ECA3, a Golgi-Localized P_{2A}-Type ATPase, Plays a Crucial Role in Manganese Nutrition in Arabidopsis

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Calcium (Ca) and manganese (Mn) are essential nutrients required for normal plant growth and development, and transport processes play a key role in regulating their cellular levels. Arabidopsis (Arabidopsis thaliana) contains four P_{2A}-type ATPase genes, AtECA1 to AtECA4, which are expressed in all major organs of Arabidopsis. To elucidate the physiological role of AtECA2 and AtECA3 in Arabidopsis, several independent T-DNA insertion mutant alleles were isolated. When grown on medium lacking Mn, eca3 mutants, but not eca2 mutants, displayed a striking difference from wild-type plants. After approximately 8 to 9 d on this medium, eca3 mutants became chlorotic, and root and shoot growth were strongly inhibited compared to wild-type plants. These severe deficiency symptoms were suppressed by low levels of Mn, indicating a crucial role for ECA3 in Mn nutrition in Arabidopsis. eca3 mutants were also more sensitive than wild-type plants and eca2 mutants on medium lacking Ca; however, the differences were not so striking because in this case all plants were severely affected. ECA3 partially restored the growth defect on high Mn of the yeast (Saccharomyces cerevisiae) pmr1 mutant, which is defective in a Golgi Ca/Mn pump (PMR1), and the yeast K616 mutant (Δpmc1 Δpmr1 Δcnb1), defective in Golgi and vacuolar Ca/Mn pumps. ECA3 also rescued the growth defect of K616 on low Ca. Promoter:β-glucuronidase studies show that ECA3 is expressed in a range of tissues and cells, including primary root tips, root vascular tissue, hydathodes, and guard cells. When transiently expressed in Nicotiana tabacum, an ECA3-yellow fluorescent protein fusion protein showed overlapping expression with the Golgi protein GONST1. We propose that ECA3 is important for Mn and Ca homeostasis, possibly functioning in the transport of these ions into the Golgi. ECA3 is the first P-type ATPase to be identified in plants that is required under Mn-deficient conditions.

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2004). Mn^{2+} is essential throughout all stages of plant development; it activates a number of different enzymes, such as decarboxylases and dehydrogenases in the Krebs cycle (Marschner, 1995), and several glycosyltransferases in the Golgi (White et al., 1993; Nunan and Scheller, 2003). Mn^{2+} is associated with PSII, where it is required for light-induced water oxidation through which oxygen is produced. Mn is also required for mitochondrial superoxide dismutase (MnSOD), which is involved in scavenging of superoxides (Bowler et al., 1991) and is important in the biosynthesis of fatty acids and carotenoids (Marschner, 1995). As well as being an essential element, Mn^{2+} is also toxic at higher concentrations causing chlorosis, brown speckles on mature leaves, and necrosis (Marschner, 1995).

Transport proteins play a crucial role in maintaining the correct concentrations of Mn in the cytoplasm and also in supplying Mn to particular subcellular compartments where it may be required to act as an enzyme cofactor. In yeast (Saccharomyces cerevisiae) and also in many bacterial systems, several pathways for Mn^{2+} transport have been identified (Kehres and Maguire, 2003; Culotta et al., 2005). In plants, the situation is less clear, but several proteins that may transport Mn have been identified in Arabidopsis (Arabidopsis thaliana), including AtECA1, an endoplasmic reticulum...
(ER) P2A-type ATPase; AtCAX2, a vacuolar cation/H\(^+\) antiporter; AtIRT1, a plasma membrane ZIP transporter; and AtNramp3, a vacuolar Nramp (for review, see Williams et al., 2000; Hall and Williams, 2003; Pittman, 2005). Certain members of the cation diffusion facilitator family also appear to transport Mn, including AtMTP11 (Delhaize et al., 2007; Peiter et al., 2007).

Ca is required at much higher concentrations than Mn and plays many fundamental roles in plants, including stabilization of cell walls and membranes, facilitating root extension, and protection against potentially damaging factors in the soil, such as salinity (Sanders et al., 1999). Ca deficiency is rare in nature, but disorders that are observed in horticulture tend to be seen in fast-growing tissues and include blossom-end rot in tomato (Solanum lycopersicum), tip-burn in leafy vegetables, and fruit cracking (Marschner, 1995). Ca is also important in signaling, coupling a wide range of extracellular stimuli to a vast array of intracellular responses (Sanders et al., 1999). As such, the cytosolic Ca concentration must be carefully regulated and resting levels are usually around 0.1–0.2 \(\mu\)M. Ca\(^{2+}\) enters the cytoplasm through calcium channels present at both the plasma membrane and intracellular membranes (White, 2002). Ca\(^{2+}\) efflux is mediated by high-affinity P-type Ca-ATPases and lower affinity Ca/H antiporters (Evans and Williams, 1998; Geisler et al., 2000, Cheng et al., 2005). The complexity of Ca signaling and homeostasis is reflected in the diversity of Ca\(^{2+}\) transporters. Molecular analyses of Ca-ATPases in plants indicate that they divide into two types: P2A-type Ca-ATPases (also known as ECAs), which show homology to the sarcoplasmic/ER Ca-ATPases (SERCA), and P2B-type Ca-ATPases (also known as ACAs), which are generally stimulated by calmodulin and show homology to the calmodulin-binding Ca-ATPases found at the plasma membranes of animal cells (PMCA; Axelsen and Palmgren, 1998; Geisler et al., 2000). A subset of P2A-type Ca-ATPases, the secretory pathway Ca-transport ATPases (SPCA), is found in other organisms (PMR1 is an example in yeast), but these do not appear to exist in plants.

