A Distinct Endosomal Ca^{2+}/Mn^{2+} Pump Affects Root Growth through the Secretory Process

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Ca^{2+} is required for protein processing, sorting, and secretion in eukaryotic cells, although the particular roles of the transporters involved in the secretory system of plants are obscure. One endomembrane-type Ca-ATPase from Arabidopsis (Arabidopsis thaliana), AtECA3, diverges from AtECA1, AtECA2, and AtECA4 in protein sequence; yet, AtECA3 appears similar in transport activity to the endoplasmic reticulum (ER)-bound AtECA1. Expression of AtECA3 in a yeast (Saccharomyces cerevisiae) mutant defective in its endogenous Ca^{2+} pumps conferred the ability to grow on Ca^{2+}-depleted medium and tolerance to toxic levels of Mn^{2+}. A green fluorescent protein-tagged AtECA3 was functionally competent and localized to intracellular membranes of yeast, suggesting that Ca^{2+} and Mn^{2+} loading into internal compartment(s) enhanced yeast proliferation. In mesophyll protoplasts, AtECA3-green fluorescent protein associated with a subpopulation of endosome/prevacuolar compartments based on partial colocalization with the Ara7 marker. Interestingly, three independent eca3 T-DNA disruption mutants showed severe reduction in root growth normally stimulated by 3 mM Ca^{2+}, indicating that AtECA3 function cannot be replaced by an ER-associated AtECA1. Furthermore, root growth of mutants is sensitive to 50 μM Mn^{2+}, indicating that AtECA3 is also important for the detoxification of excess Mn^{2+}. Curiously, Ateca3 mutant roots produced 65% more apoplastic protein than wild-type roots, as monitored by peroxidase activity, suggesting that the secretory process was altered. Together, these results demonstrate that the role of AtECA3 is distinct from that of the more abundant ER AtECA1. AtECA3 supports Ca^{2+}-stimulated root growth and the detoxification of high Mn^{2+}, possibly through activities mediated by post-Golgi compartments that coordinate membrane traffic and sorting of materials to the vacuole and the cell wall.

The dynamic endomembrane system is emerging as a central coordinator of plant growth, development, and adaptation to abiotic and biotic stress. Cell biologists have long studied the biogenesis and roles of organelles, like the vacuoles and the Golgi, and the cellular and biochemical bases of protein sorting, membrane trafficking, and secretion (Battey and Blackbourn, 1993; Battey et al., 1999). For instance, noncellulosic cell wall precursors are synthesized in the Golgi and delivered in secretory vesicles to the outside of the cell (Carpita and McCann, 2000; Nebenfuhr and Staehelin, 2001). Newly synthesized secreted and membrane proteins at the endoplasmic reticulum (ER) are sorted to their destinations via vesicle or tubular trafficking, although the specific players and mechanisms are for the most part unclear. Recent advances were stimulated by numerous new findings emerging from molecular genetic investigations and proteins predicted by the completed genome of Arabidopsis (Arabidopsis thaliana). For example, the dynamics of PIN auxin transporters are revealing the intriguing complexity of relationships between endosomal trafficking, signaling, and development (Geldner, 2004). Endosomal trafficking refers to the movement of vesicles formed from endocytosis to remove functional plasma membrane proteins or to retrieve proteins for recycling purposes. The biosynthetic and endosomal vesicle traffic merge inside cells, although the molecular bases that ensure the smooth operation of multiple trafficking patterns are poorly understood.

Ca^{2+} has long been recognized as an important ion for plant growth and the secretory process (Steer, 1988; Marschner, 1995; Brandizzi and Hawes, 2004). Ca^{2+} is required for root growth, although most of the Ca^{2+} in the plant body is associated with pectins outside the cell. Whether wall-associated Ca^{2+} is supplied from intracellular or external sources is debatable. Inside plant cells, membrane compartments forming the secretory pathway, such as the ER and Golgi apparatus, are filled with Ca^{2+} (Dauwalder et al., 1985; Sakai-Wada...
RESULTS

that AtECA3 has roles in plants beyond Mn\textsuperscript{2+} trafficking, and secretion. Our findings differ in several activities of endosomes involved in sorting, membrane pump promotes root growth, possibly through the ac-

pump critical for Ca\textsuperscript{2+} plasma membrane (ACA8 and ACA9), where extrusion of Ca\textsuperscript{2+} would decrease cytosolic Ca\textsuperscript{2+} concentration in vegetative tissues (Bonza et al., 2000) or in pollen tubes (Schiott et al., 2004). Another Ca\textsuperscript{2+} pump, ACA4, local-

ized to the vacuole or perhaps the prevacuolar compart-

ment (PVC) could fill vacuolar stores and may have a role in stress tolerance (Geisler et al., 2000). Intrigu-

ingly, there is no secretory pathway-like Ca\textsuperscript{2+} pump (SPCA) gene in plants to date, based on phylogenetic analyses of Ca\textsuperscript{2+} pumps from cyanobacteria, fungi, and animals (Baxter et al., 2003; Li, 2006). SPCA Ca\textsuperscript{2+} pumps like the yeast (Saccharomyces cerevisiae) PMR1 or human SPCA1 are localized to the Golgi and participate in protein modification, sorting, and secretion (Durr et al., 1998; Wuytack et al., 2003). Here, we show that AtECA3 is a distinct Ca\textsuperscript{2+}/Mn\textsuperscript{2+} pump critical for Ca\textsuperscript{2+}-enhanced root growth and for tolerance to toxic levels of Mn\textsuperscript{2+}. Unlike the ER-bound AtECA1, it is localized to post-Golgi compartments. Furthermore, mutants showed enhanced secretion of peroxidases, suggesting that an adequate supply of Ca\textsuperscript{2+} and Mn\textsuperscript{2+} in post-Golgi compartments is critical for regulated protein sorting. Thus, a novel Ca\textsuperscript{2+}/Mn\textsuperscript{2+} pump promotes root growth, possibly through the activities of endosomes involved in sorting, membrane trafficking, and secretion. Our findings differ in several respects from a recent report (Mills et al., 2008) and show that AtECA3 has roles in plants beyond Mn\textsuperscript{2+} nutrition.

RESULTS

AtECA3 Shares High Sequence Identity with Animal Sarcoplasmic/ER Ca\textsuperscript{2+}-ATPase

Several ECAs from Arabidopsis, rice, and tomato (Solanum lycopersicum) clustered on a phylogenetic tree with mammalian sarcoplasmic/ER Ca\textsuperscript{2+}-ATPase (SERCA; Fig. 1A); however, only AtECA3 and OsECA3 shared higher identity (53%) with mammalian SERCA1a than with other members of the ECA subfamily (44.6%–45.1%). Intriguingly, none of the plant ECAs grouped with SPCA or secretory pathway Ca\textsuperscript{2+}-ATPases (Pittman et al., 1999; Ton and Rao, 2004), which are represented by yeast Pmr1 and human SPCA (Fig. 1A).

Sequence alignments revealed further insights. AtECA3 shared considerable amino acid sequence identity with transmembrane 3 (TM3), TM5, and TM7 of mammalian SERCA. The \(\alpha\)-helices of TM3, TM5, and TM7 form the cavity that binds thapsigargin in the E2 conformation state of rabbit skeletal muscle SERCA (Toyoshima and Nomura, 2002). Interestingly, among the hydrophobic residues that face thapsigargin, Val-263, Val-773, and Phe-834 of rabbit OcSERCA (P04191) and human HsSERCA1 (O14983) are conserved and identical in the plant ECA3 from Arabidopsis and rice (Fig. 1B; Supplemental Fig. S1). In contrast, these residues are not found in yeast Pmr1p (SPCA) or AtECA1 (Fig. 1B), both of which are insensitive to thapsigargin (Liang and Sze, 1998). In addition, an extra tripeptide sequence, Q/HEA, corresponding to residues 240 and 241 of OcSERCA1, was present in most plant ECAs but not in ECA3. This analysis suggests that AtECA3 is distinct from other Arabidopsis ECAs and that plant ECA3-like proteins are structurally the most similar to animal ERCAs.

