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Analysis of the Ca^{2+} domain in the *Arabidopsis* H^+/Ca^{2+} antiporters CAX1 and CAX3

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Abstract

Ca^{2+} levels in plants are controlled in part by H^+/Ca^{2+} exchangers. Structure/function analysis of the *Arabidopsis* H^+/cation exchanger, CAX1, revealed that a nine amino acid region (87–95) is involved in CAX1-mediated Ca^{2+} specificity. CAX3 is 77% identical (93% similar) to CAX1, and when expressed in yeast, localizes to the vacuole but does not suppress yeast mutants defective in vacuolar Ca^{2+} transport. Transgenic tobacco plants expressing CAX3 containing the 9 amino acid Ca^{2+} domain (Cad) from CAX1 (CAX3-9) displayed altered stress sensitivities similar to CAX1-expressing plants, whereas CAX3-9-expressing plants did not have any altered stress sensitivities. A single leucine-to-isoleucine change at position 87 (CAX3-I) within the Cad of CAX3 allows this protein to weakly transport Ca^{2+} in yeast (less than 10% of CAX1). Site-directed mutagenesis of the leucine in the CAX3 Cad demonstrated that no amino acid change tested could confer more activity than CAX3-I. Transport studies in yeast demonstrated that the first three amino acids of the CAX1 Cad could confer twice the Ca^{2+} transport capability compared to CAX3-I. The entire Cad of CAX3 (87–95) inserted into CAX1 abolishes CAX1-mediated Ca^{2+} transport. However, single, double, or triple amino acid replacements within the native CAX1 Cad did not block CAX1 mediated Ca^{2+} transport. Together these findings suggest that other domains within CAX1 and CAX3 influence Ca^{2+} transport. This study has implications for the ability to engineer CAX-mediated transport in plants by manipulating Cad residues.

Abbreviations: Cad, calcium domain; CAX, *Arabidopsis* cation exchanger; VCX, yeast vacuolar calcium exchanger

Introduction

Precise regulation of cytosolic Ca^{2+} levels is essential for adapted physiological responses in all organisms (McAinsh and Hetherington, 1998; Sanders *et al.*, 1999; Ueoka-Nakinishi *et al.*, 1999). Gradients of Ca^{2+} are actively maintained, with the necessary lower concentrations typically located in the cytosol, opposite higher concentrations in the organelles and outside the cell. In plants, the vacuole is an important storage compartment for Ca^{2+} and numerous ions (Marschner, 1995; Marty, 1999). Release of Ca^{2+} stores from the vacuole into the cytosol has been implicated in numerous biological responses (Alexandre *et al.*, 1990; Gilroy *et al.*, 1993). After the cytosolic Ca^{2+} burst, plant vacuolar transporters must vigilantly reset the cytosolic Ca^{2+} level.

H^+/Ca^{2+} exchange helps establish the concentration gradient of Ca^{2+} across various plant membranes including the tonoplast (vacuolar membrane) (Blumwald and Poole, 1986; Schumaker and Sze, 1985; Kasai and Muto, 1990; Ettinger *et al.*, 1999). H^+/Ca^{2+} antiporters have been cloned from bacteria, fungi and plants (Ivey *et al.*, 1993; Cunningham and Fink, 1996; Hirschi *et al.*, 1996). In *Escherichia coli*

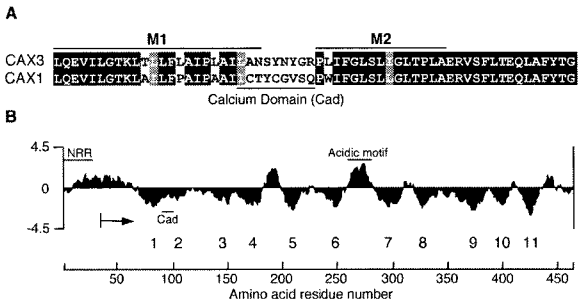

Figure 1. Ca^{2+} domains in CAX3 and CAX1. Alignment of deduced amino acid sequences between the deduced first and second membrane spanning domains (M1 and M2) of polypeptides encoded by *A. thaliana* CAX1 and CAX3. Alignments were performed by using CLUSTALW 1.8 program (Baylor College of Medicine; Thompson *et al.*, 1994). Consensus amino acid residues are boxed in black (identical) or gray (similar). The region shown is put into context of the entire CAX open reading frame by showing a hydropathy plot of CAX1 (Shigaki and Hirschi, 2000). The positions of the 36 amino acid N-terminal regulatory region (NRR), the Ca^{2+} domain (Cad) and the acidic motif are highlighted. The arrow indicates the start of the truncated CAX1 open reading frame used in this study.

and yeast, structure/function studies have only hinted at motifs involved in H^+/cation specificity.