Ca pumps may have a variety of functions: loading of Ca into intracellular stores, such as the ER and vacuole, for them to act as sources of Ca for signaling; shaping and terminating a Ca signal; providing Ca for vesicle trafficking and fusion; and supplying Ca and possibly other diveralent cations, such as Mn and zinc (Zn), to organelles to support their biochemical reactions (Sze et al., 2000). Genetic studies using mutants are now starting to reveal specific functions for individual Ca pumps (Wu et al., 2002; Schiott et al., 2004). There are four P2A-type ATPases in Arabidopsis (AtECA1–AtECA4). Of these, AtECA1 was the first to be studied (Liang et al., 1997) and was shown to function at the ER in the transport of Ca and Mn (Liang et al., 1997; Liang and Sze, 1998). The growth of the eca1-1 mutant is sensitive to low Ca and high Mn, suggesting that pumping of these cations into the ER is required to support plant growth under conditions of Ca deficiency and Mn toxicity (Wu et al., 2002). Having shown that additional P2A-type ATPases, ECA2 and ECA3, are expressed in Arabidopsis (Pittman et al., 1999), the main objective of this study was to investigate their physiological role. This was achieved by

**Figure 1.** Isolation of mutant lines. A, Determining the genotype of plants from Salk line 045567. PCR was carried out on genomic DNA from individual plants (1–3) using gene-specific primers ECA3Ex10.F and ECA3.R to detect plants that contained a wild-type copy of the gene; these amplified a band of the predicted size, 2.08 kb (top). Salk LBa1 primer for the T-DNA and the gene-specific primer ECA3.R were used to detect the presence of the T-DNA in ECA3. They amplified a band of the approximate predicted size, 1.96 kb (bottom). The zygosity of plants 1 to 3 determined from this analysis is indicated: wt, Wild-type; hom, homozygous for the insert; het, heterozygous for the insert. B, RT-PCR using RNA isolated from wild type and eca mutants. Primers designed to amplify a product across the insertion site gave products for ECA2 and ECA3 of 0.67 and 1.67 kb, respectively, only in wild-type plants. Actin 2 primers were used to amplify a product of 0.2 kb as a positive control in all samples. C, Insertion sites for mutants isolated in this study.
isolating T-DNA insertion mutants in which the genes encoding these proteins had been disrupted and comparing the phenotype of the mutants to wild-type Arabidopsis. We present evidence showing that eca3 mutants have a unique and distinct phenotype from eca1 and eca2 mutants and demonstrate that ECA3 has a crucial role in Mn nutrition.

RESULTS

Primary Structure of Arabidopsis ECA3

AtECA3 from ecotype ‘Columbia-0’ contains an open reading frame of 998 amino acids with a predicted molecular mass of 109.062 kD. It is identical to the sequence predicted from genome sequencing (The Arabidopsis Information Resource; http://arabidopsis.org), but differs from the ECA3 sequence (AJ132388) that we cloned previously from ‘Landsberg erecta’ (Pittman et al., 1999) in four bases: A336G, T453A, T1647G, and C2934T. In the ECA3 sequence from ‘Landsberg erecta’, the second- and fourth-base changes are silent, but the first and third give changes in the amino acid sequence: R46G and S549R. Conpred II, a consensus prediction method for obtaining transmembrane (TM) topology models (http://bioinfo.si.hirosaki-u.ac.jp/~ConPred2), indicates that AtECA3 contains 10 TM domains, a small cytoplasmic loop between TMs 2 and 3 and a large cytoplasmic loop between TMs 4 and 5. The latter contains the highly conserved phosphorylation domain CSDKTGTLT, which includes the phosphorylated Asp residue (Asp-347 for AtECA3) that is phosphorylated during the reaction cycle in all P-type ATPases. This model is consistent with structural models that have been presented for the sarcoplasmic reticulum Ca-ATPase from animal cells (Toyoshima et al., 2000).

Isolation of T-DNA Insertion Mutant Alleles of ECA2 and ECA3

To determine the physiological function of ECA2 and ECA3, we obtained mutant lines in which ECA2 and ECA3 were likely to be functionally disrupted. We isolated two independent mutant alleles for each (eca2-1, eca2-2, eca3-1, and eca3-2). These T-DNA insertion alleles were identified using the reverse-genetics approach outlined in “Materials and Methods.” Figure 1A illustrates genotyping of plants from the Salk line 045567; Figure 1B shows that, for each particular mutant, no product with the appropriate gene-specific primers could be detected in reverse-transcribed cDNA from plants homozygous for the insert. Figure 1C and Table I summarize the T-DNA insertion positions in the resulting mutant alleles and indicate where in the protein the insertion would occur. The position of the inserts in each of the mutants means that these are predicted loss-of-function mutants.

Effect of Low Ca on eca Mutants

To elucidate the biological function of ECA2 and ECA3 in Arabidopsis, we monitored insertion mutants under a wide range of conditions comparing their growth and development to wild-type plants. When grown on soil, no major differences from wild-type plants were observed (Supplemental Fig. S1).

Because eca1-1 mutants were previously shown to be extremely retarded when grown on low-Ca medium (0.2 mM; Wu et al., 2002), eca2 and eca3 mutants were tested in this range. At basal levels of Ca (1.5 mM), eca3-1, eca3-2, eca2-1, and eca2-2 mutants were similar in their fresh weight and chlorophyll content to wild-type plants; results are shown only for fresh weight for eca2-1 and eca3-1 (Fig. 2A). In contrast to results reported previously for eca1-1 mutants (Wu et al., 2002), eca2 and eca3 mutants were no more retarded in growth than wild-type plants at low Ca (in this case, 0.1 mM). We next tested whether there was a difference in the growth of the mutants when Ca was omitted from the medium. Under these conditions, both wild-type and mutant seedlings were adversely affected (Fig. 2B). They became chlorotic and stopped growing at an early stage. In general, both eca3 mutants were slightly more sensitive than wild-type and eca2 mutants, but in this experiment only eca3-1 was significantly lower in fresh weight (P < 0.05; Fig. 2B).

eca3 Mutants Are Susceptible to Growth in Mn-Deficient Conditions

eca1-1 mutants were previously shown to be more adversely affected in growth on high (0.5 mM) Mn medium (Wu et al., 2002). We found no difference in the response of eca2 and eca3 mutants to this level of Mn compared to wild-type plants (data not shown). Therefore, we investigated whether the mutants showed any responses under Mn-deficient conditions. A striking difference was observed in the eca3 mutants compared to eca2 and wild type when grown on

Table I. eca mutant alleles identified in this study

| Allele | Salk Line | Introns and exons are numbered so that the first exon begins with the start ATG and is followed by the first intron. |
|--------|-----------|------------------------------------------------------------------------------------------------------------------|
| eca2-1 | 0576961   | First of five exons. In large cytoplasmic loop between phosphorylated Asp and KGAAE motif. |
| eca2-2 | 0539146   | First of four introns. In large cytoplasmic loop between phosphorylated Asp and KGAAE motif. |
| eca3-1 | 0545567   | Twelfth of 33 introns. Middle of third transmembrane domain. |
| eca3-2 | 0570619   | Sixteenth of 33 introns. In large cytoplasmic loop between phosphorylated Asp and KGAPE motif. |