AtECA3 Confers Tolerance of Yeast Growth on Medium with Low Ca\textsuperscript{2+} or High Mn\textsuperscript{2+}

To determine the transport function of AtECA3 (or ECA3 for simplicity), a cDNA containing the complete open reading frame (ORF; AY650902) was cloned (see “Materials and Methods”) and expressed in yeast strain K616. The complete ORF of 2,994 bases encoded a protein of 997 residues. K616 is incapable of loading Ca\textsuperscript{2+} and Mn\textsuperscript{2+} into specific endomembrane compart-

ments with high affinity (Cunningham and Fink, 1994), as it lacks a functional Golgi Ca\textsuperscript{2+}/Mn\textsuperscript{2+} pump (Pmr1p) and a vacuolar Ca\textsuperscript{2+}-ATPase (Pmc1), respect-

ively. Therefore, growth of K616 is retarded or arrested when the culture medium contains submicromolar Ca\textsuperscript{2+} or toxic levels of Mn\textsuperscript{2+}. However, the strain pro-

iferates on medium containing high Ca\textsuperscript{2+} (0.1–1 mM) using Vcx1p, a vacuolar Ca\textsuperscript{2+}/H\textsuperscript{+} exchanger that se-

questers Ca\textsuperscript{2+} into endomembrane compartments (Cunningham and Fink, 1996).

The K616 strain expressing AtECA3 grew slower than yeast expressing the empty p426 vector in synthetic complete (SC) medium containing no EGTA (Fig. 2A). However, when free Ca\textsuperscript{2+} was reduced to approximately 0.8 \(\mu\)M by 5 mM EGTA (Fortzehl et al., 1964), growth of the K616 strain was severely curtailed. AtECA3 partially restored yeast K616 growth on Ca\textsuperscript{2+}-depleted medium relative to wild-type cells (Fig. 2A). Curiously, under the same Gal1 promoter, AtECA3
was less effective than ECA1 (Fig. 2B) and AtECA4 (data not shown) in restoring mutant growth on Ca\(^{2+}\)-depleted medium. AtECA1 rescued K616 growth in medium containing 10 mM EGTA, as shown before (Liang et al., 1997), while AtECA3 did not (data not shown). However, AtECA3 effectively restored mutant growth on medium containing 1 mM EGTA (Fig. 3), indicating that the two ECAs, although similar in transport activity, might differ in their kinetic properties, membrane localization, or expression level, as suggested below.

We tested whether AtECA3 might support yeast growth at high Mn\(^{2+}\). AtECA3 expression under the Gal1 promoter on vector p426 failed to suppress the Mn\(^{2+}\) sensitivity of strain K616 (data not shown). Consequently, AtECA3 was expressed using pDR196, which contains a very strong constitutive promoter of the plasma membrane H\(^{+}\)-ATPase1 (PMA1; Rentsch et al., 1995). Under the PMA1 promoter, AtECA3 expression improved K616 growth on medium containing 1 mM Mn\(^{2+}\) (Fig. 2C). This observation suggested that AtECA3 can also function as a Mn\(^{2+}\) pump to remove excess Mn\(^{2+}\) from the cytosol, similar to the ER-bound AtECA1 (Liang et al., 1997).

To localize AtECA3 protein in yeast, a construct was made to generate a GFP fusion to the C terminus of AtECA3. Unlike other AtECAs, AtECA3 lacks a typical ER-retention motif, KxKxx, at its extreme C terminus, although it is rich in basic amino acid residues (KDRRDK). AtECA3-GFP fluorescence was observed in intracellular tubular and granular structures (Supplemental Fig. S2) that resembled the distribution of Pmr1p-GFP, a yeast Ca\(^{2+}\) pump localized to the ER and Golgi (Huh et al., 2003). The GFP-tagged protein restored the growth of K616 transformants on medium containing 1 mM EGTA or 1 mM Mn\(^{2+}\) (Fig. 3). These results indicate that the functionally active ECA3-GFP is localized to endomembranes in yeast.

Figure 1. AtECA3 shares high similarity with animal SERCA. A, Phylogenetic relationships of type 2A Ca\(^{2+}\)-ATPases from plant, cyanobacterium, yeast, and animal. SPCA, SERCA, and ECA refer to secretory pathway, sarcoplasmic reticulum/ER, and endomembrane Ca-ATPase, respectively. Protein names shown are preceded by genus and species initials. Arabidopsis (At) and rice (Os) ECA proteins are identified by their accession numbers in parentheses: AtECA1 (AAC68819; At1g07810), AtECA2 (CAA10659; At4g00900), AtECA3 (AAT68271; At1g10130), AtECA4 (AAD29957; At1g07670), OsECA1 (AAN64492; Os03g52090), and OsECA3 (ABF98693; Os03g52090). Other Ca\(^{2+}\) pumps aligned are Synechococcus elongatus SyoPacL (BA03906); Saccharomyces cerevisiae ScPmr1 (P13586) and ScPmc1 (P38929); tomato LCA1 (Q42883); human (Hs) SERCA1 (O14983), SERCA2 (P16615), SERCA3 (Q93084), SPCA1 (AAH28139), and SPCA2 (AAT54193); Dro sophila melanogaster (Dm) Ca-P60A (P22700) and SpOCk (AAN12202); and Oryctolagus cuniculus (rabbit) OcSERCA1b (PDB_ID 1SU4_A). The tree was aligned by T-Coffee. Values shown indicate the number of times (percent) that each branch topology was found in 1,000 replicates of the performed bootstrap analysis using PAUP* 4.0b10. B, Partial alignment of plant and other Ca\(^{2+}\)-ATPases containing thapsigargin-interacting regions. Residue numbers correspond to rabbit SERCA1a or HsSERCA1. Black triangles indicate amino acid residues in direct contact with thapsigargin of rabbit SERCA1a (1SU4_A; Toyoshima and Nomura, 2002). Identical residues are shaded in black, while similar residues are boxed. TM3, TM5, and TM7 correspond to transmembrane regions of HsSERCA1. This alignment was generated in ESPript 2.2 (Gouet et al., 1999) using a T-Coffee-generated alignment. α-Helix, β-sheet, and turn are indicated as helix, black arrow, and Τ, respectively. The full alignment is shown in Supplemental Figure S1.

Endosomal Ca\(^{2+}\)/Mn\(^{2+}\) Pump Affects Root Growth
Identification and Analyses of T-DNA Insertional Mutants of AtECA3

To test the biological function of AtECA3 in planta, several T-DNA insertional lines (3-1, 3-4, and 3-5) were identified from the Salk collection (Alonso et al., 2003) and from Syngenta (McElver et al., 2001). Homozygous lines of each allele were obtained and their genotypes confirmed by PCR. Left border sequencing of the T-DNA was conducted to verify the insertion site of each allele (Fig. 4A). Mutants Ateca3-5 and Ateca3-1b hosted T-DNA insertions in intron 11 and intron 12, respectively, while Ateca3-4 had an insertion in exon 18.