Two *Arabidopsis thaliana* H^+/Ca^{2+} transporters, CAX1 (cation exchanger 1) and CAX2 were identified (Hirschi *et al.*, 1996; Hirschi, 2001) by their ability to sequester Ca^{2+} into yeast vacuoles in *Saccharomyces cerevisiae* mutants deleted for the vacuolar Ca^{2+} ATPase (*PMC1*) and H^+/Ca^{2+} antiporter (*VCX1*). Using yeast as an experimental tool, two domains have been identified which modulate CAX activity (Pittman and Hirschi, 2001; Shigaki *et al.*, 2001). The first domain that regulates CAX function has been termed the N-terminal regulatory region (NRR) (Pittman and Hirschi, 2001; Figure 1B). The CAX1 open reading frame contains an additional 36 amino acids at the N-terminus that were not found in the original clone (identified by suppression of yeast vacuolar Ca^{2+} transport mutants). This longer version of CAX1 does not suppress the yeast Ca^{2+} transport defects despite localization to the yeast vacuole. Minor alterations in the 36 amino acid region restore H^+/Ca^{2+} transport (Pittman and Hirschi, 2001). Sequence analysis suggests that an N-terminal regulatory domain may be present in all *Arabidopsis* CAX-like transporters; however, in this study we utilize CAX1 constructs that do not contain this N-terminal regulatory region.

The second domain, termed the Ca^{2+} domain (Cad), is located between amino acids 87 and 95 in CAX1 (Shigaki *et al.*, 2001; Figure 1). CAX3 is highly similar to CAX1, yet it fails to suppress yeast vacuolar Ca^{2+} transport mutants (Shigaki and Hirschi, 2000). However, CAX3 can suppress the mutants if this 9 amino acid region of CAX1 is inserted into CAX3 which lacks the N-terminal regulatory region (CAX3-9). A single change of leucine to isoleucine within this region caused CAX3 to weakly suppress the yeast Ca^{2+}sensitivity (CAX3-I). These findings suggest structural features involved in regulation of H^+/cation antiport; however, the extent to which the Cad can be engineered to alter Ca^{2+} transport has not been systematically addressed.

In this study, we have tried to further define the modifications that can alter the Ca^{2+} transport properties of the CAX transporters. To confirm that yeast CAX structure-function studies have relevance to changing plant ion uptake, we analyzed transgenic tobacco plants expressing mutant CAX3 constructs harboring the entire Cad from CAX1 (CAX3-9). We analyzed yeast strains harboring various point mutations at amino acid 87 within the Cad of CAX3. We then analyzed several modifications of CAX1 and CAX3 Cad residues. These findings suggest that CAX3-9 is sensitive to modifications within the Cad while changes in the Ca^{2+} transport properties of CAX1 may require multiple amino acid changes within the Cad. These findings may have broad implications for altering the ion uptake of CAX-like transporters.

Materials and methods

Yeast strains, vectors, and DNA manipulations

K667 (*cnb1::LEU2 pmc1::TRP1 vcx1*) was the yeast strain used to express wild-type and mutant genes. The genes were propagated in either pBluescript II SK(+) (Stratagene, La Jolla, CA) or pGEM-7Zf(+) (Promega, Madison, WI) and inserts were transferred to the shuttle vector piHGpd (Nathan *et al.*, 1999) for their expression in the yeast. The plasmids were introduced into yeast by the lithium acetate/single-stranded DNA/polyethylene glycol transformation method (Gietz *et al.*, 1995). Standard techniques were used to manipulate DNA used in this study (Ausubel *et al.*, 1998). All CAX1 and CAX3 variants used in this study lack the N-terminal regulatory region.

Site-directed mutagenesis

Site-directed mutagenesis was performed with a QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA) or a method described by Shigaki

Table 1. Primers used for site-directed mutagenesis.