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medium with Mn omitted (Fig. 3). Under these conditions, eca3 mutants showed a marked reduction in growth, which first became apparent after approximately 8 to 9 d. Both root and shoot growth were reduced (Fig. 3, A and B). In addition, the cotyledons became yellow and the marked reduction in chlorophyll is indicated in Figure 3B. There were some interesting features observed when individual seedlings were examined closely (Fig. 3A; Supplemental Fig. S2). In some seedlings, dark areas were seen at the tips of developing leaves (Supplemental Fig. S2). On leaves where the lamina was expanding, a brown band was often observed across the petiole or lamina with bleaching occurring distal to the band. In many cases, the leaf lamina failed to expand further and senescing areas were observed. When leaves did develop, these and the expanded cotyledons were seen to bleach. Addition of Mn to the medium at a concentration of around 1 μM was sufficient to suppress these phenotypes (Fig. 4).

The response of Arabidopsis mutants to other ion deficiencies was also investigated. When Zn was omitted from the growth medium, there was no significant difference in the performance of the eca3 (Fig. 5A) or eca2 mutants (data not shown) compared to wild type. eca3 mutants were inhibited in their growth to the same extent as wild type and there were no significant differences in terms of fresh weight of shoot or chlorophyll content. Omission of iron (Fe) from the medium had a marked effect on growth and chlorophyll content of all plants, but there was no specific detrimental effect on the mutants compared to wild-type plants (Fig. 5B). Omitting copper (Cu) from the medium also had no specific effect on mutants compared to wild type (Fig. 5B).

**Elemental Analysis of eca3 Mutants**

Because the eca3 mutants showed a clear phenotype under Mn-deficient conditions, Mn content was determined under nutrient-sufficient (basal; 50 μM Mn) and Mn-deficient (0 μM Mn) conditions. The levels observed in each genotype are shown in Figure 6. To make this clearer, the level relative to the wild type (expressed as a percentage) is shown in Supplemental Figure S3, with the level in the wild type (100%) indicated as a dashed line. Despite the marked differences observed in the growth phenotype of eca3 mutants compared

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**Figure 2.** Effect of low Ca on growth of Arabidopsis eca2 and eca3 mutants compared to wild-type plants. Plants were grown for 17 d on plates in 0.5× Murashige and Skoog medium containing varying levels of Ca: 0.1 mM and 1.5 mM (A); 0 mM (B). White line = 1 cm. Fresh-weight values are shown for eca2-1 (top) and eca3-1 (bottom) compared to wild-type (wt) in A and for wild type and all mutants in B; data represent the means ± se from five separate plates, each having six seedlings per genotype per plate (wild type, black bar; mutant, gray bar). * Significant difference between mutant and wild-type plant (P < 0.05); Student’s t test (paired). Results are from representative experiments repeated at least twice for each mutant. [See online article for color version of this figure.]
to wild-type plants when grown under Mn-deficient conditions, there were no major differences in metal content changes when *eca3* mutants and wild-type plants were compared. Generally, Mn content of *eca3* mutants was slightly lower than wild-type plants under both conditions (i.e. basal Mn supplied and Mn omitted). Ca content was slightly higher under both conditions in the *eca3* mutants compared to wild-type plants and

![Figure 3](image_url)

*eca3* mutants show reduced growth and chlorosis on Mn-deficient medium. Plants were grown on plates in 0.5× Murashige and Skoog* medium containing normal levels of Mn (50 μM) or without Mn present (0 μM) for 17 d. A, Seedling phenotype of *eca2-1*, *eca3-1*, and *eca3-2* mutants compared to wild type (wt). Black line = 1 cm. B, Fresh-weight and chlorophyll content for *eca3-1* and *eca3-2* mutants (gray bars) compared to wild type (black bars). C, Fresh-weight and chlorophyll content for *eca2-1* mutant (gray bars) compared to wild-type (black bars). Results in B and C represent the means ± se from at least five separate plates, each having six seedlings per genotype per plate. *, Significant difference between mutant and wild-type plants (*P* < 0.05); Student’s *t* test (paired). Results are from a representative experiment repeated at least three times. [See online article for color version of this figure.]
Zn was similar or slightly lower (Fig. 6). Again, for both Ca and Zn, there was little difference in the response to reducing the Mn concentration in the medium of eca3 mutants compared to wild-type plants. We cannot rule out that extended growth under Mn-deficient conditions could lead to metal content changes, but we chose a point at which the symptoms, although apparent, were not too severe.

Expression Analysis of Type 2A Ca-ATPases

Semiquantitative reverse transcription (RT)-PCR was used to determine whether ECAs were present in various organs of hydroponically grown Arabidopsis. Primers designed to regions of AtECA1 to AtECA4 amplified products of 127, 325, 279, and 127 bp, respectively (Fig. 7A). Amplification of a 122-bp product with primers to the constitutively expressed ribosomal protein S16, a component of the 40S subunit (Kim et al., 2006), was used as a control. Transcripts for all ECAs are present in all major organs (Fig. 7A). Results for ECA2 and ECA3 are consistent with data from publicly available microarray studies (https://www.genevestigator.ethz.ch; Zimmermann et al., 2004), which also show that these genes are expressed in all major organs (Fig. 7B). Expression of ECA1 and ECA4 cannot be determined from microarray analysis because the probe sets are not distinct.

To confirm these results and obtain further information about the tissue-specific expression of ECA3 within the different plant organs, a GUS reporter gene was cloned under the control of the promoter region for ECA3. GUS staining of seedlings showed high expression levels in guard cells, hydathodes, and the vascular tissue in leaves. Transverse sections of roots showed high GUS activity in the stele, particularly in pericycle and xylem parenchyma cells. Staining was also observed in developing lateral roots and tip and vascular staining was seen in more mature lateral roots. Strong staining was also found in different flower tissues, including stamens, petals, and sepals, as well as in siliques (Fig. 8).