To test for AtECA3 transcript, total RNA was extracted from 2-week-old wild-type and mutant plants grown on half-strength Murashige and Skoog (MS) medium (Fig. 4B) and reverse transcribed. Primers were designed to amplify any transcript upstream or downstream of the insertion site. Primers flanking the T-DNA insertion site and primer set E3F and E3R failed to amplify any product from all three mutants. Nevertheless, primers E5F and E5R amplified a product of 638 bp upstream of the insertion site in all three mutant alleles, indicating the presence of a truncated mRNA. Although the length of these transcripts is unclear, any potential translated protein would likely include only four TM regions (Lys-293) out of eight to 10 TM found in P-type 2A ATPases. A protein lacking a complete hydrophilic domain and TM5 to TM8 is unlikely to have any catalytic activity. These T-DNA insertional lines were used in phenotypic analyses as nonfunctional AtEca3 mutants. Siblings without T-DNA insertions from the same segregating population were used as wild-type controls. All three homozygous mutant lines were viable and completed their life cycle under normal conditions. No obvious growth or developmental defects were observed in the vegetative plants grown on soil.

Role of AtECA3 in Root Growth

Root Growth of eca3 Mutants Is Sensitive to High Mn2+

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K616 conferred tolerance to toxic levels of Mn$^{2+}$ (1 mM; Fig. 2). So we examined the effect of Mn$^{2+}$ levels on root growth. Plants were first grown in quarter-strength Hoagland nutrient medium containing from 3 to 100 μM Mn$^{2+}$. The root length of mutants was similar to that of wild-type plants in medium containing Mn$^{2+}$ at 3.5 (Fig. 5A), 10, or 20 μM (data not shown). But root growth of eca3-4 was clearly reduced compared with that of wild-type plants when Mn$^{2+}$ was increased to 50 μM. Moreover, mutants manifested little or no lateral roots after 3 d of growth, suggesting inhibition of lateral root initiation. The compromised root growth also caused changes in shoot growth, such as reduced leaf expansion and rosette size (data not shown). Therefore, as in yeast, AtECA3 conferred tolerance to high Mn$^{2+}$ in plants.

Similar results were obtained when plants were nourished by half-strength MS medium, which contains 50 μM Mn$^{2+}$. Primary roots of wild-type plants grew, but eca3-4 mutant growth was inhibited (Fig. 5B). Wild-type plants also became sensitive when Mn$^{2+}$ was increased to 100 μM (Fig. 5B). It is noted that a concentration of 50 μM Mn$^{2+}$ in half-strength MS medium is considered unusually high compared with a recommended Mn$^{2+}$ of less than 10 μM in Hoagland's nutrient medium (Hoagland and Arnon, 1950). Therefore, 50 μM probably imposes a Mn$^{2+}$ stress that is tolerated by the wild type but not by mutant seedlings. In contrast, increasing Cu$^{2+}$ inhibited the root growth of both wild-type and eca3-4 plants similarly (Fig. 5C). These results indicate that AtECA3 could alleviate Mn$^{2+}$ toxicity through a mechanism distinct from that which guards against Cu$^{2+}$ toxicity, another essential yet potentially toxic divalent heavy metal. These results are consistent with the notion that AtECA3 transports Mn$^{2+}$ but is not involved in transporting Cu$^{2+}$.

**Ca-Stimulated Root Growth Is Reduced in eca3 Mutants**

Ca$^{2+}$ at a few millimolar concentration is required for root growth (Marschner, 1995), so we tested the effect of Ca$^{2+}$ on root growth in mutant lines containing a T-DNA insertion in the *AtECA3* gene. The effect of external Ca$^{2+}$ on root growth in wild-type seedlings was first tested using half-strength MS medium in which the Ca$^{2+}$ concentration was specifically varied. Calcium at 3 mM stimulated root growth (Fig. 6B), as shown before (Marschner 1995). Reduced root length at 0.1 mM or less Ca$^{2+}$ confirmed that this level of Ca$^{2+}$ limited growth. Similarly, root growth at 20 mM Ca$^{2+}$ was decreased, demonstrating that excess Ca$^{2+}$ was stressful to the seedling (Fig. 6B).

Intriguingly, the 3 mM Ca$^{2+}$-stimulated root growth was not observed in *eca3-1b* or *eca3-4* mutants (Fig. 6B), indicating that mutants were nearly insensitive to the beneficial effects of Ca$^{2+}$. Similar results were observed in the *eca3-5* mutant (Fig. 6C). At 3 mM Ca$^{2+}$, root lengths of *eca3-1b* and *eca3-4* mutants were reduced by 33% and 34%, respectively, compared with wild-type controls. With 3 mM external Ca$^{2+}$, it is reasonable to assume that Ca$^{2+}$ entry pathways via cation-permeable channels are functional in *eca3* mutants. Thus, the results indicate that reduced growth is due to defective sorting of cytosolic Ca$^{2+}$ into one or more intracellular compartments or organelles.
Spatial Expression of AtECA3 in Root and Leaf

The tissue-specific pattern of expression for AtECA3 was examined by GUS reporter activity driven by a 5-kb intergenic region upstream of the ORF. In vegetative tissues, AtECA3 promoter::GUS activity was enriched in vascular tissues of primary roots (Fig. 7A), lateral roots (Fig. 7B), and young expanding leaves (Fig. 7E). At the root tip, GUS activity first appeared in the elongation and differentiation zones (Fig. 7, A and B) but was not detectable in the cell division zone under the conditions tested. GUS activity was not detected in the shoot apex (Fig. 7, C and D) or in fully expanded cotyledons (Fig. 7D). Promoter activity was also detected in vascular tissues of floral pedicels and of the style (Fig. 7, F–H). Expression of AtECA3 in other cell types is not excluded by this analysis, as the GUS gene was transcriptionally fused to the 5′ regulatory region only, so that any transcriptional regulation by cis-acting elements within introns would be missed, as would 3′ or chromosomal positional effects.

RT-PCR of total RNA verified the presence of AtECA3 transcripts in root and leaf (Li, 2006). Using a smaller 1.5-kb promoter fragment upstream of AtECA3, Mills et al. (2008) showed that expression in vascular tissues was enriched in xylem parenchyma and the pericycle. In addition, GUS activity was detected in root caps, hydathodes, and guard cells (Mills et al., 2008). Thus, although minor differences exist in the two studies, the results using either a 1.5- or 5-kb promoter show similarities that are supported in general by whole genome ATH1 chip data (http://www.geneinvestigator.ethz.ch).

The AtECA3 promoter activity appeared to be lower than that of AtECA1 or AtECA4 based on 2 d of 5-bromo-4-chloro-3-indolyl β-D-glucuronide (X-Gluc) staining required to visualize GUS activity. In contrast, AtECA1 promoter::GUS activity was detected after 3 h of incubation in X-Gluc in roots and leaves, including the root tip, lateral roots, vascular tissues, ground tissues, and guard cells (Supplemental Fig. S3). The higher expression of AtECA1 relative to AtECA3 is supported by the root expression map from the Benfey laboratory (Supplemental Fig. S4). Together, these results confirm that AtECA3 is expressed in all

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**Figure 5.** Root growth of mutants is sensitive to 50 μM Mn2⁺. A, Effect of Mn2⁺ on root growth in Hoagland medium. Arabidopsis seeds were germinated on quarter-strength Hoagland’s medium containing 0.5 μM Mn2⁺ and 2.5 mM Ca2⁺ (1% agar, 10 mM MES-K⁺, pH 5.7). Three days later, seedlings were transferred either to the same medium or to one supplemented with MnCl₂ to 50 μM. Photographs were taken 3 d after transfer. B, Effect of Mn2⁺ on root growth in half-strength MS medium. Arabidopsis seeds were germinated on half-strength MS medium containing 50 μM Mn2⁺ with 1% Suc after 5 d of cold treatment. Seven-day-old seedlings were transferred to half-strength MS medium with 1% Suc containing 50 μM or 0.1 mM Mn2⁺. Root length was scored 3 d after transfer. Six to 10 seedlings were measured in each treatment. Results represent three independent experiments. Error bars indicate SE. C, Effect of Cu2⁺ on root growth in half-strength MS medium. Seeds were germinated as in B except that seedlings were transferred to half-strength MS medium alone (approximately 0.1 μM Cu2⁺) or medium containing 10, 50, or 100 μM Cu2⁺. Root lengths were scored 3 d after transfer. Six to 10 seedlings were measured in each treatment. Results represent three independent experiments. Error bars indicate SE.
cell types found in roots but that its expression is consistently much lower than that of AtECA1.