Introduction of a random amino acid at residue number 87 of CAX3[a]
F 5′-GAATTCCGTCTCCCATT**NNN**GCCAATTCTTACAACTACGGTC-3′
R 5′-GAATTCCGTCTCCAATGGCGAGAGGGATGGCGAG-3′
CAX3-6 (CAX3-3 used as the template)[b]
F 5′-CCTCTCGCCATTATTTGCACTT**A**TT**GC**GCTACGGTCGTCCGTTGATATTTG-3′
R 5′-CAAATATCAACGGACGACCGTAG**CC**G**C**AA**T**AAGTGCAAATAATGGCGAGAGG-3′
CAX3-3X3[a]
F 5′-GAATTCCGTCTCCCATT**ATTTGCACC**TCTTACAAC**GTCAGTCAG**CCGTTGATATTTGGACTGAGC-3′
R 5′-GAATTCCGTCTCCAATGGCGAGAGGGATGGCGAG-3′
CAX1-3[b]
F 5′-CCGGCCATACCTGCCGCCATT**C**TT**GC**CA**A**CTATTGTGGCGTCAGTCAGCC-3′
R 5′-GGCTGACTGACGCCACAATAG**T**TG**GC**AA**G**AATGGCGGCAGGTATGGCCGG-3′
CAX1-X3[b]
F 5′-CCTGCCGCCATTATTTGCACCT**C**TT**A**T**AA**CGTCAGTCAGCCTTGGATATTTG-3′
R 5′-CAAATATCCAAGGCTGACTGACG**TT**A**T**AA**G**AGGTGCAAATAATGGCGGCAGG-3′
CAX1-XX3[a]
F 5′-GAATTCCGTCTCCTGGC**TACGGTCGT**CCTTGGATATTTGGACTTAGC-3′
R 5′-GAATTCCGTCTCCGCCACAATAGGTGCAAATAATG-3′
CAX1-6[a]
F 5′-GAATTCCGTCTCCCATT**CTTGCCAATTCTTACAAC**GTCAGTCAGCCTTGGATATTTG-3′
R 5′-GAATTCCGTCTCCAATGGCGGCAGGTATGGCCGG-3′
CAX1-X6[a]
F 5′-GAATTCCGTCTCCCACC**TCTTACAACTACGGTCGT**CCTTGGATATTTGGACTTAGC-3′
R 5′-GAATTCCGTCTCCGGTGCAAATAATGGCGGCAGG-3′
CAX1-C88A/C91Y[a]
F 5′-GAATTCCGTCTCCTATT**GCCACCTATTAC**GGCGTCAGTCAGCCTTGGATATTTG-3′
R 5′-GAATTCCGTCTCCAATAATGGCGGCAGGTATGGC-3′
CAX1-T89N/Y90S[a]
F 5′-GAATTCCGTCTCCTTGC**AATTCT**TGTGGCGTCAGTCAGCCTTGG-3′
R 5′-GAATTCCGTCTCCGCAAATAATGGCGGCAGGTATG-3′
CAX1-A76T[b]
F 5′-GTCATTCTCGGAACAAAGCTT**A**CCATTCTTTTTCCGGCCATACC-3′
R 5′-GGTATGGCCGGAAAAAGAATGG**T**AAGCTTTGTTCCGAGAATGAC-3′
CAX1-P80L[b]
F 5′-CTTGCCATTCTTTTTC**TC**GCCATACCTGCCGCC-3′
R 5′-GGCGGCAGGTATGGC**GA**GAAAAAGAATGGCAAG-3′
CAX1-A84L[b]
F 5′-GCCATTCTTTTTCCGGCCATACCT**CT**CGCCATTATTTGCACCTATTGTGGC-3′
R 5′-GCCACAATAGGTGCAAATAATGGCG**AG**AGGTATGGCCGGAAAAAGAATGGC-3′
CAX1-W97L[b]
F 5′-GCGTCAGTCAGCCTTT**T**GATATTTGGACTTAG-3′
R 5′-CTAAGTCCAAATATC**A**AAGGCTGACTGACGC-3′

F and R indicate a forward and a reverse primer, respectively.

[a]Mutagenesis with primers with a Class IIS restriction enzyme site (Shigaki and Hirschi, 2001). Double underlines indicate *Bsm*BI (a Class IIS restriction enzyme) site. Bases to be altered are highlighted in bold, and the sequence that is used for annealing with the template is shown underlined.

[b]Mutagenesis with a QuikChange kit (Stratagene, La Jolla, CA). Bases to be altered are highlighted in bold.

and Hirschi (2001). All mutant clones analyzed in this study were completely sequenced to confirm that the PCR mutagenized DNA contained no errors. The primers used in the study are shown in Table 1.

Assay for yeast suppression

The assay for Ca^{2+} tolerance on solid agar media was performed as previously described by Hirschi *et al.* (1996). K667 strains expressing various constructs were grown in a selective liquid medium for 16 h, and 5 μl of diluted cells were spotted on YPD agar medium containing 200 mM $CaCl_2$, unless otherwise specified. The cultures were then allowed to grow for 48 h at 30 °C.