Some metal transporters are regulated at the transcriptional level by the metals they transport. For example, AtHMA4, a Zn-transporting ATPase, is up-regulated by elevated levels of Zn (Mills et al., 2003; Williams and Mills, 2005), and the Fe transporter IRT1 is up-regulated under Fe-deficient conditions (Vert et al., 2001). Others, such as MTP1/ZAT, show no high GUS activity in the stele, particularly in pericycle and xylem parenchyma cells. Staining was also observed in developing lateral roots and tip and vascular staining was seen in more mature lateral roots. Strong staining was also found in different flower tissues, including stamens, petals, and sepals, as well as in siliques (Fig. 8).
obvious regulation by metals at the level of transcription (van der Zaal et al., 1999). Real-time PCR was performed to determine whether expression of ECA2 and ECA3 changed under conditions of metal deficiency. Root and shoot expression levels of ECA2 and ECA3 relative to the S16 component of ribosomal 40S are shown for seedlings grown on basal medium (control) or in the absence of either Mn or Zn (Fig. 9). There were no significant differences in the expression of either gene when grown under these conditions. For a comparison, we measured expression levels of the transporter ZIP4 and in this case there was marked up-regulation under Zn-deficient conditions as reported previously (Wintz et al., 2003; van de Mortel et al., 2006).

ECA3 Is Localized to the Golgi

To determine the subcellular localization of ECA3, fusions with yellow fluorescent protein (YFP) were generated. Transient expression in tobacco (*Nicotiana tabacum*) epidermal cells followed by confocal laser-scanning microscopy revealed small motile organelles characteristic of Golgi (Fig. 10). There was a high level of colocalization with the Golgi protein GONST1 (Handford et al., 2004) visualized using GONST1-GFP fusions.

ECA3 Restores Growth Defects of the Yeast K616 Mutant on Ca-Depleted Medium and of K616 and pmr1 Mutants on High-Mn Medium

Heterologous expression in yeast has been used in a number of studies to functionally characterize animal and plant transporters. The K616 (Δpmr1, Δpmc1, and Δcnb1) yeast mutant is defective in endogenous Ca/Mn pumps and has been very useful in functional

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**Figure 6.** Elemental analysis of eca3 mutant Mn, Ca, and Zn concentration in root and shoot tissue of wild type (black bars), eca3-1 (gray bars), and eca3-2 mutants (white bars) grown on plates on basal (0.5× Murashige and Skoog*) or 0 Mn (0.5× Murashige and Skoog* with Mn omitted) for 17 d. Results are means ± se from four independent pools of plants from different experiments and each pool is from at least five replicate agarose plates. *, Significant difference between mutant and wild-type plant (P < 0.05); Student’s t test (paired).

**Figure 7.** *ECA* expression analysis. A, Organ distribution of *ECA*s in hydroponically grown plants determined using RT-PCR. B, *ECA2* and *ECA3* expression in different organs determined from microarray analysis. Graphs show data (mean and se) extracted from Genevestigator (ATH1: 22k array) for the same organs (https://www.genevestigator.ethz.ch).
analysis of plant Ca pumps (Harper et al., 1998; Liang and Sze, 1998). Growth of K616 yeast is inhibited in the presence of elevated Mn or Zn in the medium (Wu et al., 2002). ECA3 rescues K616 on high Zn, restoring growth almost to the level of control K601 cells (Fig. 11A). ECA3 also partially restored growth of K616 on elevated Mn, but not to the extent of the K601 cells. Increased sensitivity to elevated Mn concentrations is also observed in the pmr1 mutant (Lapinskas et al., 1995) and ECA3 reduced this growth defect compared to vector controls (Fig. 11C). K616 vector transformants are also unable to grow on medium containing reduced levels of Ca (i.e. in the presence of EGTA). ECA3 suppresses the EGTA-sensitive phenotype, allowing growth in the presence of EGTA to levels similar to those seen in the control K601 strain (Fig. 11B).

DISCUSSION

Arabidopsis contains four P2A-type ATPases (ECA1–ECA4), which are related to the well-characterized SERCA Ca-ATPases in mammals, and 10 P2B-type ATPases related to the mammalian plasma membrane calmodulin-stimulated Ca$^{2+}$-ATPases. These are all members of the P-type superfamily of ATPases. Understanding the functional properties, expression patterns, and localization of each of these pumps is important if we are to understand how they contribute to ion homeostasis and signaling in plants. The aim of this study was to gain insight into the physiological roles of ECA2 and ECA3 by studying Arabidopsis T-DNA insertion mutants in which the genes encoding these ATPases have been disrupted. In addition, we have used heterologous expression in yeast to study functional properties of ECA3.

The studies conducted here with eca3 mutants have revealed a crucial role for ECA3 in Mn nutrition. eca3 mutants showed poor growth on Mn-deficient medium compared to wild-type plants (Fig. 3; Supplementary Fig. S2). The response to Mn deficiency is first seen after about 8 to 9 d on this medium; prior to this, growth is similar to wild type. Omitting Zn, Fe, or Cu from the medium had little specific effect on the mutant compared to wild type (Fig. 5). In the absence of Mn, eca3 mutants showed a reduction in both root and shoot fresh weight and in chlorophyll content; this phenotype could be rescued by adding 1 $\mu$M Mn to the medium (Fig. 4). The fact that eca3 mutants show little difference from wild-type plants when Mn was present in the medium at normal levels indicates that other genes can compensate for the loss of ECA3 under these conditions. Notably, on soil there were no marked differences in the development of eca3 mutants, which grew similarly to wild type; in this case, there was probably sufficient Mn for normal growth and development.
analysis showed that ECA3 confirmed this and showed that ECA3 organs of Arabidopsis (Fig. 7). Promoter:GUS studies observed in The markedly different metal-dependent phenotypes tips, hydathodes, guard cells, and vascular tissue (Fig. 8). pressed in a range of tissues and cells, including root from the medium. differences in the mutants and wild type were not as conditions, all plants were severely affected and the suggesting a role for ECA3 in Ca homeostasis (Fig. 2). be related to differences in their membrane location.

There appear to be distinct differences in the roles ECAs play in Mn and Ca tolerance. Wu et al. (2002) found that eca1 mutants were sensitive to high Mn and suggested that ECA1 may confer tolerance to this metal by removing it from the cytosol. Physical symptoms of Ca deficiency have also been reported in eca1-1 mutants when external Ca was reduced to 0.2 to 0.4 mM, suggesting that ECA1 may be the main pump capable of loading Ca into the ER lumen under these conditions (Wu et al., 2002). Both eca2 and eca3 mutants grew normally in this range, but, when Ca was omitted from the medium altogether, eca3 mutants seemed slightly more affected than eca2 mutants or wild type, suggesting a role for ECA3 in Ca homeostasis (Fig. 2). However, it should be stressed that, under these conditions, all plants were severely affected and the differences in the mutants and wild type were not as marked as seen for the situation when Mn was omitted from the medium.