AtECA3-GFP Is Localized with Endosome/PVC Markers in Plant Cells

Although functional studies from yeast and plants suggest that AtECA3 is localized to endomembrane compartments, the identity of the compartment is unclear. We determined the membrane location of AtECA3 after transient expression using the S35 cauliflower mosaic virus (CaMV) promoter in Arabidopsis mesophyll protoplasts (Jin et al., 2001; Kim et al., 2001). We first demonstrated that the AtECA3 fused at the C terminus to GFP retained native activity, judging by its ability to restore yeast mutant growth on Ca\(^{2+}\)-depleted medium or medium containing toxic levels of Mn\(^{2+}\) (Fig. 3).

Cells expressing ECA3-GFP alone (Fig. 8Ae) had small punctate fluorescence that resembled the puncta seen in Golgi or post-Golgi compartment markers (Fig. 8A, f–h), including sialyl transferase, Sy41, or Ara7 (Ueda et al., 2004; Uemura et al., 2004). The puncta from ECA3-GFP were dissimilar from those emitted from the ER or a vacuolar marker (Sy22). A GFP tag at the C terminus apparently did not interfere with potential retention signals for another calcium pump, as AtECA1 tagged with GFP at the C terminus gave a reticulate fluorescent pattern characteristic of ER when it was transiently expressed in protoplasts (Fig. 8Aa). AtECA1 was previously localized to the ER by cell fractionation and immunostaining (Liang et al., 1997).

To clarify the membrane location, ECA3-GFP was coexpressed with markers tagged with a red fluorescent protein (RFP) in protoplasts. The fluorescence signals were carefully collected with a Zeiss LSM 510 confocal microscope using different emission wavelengths. ECA3-GFP did not colocalize with ST-RFP or with RFP-Sy41 (Fig. 8B), which are markers for the trans-Golgi or the trans-Golgi network, respectively (Ueda et al. 2001, 2004). ECA3-GFP colocalized in part with RFP-Ara7, an endosome/PVC marker (Lee et al., 2004; Ueda et al., 2004), as indicated by the extent of yellow fluorescence in merged images (Fig. 8Bc$^\text{m}$). These results suggest that

Figure 6. Ca\(^{2+}\)-stimulated root growth is abolished in Ateca3 mutants. A, A representative image of Ateca3-4 and its wild-type (Col) seedlings.

Wild-type and mutant seeds were germinated side by side on plates containing half-strength MS medium with 3 mM Ca\(^{2+}\). Photographs were taken at 3 d. B, Effect of Ca\(^{2+}\) concentration. Seeds from wild-type (black circles), eca3-1b (white circles), and eca3-4 (white triangles) plants were germinated on plates containing half-strength MS with either no added Ca\(^{2+}\) or supplemented with Ca\(^{2+}\) to final concentrations of 0.1, 3, and 20 mM. Each experiment consisted of 20 seedlings per treatment. Primary root length was measured at 3 to 4 d. Data represent at least three independent experiments. Error bars indicate se. C, Differential effect of Ca\(^{2+}\) on root growth of eca3 and eca1 mutants. Seeds from wild-type (Col), eca3-1b, eca3-4, eca3-5, wild-type (Wassilewskija [WS]), eca1-1, and 35S-ECA1 (Wu et al., 2002) plants were germinated on plates containing half-strength MS with 3 mM Ca\(^{2+}\). Each experiment consisted of 12 to 18 seedlings per treatment. Primary root length was measured at 4 d. Data represent at least two independent experiments. Error bars indicate se. [See online article for color version of this figure.]
ECA3-GFP is localized to a subpopulation of post-Golgi compartments, including endosomes and PVCs.

Enhanced Apoplastic Peroxidase Activity and Protein Secretion in eca3 Mutants

Because plant post-Golgi compartments are involved in the processing, sorting, and exocytosis of proteins, we wondered whether secretory activities might be compromised in eca3 mutants. The activities of secreted apoplastic peroxidases (APXs) were examined in roots from hydroponically grown plants. Apoplastic washing fluid (AWF) was extracted from roots by first vacuum infiltrating the tissue with buffer and then collecting the fluid by centrifugation. Guaiacol-dependent peroxidase activity was then monitored spectrophotometrically by following the oxidation of guaiacol by hydrogen peroxide to form tetraguaiacol.

We first established that the enzyme reaction, monitored by the appearance of tetraguaiacol, was linear for 2 min (Fig. 9A). The initial rate of the reaction was then estimated from the slope. Increasing aliquots of AWF from wild-type roots produced an enhanced rate of guaiacol oxidation, indicating that Arabidopsis roots contained extracellular peroxidase activity (Fig. 9B). Based on four independent experiments, the extracellular peroxidase activity of wild-type roots was estimated to be 79 nmol min$^{-1}$ g$^{-1}$ fresh weight of root. When AWF of mutants was analyzed, an increase in APX was consistently observed when activity was expressed per gram fresh weight of roots. The APX activity of eca3-4 mutants was approximately 147 nmol min$^{-1}$ g$^{-1}$ fresh weight of root.

In three independent experiments, the activity of the mutants was 80%, 86%, and 86% higher than that of wild-type controls (Fig. 10). The increase in activity was accompanied by an increased amount of apoplastic protein by the mutants. eca3-4 mutant roots produced 39%, 60%, and 95% (in three separate experiments) more protein per gram fresh weight of tissue than did wild-type roots. Similar results were obtained with the eca3-5 mutant, which produced 2-fold more apoplastic protein than did wild-type roots (data not shown). Thus, the specific activity of peroxidase (nanomoles per minute per microgram of protein) was relatively unchanged in the apoplastic fluid between the wild type and mutants. Interestingly, the increase in both activity and protein in the mutants was still detected when they were based on milliliters of AWF (Fig. 10). These results indicate that the concentration of protein and peroxidase was increased in the extracellular fluid of mutants. The volume of fluid collected per gram fresh weight of tissue was quite similar, although slightly more (19%) was recovered from the mutant than from the wild type. Together, these results suggest that the secretory process of the eca3 mutant is altered, causing an increase in total apoplastic protein and a proportional increase in the activity of secreted peroxidase activity.

DISCUSSION

Maintaining divalent cation homeostasis for plant growth and adaptation depends on a remarkable coordination of transport activities; however, the cellular and biochemical bases of Ca$^{2+}$ and Mn$^{2+}$ distribution and their dynamics in plants are still poorly understood. Here, we provide evidence for a special endosomal Ca$^{2+}$/Mn$^{2+}$ pump that is involved in multiple functions, including the secretory process, root growth, and ion detoxification. Phylogenetic analyses
revealed that of four ECAs in Arabidopsis, ECA3 represents a unique subbranch and showed the highest identity and structural similarities to the subfamily of animal SERCA Ca$^{2+}$-ATPase. This unique ECA3 subgroup is conserved among higher plants, as shown by the high identity/similarity (79%/87.3%) of Arabidopsis ECA3 (At1g17310) with rice (japonica) ECA3 (Os03g52090). Moreover, like Arabidopsis, only one ECA3-like gene is found in the rice genome, strongly suggesting that this ion pump is functionally conserved in flowering plants.