Preparation of membrane vesicles for Ca^{2+} transport assays

Vacuolar membrane-enriched microsomes were prepared as previously described by Nakanishi *et al.* (2001). The yeast cells expressing various constructs were grown in yeast and assayed as previously described (Pittman and Hirschi, 2001).

Measurement of $^{45}Ca^{2+}$ uptake

For the measurement of the time dependence of Ca^{2+} uptake, membrane vesicles were prepared and analyzed as previously described (Pittman and Hirschi, 2001; Shigaki *et al.* 2001).

Plant transformation and ion sensitivity assay

The coding region of *CAX3* and *CAX3–9* were cloned into pBIN19 (Clontech, Palo Alto, CA), which contained the cauliflower mosaic virus 35S-promoter fragment (35S::CAX3 and 35S::pCAX3-9) (Hull *et al.*, 2000). The recombinant plasmids, or vector controls, were introduced into *Agrobacterium tumefaciens* strain LBA4404 (Life Technologies, Grand Island, NY). Tobacco leaf disc transformation were carried out as previously described with tobacco cultivar KY160 (Hirschi, 1999). Transformants were selected on MS medium (Murashige and Skoog, 1962) containing 50 μg/ml kanamycin. About 20 primary transformants harboring the 35S::*CAX3* or 35S::*CAX3-9* construct were planted in soil. Expression of the introduced genes was confirmed by reverse transcription-PCR (RT-PCR) as previously described (Cheng *et al.*, 2002).

Surface-sterilized tobacco seeds were plated on standard media and maintained as previously described (Hirschi, 1999). For ion sensitivity analysis, surface-sterilized seeds were germinated and grown in standard medium. The 10-day old seedlings were transferred to standard medium supplemented with the appropriate ion.

Results

CAX3 and CAX3-9 expression in tobacco

Expression of the *Arabidopsis CAX1* gene in tobacco causes altered Ca^{2+} homeostasis which leads to various stress sensitivities (Hirschi, 1999, 2001). Although the precise mechanisms of these stress sensitivities require further studies, the CAX1-expressing tobacco plants show phenotypes which are distinct from CAX2-expressing plants. In fact, even though CAX2-expressing plants accumulated Ca^{2+} levels comparable to those seen with CAX1, these plants were, for the most part, as vigorous as controls (Hirschi *et al.*, 2000). We were interested in testing the phenotypes of heterologous expression of CAX3 and CAX3-9 in tobacco.

Ten transgenic lines of tobacco were generated with the *CAX3* and *CAX3-9* open reading frames expressed in the sense orientation in front of the 35S promoter. As a control, several transgenic lines harboring only the expression vector were made. The expression of *CAX3* and *CAX3-9* RNA was verified in T_2 transgenic lines by RNA gel blot analysis or RT-PCR (data not shown). CAX3 RNA accumulated in all 35S::*CAX3* and 35S::*CAX3-9* transgenic lines analyzed. *CAX3*-specific RNA was not present in the vector control lines (data not shown). As shown in Figure 2, all transgenic lines grew well on normal media. In media containing elevated levels of magnesium, the vector control and all ten CAX3-expressing lines analyzed showed no altered growth; however, CAX1 expressing plants were extremely sensitive to these growth conditions and all ten CAX3-9 lines analyzed showed increased stress sensitivity. All CAX3-9-expressing lines also demonstrated modest sensitivity to KCl and NaCl that was not evident in any of the CAX3-expressing lines (data not shown).

Single amino acid changes within the CAX3 Cad

Previously, we have shown that a Leu-to-Ile change at amino acid 87 of CAX3 can cause weak suppression