Results from semiquantitative RT-PCR and microarray analysis showed that ECA3 is expressed in all major organs of Arabidopsis (Fig. 7). Promoter:GUS studies confirmed this and showed that ECA3 is highly expressed in a range of tissues and cells, including root tips, hydathodes, guard cells, and vascular tissue (Fig. 8). The markedly different metal-dependent phenotypes observed in eca1 mutants (Wu et al., 2002) and eca3 mutants (this study) indicate that these ATPases have different physiological roles in Arabidopsis. This could be related to differences in their membrane location.

ECA1 is associated primarily with the ER functioning in the transport of Ca and Mn into this compartment (Liang et al., 1997). Recent proteomic analysis (Dunkley et al., 2006) confirms ER localization for ECA1 and Golgi localization for ECA3 (P. Dupree, unpublished data). Using transient expression of YFP-ECA3 fusion proteins in tobacco, we show here that ECA3 colocalizes with GONST1 (Fig. 10), a protein previously shown to be located in the Golgi (Handford et al., 2004).

From the data we have presented in this article, we propose that ECA3 could serve a similar function to Golgi-associated SPCAs found in other organisms. In mammalian cells, there are three distinct classes of P_{1}-type Ca-ATPases, SERCA type, PMCA type, and SPCAs (also referred to as PMR1 type), and these three groups can be clearly distinguished in phylogenetic analysis (Pittman et al., 1999; Schmidt et al., 2002; Supplemental Fig. S4). SPCAs transport Ca, but also Mn, with high affinity, and they function both in Mn detoxification via exocytosis and also in providing Mn for Golgi-localized enzymes (Yadav et al., 2007). In yeast, the SPCA, PMR1, functions as a Ca and Mn transporter at the Golgi, transporting these ions into the secretory pathway. Both ions can exert distinct functions: Ca supplied by PMR1 is required to sustain vacuolar protein sorting, whereas Mn is required to activate various Mn-requiring enzymes involved in the addition of complex carbohydrates onto N- and O-linked glycosylated proteins (Lapinskas et al., 1995; Durr et al., 1998). The fact that, on low Ca medium, ECA3 complements the yeast triple null mutant strain K616 (Δpmr1 Δpmc1 Δcnb1), lacking its endogenous Golgi (PMR1) and vacuolar (PMC1) Ca pumps, is consistent with ECA3 functioning as a Ca pump in yeast (Fig. 11). K616 also exhibits growth sensitivity to high levels of extracellular Mn, which results from toxicity of excessive cellular levels due to the lack of functional endogenous pumps (Lapinskas et al., 1995, Culotta et al., 2005). Expression of ECA3 reduces the Mn-sensitive phenotype of the triple yeast mutant K616 and also the single pmr1 mutant, indicating that ECA3 could serve a similar function to PMR1 (Fig. 11). ECA3 is particularly effective in reducing the Zn-sensitive phenotype in yeast, suggesting that it may...
have the capability of transporting Zn. However, ECA3 does not seem to play a major role in Arabidopsis under Zn deficiency because wild-type plants and eca3 mutants were similarly affected under such conditions.

There are SPCAs in fungi, animal, and bacteria, but they have not been identified in higher plants. Phylogenetically, AtECA3 clusters with animal-type SERCA pumps, whereas AtECA1 and AtECA2 cluster more closely with plant-type SERCA pumps (Pittman et al., 1999; Supplemental Fig. S4). Our data indicate that, in plants, ECA3-type pumps could have a comparable physiological role to SPCA types and function in transporting Ca and Mn into the Golgi-associated secretory pathway. The partial rescue of metal-dependent growth phenotypes displayed in yeast mutants would support a transport function for ECA3, but more direct transport assays would be required to show that ECA3 functions as a Ca and Mn transporter. Future experiments will be aimed at testing the hypothesis that ECA3 transports Mn into the Golgi to supply various Mn-requiring enzymes for biochemical processes required for normal growth of Arabidopsis.

Mn deficiency is a serious nutritional disorder for plants in many areas of the world, and in arable crops this can lead to reduced yield. Not only is there variability between plant species in their ability to grow on low Mn, but there are also considerable differences among genotypes of the same species (Pedas et al., 2005). Varieties displaying tolerance to Mn deficiency have been termed Mn efficient (Pedas et al., 2005; Jiang, 2006), but the mechanisms underlying this have not been clearly defined. In barley, differential capacity for high-affinity Mn influx is thought to contribute to Mn efficiency; in wheat, it may be due to improved internal utilization rather than higher Mn accumulation (Jiang, 2006). ECA3 homologs could play a role in the Mn efficiency of crop species and there is the potential in the future to test whether expression of AtECA3 could improve Mn efficiency.

In summary, this study has provided evidence that AtECA3 plays a crucial role in Mn nutrition in Arabidopsis. Golgi localization of ECA3 could explain why disruption of ECA3 does not lead to a major effect on the overall Mn content of the plant. The severe phenotype observed under Mn-deficient conditions may be due to a reduction in the Mn content of the Golgi. Our observations indicate that other secretory pathway/endomembrane-localized Mn transporters, such as AtMTP11, cannot compensate for the lack of ECA3 under Mn deficiency. AtECA1, AtCAX2, and AtMTP11 have previously been reported to confer tolerance to high-Mn concentrations, but AtECA3 is the first to be identified that is required for growth under low-Mn conditions.

**Figure 11.** AtECA3 expression alleviates Mn-, Zn-, and Ca-dependent growth defects in yeast K616 (∆pmr1, ∆pmcl, ∆cnb1; A and B) and pmr1 (∆pmr1) mutants (C) transformed with p426-ECA3 (K616 + ECA3; pmr1 + ECA3) or p426 vector (K616 + vector; pmr1 + vector) were grown overnight in SC – uracil with 2% (w/v) Glc. K601 cells are shown as a positive control (wt). A and B, Cultures were adjusted to OD₆₀₀ = 0.25 and used undiluted at this concentration (1) or at a 100-fold dilution (1/100). Five-microliter aliquots were spotted onto SC – uracil plates containing 2% (w/v) Gal as the carbon source (p426 uses a Gal-inducible promoter), with the following additions: A, either MnCl₂ (0.3 mM) or ZnSO₄ (3 mM); and B, Ca (20 mM) and EGTA (10 mM) or EGTA (10 mM). C, Cultures were adjusted to OD₆₀₀ = 0.4 and used at this concentration (1) or 10-fold dilution (1/10). Seven-microliter aliquots were spotted onto SC – uracil plates containing 2% (w/v) Gal with 0, 0.3, or 1 MnCl₂. Plates were incubated for 3 to 4 d at 30°C.