**AtECA3 Is a Ca$^{2+}$/Mn$^{2+}$-ATPase Functionally Distinct from AtECA1**

We provide evidence that AtECA3 behaves like a Ca$^{2+}$/Mn$^{2+}$ pump, as it functionally replaced two endogenous Ca$^{2+}$ pumps of yeast, Pmr1 and Pmc1p, localized on the Golgi/ER and vacuole, respectively. AtECA3 restored the ability of strain K616 to grow on Ca$^{2+}$-depleted medium. As AtECA3 is localized to endomembranes in yeast, these results (Fig. 2) are consistent with the idea that AtECA3 loads Ca$^{2+}$ into endomembrane compartments and that the accumulated Ca$^{2+}$ activates processes needed for growth. AtECA3 also conferred tolerance of K616 yeast to toxic levels of Mn$^{2+}$, suggesting that the pump is able to remove Mn$^{2+}$ from the cytosol. These results of AtECA3 activity are consistent with those reported by Mills et al. (2008). Thus, biochemically, AtECA3 appears very similar to AtECA1 (Liang et al., 1997). We showed before that AtECA1 pumps $^{45}$Ca$^{2+}$ with selectivity for divalent cations, like Mn$^{2+}$ (Liang and Sze, 1998; Wu et al., 2002).

**Figure B. Localization of AtECA3-GFP to endosomes.** A, Differential patterns of AtECA1, AtECA3, and GFP-tagged markers transiently expressed in mesophyll protoplasts. Arabidopsis leaf protoplasts were transfected separately with a single GFP construct driven by the 35S CaMV promoter. After 14 to 24 h of incubation, GFP signal was observed by confocal microscopy in cells transfected with AtECA1-GFP (a), free soluble eGFP (b), ER retention sequence GFP-HDEL (c), vacuolar syntaxin GFP-AtSyp22 (d), AtECA3-GFP (e), trans-Golgi enzyme sialyl transferase ST-GFP (f), trans-Golgi network syntaxin GFP-AtSyp41 (g), and endosomal Rab-GTPase GFP-Ara7 (h). Bars = 5 μm. Panels reflect representative images from three to six independent experiments. B, ECA3-GFP is associated with a population of endosomes. Arabidopsis leaf protoplasts were cotransfected with ECA3-GFP and a marker protein tagged with RFP. After 18 to 24 h of incubation, fluorescent signals were observed. Markers used are Golgi ST-RFP (a), trans-Golgi network RFP-Syp41 (b), and endosomal RFP-Ara7 (c). Corresponding cells coexpressing ECA3-GFP are shown in a', b', and c' (column 2). Merged image (column 3) c'' shows AtECA3 colocalized partially with Ara7. Bright-field images of the cells are shown at far right (column 4). Bars = 5 μm.
However, several observations suggest that AtECA3 differs in properties and function from AtECA1. First, AtECA1 was more effective than AtECA3 in promoting the growth of the K616 mutant on Ca\(^{2+}\)-depleted medium containing 5 mM EGTA (Fig. 2B). Second, AtECA3 expression in strain K667 caused a hypersensitive response to 150 mM Ca\(^{2+}\), whereas AtECA1 expression had no effect (Fig. 2D). Strain K667 has a functional Pmr1p but is defective in the vacuolar Ca\(^{2+}\)/H\(^+\) exchanger (Vcx1) and the vacuolar Ca\(^{2+}\)-ATPase (Pmc1); thus, the strain is highly sensitive to very high levels of Ca\(^{2+}\) (Cunningham and Fink, 1994). Reduced K667 yeast growth by expression of AtECA3, but not by AtECA1, suggests that AtECA3 attenuated Pmr1p activity. Although the mechanism is unknown at this time, AtECA1 and AtECA3 clearly affect K667 yeast growth differentially. Thus, the function of AtECA3 in yeast appeared to be distinct from that of AtECA1, possibly due to distinct subcellular locations, as shown for plants. This idea is further supported by the differential phenotype of the T-DNA insertion mutants in plants. For example, root growth of the eca1-1 mutant was similar to that of the wild type at 1.5 mM (Wu et al., 2002) or 3 mM external Ca\(^{2+}\) (Fig. 6C); however, three alleles of eca3 displayed reduced root growth.

**AtECA3 Encodes an Endosomal Ca\(^{2+}\)/Mn\(^{2+}\) Pump**

Our studies suggest that AtECA3 is a Ca\(^{2+}\)/Mn\(^{2+}\) pump associated with plant endosomal membranes. We showed that AtECA3 fused at the C terminus to
GFP retained its activity as a Ca\(^{2+}\) as well as a Mn\(^{2+}\) pump (Fig. 3) in yeast. AtECA3-GFP, expressed transiently in mesophyll protoplasts, emitted punctate fluorescent patterns initially thought to resemble Golgi compartments (Li, 2006), as reported by Mills et al. (2008). However, in further studies, the AtECA3-GFP signal did not colocalize with markers of the trans-Golgi (sialyltransferase) or the trans-Golgi network (Syp41; Fig. 8B). Instead AtECA3-GFP partially colocalized with an endosome/PVC marker, Ara7. Ara7 is a conventional-type ortholog of Rab5, a Rab GTPase involved in regulating vesicular transport in the endocytic pathway of mammalian cells. In Arabidopsis cells, Ara7 was shown to be associated with earlier endocytic compartments involved in recycling plasma membrane proteins (Ueda et al., 2004). However, another study suggested that Ara7 localized to the PVC (Lee et al., 2004). Thus, these studies to localize AtECA3 suggest that the Ca\(^{2+}\)/Mn\(^{2+}\) pump is associated with vesicles involved in one or more possible functions, including maturation/differentiation of the trans-Golgi network, protein sorting at the PVC, machinery (e.g. cytoskeletal dynamics) involved in vesicle trafficking, and events that promote membrane fusion, secretion, or exocytosis to the plasma membrane or vacuole (Samaj et al., 2005).

In other experiments, we observed AtECA3-GFP protein localized to cortical structures at the tip of growing pollen tubes (data not shown). This distribution pattern resembles that of secretory vesicles and Golgi-derived compartments that readily fuse with the plasma membrane during fast pollen tube growth. These results together are consistent with the idea that AtECA3 is located on endosomal membranes, including those involved in endocytosis and/or exocytosis in plant cells. Previously, a Golgi-purified fraction from pea (Pisum sativum) epicotyl was shown to have Ca\(^{2+}\) pump activity (Ordenes et al., 2002), although without sequence information it is unclear whether the pea pump is orthologous to AtECA3. Whether the subcellular distribution of AtECA3-GFP is predominantly Golgi alone (Mills et al., 2008), post-Golgi (Fig. 8), or varies according to the physiological state of the cell will need further investigation. However, it is clear that the punctate pattern of AtECA3-GFP is strikingly distinct from the reticulate pattern of AtECA1-GFP, indicating that these two pumps are localized to separate compartments.

