A Plant Ca\(^{2+}\) Pump, ACA2, Relieves Salt Hypersensitivity in Yeast

MODULATION OF CYTOSOLIC CALCIUM SIGNATURE AND ACTIVATION OF ADAPTIVE Na\(^{+}\) HOMEOSTASIS\(^{\dagger}\)

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Stress responses in both plants and yeast utilize calcium-mediated signaling. A yeast strain, K616, which lacks Ca\(^{2+}\) pumps, requires micromolar Ca\(^{2+}\) for growth. In medium containing 100 \(\mu\)M Ca\(^{2+}\), K616 can withstand osmotic stress (750 mM sorbitol) and ionic stress (300 mM KCl) but not hypersodic stress (300 mM NaCl). Heterologous expression of the endoplasmic reticulum-located Arabidopsis thaliana Ca\(^{2+}\)-ATPase, ACA2, permits K616 to grow under NaCl stress even in Ca\(^{2+}\)-depleted medium. All stresses tested generated transient elevation of cytosolic Ca\(^{2+}\) in wild type yeast, K601, whereas NaCl alone induced prolonged elevation of cytosolic Ca\(^{2+}\) in K616. Both the Ca\(^{2+}\) transient and survival of cultures subjected to NaCl stress was similar for the ACA2 transformant and K601. However, whereas K601 maintained low cytosolic Na\(^{+}\) predominantly by pumping it out across the plasma membrane, the transformant sequestered Na\(^{+}\) in internal organelles. This sequestration requires the presence of an endomembrane Na\(^{+}\)/H\(^{+}\)-antiporter, NHX1, which does not play a significant role in salt tolerance of wild type yeast except at acidic pH. Transcript levels of the plasma membrane Na\(^{+}\)-ATPase, ENA1, were strongly induced only in K601, whereas NHX1 was strongly induced in both K601 and the ACA2 transformant. The calmodulin kinase inhibitor KN62 significantly reduced the salt tolerance of the ACA2 transformant and the transcriptional induction of NHX1. Thus, the heterologous expression of a plant endoplasmic reticulum Ca\(^{2+}\) pump results in the rapid depletion of cytosolic Ca\(^{2+}\) and the activation of an alternate mechanism for surviving saline stress.

Eukaryotic cells regulate a variety of cellular processes, including responses to abiotic stresses using calcium-mediated processes. Soil salinity adversely affects plant yields worldwide (1), salt-tolerant cultivars being reported to utilize signal transduction systems involving calcium (2–4). NaCl-induced transient and survival of cultures subjected to NaCl stress were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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\(^{\dagger}\) The online version of this article (available at http://www.jbc.org) contains supplemental Figs. 1–3.

\(^{2}\) The abbreviations used are: CaM, calmodulin; SOS, salt overly sensitive; ER, endoplasmic reticulum; RT, reverse transcription; ACA, N-(p-amyl-cinnamoyl) anthranilic acid; MES, 4-morpholineethanesulfonic acid.
ER \text{Ca}^{2+}-\text{ATPase Sculpts Signaling \text{Ca}^{2+} \text{Transients}}

to endosomal/vacuolar \text{Na}^+ sequestration when stressed in acidic medium (29, 30). These multiple transport pathways regulating yeast \text{Na}^+ homeostasis are under complex regulation; ENA1 is transcriptionally activated by several distinct pathways (26, 31), of which the HOG1 pathway has also been proposed to enhance activity of the plasma membrane-located NHAI (32).

A substantive understanding of the mechanisms of \text{Ca}^{2+} homeostasis makes yeast an ideal system to study how \text{Ca}^{2+} pumps could influence \text{Na}^+ homeostasis and salt tolerance. Under normal conditions, extracellular \text{Ca}^{2+} enters the yeast cytosol through an as yet unidentified transporter (33). The \text{Ca}^{2+}-\text{ATPase PMR1 pumps }[\text{Ca}^{2+}]_{\text{cyt}} into the ER and Golgi, whereas the high affinity \text{Ca}^{2+}-\text{ATPase, PMCI, and low affinity \text{Ca}^{2+}/\text{H}^+ \text{exchanger, VCX1, serve to sequester it in the vacuole (33). Hypertonic shock, induced by extracellular NaCl, induces release of vacuolar \text{Ca}^{2+} into the cytosol through the tonoplast-located \text{Ca}^{2+} channel YVC1 (34) and possibly influx of external \text{Ca}^{2+} via the CCH1/MID1 \text{Ca}^{2+} channel on the plasma membrane (35). Expression and function of PMCI, PMR1, and VCX1 are regulated by calcineurin (36), a highly conserved protein phosphatase that is activated by \text{Ca}^{2+}/\text{CaM}. In contrast to plants, there are no \text{Ca}^{2+}-\text{ATPases or exchangers detected on the yeast plasma membrane (33) and consequently little or no active efflux of \text{Ca}^{2+} across the plasma membrane.}

The ER-located ACA2 and vacuolar ACA4 are \textit{Arabidopsis} \text{Ca}^{2+}-\text{ATPases that have previously been expressed in yeast (37, 38), and the latter has been shown to ameliorate the salt hyper-sensitivity of the host strain (38). This investigation was carried out to determine whether an ER-located plant \text{Ca}^{2+} pump could facilitate a salt tolerance response and, if so, to understand the underlying cellular mechanism involved. Here we demonstrate that the ER-located ACA2 relieves the hypersensitivity of the salt-sensitive triple mutant yeast strain K616, in which genes encoding the \text{Ca}^{2+} pumps (PMCI, PMR1, and the regulatory subunit of calcineurin, CNB1) have been deleted (39). This strain requires 10^{-4} \text{M} \text{Ca}^{2+} to grow and millimolar \text{Ca}^{2+} to survive hypertonic stress. Heterologous expression of ACA2 in this strain alleviates the calcium requirement in both cases. ACA2-expressing cells sequester \text{Na}^+ into internal stores rather than extrude it across the plasma membrane, as does wild type yeast. Using the aequorin reporter system, we demonstrate that ACA2 alters the [\text{Ca}^{2+}]_{\text{cyt}}, transient induced by NaCl stress in K616. We propose that this action of the pump contributes to the triggering of an alternative pathway, which activates the endomembrane \text{Na}^+/\text{H}^+ -antiporter, NHX1, resulting in enhanced salt tolerance.