**MATERIALS AND METHODS**

**Plant Material and Growth Conditions**

Seeds of wild-type and transgenic Arabidopsis (Arabidopsis thaliana ‘Columbia-0’) were sterilized in 10% (v/v) bleach for 20 min, rinsed five times with sterile water, and inoculated onto plates containing 0.8% (w/v) agarose
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(Melford), 1% (v/v) Suc, and either one-half-strength (0.5×) Murashige and Skoog medium (Murashige and Skoog, 1962) or the same medium but with Ca levels reduced from 1.5 mM to 0.1 mM (0.5× Murashige and Skoog). For experiments investigating the effect of Mn deficiency on phenotype, Mn was omitted from the 0.5× Murashige and Skoog medium. Seeds were stratified at 4°C for 48 h prior to transfer to a controlled-environment cabinet (23°C, 16 h light; 18°C, 8 h dark) and plates were incubated vertically or horizontally as indicated. To examine the development of the plants to maturity, seeds were sown in soil (one part Levingtons F5 no. 2; one part John Innes no. 2; one part Vermicrop-graded horticultural vermiculite, medium grade) sterilized by autoclaving at 121°C for 15 min at 1 bar pressure, and plants were grown in a controlled-environment cabinet (22°C, 16 h light; 20°C, 8 h dark cycle) or under glasshouse conditions. To obtain organ material from mature plants for gene expression analysis, plants were grown hydroponically as described previously (Mills et al., 2003).

Chlorophyll Determination and Fresh-Weight Measurements of Seedlings

Fresh-weight and chlorophyll measurements were determined using seedlings grown on five separate plates, each plate having six wild-type seedlings and six mutant seedlings. The data presented are generally the means from the five plates ±SE expressed on a per-seedling basis. Student’s t test (paired) was used to determine significant differences between mutant and wild-type plants and an asterisk (*) indicates a significant difference (P < 0.05). Chlorophyll was determined following extraction in N,N-dimethylformamide (Moran, 1982).

Elemental Analysis

Shoots and roots were harvested separately from plants grown on agarose plates (see above). Plant samples were dried for 2 d at 70°C and six mutant seedlings. The data presented are generally the means from the five plates ±SE expressed on a per-seedling basis. Student’s t test (paired) was used to determine significant differences between mutant and wild-type plants and an asterisk (*) indicates a significant difference (P < 0.05). Chlorophyll was determined following extraction in N,N-dimethylformamide (Moran, 1982).

RNA Isolation, RT-PCR, and Real-Time PCR

Total RNA was extracted from different plant organs (roots, leaves, stems, flowers, and silicules) of hydroponically grown plants and from root and shoot tissue from plants grown on agarose plates (see above) using a phenol:SDS extraction and LiCl precipitation method based on Verwoerd et al. (1989). RNA was checked for purity, integrity, and quantity using RNA gel electrophoresis and spectrophotometry (Sambrook et al., 1989). First-strand cDNA synthesis using 2 μg of total RNA was carried out using SuperScript RNase H− reverse transcriptase (Invitrogen) according to the manufacturer’s instructions with an oligo(dT)15 primer. The expression pattern of AtECA1 to AtECA4 was determined by semiquantitative RT-PCR using primers designed to specifically amplify parts of each sequence: AtECA1 and AtECA4 forward primer, 5′-GCTACCGTGATCCATGTCCG-3′; AtECA1 reverse primer, 5′-CCACGCGGAGGTAAGTAC-3′; AtECA4 reverse primer, 5′-GCAAGGATGCTAAGACACA-3′; AtECA2 forward primer, 5′-TAGGGACGACCAAAATGG-3′, and reverse primer, 5′-GCTCTAAACAACTTTCCCTTC-3′; and AtECA3 forward primer, 5′-GCTATGAGCAGTCTGTTGTT-3′, and reverse primer, 5′-GCTCATTGATCTGAGAGGAGA-3′. Control PCR was performed using primers to S16 ribosomal gene (40S component): forward primer, 5′-GATGAGTAAAGCAACAA-3′ and reverse primer, 5′-GCCTGTATTTCTAGAGAGGA-3′ formed using primers to S16 ribosomal gene (40S component): forward primer, 5′-GATGAGTAAAGCAACAA-3′ and reverse primer, 5′-GCCTGTATTTCTAGAGAGGA-3′.

YFP-ECA3 Construct

Gateway technology was used to produce the YFP-ECA3 construct. ECA3 cDNA was PCR amplified from the p426-ECA3 construct and integrated into the pENTR-TOPO vector by TOPO cloning (Invitrogen). ECA3 was then subcloned into the pEarleyGate 104 destination vector (Earley et al., 2006), which attaches YFP in-frame to the N terminus of ECA3.

EC3 Promoter:GUS Construct

For tissue-specific expression studies, 1.5 kb of the 5′-untranslated region for ECA3 was amplified by high-fidelity PCR with primers 2548 (5′-CAC- CGTGTTACAGTTGTAAGTTACCAAC-3′) and 2549 (5′-TTTGAAAACGC- CAAGGGTTTCTG-3′). The PCR product was cloned into pENTR-TOPO (Invitrogen) using Gateway technology and transferred to plasmid pMD126 (Curts and Grossniklaus, 2003) to generate a construct containing the open reading frame for a GUS reporter gene under the control of the ECA3 promoter region.

Yeast Strains, Transformation, and Growth

The K616 yeast (Saccharomyces cerevisiae) strain (MATa pom1:1; HIS3 pmc1:1; TRPI cbl1); LEU2, ura3; Cunningham and Fink, 1994) was transformed with p426-ECA3 or p426 vector alone using a Li-acetate method based on Gietz et al. (1992). Yeast transformants were selected on medium lacking uracil. Yeast strain K601/W301D1 (MATa leu2, his3, ade2, and ura3; Cunningham and Fink, 1994) transformed with the empty p426 vector was used as a positive control.