Possible Roles of Endosomal Ca\(^{2+}\) and Mn\(^{2+}\) in Vesicle Trafficking and Secretion

Intriguingly, we found that eca3 mutants secreted more total protein and more peroxidase activity than wild-type plants (Figs. 9 and 10), suggesting that perturbation of Ca\(^{2+}\) and/or Mn\(^{2+}\) homeostasis in the endosomes perturbs protein secretion. Coincidently, yeast mutants lacking Pmr1 also secrete more proteins than wild-type cells (Smith et al., 1985; Rudolph et al., 1989), although the mechanism remains unclear. In plants, Ca\(^{2+}\) mediated the association of the vacuolar sorting receptor, VSRI, to its cargo. Furthermore, a defective VSRI causes vacuolar storage proteins to be secreted (Shimada et al., 2003). Here, we provide genetic evidence linking endosomal Ca\(^{2+}\) homeostasis to protein secretion in plants. It is not yet understood why a defect in AtECA3 function would increase the secretion of peroxidase. Secreted peroxidases (class III) or apoplastic peroxidases include many isoforms and are involved in diverse functions, such as generating reactive oxygen species, forming cell wall polymers, and regulating hydrogen peroxide levels. They modify cell wall properties, such as loosening walls during growth and cross-linking walls to form a barrier in response to wounding, biotic, and abiotic stress. Interestingly, peroxidases and phenolic compounds are induced by heavy metals and appear to protect plants from heavy metal toxicity (Passardi et al., 2005). Increased peroxidases might aid in cross-linking cell walls and thus reduce or arrest wall growth. Possibly, deregulated secretion might alter cell wall composition or organization in eca3 mutants.

Function of an Endosomal Ca\(^{2+}\) in Root Growth

Almost nothing is known about the role of endosomal Ca\(^{2+}\) pumps in plants. Here, we show that the Ca\(^{2+}\)-stimulated root growth seen in wild-type plants is reduced or inhibited in eca3 mutants. In contrast, the external concentration (millimolar) of [Ca\(^{2+}\)]\(_{ext}\) had no effect on the growth of the eca1-1 mutant (Wu et al., 2002). Ca\(^{2+}\) entry into the eca3 mutant should not be limited when external Ca\(^{2+}\) is 3 mm. This idea is supported by the similar Ca\(^{2+}\) content of eca3 and wild-type plants (Mills et al., 2008). Thus, the requirement for millimolar levels of Ca\(^{2+}\) for root growth in wild-type plants indicates that sorting internal Ca\(^{2+}\) into particular endosomal compartments, like post-Golgi compartments, restricts the growth of mutants. We suggest that AtECA3 promotes growth by loading an adequate level of Ca\(^{2+}\) into a subpopulation of endosomal compartments. The sequestered Ca\(^{2+}\) could perform one or more functions, such as (1) to modulate enzyme or protein activities in the lumen, (2) to fill Ca\(^{2+}\) stores that can be released via gated Ca\(^{2+}\) channels to enhance local [Ca\(^{2+}\)]\(_{lum}\) in a temporal manner, and (3) to supply Ca\(^{2+}\) to the extracellular medium for biochemical, signaling, or polarity purposes. Endosomal luminal [Ca\(^{2+}\)]\(_{lum}\), might modulate protein and enzyme activities that are responsible for vesicle transport or for modification of its polysaccharide and protein cargo. An induced local rise in [Ca\(^{2+}\)]\(_{lum}\) may allow (1) in directing the movement of secretory vesicles via the cytoskeleton to their destination and (2) in modulating exocytosis, including membrane docking and fusion events, and perhaps subsequent endocytosis to recycle essential components of secretory pathways.

Studies using yeast and animal cells have shown that Ca\(^{2+}\) in the Golgi and Golgi-derived vesicles has roles in protein processing, sorting, glycosylation,
secretion (Durr et al., 1998; Ton et al., 2002; Rizzuto and Pozzan, 2006), and, more recently, Ca$^{2+}$ signaling (Van Baalen et al., 2004). In plants, the vacuolar sorting receptor (AtVSR1 or pumpkin [Cucurbita pepo] VP72) binds its cargo in a Ca-dependent manner (Watanabe et al., 2002; Shimada et al., 2003). Thus, depleting luminal Ca$^{2+}$ concentration in plant endosomes would conceivably disrupt sorting and the synthesis and delivery of wall proteins and noncellulosic polysaccharides (Nebenfuhr and Staehelin, 2001; Robinson, 2003) and therefore affect polarized growth and cell patterning.

**Role of AtECA3 in Detoxifying Excess Mn$^{2+}$**

While a few micromolar Mn$^{2+}$ in the soil is sufficient to sustain plant growth (Marschner, 1995), high Mn$^{2+}$ is detrimental. We show that AtECA3 plays a role in Mn$^{2+}$ detoxification in both yeast and Arabidopsis seedlings, based on the sensitivity of eca3 mutants to high Mn$^{2+}$ (Fig. 2). Curiously, Mills et al. (2008) did not see evidence for a role of ECA3 in detoxifying excess Mn$^{2+}$ in plants, which was inconsistent with their yeast result, where AtECA3 conferred tolerance to high Mn$^{2+}$ in the K616 mutant. The difference between their findings and our results showing a Mn-sensitive eca3 mutant plant phenotype is most likely due to the experimental conditions used in the two studies. Importantly, Mills et al. (2008) showed that ECA3 is critical for Mn$^{2+}$ nutrition in plants. They found that 9-d-old eca3-2 mutants (SALK_0570619) had reduced fresh weight when grown on Mn$^{2+}$-deficient medium and that a few micromolar Mn$^{2+}$ restored growth to wild-type levels. Taken together, these results suggest that ECA3 serves at least two biological functions: (1) to accumulate Mn$^{2+}$ into a Golgi-related compartment to satisfy Mn$^{2+}$ nutrition needed for growth; and (2) to load Mn$^{2+}$ into a subset of post-Golgi compartments that participate in detoxifying excess Mn$^{2+}$.

The cellular basis of detoxification is unclear. Curiously, the presence of AtECA1, an abundant divalent cation transporter, is inadequate to reduce Mn$^{2+}$ toxicity in eca3 mutants when [Mn$^{2+}$]$_{ext}$ is 50 μM or greater. As AtECA1 pumps Mn$^{2+}$ and Ca$^{2+}$ mainly into the ER lumen, a relatively extensive endomembrane system, the impaired growth is unlikely caused by an inability to lower cytosolic [Mn$^{2+}$]$_{cyt}$ by divalent pumps or H$^{+}$-coupled cotransporters into ER or vacuolar compartments (Sze et al., 2000). Instead, the machinery to alleviate Mn$^{2+}$ toxicity is dependent on the proper functioning of additional endomembranes, such as the PVC and endosomal vesicles. It is possible that inadequate loading of Ca$^{2+}$, Mn$^{2+}$, or both into endosomes causes protein missorting and deregulated membrane trafficking in eca3, as shown by increased apoplastic peroxidase, and thus interferes with the detoxification machinery that might involve one or more processes, including delivering Mn$^{2+}$ into vacuoles and the release of Mn$^{2+}$ and stress-induced proteins into the apoplast (Fecht-Christoffers et al., 2006). It is possible that Mn$^{2+}$-entering the cytosol is pumped into the ER by AtECA1 and then removed by exocytosis via endosomes with AtECA3.

**Summary and Model**

The apparently distinct physiological effects of an endosomal cation pump, AtECA3, in Ca$^{2+}$-stimulated root growth, in Mn$^{2+}$ nutrition, and in Mn$^{2+}$ detoxification appear to converge on the highly regulated endosomal trafficking pathway and secretory processes of cells. When Ca$^{2+}$ and Mn$^{2+}$ are present at near optimal range, loading of these divalent cations into endosomal compartments promotes growth, possibly through the synthesis and delivery of wall proteins and polysaccharides. When wild-type plants are subject to Mn$^{2+}$ toxicity stress, the endosomal/secretory trafficking machinery is likely engaged in the dynamic process of moving excess Mn$^{2+}$ either to or from the vacuole and/or to the cell exterior. Taken together, the results of this study suggest that loading Ca$^{2+}$ and Mn$^{2+}$ into a subpopulation of post-Golgi compartments by AtECA3 affects activities critical for highly regulated endosomal trafficking that determine intracellular sorting (to and from the vacuole) and the extent of exocytosis and secretion. Given the central role of membrane trafficking in plant responses to hormones and to stress, it is very likely that AtECA3 will affect many other growth and developmental processes.