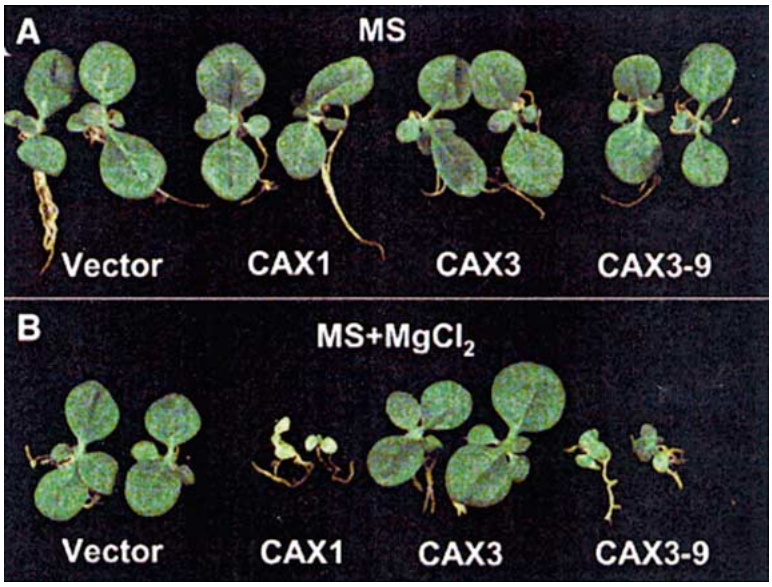

Figure 2. Ion sensitivity of p*CAX3-9*-expressing plants. Two vector control transgenic tobacco plants are at the left, followed by two *CAX1*-expressing plants (35S::*CAX1*) then two CAX3-expressing plants (35S::*CAX3*) and at the far right two CAX3-9-expressing plants (35S::p*CAX3-9*). A. Tobacco plants transferred 10 days after germination to standard medium (MS) and grown for 10 additional days. B. Tobacco plants transferred 10 days after germination to standard medium supplemented with 30 mM $MgCl_2$ and grown for 10 additional days.

of the yeast vacuolar Ca^{2+} transport defect (Shigaki *et al*., 2001). This is the only single residue change in CAX3 that appears to confer yeast vacuolar Ca^{2+} transport; however, this variant of CAX3 has less than 10% of the Ca^{2+} transport capacity of CAX1. We were interested in altering this residue in CAX3 in an attempt to heighten transport and elucidate the nature of the Cad. Through a mutagenesis approach, we made multiple changes at residue 87. As shown in Figure 3, analysis of yeast strains harboring different CAX3 variants suggests that none of the strains analyzed conferred higher levels of H^+/Ca^{2+} antiport than did CAX3-I.

Multiple amino acid changes in the CAX3 Cad

Previously, we demonstrated that CAX3-9-expressing vesicles had ca. 36.5% of the uptake capability of CAX1-expressing vesicles and the CAX3-I-expressing vesicles had 7.1% of the uptake of the CAX1-expressing vesicles (Shigaki *et al*., 2001). We were interested in determining the transport properties of CAX3 variants containing the first 3 (previously termed CAX3-ICT now termed CAX3-3) or 6 amino acids of the CAX1 Cad (CAX3-6). Addition of $MgCl_2$/ATP and establishment of a steady-state ΔpH by the V-ATPase associated with the vacuolar membrane followed by the addition of $^{45}Ca^{2+}$ re-

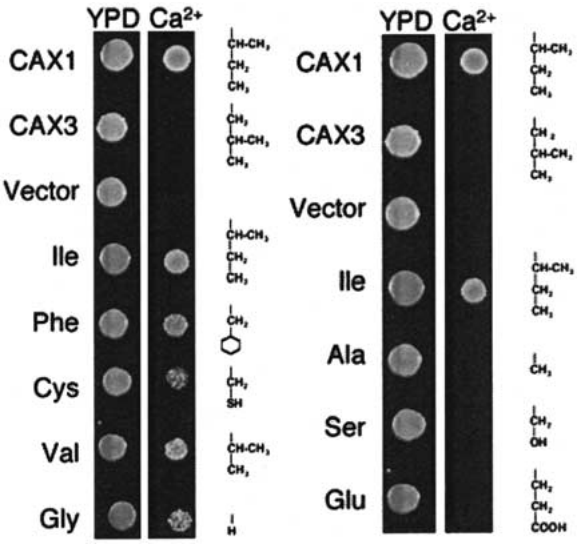

Figure 3. Effects of changes made at amino acid residue number 87 of CAX3 on Ca^{2+} sensitivity of K667. Saturated liquid cultures of *pmc1vcx1cnb* yeast strains (K667) harboring CAX3 plasmids with a change of amino acid residue number 87 from Leu to the indicated amino acid. The cultures were spotted onto yeast-peptone-dextrose media (YPD) or medium that selects for the presence of plasmid-borne vacuolar Ca^{2+} transport, i.e. YPD containing 175 mM $CaCl_2$ (Hirschi *et al.*, 1996). The photographs were taken after 2 days.

sulted in uptake into endomembrane vesicles from CAX3-9-, CAX3-6- and CAX3-3-expressing K667 vesicles. The CAX3-6-and CAX3-3-expressing vesicles both had ca. 60% of the uptake capability of CAX3-9-expressing vesicles (Figure 4A). In CAX3-9- , CAX3-6- and CAX3-3-expressing K667 vesicles, the inclusion of gramicidin (an uncoupler of the proton gradient) in the uptake medium decreased $^{45}Ca^{2+}$ uptake to a level similar to that seen in the absence of $MgCl_2$/ATP (data not shown). As previously reported, the low rate of uptake found in vesicles from K667 cells transformed with control vector was not inhibited by gramicidin or by the V-ATPase inhibitor, bafilomycin (data not shown). These results, together with the release of $^{45}Ca^{2+}$ seen with the addition of the Ca^{2+} ionophore A23187 to CAX3-9, CAX3-6 and CAX3-3 vesicles, versus the small increase seen in $^{45}Ca^{2+}$ uptake with vector control vesicles, demonstrate that CAX3-9, CAX3-6- and CAX3-3-generated uptake is concentrative.