**EXPERIMENTAL PROCEDURES**

\textit{Yeast Strains and Growth Media—}\textit{S. cerevisiae} wild type strain K601 (MATa, leu2, his3, ade2, trp1, and ura3) and mutant strain K616 (MATa \text{pmr1::His3 pmc1::Trp1 cnb1::Leu12, ura3}) were grown in complete synthetic medium (SC) and SC minus Trp, His, and Leu, respectively, throughout this study. For the K616 strain, the medium was supplemented with 10 mM \text{CaCl}_2. SC medium consisted of 1.7 g/liter yeast nitrogen base without amino acids, 5 g/liter (NH_4)_2SO_4, 1.3 g/liter drop-out mix without Ura, Trp, His, and Leu (these amino acids were added from separate stock solutions when required), and 2% (w/w) dextrose as a carbon source. Medium pH was adjusted to 6 (buffered with 5 mM MES) prior to the addition of agar and sterilization. Buffering by MES was found to be efficient, since autoclaving did not introduce significant change in medium pH. In all experiments under hyperosmotic stress, a Ca-EGTA buffer was used to maintain the free \text{Ca}^{2+} of SC medium at 100 \mu M, unless otherwise mentioned.

\textit{Yeast Transformation—}For transformations with pYX112 constructs, K616 was transformed by the lithium acetate/polyethylene glycol method (40), and transformants were selected for their ability to grow in the absence of uracil on plates containing SC medium minus Trp, Leu, His, and Ura. For complementation studies, Ura-positive colonies were streaked on complete SC plates containing 10 mM EGTA and incubated for 3 days at 30°C. For transformations with yeast vector pKC147/AEQ, K601 and K616 were transformed and selected for Ura autotrophy as mentioned above. A Ura-positive K616 colony was then subjected to a second transformation with pYX112-ACA2. Transformants were then selected based on their ability to complement on low calcium medium by spreading on SC minus Trp, Leu, His, and Ura, containing 5 mM EGTA. Controls for this selection included a transformation with the same volume of water and a vector alone, both of which showed no growth on the EGTA selection plate. Double transformants were analyzed for the expression of aphaequorin by monitoring luminescence before and after lysis with 5% Triton X-100 and 2 mM CaCl\(_2\) and by colony PCR to confirm the presence of the ACA2 gene. They were also tested for salt tolerance in SC-medium containing 400 mM NaCl.

\textit{NHX1 Knock-out Mutants in K616 Background—}A forward primer (5’TAAAGCCGTATGCAAGGAACTG-3’) that hybridizes 200 bp upstream of the start codon of \text{NHX1} and a reverse primer (5’TGGCGTTGAAGAGAGAATG-3’) that hybridizes 80 bp downstream of the \text{NHX1} stop codon were used to amplify the \text{NHX1}-\text{KanMX} cassette from the appropriate deletion strain in the background of the yeast strain, BY4742 (a gift from Dr. R. Rao). The 1.82-kb PCR product thus obtained was gel-eluted and used to transform K616. This strategy allows specific gene disruption by homologous recombination and chromosomal integration. Recombinants were selected on SC medium containing 1 mg/ml G418. A positive transformant was then subjected to a second round of transformation with pYX112-ACA2 and selected on SC minus Ura, Trp, Leu, and His plates containing 1 mg/ml G418. Genomic DNA was isolated from these colonies and analyzed for the presence of ACA2 and the \textit{KAN-NHX1} sequences by PCR using specific primers followed by DNA sequencing.

\textit{Monitoring Expression of ENA1 and NHX1 by RT-PCR—}Total RNA was extracted from yeast cells exposed to 400 mM NaCl for a range of time periods (0–50 h). The RNA was first treated with RNase-free DNase (Promega, Madison, WI) to eliminate contaminating DNA. 500 ng of RNA each was then subject to a reverse transcriptase reaction using 100 units of Moloney murine leukemia virus reverse transcriptase (Invitrogen) for 1 h at 37°C in buffer containing 50 mM Tris-HCl, pH 8.3, 75 mM KCl, 3 mM MgCl\(_2\), 10 mM dithiothreitol, and 0.5 mM each of dNTPs. The cDNA thus obtained was subjected to a 30-cycle
PCR reaction using standard conditions with primers specific for ENA1 (forward primer, 5’-ATG GGC GAA GGA ACT ACT AA-3’; reverse primer, 5’-CAC ACA AAT GGC ATA GAT AGC-3’) amplifying a 960-bp product and for NHX1 (forward primer, 5’-ATG CTA TCC AAG GTA TGG CTG-3’; reverse primer, 5’-AAC CGA CGA AAA TGT TGC-3’) amplifying a 759-bp product. The constitutively expressed ACTI (actin) was amplified under identical PCR conditions using specific primers (forward primer, 5’-GAG GTT GCT GTT TGG AT-3’; reverse primer, 5’-GGG GTC GTC ATT TCT GGT-3’) amplifying a 680-bp product.