**MATERIALS AND METHODS**

**Plant Materials, Growth, and Measurement**

Wild-type and eca3 Arabidopsis (Arabidopsis thaliana) plants (Columbia [Col] ecotype) were used in this study. Homozygous seed stocks for eca3-1b, eca3-4, and eca3-5 correspond to SALK_537_C07, SALK_032082, and SALK_045567 lines, respectively (McElver et al., 2001; Alonso et al., 2003).

For growth on plates, Arabidopsis seeds were surface sterilized by soaking in 20% (v/v) Clorox and 0.05% Tween 20 for 10 min followed by five rinses in sterile water. The seeds were placed on half-strength MS medium (Murashige and Skoog, 1962) containing 0.8% agar and 2.5 mM MES-K at pH 5.7, followed by stratification at 4°C for 3 d in the dark. The plates were then positioned vertically in Conviron ATC60 growth chambers with a photoperiod of 16 h of light of 120 to 180 μmol m$^{-2}$ s$^{-1}$ and 8 h of dark. The temperature was set at 22°C in the day and 20°C at night.

For soil-grown plants, Arabidopsis seeds were planted on synthetic soil mixture containing Miracle-Gro potting mix and perlite, followed by 3 d at 4°C in the dark, before being taken to growth chambers with a photoperiod of 16 h of light of 120 to 180 μE m$^{-2}$ s$^{-1}$ at 22°C and 8 h of dark at 20°C and 60% relative humidity, or with a short-day photoperiod of 10 to 12 h of light of 120 μE m$^{-2}$ s$^{-1}$. Plants were watered twice per week or as needed. Wild-type and mutant plants were always grown side by side in the same tray and growth chamber for consistency.

**Root Length Measurement**

Three days after germination on square plates containing standard half-strength MS medium, the seedlings were transferred onto half-strength MS medium plates containing different Mn$^{2+}$ concentrations as specified in “Results.” The plates were then positioned in an inverted orientation for another few days before photographs were taken by scanning the plates using a Umax Astra 1200S scanner. Root measurement was conducted using ScionImage software (Scion). The wild-type and mutant plants were treated with the same conditions by placing them side by side on the same plate.
Hydronic Plant Growth for Extraction of Root AWF

Arabidopsis seeds were dispersed on quarter-strength Hoagland medium (Hoagland and Arnon, 1950) solidified with 0.5% agar on the bottom of a 1-mL tip box (approximately 0.5 cm depth). After germination, the tip boxes were floated on quarter-strength Hoagland medium (pH 5.5, 10 mM MES-K) aerated by a fish tank air pump. Medium was replaced twice per week to prevent algal contamination. Roots penetrating the medium were excised after 3 to 4 weeks.

DNA Manipulations

cDNA Cloning and Molecular Constructs

ECA3 cDNA was amplified by PCR from first-strand cDNA. The template was reverse transcribed from total RNA of 3-week-old rosette leaves using primers ECA3-α and ECA3-α (Supplemental Table S1). Proofreading High-Fidelity Taq DNA Polymerase Deep Vent (New England Biolabs) was used to amplify the DNA. The PCR product was purified by gel extraction and ligated to an EcoRV-linearized vector pBluescript SK+ (Stratagene). The cloned sequence was verified by sequencing (GenBank accession no. AF550902) and used for subsequent cDNA cloning.

AIECA Promoter::GUS Constructs

Genomic DNA was isolated from 4-week-old plants using the cetlytrimethyl-ammonium bromide method (Ausubel et al., 1988). Based on the annotation of bacterial artificial chromosome clone T271 (AC0004122), the region between the AIECA3 start codon and its immediate upstream ORF (AK230347) was taken as the AIECA3 promoter. This region was amplified using primer sequences with appended restriction enzyme sites, ECA3-pf and ECA3-pr (Supplemental Table S1). The PCR-amplified product of 5.2 kb was digested with XhoI and NotI and cloned into a GUS ORF-containing vector pRTITA to make a promoter::GUS construct, AIECA3-RITA. The construct was verified by sequencing. The NotI-Norf fragment of AIECA-RITA was ligated to the binary vector pMLBART. The resulting pECA3-GUS-MLBART construct was introduced into plants by the floral dip method (Clough and Bent, 1998), and plants homozygous for a Bara resistance marker were analyzed for promoter::GUS activity. Transgenic plants containing AIECA promoter::GUS were obtained using a similar method. Primers ECA1-pf and ECA1-pr (Supplemental Table S1) were used to amplify a 3.1-kb fragment upstream of the ECA1 ORF.

GFP-Tagged AIECA3

Conventional cloning and Gateway cloning methods were used to obtain GFP-tagged AIECA3. For conventional cloning, AIECA3 cDNA was amplified by PCR using Platinum Pfx DNA polymerase (Invitrogen) and primers ECA3-pf and ECA3-pr (Supplemental Table S1). The resultant PCR-amplified product for the C-terminal tailed GFP was digested with BglII and ligated to the Ncol-cut vector pAVA393 (von Arnim et al., 1998). The resultant construct, ECA3-GFP-393, was verified by sequencing of the AIECA3 coding region and contains a Cm4V 35S promoter.

For Gateway cloning, the AIECA3 cDNA was amplified by PCR using Platinum Pfx DNA polymerase (Invitrogen) and primers ECA3-α and ECA3-Gr. The PCR-amplified fragment of AIECA3 (stop codon removed) was cloned into the Gateway vector pDONR221 (Invitrogen) to produce an entry clone, AIECA3-DONR221, using BP recombination cloning strategy. The sequence of the AIECA3 insert was verified. The clone ECA3-DONR221 was then cloned into the Gateway destination binary vector pK7FWG2 by LR recombination (Kanamori et al., 2002). The resultant construct, ECA3-FWG, contains an expression cassette of Cm4V P35S-ECA3-GFP-T35S. Both conventional clones and Gateway clones were used in localization experiments.

Agrobacterium-Mediated Plant Transformation and Histochemical GUS Staining

Agrobacterium tumefaciens strain GV3101 was transformed with the binary vectors via electroporation and transformants were selected on Luria-Bertani plates with gentamicin and spectinomycin. Arabidopsis ecotype Col plants were transformed with Agrobacterium using the floral dip method (Clough and Bent, 1998). Plant transformants were selected on plates containing Basta (50 mg L−1 glufosinate ammonium [Crescent Chemical]) or 50 μg mL−1 kanamycin.

At least five T3 lines were checked for a consistent GUS staining pattern (Lagarde et al., 1996). Samples at various stages were harvested in 90% acetone, rinsed once with staining buffer containing 50 mM Na2 phosphate (pH 7.2), 0.5 mM KFe(CN)6 and 0.5 mM K4Fe(CN)6, and then incubated for 48 h at 37°C in buffer containing 1 mM X-Gluc. The reaction was stopped in 70% ethanol, and chlorophyll was cleared in 95% ethanol. GUS activity was visualized with a Nikon Eclipse E600 microscope equipped with differential interference contrast (Nikon Instruments), and images were recorded using a Nikon DSM1200 digital camera.

Yeast Strains, Plasmids, Transformation, and Growth

The yeast (Saccharomyces cerevisiae) strains used were W303-1A (MATa, ade2-1 can1-100 his3-200 leu2-3,112 trpl-1 leu2-3), K616 (PMA1::HIS3 pmc1:: TRP1 URA3::LEU2), and K607 (Ura1::LEU2 pmc1:: TRP2 pmc1::LEU2; Cunningham and Fink, 1994, 1996). Yeast was transformed with AIECA3 constructs by the lithium acetate method (Gietz et al., 1992).