CAX3-9-expressing yeast cells had ca. 40% more Ca^{2+} transport activity than CAX3-3- and CAX3-6-expressing yeast cells. Since there is no difference between CAX3-3- and CAX3-6-mediated Ca^{2+} transport, one may infer that the middle 3 amino acids of the CAX1 Cad are not important for mediating H^+/Ca^{2+} transport. To test this hypothesis, we analyzed the effect of changing the first 3 and last 3 amino acids of the CAX3 Cad. We have termed this construct CAX3-3X3. Given our previous results, we speculated that this chimeric construct would be as active as CAX3-9. Interestingly, yeast cells expressing CAX3-3X3 could not suppress the vacuolar Ca^{2+} transport defect (Figure 4B).

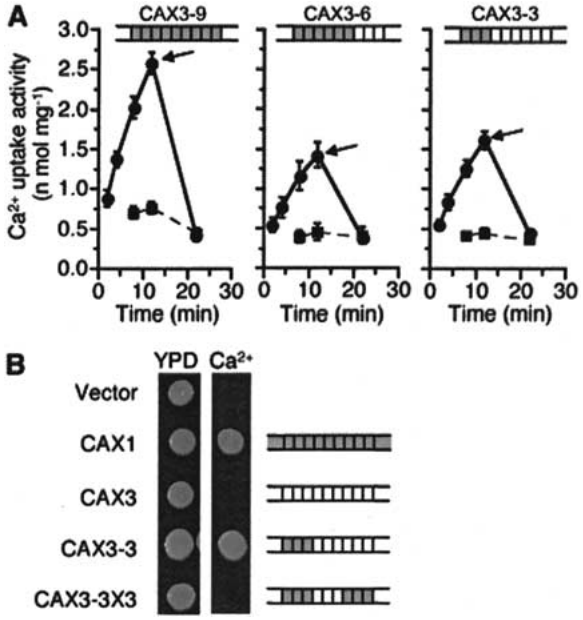

Figure 4. Effects of alteration within Cad of CAX3. A. Time-course of $^{45}Ca^{2+}$ uptake into endomembrane vesicles prepared from *S. cerevisiae* strain K667 after transformation with CAX3-9, CAX3-6, and CAX3-3. CAX3-9, CAX3-6, and CAX3-3 are CAX3 derivatives with the entire nine, first six, or first three amino acids of Cad replaced with the corresponding amino acids from CAX1, respectively. Schematic diagrams of the constructs are also shown. Each box represents an amino acid residue within the Cad. Gray and white boxes represent CAX1 and CAX3 residues, respectively. Uptake in the absence of protonophore gramicidin (●); uptake in the presence of gramicidin (■). The Ca^{2+} ionophore, A23187 (5 μM), was added at the times indicated by an arrow. $^{45}Ca^{2+}$ was added at a concentration of 10 μM throughout. The data represent means of three replications, and the bars indicate standard errors of mean. B. Suppression of Ca^{2+} sensitivity by CAX3 derivatives with mutations within Cad. CAX3X3 is a CAX3 mutant that has the first and the last three amino acids of Cad from CAX1 (a six amino acid change). The same assay conditions were used as in Figure 3 except that the concentration of calcium was 200 mM.

Inhibiting CAX1-mediated H^+/Ca^{2+} transport through alterations in the Cad

The Cad of CAX3 fused in frame to CAX1 (termed CAX1-9) inhibits CAX1's ability to suppress the K667 Ca^{2+}-sensitive phenotype (Shigaki *et al.*, 2001). We were interested in further defining which residues within the CAX3 Cad could inhibit CAX1 function. As shown in Figure 5, yeast vacuolar transport mutants expressing chimeric CAX constructs containing the first 3 amino acids of the Cad from CAX3 (CAX1-3), the second 3 amino acids of the Cad from CAX3 (CAX1-X3), and the third 3 amino acids of the Cad from CAX3 (CAX1-XX3) all suppressed yeast vacuolar Ca^{2+} transport defects. Yeast vacuolar transport mutants expressing chimeric CAX constructs containing the first 6 amino acids of the Cad from CAX3 (CAX1-6) did not suppress the Ca^{2+} sensitivity phenotype. However, yeast vacuolar transport mutants expressing a chimeric CAX constructs containing the last 6 amino acids of the Cad from CAX3 (CAX1-X6) could suppress the Ca^{2+} phenotype.