**Stress Conditions**—For drop tests, midlog yeast cultures were washed twice with water, and $A_{600}$ was adjusted to 0.5 with sterile double-distilled water. Cells were serially diluted with water to obtain 10-, 10²-, and 10³-fold dilutions. Five microliters of each dilution was then spotted on SC medium buffered at 20 mM, 100 mM, or 5 mM Ca²⁺ and supplemented with 300 mM KCl, 400/800 mM NaCl, or 750 mM sorbitol concentrations. Growth was recorded after a 3-day incubation at 30 °C.

For growth curves, equal cell inocula were added into liquid SC medium buffered for Ca²⁺ at 100 μM with or without 400 mM NaCl. In some experiments, medium was also supplemented with amiloride (500 μM). Cells were incubated on an incubator shaker at 30 °C. Change in $A_{600}$ was monitored over a period of 2 days. Alternatively, sensitivity curves were generated using log phase cells with or without preincubation with inhibitors such as 5 μM bafilomycin, N-(p-amylcinnamoyl) anthranilic acid (ACA) (30 and 40 μM), KN62 (20, 50, and 100 μM), and SB 203580 (10 and 200 μM) prior to administering the NaCl insult. $A_{600}$ was determined at the 18th hour of salt stress.

**Estimation of Total Cellular Na⁺ and Ca²⁺**—Yeast strains were grown to an $A_{600}$ of 0.6 in SC medium buffered at 100 μM Ca²⁺. Cells were either taken as such or stressed with 400 mM NaCl, and 2-ml aliquots were collected over a range of time points extending up to 18 h. Cells were collected by centrifugation, washed four times in ice-cold Buffer B (10 mM Tris-HCl, pH 6.0, 2 mM MgCl₂, 1% glucose, 0.6 M sorbitol), and dried at 50 °C for 4 days. Dried cells were acid-digested in a 4:1 diacid mixture of perchlorate, and nitrate and Na⁺ levels were estimated by flame photometry using a Systronics Flame Photometer 128 (Ahmedabad, India) (41). In a few experiments, growth was carried out at Ca²⁺ concentrations of 100 μM and 5 mM. At an $A_{600}$ of 0.6, a set of cells grown in 100 μM Ca²⁺ was washed three times with SC medium and adapted to 20 mM Ca²⁺ for 1 h before processing these cells, as well as those at 100 μM and 5 mM for total Ca²⁺ estimation using a Zeeman atomic absorption spectrophotometer (model Z-6100; Hitachi).

**Permeabilization of Plasma Membranes with Cu²⁺**—Yeast strains were grown in 300 ml of SC medium, to $A_{600}$ of 0.6 and stressed for 3 h with 400 mM NaCl. Cells were harvested by centrifugation, washed twice in ice-cold Buffer B, and resuspended in the same buffer at room temperature at a density of $1 \times 10^8$ cells/ml. After withdrawing 2-ml aliquots for total Na⁺ estimation, the remaining cell suspension was subjected to plasma membrane permeabilization with Cu²⁺ as described by Anraku and co-workers (42). CuSO₄ was added to a final concentration of 500 μM and incubated at 23 °C. At the indicated times, aliquots were withdrawn and washed twice with buffer B, and cells were dried for acid digestion and flame photometry.

**Aequorin Luminescence Measurements and [Ca²⁺]cyt Quantification**—Aequorin luminescence was determined using a luminometer (TD-20/20; Turner Designs, Sunnyvale, CA) following procedures used to quantify [Ca²⁺]cyt in yeast (19). Cells were transformed with the 2 μ plasmid pKC147/AEQ (provided by Dr. K. Cunningham) containing the APOAE-QUORIN gene. Transformants, grown to an $A_{600}$ of ~0.4–0.6, were harvested by centrifugation, resuspended in fresh medium (SC-Ura + 1 mM EGTA) at an $A_{600}$ of 10, and loaded with 25 μg/ml coelenterazine (Molecular Probes, Inc., Eugene, OR) for 3 h in the dark at room temperature. Cells were then collected by centrifugation and resuspended in the same volume of fresh medium buffered at 100 μM Ca²⁺. After a preincubation of 1 h, the cells were diluted with the same medium to an $A_{600}$ of 0.5, and a cellular luminescence baseline was determined by 2–3 min of recordings at 10-s intervals.

Hypertonic/ionic shock was administered to yeast cells by the addition of a range of NaCl (100–1200 mM), 300 mM KCl, 50 μM CaCl₂, or 750 mM sorbitol. When required, loaded cells were preincubated for 20 min with 5 μM bafilomycin A1 or 30 μM ACA dissolved in Me₂SO or 10 mM LaCl₃ dissolved in water prior to the acquisition of the basal luminescence readings and the subsequent osmotic/ionic shock. Luminescence from aequorin that remained in cells at the end of an experiment was determined by diluting the cell suspension 1:1 with a solution containing 10% Triton X-100 and 4 M CaCl₂. [Ca²⁺]cyt was calculated using Equation 1,

$$[Ca^{2+}] = \left( \frac{L}{L_{\text{max}}} \right)^{1/3} + \left( \frac{118}{\left( \frac{L}{L_{\text{max}}} \right)^{1/3}} - 1 \right) \left( \frac{7 \times 10^6 - 7 \times 10^5 \left( \frac{L}{L_{\text{max}}} \right)^{3/2}}{7 \times 10^5} \right)$$

where $L$ represents the luminescence intensity at any time point, and $L_{\text{max}}$ is the integrated luminescence intensity (34). $L$ values did not exceed ~1% of the maximal light emission capacity in all three strains. Figures are representative of a minimum of three experiments in each case.