To compare the growth of K616 cells containing p426-ECA3 or p426 alone, cells were first grown in liquid culture overnight at 30°C in synthetic complete (SC) without uracil (pH 5.0) and containing 20 g L−1 Glc as the carbon source. Cultures were adjusted to the same OD600 and aliquots were inoculated onto SC medium with uracil (pH 4.9) with Gal or Glc and various additions as specified in “Results.” Plates were incubated at 30°C for 3 d to 5 d.

Transient Expression of ECA3 in Tobacco

Four-week-old tobacco (Nicotiana tabacum ‘Petit Havana’) plants grown at 22°C on a 16-h light/8-h-dark cycle were used for Agrobacterium tumefaciens (strain GV3101)-mediated transient expression (Batoko et al., 2000). pVK1H18Em6-GONST1-GFP (Handford et al., 2004) or pEG104-ECA3-YFP-transformed A. tumefaciens was cultured at 28°C, until the stationary phase (approximately 24 h), washed, and resuspended in infiltration medium (MES 50 mM, pH 5.6, Glc 0.5% [w/v], NaPO4 2 mM, acetosyringone 300 μM [from 750 mM stock in dimethyl sulfoxide]). The bacterial suspension was inoculated using 1-mL syringe without a needle by gentle pressure through the stomata on the lower epidermal surface (Brandizzi et al., 2002). Transformed plants were then incubated under normal growth conditions for 3 d at 22°C.

Cloning of AtECA3 and Plasmid Constructs

p426-ECA3 Yeast Expression Construct

AtECA3 from Arabidopsis ‘Columbia-0’ was cloned in two overlapping parts as initial attempts to amplify full-length cDNA failed. The 5′ part was amplified from clone RZL01a05 (AV545725/AV545775) obtained from Kazusa DNA Research Institute (Asamizu et al., 2000) using the primers ECA3.F (5′-CGCGATGTTAAGCTTTAGTCCAC-3′) and ECA3-5′R (5′-CATCCAACCTGGTCAAGACGATTTTACC-3′). The ECA3.F primer introduced a BamHI site, allowing the product to be cut with BamHI, and at an EcoRI site within the ECA3 sequence for ligation into the same sites of p426 (Mumberg et al., 1994) giving p426-5′-ECA3. The 3′ part of ECA3 was amplified from reverse-transcribed RNA from leaf tissue using the primers ECA3-3′F (5′-TACATGCTGTTCAAGATATTGTGTT-3′) and ECA3-5′R (5′-AGGAATCCCTGAAAGGACTCTTCTG-3′) cloned into pGEMTe. The primer ECA3-3′F overlaps the end of the 5′ part and the ECA3HR primer introduced a HindIII site, allowing the ECA3 3′ fragment to be excised with EcoRI and HindIII and ligated into the same sites of p426-5′-ECA3 to produce the full-length ECA3 DNA in p426 (p426-ECA3). Sequencing of the final product confirmed that the full-length ECA3 was identical to the sequence predicted from genome sequencing. The GentiBank accession number for ECA3 cloned in this study is EU082212.
Confocal Microscopy

Transformed leaves were analyzed 72 h after infection of the lower epidermis. Confocal imaging was performed using an inverted Zeiss LSM510 META laser-scanning microscope with a 63× oil immersion objective. For imaging expression of GFP and YFP, excitation lines of an argon ion laser of 458 nm for GFP and 514 nm for YFP were used. For YFP, a 475/525-nm band-pass filter for GFP and a 530/600-nm band-pass filter for YFP were used alternately with line switching using the multitrack facilities of the microscope. Appropriate controls were performed to exclude any cross talk and bleed through of fluorescence.

**ECA3 Promoter:GUS Expression Analysis**

*A. tumefaciens* strain C5851 (Koncz and Schell, 1986) was transformed by electroporation and transformants were selected on YEP plates (1% yeast extract, 2% peptone, 1.5% agar) containing 25 µg mL⁻¹ gentamycin and 50 µg mL⁻¹ kanamycin. Wild-type Arabidopsis ecotype 'Colombia-0' plants were transformed by floral dipping (Clough and Bent, 1998). Seeds were selected on 0.5× Murashige and Skoog plates without Suc and supplemented with 25 µg mL⁻¹ hygromycin B under short-day (8-h-light) conditions. After 2 weeks, transformants were tested for GUS activity as described by Haritatos et al. (2000). For flower and silique staining, plants were selected on plates as before and then transferred to soil under long-day (16-h-light) conditions for 2 weeks. Transverse sections of GUS-stained roots were obtained as described by Haritatos et al. (2000) and visualized in a Nikon Eclipse 80i light microscope. At least four independent transformant lines were tested in each case.

**Isolation of Insertional Mutants for AtECA2 and AtECA3**

Seed for putative T-DNA insertion lines for AtECA2 and AtECA3 from the Salk collection (http://signal.salk.edu/) were obtained from the Nottingham Arabidopsis Stock Centre. These were seed lines N579691 (eca2-1), N593146 (eca2-2), N545567 (eca1-1), and N570619 (eca1-2). To isolate eca mutants, homozygous for the insert, a similar procedure to that previously described for hom3 mutants was followed (Mills et al., 2005). Briefly, genomic DNA was isolated from soil-grown plants using the DNAMITE plant kit (Microzone). To genotype the plants with respect to the T-DNA insert, PCR was carried out on genomic DNA using the primer for the T-DNA left border (Salk LB1, 5'-TGTTATCCAGTATGGCCCATGC-3') and gene-specific primers ECA2.F (5'-TACGGACAGGAAAAATGG-3') for eca2-1 and eca2-2, ECA3.R (5'-CATCCATTTGGTCTCAAAAGCAGTATTCT-3') for eca1-1, and ECA3.F (5'-TCC-AATCCTGACAGATGGCTTACTGTA-3') for eca1-2. PCR conditions were 94°C for 2 min followed by 40 cycles of 94°C for 30 s, then 54°C (ECA2.F), 56°C (ECA3.F), or 58°C (ECA3.R) for 1 min, then 72°C for 1 min (except 2 min for ECA3.R), and then a final elongation step of 72°C for 5 min. For N579691 (eca2-1), N545567 (eca1-1), and N570619 (eca1-2), sequencing confirmed the original Salk-predicted T-DNA locations, whereas for N593146 (eca2-2) the insert location was confirmed at 139 bp 3' of the predicted location. Mutant alleles for ECA2 (eca2-1 and eca2-2) and ECA3 (eca1-1 and eca1-2) were obtained and lines homozygous for the insert were selected for phenotypic analysis. The lack of a transcript was confirmed at the RNA level by RT-PCR using primers that span the insertion site: for ECA2, ECA2.F (see above) and ECA2.R (5'-CTTAAGGATCTTGTGCT-3') and ECA3, ECA3.ex10F (5'-GCATACACGATTCTATGTTGCAGACAGATGAT-3') and ECA3.SGR (5'-TACGGACAGGAAAAATGG-3'). Amplification of Actin2 was used as a positive control using primers Actin2.F, 5'-GGTAACTTGTTCTCAGTATG-3' and Actin2.R, 5'-TCTGATCCGATGACAGTACTC-3'. After denaturation at 94°C for 2 min, PCR was cycled as follows: 24 cycles of 94°C, 30 s; 50°C, 1 min; 72°C, 1 min for ECA2; and 40 cycles of 94°C, 30 s; 55°C, 1 min; 72°C, 2 min for ECA3. A final elongation step of 72°C for 5 min was used in all. Sequence data from this article can be found in the GenBank/EMBL data libraries under accession number EU082212.