We also made several changes at amino acids in the regions adjacent to Cad of CAX1 to corresponding amino acids found in CAX3, namely, A76T, P80L, A84L, and W97L. The mutants with these changes suppressed Ca^{2+} sensitivity, and were indistinguishable from the wild-type phenotype (data not shown).

These findings implicated multiple residues from the first 6 amino acids of the CAX3 Cad being in-

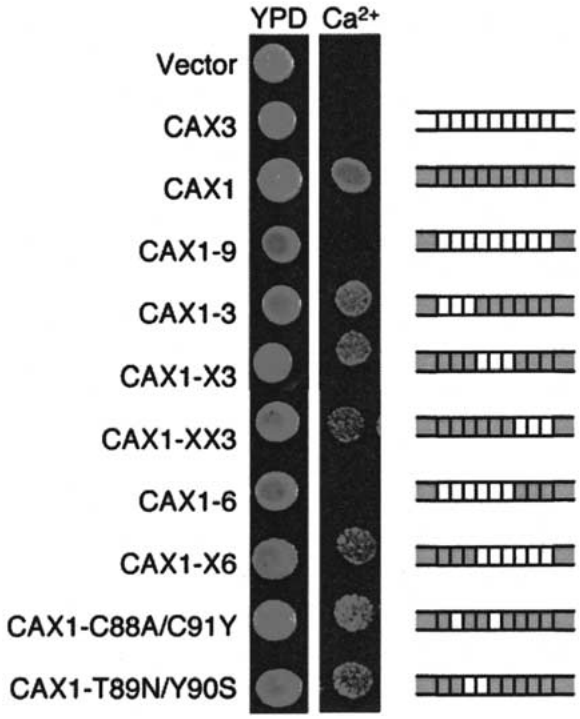

Figure 5. Suppression of Ca^{2+} sensitivity by various CAX1 Cad mutants. The assay conditions were the same as in Figure 3, with Ca^{2+} medium containing 200 mM $CaCl_2$. CAX1-9 has an entire Cad region from CAX3. CAX1-3, CAX1-X3, and CAX1-XX3 have the first, second, and third three amino acid regions from the Cad of CAX3, respectively. CAX1-6 and CAX1-X6 have the first and last six amino acids from the Cad of CAX3, respectively. Schematic diagrams of the constructs are also shown. Each box represents an amino acid residue within the Cad. Gray and white boxes represent CAX1 and CAX3 residues, respectively.

volved in inhibiting CAX1-mediated H^+/Ca^{2+} transport. To directly test this, we exchanged several amino acids within this six amino acid region simultaneously. Yeast mutants expressing either a chimeric CAX1 constructs containing changes at amino acids 88 and 91 (CAX1-C88A/C91Y) or changes at amino acids 89 and 90 (CAX1-T89N/Y90S) were both capable of suppressing the vacuolar Ca^{2+} transport defect (Figure 5).

Discussion

Regulation of cytosolic Ca^{2+} levels in plants has been attributed in part to H^+/Ca^{2+} antiport activity. We initially utilized yeast to rapidly identify the Ca^{2+} domain (Cad) in the H^+/Ca^{2+} exchanger of CAX1 (Shigaki *et al.*, 2001), we have also demonstrated that this domain can cause alterations in CAX-mediated transport in plants (Figure 2). CAX3 expression in yeast does not produce changes in Ca^{2+} transport (Shigaki *et al.*, 2001) and transgenic tobacco expressing CAX3 did not produce any alterations in ion sensitivities. When the Cad of CAX1 was fused to CAX3 (CAX3-9), yeast vesicles exhibit increased Ca^{2+} transport and transgenic tobacco plants expressing CAX3-9 demonstrated ion sensitivities that were similar, but less severe, than tobacco plants expressing CAX1 (Hirschi, 1999). The CAX1 induced ion sensitivities in transgenic tobacco were correlated with altered Ca^{2+} homeostasis (Hirschi, 1999). We speculate that the CAX3-9 ion sensitivities are also caused by altered Ca^{2+} homeostasis. In short, the domains that appear to mediate CAX-dependent Ca^{2+} transport are similar in our plant and yeast studies.