**RESULTS**

**Complementation by Full-length ACA2**—K616 transformed with pYX112-ACA2 was selected by Ura autotrophy. K616 is a mutant for the endogenous Ca²⁺ pumps PMC1 and PMR1, a potentially lethal combination but for a third knock-out mutation of CNB1. In the absence of CNB1, the Ca²⁺/H⁺-antiporter (VCX1) located in the vacuolar membrane is active and suffices to populate intracellular calcium stores, provided K616 is grown in adequately high Ca²⁺. Ura-selected colonies expressing ACA2 exhibited growth on complementation plates comprising SC medium depleted of calcium with 10 mM EGTA, unlike K616 and K616 transformed with the vector alone (Fig. 1A). The ACA2 transformant strain will be referred to as K616-ACA2 henceforth for simplicity, whereas the strain transformed with vector alone will be referred to as K616-V. K616, in
ER Ca\(^{2+}\)-ATPase Sculpts Signaling Ca\(^{2+}\) Transients

![Diagram](Image)

**FIGURE 1. Growth under Ca\(^{2+}\)-depleted and hyperosmotic conditions.** Triple mutant yeast strain, K616 (pmc1,pmr1,crn1) was transformed with either pYX112-ACA2 or the vector pYX112 using standard conditions as described under “Experimental Procedures.” A growth of ACA2 transformants (K616-ACA2), K616, vector control (K616-V), and wild type (K601) on SC medium depleted of Ca\(^{2+}\). B, drop test showing growth of K616-V (left column in each panel) and K616-ACA2 (right column in each panel) at 20 nM, 100 \(\mu\)M, and 5 mM free Ca\(^{2+}\) with and without 400 mM NaCl. C, growth of K616-V in SC medium containing 0 mM ( ), 100 mM ( ), 200 mM ( ), 300 mM ( ), 400 mM ( ), and 800 mM NaCl. D, drop test showing growth in SC medium (control) and in SC supplemented with KCl (300 mM), NaCl (400 and 800 mM), or sorbitol (750 mM). Free Ca\(^{2+}\) was buffered at 100 \(\mu\)M in C and D. The arrows in B and D represent serial dilution of 10\(^{-3}\), 10\(^{-2}\), and 10\(^{-1}\)-fold of cells adjusted to an \(A_{500}\) of 0.5.

turn, is derived from the strain K601, which will be treated as wild type for this study.

**Saline and Osmotic Sensitivity of Yeast Strains**—At very low Ca\(^{2+}\) of 20 nM, K616-V grew very poorly on SC medium (Fig. 1B) and not at all under 400 mM NaCl stress. Interestingly, at a moderate free Ca\(^{2+}\) level of 100 \(\mu\)M, K616-V showed effective growth under unstressed conditions but failed to grow under salt stress (Fig. 1B). With 5 mM Ca\(^{2+}\) in the medium, however, K616-V was able to grow effectively under unstressed conditions, as well as mount a reasonable salt tolerance response when stressed with NaCl (Fig. 1B). Thus, K616-V requires two levels of media Ca\(^{2+}\): a lower concentration (~100 \(\mu\)M) for growth and millimolar calcium for reasonable salt tolerance. K616 and K616-V behaved identically in Ca\(^{2+}\)-depleted medium (Fig. 1A) and under hyperosmotic stress (supplemental Fig. 1D). The expression of ACA2 in K616 eliminated both requirements, transformants growing well and exhibiting salt tolerance even at 20 nM Ca\(^{2+}\) (Fig. 1B). NaCl titration carried out at 100 \(\mu\)M medium Ca\(^{2+}\) showed that K616 did not grow in 300 mM NaCl (Fig. 1C). Sensitivity to NaCl was detected even at 100 mM, where a significant lag was observed in the growth curve (Fig. 1C). K601 and K616-ACA2, on the other hand, grew well in medium containing up to 1.2 M NaCl (growth curves not shown).

To evaluate whether other hyperosmotic stresses affect these strains similarly, they were subjected to saline and osmotic stress comprising 300 mM KCl or 400/800 mM NaCl or 750 mM sorbitol in SC medium containing 100 \(\mu\)M free Ca\(^{2+}\). Although both K601 and K616-ACA2 grew well on plates containing 400 or 800 mM NaCl, K616-V did not grow on these plates (Fig. 1D). However, all three strains grew reasonably well on plates containing either 750 mM sorbitol or 300 mM KCl, indicating that purely osmotic stresses and ionic stresses up to 300 mM were tolerated by all of them (Fig. 1D). Curiously, the K616-ACA2 strain of pYX112-ACA2 generated colonies that did not grow on calcium-depleted media and were as sensitive to NaCl stress as was K616 (supplemental Fig. 1, A–D). ATP-induced Ca\(^{2+}\) pumping and vanadate-sensitive-ATPase activities were detected in ER-enriched vesicles prepared from K616-ACA2 but not in those from K616, indicating the expression of a functional Ca\(^{2+}\)-ATPase in K616-ACA2 (supplemental Fig. 1, E–G). An N-terminal, autoinhibitory domain in ACA2 (43) does not appear to prevent activity of the full-length construct used here.

**ACA2 Changes Ca\(^{2+}\) Homeostasis in K616**—To test the degree to which internal calcium stores get filled, cellular Ca\(^{2+}\) of yeast preincubated with external Ca\(^{2+}\) of 20 nM, 100 \(\mu\)M, or 5 mM was estimated by atomic absorption spectrophotometry. Total cellular calcium is tightly controlled in wild type, K601, and K616-ACA2, remaining below 0.2 mg/g, dry weight, under all of the Ca\(^{2+}\) concentrations tested (Fig. 2A). K616 showed >8-fold higher total calcium than K601 when subjected to 20 nM external Ca\(^{2+}\) (Fig. 2A). However, at both 100 \(\mu\)M and 5 mM calcium, cellular Ca\(^{2+}\) levels were comparable among all of the yeast strains tested, suggesting that internal stores of Ca\(^{2+}\) are adequately filled in all. We focused on 100 \(\mu\)M Ca\(^{2+}\), since it is the concentration that is adequate for growth of K616 but insufficient for mounting a salt tolerance response.