AtECA3 cDNA was initially subcloned into the yeast expression vector p426 (Mumberg et al., 1994) by ternary ligation of three fragments: 1.2-kb fragment of a Kpn1/HindIII-digested ECA3-BSK clone, 1.6-kb fragment of a SpeI/SalI-digested ECA3-BRK, and 6.3-kb fragment of a SpeI/SalI-digested p426. This strategy was taken due to limited restriction enzyme sites in the p426 vector and an internal Kpn1 site in AIECA3. The final vector, p426-ECA3, contains a GAI1 promoter::AIECA3 expression cassette and was verified by enzyme digestion.

For Gateway cloning, ECA3-DONR221 was recombined with three destination vectors, pYES-DEST32 (Invitrogen), pYESDR196, and pGWD196 (Supplemental Materials and Methods S1). The first two cassettes drive strong expression of ECA3 under the GAL1 promoter (GAL1::ECA3) and the PMA1 promoter (PMA1::ECA3). In pYESDR196, the GAL1 promoter was replaced by the PMA1 promoter (Supplemental Materials and Methods S1). The pGWD196 vector uses the PMA1 promoter to drive the expression of ECA3 fused at its C-terminal tail to GFP.

At least three independent transformants from each construct were inoculated in synthetic complete medium with Glc but no uracil (SC-URA/glu). An overnight culture (5–10 mL) was diluted to an OD600 of 0.5, washed twice, and suspended in sterile water. A six-step serial dilution of 5-fold was prepared in sterile water for each culture. Five microliters of each was spotted onto the SC-URA plates (with Glc or Gal) supplemented with different ions or EGA. The pH of the medium was maintained with 10 mM MES-KOH at pH 5.5 or 6.4. Growth at 30°C was recorded at varying times.

Transient Expression in Arabidopsis Mesophyll Protoplast

Protoplast Isolation

Transient expression was conducted according to published protocols with modifications (Jin et al., 2001; Sheen, 2001; Uemura et al., 2004; Yoo et al., 2007). Arabidopsis leaves from approximately 3-week-old plants grown under continuous low light (40-50 μE m−2 s−1) and constant temperature (21°C) were cut into small strips and digested in 2.5 mL of enzyme solution for 3 h. The digestion solution consisted of 1.3% cellulose R10, 0.25% macerozyme R100 (Yakult), 0.4 mM mannitol, 20 mM KCl, 20 mM MES at pH 5.7, 10 mM CaCl2, 0.1% fetal bovine serum (Sigma; no. F6778), and 2.5 mM β-mercaptoethanol. Protoplasts were filtered through 70-μm nylon mesh and centrifuged at 100g for 2 min. The pellets were washed twice in W5 solution containing 0.3 mM mannitol, 125 mM Suc, 8 mM CaCl2, and 3 mM MES at pH 5.7 prior to transfection. Plasmids were purified by Miniprep kit (QIAGEN; no. 27160) or Plasmid Midi kit (QIAGEN; no. 12143) and resuspended in 0.4 mM mannitol.

For each transfection, 10 μL of protoplast suspension (~2 × 104 cells) was added to a mixture of 40 μL of plasmids (5–10 μg per plasmid), 5 μL of Mobilinfection (0.4 mM mannitol, 150 mM MgCl2, 5 mM CaCl2, and 40 mM MES), and 55 μL of PEG-Ca solution (40% PEG4000 [Fluka; no. 81240], 0.2 mM mannitol, and 100 mM Ca(NO3)2). After incubation at room temperature for 20 to 30 min, the transfection mixture was diluted with 700 μL of W5 solution and centrifuged at 100g for 45 s. After supernatant removal, protoplasts were resuspended in

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300 μL of WI solution (0.5 mM mannitol, 8 mM KH₂PO₄, and 2 mM MES) and 400 μL of MS solution (0.45 mM mannitol, 1× MS salt mixture [GIBCO; no. 11117-058], 1× Gamborg’s vitamin solution [Sigma; no. G1019], 2% Suc, 2 mM MES, and 100 μg/mL ampicillin). Transfected protoplasts were incubated in the dark at room temperature for 18 to 24 h before microscopy. In general, the efficiency of a single transfection of marker proteins is 50% or more; however, the efficiency is lower in cotransfections due to differences in fluorescent signals and the expression level of each gene.

**Confocal Microscopy**

Fluorescent signals in protoplasts were examined on the Zeiss LSM510 confocal microscope (Carl Zeiss), usually 18 to 24 h after transfection. The filter sets used for excitation (Ex) and emission (Em) are as follows: GFP, 485 nm (Ex)/BP505 to 530 nm (Em); RFP, 543 nm (Ex)/BP560 to 615 nm (Em); chlorphyll, 543 nm (Ex)/LP560 nm (Em); bright field, 633 nm. Signals were captured in multichannel mode. Images were analyzed and processed in the Zeiss LSM image browser (Carl Zeiss) and Adobe Photoshop (Adobe Systems). To examine cells expressing GFP, liquid cultures grown overnight were cultured in SC-URA Glc medium with 3,000 L of MS solution (0.45 M mannitol, 1 mM Suc, 2 mM MES, and 300 μL of 0.2 to 0.4 were used.

**Apoplastic Wall Fluid and Peroxidase Activity**

AWF was prepared from roots of Arabidopsis grown hydroponically in modified quarter-strength Hoagland medium (5 mM MES-K, pH 5.7; Hoagland and Arnon, 1950). The method was modified from that used to extract apoplastic wall fluid from leaves (Rathmell and Sequeira, 1974; Hoagland and Arnon, 1950). The method was modified from that used to extract apoplastic wall fluid from leaves (Rathmell and Sequeira, 1974; Hoagland and Arnon, 1950). The method was modified from that used to extract apoplastic wall fluid from leaves (Rathmell and Sequeira, 1974; Hoagland and Arnon, 1950). The method was modified from that used to extract apoplastic wall fluid from leaves (Rathmell and Sequeira, 1974; Hoagland and Arnon, 1950).

Peroxidase activity of the AWF fraction was determined from the rate of oxidation of guaiacol to tetraguaiacol (Maehly and Chance, 1954; Cordoba-Pedregosa et al., 1996). In the presence of peroxidases, the artificial substrate guaiacol (Kasei Kogyo) was oxidized by hydrogen peroxide to tetraguaiacol, which was monitored as an increase in A470. Briefly, 10-, 20-, and 300-μL AWF samples containing 0.1 to 2 μg of total protein were added to a 1-mL reaction mixture containing 0.3% (v/v) hydrogen peroxide and 0.1% (v/v) guaiacol in 10 mM sodium phosphate at pH 6.0. The reaction was started by adding guaiacol, and the increase in A470 was recorded for an initial 2 min using a Beckman DU640 spectrophotometer at room temperature. The rate of A470 change was expressed as nanomoles per minute per gram of tissue (fresh weight) using a molecular extinction coefficient of 26.6 μmol−1 cm⁻1. At least three independent experiments were conducted for each set of wild-type and mutant plants.

Sequence data from this article can be found in the GenBank/EMBL data libraries under accession number AY663002.

**Supplemental Data**

The following materials are available in the online version of this article.

**Supplemental Figure S1.** Full alignment of ECAs and SERCAs.

**Supplemental Figure S2.** Localization of Antica3-GFP in yeast.

**Supplemental Figure S3.** Antica1 promoter–GUS activity.

**Supplemental Figure S4.** Root map expression of Antica1, ECA2, and ECA3.

**Supplemental Table S1.** Primers used.

**Supplemental Materials and Methods S1.** Gateway vectors for expression in yeast.

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