The mechanism by which the Cad may regulate ion transport is currently unknown. However, the message from this study is that the manipulation of Cad residues is extremely 'context-specific'. In other words, other regions of the CAX1 and CAX3 transporters are influencing Ca^{2+} transport. The Cad appears to be a hydrophilic loop that separates membrane-spanning domains 1 and 2 (Shigaki *et al.*, 2001). Currently we do not know the topology of the CAX transporters within the membrane and how this Cad relates to the structure (context) of the entire protein. A Leu-to-Ile change in this region (CAX3-I; amino acid 87) allows the CAX3 gene product to weakly suppress the yeast vacuolar transport mutant (Shigaki *et al.*, 2001). We made multiple different amino acid substitutions at this residue and no change increased the activity of CAX3 more than the Leu-to-Ile change. Yeast strains harboring CAX3 variants with particular aliphatic and neutral amino acid changes at position 87 caused growth on Ca^{2+} containing media. The suppression of Ca^{2+} sensitivity with these CAX3 variants was equal to (or weaker than) the CAX3-I mutant (Figure 3). Other CAX3 variants harboring aliphatic, neutral or acidic amino acids at position 87 were not active. Although it is difficult to rationalize the mode of action of the Cad from these studies, it is apparent that the Leu residue at position 87 is not itself inhibitory and the Ile is not specifically required for activity. Conceivably, the amino acids that are conferring H^+/Ca^{2+} transport to CAX3 are permitting a particular three-dimensional motif to be obtained while the inhibitor residues confer a different structure to the protein. It should be noted that because we did not sequence all 35 CAX3 variants made, we can not rule out the possibility that some

PCR errors may have masked the positive effect of changing this Leu to another residue not reported in Figure 3.

The inability of single or multiple amino acid changes within the Cad of CAX3 to confer high levels of Ca^{2+} transport (less than 30% of CAX1; Figure 4) suggests that the amino acids in this region may form a functional unit which is difficult to decipher through the experimental approaches utilized here. Replacement of the CAX3 Cad with amino acids from the Cad from CAX1 suggest that the Ile residue (87) is necessary but does not confer high levels of Ca^{2+} transport unless other residues from the CAX1 Cad are present (Shigaki *et al*., 2001; Figure 4). However, it is difficult to identify which particular residues within this region are also important for CAX-mediated Ca^{2+} transport. An example of this difficulty is readily apparent from the results obtained with the CAX3-3X3 construct. Both CAX3-3 and CAX3-9 can suppress yeast mutants defective in vacuolar Ca^{2+} transport; however, if the last three amino acids from the Cad of CAX1 were added to CAX3-3, it abolished Ca^{2+} transport. This finding suggests that CAX3-9 is sensitive to changes within the Cad and that caution should be exercised when making chimeric constructs of different Cad residue from various CAX-like transporters.

Reciprocal experiments where we attempted to abolish the Ca^{2+} transport of CAX1 through alterations at the Cad suggest that this transporter is quite 'sturdy'. CAX1-mediated Ca^{2+} transport is not easily abolished through simple substitutions between CAX1 and CAX3 in the Cad. Loss of the Ile at residue 87 did not inhibit CAX1 function (data not shown). Furthermore, when the three amino acids that form the core of the Cad from CAX3 were inserted into CAX1 (CAX1-X3X), they did not inhibit CAX1 function. The residues that confer activity to CAX3 when mutated in CAX1 did not abolish activity. The two Cys residues in the Cad of CAX1 may form a stable disulfide bond important for CAX1 function; however, when both these amino acids were changed to the amino acids found in the Cad of CAX3, the altered protein still functioned (Figure 5). In a similar fashion, Thr and Tyr residues within the Cad may be phosphorylated to alter CAX1 function, yet mutations in both these residues did not inhibit CAX1 function (Figure 5).

Altering the Ca^{2+} content of agriculturally important crops may be feasible by manipulating the CAX transporters (Hirschi, 1999, 2001). In animal cells and yeast, capacitative calcium entry (CCE) mechanisms become activated when vacuolar Ca^{2+} transporters are over-expressed (Locke *et al*., 2000). This CCE response requires putative plasma membrane voltage-gated Ca^{2+} channels. A model for increasing the Ca^{2+} content in agricultural crops would be to manipulate various endomembrane transporters to transport Ca^{2+} more efficiently. Once we understand the structural determinants of Ca^{2+} antiport, it could lead to the ability to make 'designer' transporters which preferentially sequester this nutrient into edible portions of plants. This present study suggests that any of the CAX transporters can be modified within the Cad; however, the biological implications of these changes may not be easily transferable between the CAX transporters.

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