At 100 \(\mu\)M Ca\(^{2+}\), the sensitivity of K616 to hypersodic media could be a consequence of altered signaling due to an aberrant \([\text{Ca}^{2+}]_{\text{cyt}}\) transient in response to saline stress. To test this hypothesis, we used aequorin to monitor \([\text{Ca}^{2+}]_{\text{cyt}}\). The addition of 400 mM NaCl to the yeast suspension resulted in rapid increases in aequorin luminescence in all three strains (Fig. 2B). In the case of K601 and K616-ACA2, the rise in \([\text{Ca}^{2+}]_{\text{cyt}}\) was limited to a peak value between 0.6 and 0.8 \(\mu\)M, from which maximum it decayed to basal levels in 2–3 min (Fig. 2B). In K616, on the other hand, \([\text{Ca}^{2+}]_{\text{cyt}}\) rose to over 1.2 \(\mu\)M over the course of 1 min. Thereafter, it declined very slowly, not reaching basal values even after 20 min (Fig. 2B).
among the stresses used in this study. K601 and K616-ACA2 showed transient elevations of \([\text{Ca}^{2+}]_{\text{crt}}\) and were tolerant to all hyperosmotic stresses tested (Fig. 1D and supplemental Fig. 2). K616 was reasonably tolerant to sorbitol, KCl, and CaCl\(_2\) stresses (Fig. 1D; data for 50 mM CaCl\(_2\) not shown) and also mounted a transient \([\text{Ca}^{2+}]_{\text{crt}}\) elevation for these stresses, reaching base-line values in 5–7 min (supplemental Fig. 2). In contrast, NaCl stress resulted in a large and prolonged \([\text{Ca}^{2+}]_{\text{crt}}\) rise in this strain with an estimated \(t_{1/2}\) of \(~11\) min. \([\text{Ca}^{2+}]_{\text{crt}}\) did not reach base line over the 20-min duration of the experiment (Fig. 2B).

**NaCl Stress Mobilizes \([\text{Ca}^{2+}]_{\text{crt}}\) from an Intracellular Store**—Pretreatment of the three strains with 10 mM LaCl\(_3\), 5 mM NiSO\(_4\), or MgCl\(_2\), all of which have been shown to block yeast plasma membrane \(\text{Ca}^{2+}\)-channels (35, 44), did not show any significant effect on the \([\text{Ca}^{2+}]_{\text{crt}}\) rise with NaCl stress (supplemental Fig. 3, A and B), suggesting that most, if not all, of the \(\text{Ca}^{2+}\) that enters the cytosol is released from internal stores. Chelating external \(\text{Ca}^{2+}\) with 5 mM EGTA for 60 min prior to the NaCl insult also had no effect on the induced \([\text{Ca}^{2+}]_{\text{crt}}\) transient (supplemental Fig. 3B, inset), confirming that the \([\text{Ca}^{2+}]_{\text{crt}}\) rise is not due to influx from the medium. Of the stresses used, only the transient induced by \(\text{Ca}^{2+}\) stress (50 mM CaCl\(_2\)) was reduced by La\(^{3+}\) (supplemental Fig. 3C), demonstrating that the \([\text{Ca}^{2+}]_{\text{crt}}\) in the other cases (data for KCl and sorbitol not shown) was mobilized from internal sources.

**ACA2 Changes \(\text{Na}^+\) Homeostasis in K616**—Cells were grown to midlog phase and stressed for up to 18 h with 400 mM NaCl. Total cellular \(\text{Na}^+\) in K601 did not increase significantly over the first 3 h of stress and increased gradually thereafter until the 18 h time point (Fig. 3A). In contrast, both K616-ACA2 and K616 showed significant increases in intracellular \(\text{Na}^+\) after 3 h and still greater increase after 6 h and 18 h (Fig. 3A). K616 survived poorly after 18 h of NaCl stress, with close to 60% of the cells dead (data not shown). Consequently, data at the 3 h time point, where all three strains were viable, have been taken for comparative analysis. K616-ACA2 accumulated about twice as much \(\text{Na}^+\) as the wild type strain K601 after 3 h (Fig. 3A).

Treatment with CuSO\(_4\) permeabilizes the plasma membranes to small solutes but does not permit release from \(\text{Cu}^{2+}\)-resistant intracellular compartments (42), which are termed the
“nonexchangeable pool.” This assay has been used earlier to estimate the sequestration of Na\(^+\) and K\(^+\) into late endosomal/vacuolar compartments of yeast (29, 45). Only \(-25\%\) of total cellular Na\(^+\) was released from cells stressed for 3 h in the case of K616-ACA2, whereas \(-75\%\) was released from both K601 and K616, indicating that K616-ACA2 accumulates a larger fraction of Na\(^+\) in internal compartments (Fig. 3B).

Requirement of NHX1 for Survival of K616-ACA2—The sequestration of Na\(^+\) into a nonexchangeable pool suggests active transport into intracellular organelles. The yeast pre-vacuolar Na\(^+\)/H\(^+\)-antiporter NHX1 has an amiloride-binding domain, and its activity is reduced by amiloride (46). Amiloride affected the growth of K616-ACA2 in the presence of 400 mM NaCl (Fig. 3C) but not that of K601 (data not shown). Amiloride affected neither strain in the absence of salt, indicating that transporters critical for the tolerance response in the transportant are sensitive to amiloride, whereas those in K601 are not. Further, treatment with amiloride reduced the fraction of total cellular Na\(^+\) that was not released from K616-ACA2 upon Cu\(^{2+}\)-induced permeabilization of the plasma membrane (Fig. 3B), indicative of a corresponding decline in Na\(^+\) sequestration in intracellular organelles.

