, Y., Sugawara, M., Nakazishi, T., Huang, W., Wang, H., Prasad, P. D., Leibach, F. H., and Ganapathy, V. (2000) J. Biol. Chem. 275, 23707–237130