Bafilomycin A1, an inhibitor of the vacuolar H\(^+\)-ATPase, affected growth of K616-ACA2 when subjected to 18 h of exposure to 400 mM NaCl, without any major effect on K601 growth (Fig. 3D). Survival thus requires the building up of pH gradients across internal membranes, consistent with the use of a transporter coupled to H\(^+\) movement.

Inserting a kanamycin cassette into the NHX1 gene (NHX1::KanMX) generates yeast strains that are nulls for the transporter. NHX1 knockout (Anhx1) was generated in the background of K616, which we named as KNX161, to more directly assess the contribution of this transporter to the alleviation of salt hypersensitivity. The knockout was selected on G418 and analyzed by sequencing, which confirmed a disruption of NHX1 (Ydr456w) in chromosome IV. Under control conditions, all of the strains tested grew well. Under both Ca\(^{2+}\)-depleted and NaCl-stressed conditions, K616 and KNX616 failed to grow (Fig. 3E). KNX161-ACA2, on the other hand, grew well in Ca\(^{2+}\)-depleted medium (Fig. 3E), but in the presence of 400 mM NaCl, its growth was severely compromised (Fig. 3E and F), indicating that the activity of the transporter is critical to the survival of this strain in high salt over the 48 h time scale of this experiment. Cu\(^{2+}\) induced release of close to 75\% of total cellular Na\(^+\) from KNX616-ACA2 (Fig. 3B), indicating that the sequestration seen in K616-ACA2 is dependent on NHX1.

Transcriptional Regulation of ENA1 and NHX1—RT-PCR experiments on RNA extracted from the four strains revealed that ENA1 transcripts increased upon exposure to NaCl in all
strains (Fig. 4). The largest increase was seen in K601, with significantly smaller increases seen in the other strains. ENA1 transcripts appeared within minutes of NaCl exposure in K601, increased severalfold over 90 min, and were then sustained over several h. In contrast, a mild transient increase in transcript levels over the control was observed within minutes in K616-ACA2 and KNX616-ACA2, followed by a second modest and delayed induction after several h of salt stress; K616 also showed some induction of ENA1 with NaCl stress.

Transcript levels of NHX1 increased severalfold over the basal level with time of exposure to NaCl in both K601 and K616-ACA2, both the levels and the temporal pattern of induction being comparable in the two cases (Fig. 4). Transcriptional induction of NHX1 over control levels was not observed in K616. No RT-PCR product was observed in KNX616-ACA2, in keeping with a knock-out phenotype. Levels of ACTIN transcripts were unaffected by stress and served as a loading control (Fig. 4).

**Identifying Candidate Signaling Intermediates That Regulate Salt Tolerance of K616-ACA2**—ACA is reported to both inhibit phospholipase A2 and block TRP channels (47). ACA was preincubated with log phase cells of K601 and K616-ACA2 at sub-lethal doses (30 and 40 μM), followed by an 18-h exposure to a range of NaCl (0–1000 mM). Inhibitor alone, at 30 μM, had no significant effect, and at 40 μM, it had little effect on either strain (Fig. 5A). Significant reduction in cell viability of both K601 and K616-ACA2 was observed with increasing NaCl stress, with essentially no survival above 800 mM NaCl (Fig. 5A). Pretreatment with ACA reduced the NaCl-induced [Ca2+]cyt transient (supplemental Fig. 3D) and severely affected transcriptional induction of ENA1 and NHX1 in both K601 and K616-ACA2 (Fig. 5, B and C).

In an effort to block a downstream intermediate in the pathway, we used KN62, an inhibitor of CaM kinases, which had no effect on salt tolerance at 20 μM and was found to be lethal at 100 μM. However, at a sublethal dose of 50 μM, the inhibitor had a significant deleterious effect on salt tolerance of K616-ACA2, whereas no perturbation was observed in K601 (Fig. 5D). KN62 thus appears to affect a step in the salt tolerance pathway unique to K616-ACA2. Indeed, RT-PCR analysis showed that this inhibitor blocks the transcriptional induction of both ENA1 and NHX1 in K616-ACA2 (Fig. 5E).

**DISCUSSION**

Plants show intra- and interspecific variations in their tolerance to salinity (48, 49). Adaptive responses to maintain low [Na+]cyt, especially in photosynthesizing tissues, are complex and occur at both the whole plant and cellular levels (3, 41). When exposed to salinity, *A. thaliana* uses a pathway comprising
ER Ca\(^{2+}\)-ATPase Sculpts Signaling Ca\(^{2+}\) Transients

a Ca\(^{2+}\)-binding protein SOS3, which activates the kinase SOS2 that, in turn, phosphorylates the plasma membrane Na\(^+\)/H\(^+\) antiporter SOS1, thereby activating it. Mutations in these genes result in an SOS phenotype (6). Salinity has also been shown to induce transient elevation of Ca\(^{2+}\) in the cytosol of plant cells (5, 23) and to up-regulate Ca\(^{2+}\)-dependent protein kinases in rice (8, 9). Thus, Ca\(^{2+}\)-mediated signaling plays a pivotal role in salt tolerance of plants.

The role of endomembrane Ca\(^{2+}\)-ATPases would appear to be limited to cocking the gun, as it were, by filling the intracellular stores from which Ca\(^{2+}\) can be released in response to specific stimuli. The realization that Ca-ATPases in plants are potentially amenable to post-translational modification has led to the proposal that they may regulate the characteristics of the stimuli-induced Ca\(^{2+}\) transient, thereby dictating the pathway activated (22).

This study explores the relevance of an ER-located Ca\(^{2+}\)-ATPase from Arabidopsis (ACA2) in modulating stress responses. The study has been carried out in yeast, which, like plants, has a calcium-mediated salinity stress response mechanism and is an organism where Ca\(^{2+}\) homeostasis is relatively well understood (24, 33). ACA2 has been expressed in a triple mutant yeast strain, K616, which is deficient in Ca\(^{2+}\) pumps and the regulatory subunit of the highly conserved protein phosphatase, CNB1. Calcineurin-induced expression of the plasma membrane Na\(^+\)-ATPase, ENA1 (26), may be expected to be compromised in this strain, which is seen to be hypersensitive to salt stress. The strain does not grow in low Ca\(^{2+}\) below 100 \(\mu M\) (Fig. 1B).

At extremely low external Ca\(^{2+}\), K616 hyperaccumulates Ca\(^{2+}\) (Fig. 2A), as previously reported for strains lacking PMR1 (50). This accumulation and possible mislocalization may underlie poor survival of the strain under these conditions. Total Ca\(^{2+}\) is comparable in all three strains studied when suspended in medium containing 100 \(\mu M\) or more of Ca\(^{2+}\) (Fig. 2A), demonstrating that the only remaining Ca\(^{2+}\) transporter, the vacuolar Ca\(^{2+}\)/H\(^+\) exchanger VCX1, is capable of both filling internal stores and of regulating [Ca\(^{2+}\)]\(_{\text{cyt}}\) under optimal growth conditions. When subjected to saline stress, however, K616 is unable to grow in medium containing 100 \(\mu M\) Ca\(^{2+}\), whereas K616-ACA2, with an Arabidopsis ER-Ca\(^{2+}\)-ATPase, grows as well as does K601 (Fig. 1, B and D). K616-ACA2 (Fig. 3A) is either sequestered in internal organelles or accompanied by suitable compatible solutes. We have taken recourse to an assay that has been used to estimate Na\(^+\) in endomembrane compartments of yeast several times over the past decade (29, 45). Cu\(^{2+}\)-induced release of cytosolic solutes releases up to 75% of Na\(^+\) in K616 and K601 but only 25% from K616-ACA2 (Fig. 3B), indicating that ~75% of cellular Na\(^+\) is sequestered in endomembrane compartments. This sequestration requires the presence of the vacuolar Na\(^+\)/H\(^+\)-antiporter, NHX1, as seen by the significantly decreased fraction of nonexchangeable Na\(^+\) upon blocking NHX1 with amiloride or knocking it out (Fig. 3B). The limited sequestration (25%) observed in K601 and in KNX616-ACA2 (Fig. 3B) could possibly be ascribed to VNX1, a low affinity vacuolar Na\(^+\)/H\(^+\) and K\(^+\)/H\(^+\) antiporter (27). The \(\Delta\text{nhx}1\) strain (KNX616-ACA2) exhibits hypersensitivity to saline stress (Fig. 3, E and F). Knocking out NHX1 could prove lethal due to its role in vesicular trafficking and sorting of proteins destined to the vacuole (52). However, Stevens and co-workers (53) have demonstrated that a knock-out of NHX1 (VPS44) in yeast had no effect on growth under control conditions or under ion stress. On the other hand, the deleterious effect of bafilomycin on the salt tolerance of K616-ACA2 (Fig. 3D) indicates the involvement of an H\(^+\) gradient-driven transporter in the tolerance mechanism.

The endosomal compartments where NHX1 is located are small but have been shown to be adequate for enhancing salt tolerance under conditions of acidic pH, as demonstrated by Rao and co-workers (29). In addition, a more recent study indicates that although ENA1 to -4 are the main contributors to Na\(^+\) homeostasis in S. cerevisiae, there is some contribution of NHX1 and VNX1 mediating Na\(^+\) transport into the yeast endosomal/vacuole, respectively, even at neutral pH (27).

To address the question of how a plant ER-located Ca-ATPase could bring about changes in adaptive Na\(^+\) homeostasis in K616, we looked at [Ca\(^{2+}\)]\(_{\text{cyt}}\) profiles using aequorin as a reporter. The 3:1 stoichiometry of the functional complex and probable nonuniform expression mean that absolute concentrations estimated using the commonly used expressions for normalization should be used cautiously. The time courses of the transients monitored are, however, likely to be reliable. [Ca\(^{2+}\)]\(_{\text{cyt}}\) rises sharply in all three strains in response to stress. It decays to basal levels in a few minutes in all instances when a successful stress response is mounted, irrespective of the stress (supplemental Fig. 2 and Fig. 2, B and D), whereas [Ca\(^{2+}\)]\(_{\text{cyt}}\) remains above 400 \(\mu M\) for an extended period in all instances when cells fail to survive the stress (Fig. 2, B and D).

Rapid refilling of internal stores could promote survival in one or more of several ways. (a) Stores are now made available for subsequent release. However, the fact that all stresses stimulated robust release of Ca\(^{2+}\) in K616 suggests that initial filling is not a limiting factor. Although only a single transient increase in [Ca\(^{2+}\)]\(_{\text{cyt}}\) is observed over the time course of our aequorin experiments, we cannot rule out the possibility that subsequent spikes are required to recruit downstream effectors or to maintain the response. (b) Limiting the duration that [Ca\(^{2+}\)]\(_{\text{cyt}}\) is high could preclude initiation of mitochondrial death pathways. The time course of NaCl-induced cell death in K616 is not
supportive of death processes being directly induced by Ca\(^{2+}\) (data not shown). (c) The time course of the calcium signal could be critical for triggering downstream survival pathways. This hypothesis is appealing, particularly in view of the fact that poor survival is correlated with prolonged transients.

Surviving in high salt medium requires active depletion of Na\(^+\) from the cytosol. In K601, this is achieved mainly by the Na\(^+\)-ATPase ENA1 (26), with possible contributions from the plasma membrane Na\(^+\)/H\(^+\) antiporter NHA1. ENA1 expression is low under standard growth conditions but is rapidly induced by a number of complex pathways after exposure to saline or osmotic or alkaline stress (54). It is activated by calcineurin at high Na\(^+\) stress and by the Ca\(^{2+}\)-independent HOG pathway at low osmotic stress (below 300 mM NaCl) (55). Inhibition of the TOR pathway by saline stress also activates ENA1 (56). The sensitivity of K616 to salinity indicates that neither NHA1 nor any calcineurin-independent mechanism of activation of ENA1 contributes significantly to the depletion activity under our experimental conditions. ENA1 is transcriptionally induced in all of the strains tested (Fig. 4) but significantly more so in K601.

A microarray study found that yeast responds to saline stress by the transcriptional induction of a large number of genes, including ENA1, which shows transient activation over a time period of 20 min (57). Our data acquired over a time scale of hours detects strong induction of ENA1 in K601 after 1 h of stress, increasing for an additional 2 h and sustained for several h thereafter (Fig. 4). Among the strains lacking CNB1, K616 shows lowest activation, whereas K616-ACA2 and KNX616-ACA2 (with or without functional NHX1, respectively) show comparable mild induction of ENA1, in two phases (Fig. 4). Differences in temporal patterns of expression between the wild type strain and K616-ACA2 are indicative of differences in the mode of induction of ENA1. K616-ACA2 survives saline stress, whereas KNX616-ACA2 does not (Fig. 3, D and E), demonstrating that the levels of ENA1 induction in these strains is insufficient to confer tolerance in the absence of NHX1.

NHXI is also amenable for transcriptional induction upon saline stress (Fig. 4), the temporal pattern of NHXI induction being comparable between wild type and K616-ACA2, transcript levels increasing significantly from basal levels over several hours to comparable extents. However, accumulation of Na\(^+\) into internal stores is negligible in K601, suggesting a second level of post transcriptional control in K616-ACA2. It is to be noted that Posas et al. (57) report the rapid induction of several H\(^+\)-ATPases located on the tonoplast immediately after exposure to salinity.

We used sublethal doses of inhibitors of candidate signaling intermediates to decipher the pathway that couples the stress signal to the tolerance response in K616-ACA2. ACA, which affects both phospholipase A1 and TRP channels, dramatically reduced salt tolerance in both K601 and K616-ACA2, with negligible transcriptional induction of ENA1 in K601, and of NHXI in both K601 and K616-ACA2 (Fig. 5, A–C). The inhibitor must block a common upstream signaling event in both strains. The [Ca\(^{2+}\)]\(_{\text{cyt}}\) transient in response to NaCl arises from internal Ca\(^{2+}\) stores (supplemental Fig. 3, A–C). Ca\(^{2+}\) release from the vacuole in response to hyperosmotic stress has been earlier shown to involve YVC1, a TRP channel (34) that could be a target of ACA (47). Indeed, the [Ca\(^{2+}\)]\(_{\text{cyt}}\) transient we see in response to NaCl is attenuated in ACA-treated cells (supplemental Fig. 3D). In the absence of CNB1, the downstream pathway in K616-ACA2 must diverge from that of the wild type strain. HOG mitogen-activated protein kinase plays roles in osmotic stress signaling in yeast (55). However, SB 203580, an inhibitor of HOG mitogen-activated protein kinase, had no effect on salt tolerance of K616-ACA2 (data not shown). On the other hand, the significant and specific reduction in viability of K616-ACA2 exposed to salt stress in the presence of KN62 and the resulting block of transcriptional induction of NHXI (Fig. 5, D and E) indicates that Ca\(^{2+}\)/CaM kinase function is required in the survival pathway. Surprisingly, yeast strains lacking both CMK1 and CMK2, which encode Ca\(^{2+}\)/CaM-dependent protein kinases, are viable, as are strains in which two other closely associated genes, CLK1 and RCK1, are knocked out along with CMK1 and -2 (58). These enzymes are thus not required for a constitutive function but may have roles during stress signaling. Indeed, the Ca\(^{2+}\)/CaM kinase RCK1 has been reported to be transcriptionally up-regulated in yeast under salt stress (57).

Future studies will focus on this pathway that couples the [Ca\(^{2+}\)]\(_{\text{cyt}}\) signature with the activation of NHX1. In plants, the significant contribution of the NHX1 homologue to salt tolerance is well documented (59, 60). Interestingly, blocking a related kinase, Ca\(^{2+}\)-dependent protein kinase, results in increases in [Na\(^+\)]\(_{\text{cyt}}\) in rice cells due to the lack of activation of a likely vacuolar Na\(^+\)/H\(^+\) antiporter (3). It is, however, to be determined whether ACA2 plays a direct signaling role in activation of plant NHX1.

Thus, heterologously expressed ACA2 sculpts the Ca\(^{2+}\) transient induced by NaCl stress. K616-ACA2 survives saline stress by accumulating Na\(^+\) in endomembranous compartments in an NHX1-dependent manner. A signaling role for a Ca\(^{2+}\)/CaM kinase in this stress pathway is likely. This is in distinction to wild type yeast, where activation of calcineurin results in transcriptional up-regulation of ENA1, which then pumps Na\(^+\) out across the plasma membrane.

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