**Supplemental Data**

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

**Supplemental Figure S1.** Growth of eca mutants is similar to wild-type plants on soil.

**Supplemental Figure S2.** Phenotype of eca3 mutants grown under Mn-deficient conditions.

**Supplemental Figure S3.** Elemental analysis of eca3 mutant.

**Supplemental Figure S4.** Maximum-likelihood phylogenetic tree for P-type ATPases created using Tree-puzzle 5.2.

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**LITERATURE CITED**

Asamizu E, Nakamura Y, Sato S, Tabata S (2000) A large scale analysis of cDNA in *Arabidopsis thaliana*: generation of 12,028 non-redundant expressed sequence tags from normalized and size-selected cDNA libraries. DNA Res 7: 175–180

Axelsen KB, Palmgren MG (1998) Evolution of substrate specificities in the P-type ATPase superfamily. J Mol Evol 46: 84–101

Batoko H, Zheng HQ, Hawes C, Moore J (2000) A Rab1 GTPase is required for transport between the endoplasmic reticulum and Golgi apparatus and for normal Golgi movement in plants. Plant Cell 12: 2201–2217

Bowler C, Slooten L, Vandenbransen S, De Rycke R, Bottermann J, Sybesma C, Van Montagu M, Inzé D (1991) Manganese superoxide dismutase can reduce cellular damage mediated by oxygen radicals in transgenic plants. EMBO J 10: 1723–1732

Brandizzi F, Snapp EL, Roberts AG, Lippincott-Schwartz J, Hawes C (2002) Membrane protein transport between the endoplasmic reticulum and the Golgi in tobacco leaves is energy dependent but cytoskeleton independent: evidence from selective photobleaching. Plant Cell 14: 1293–1309

Cheng NH, Pittman JK, Shigaki T, Lachmansingh J, LeClerc S, Lahner B, Salt DE, Hirschi KD (2005) Functional association of Arabidopsis CAX1 and CAX3 is required for normal growth and ion homeostasis. Plant Physiol 138: 2048–2060

Clough SJ, Bent AF (1998) Floral dip: a simplified method for Agrobacterium-mediated transformation of *Arabidopsis thaliana*. Plant J 16: 735–743

Culotta VC, Yang M, Hall MD (2005) Manganese transport and trafficking: lessons learned from *Saccharomyces cerevisiae*. Eukaryot Cell 4: 1159–1165

Cunningham WK, Fink G (1994) Calnexin-dependent growth-control in *Saccharomyces cerevisiae* mutants lacking PMC1, a homolog of plasma membrane Ca²⁺ ATPases. J Cell Biol 124: 351–363

Curts MD, Grossniklaus U (2003) A gateway cloning vector set for high-throughput functional analysis of genes in plants. Plant Physiol 133: 462–469

Delhaize E, Gruber BD, Pittman JK, White RG, Leung H, Miao Y, Jiang L, Ryan PR, Richardson AE (2007) A role for the AtMTP11 gene of *Arabidopsis* in manganese transport and tolerance. Plant J 51: 198–210

Dunkley TP, Hester S, Shadforth IP, Runions J, Weimar T, Hanton SL, Earley KW, Haag JR, Pontes O, Opper K, Juehne T, Song KM, Pikaard CS (2006) Gateway-compatible vectors for plant functional genomics and proteomics. Plant J 45: 616–629

Evans DE, Williams LE (1998) P-type calcium ATPases in higher plants—biochemical, molecular and functional properties. Biochim Biophys Acta 1376: 1–25

Geisler M, Axelsen KB, Harper JF, Palmgren MG (2000) Molecular aspects of higher plant P-type Ca²⁺ ATPases. Biochim Biophys Acta 1465: 52–78

Gietz D, Ste Jea A, Woods RA, Schiesl RH (1992) Improved method for...
high-efficiency transformation of intact yeast cells. Nucleic Acids Res 20: 1425
Hall JL, Williams LE (2003) Transition metal transporters in plants. J Exp Bot 54: 2601–2613
Handford MG, Sicilia F, Brandizzi F, Chung JH, Dupree P (2004) Arabidopsis thaliana expresses multiple Golgi-localised nucleotide-sugar transporters related to GONST1. Mol Genet Genomics 272: 397–410
Haritatos E, Ayre BG, Turgeon R (2000) Identification of phloem involved in assimilate loading in leaves by the activity of the galactosin synthase promoter. Plant Physiol 123: 929–937
Harper JE, Hong B, Hwang I, Guo HQ, Toddard R, Juang JF, Palmgren MG, Sze H (1998) A novel calmodulin-regulated Ca2+–ATPase (ACA2) from Arabidopsis with an N-terminal autoinhibitory domain. J Biol Chem 273: 1099–1106
Jiang WZ (1995) Mineral Nutrition of Higher Plants, Ed 2. Academic Press, London
Kang CY, Hong BY, Ji DH, Lee Y (2003) Expression profiles of Arabidopsis thaliana in mineral deficiency and parallel computing. Bioinformatics 19: 502–504
Shenker M, Plessner OE, Tel-Or E (2004) Manganese nutrition effects on tomato growth, chlorophyll concentration and superoxide dismutase activity. J Plant Physiol 161: 197–202
Sze H, Liang F, Hwang I, Curran AC, Harper JF (2000) Diversity and regulation of plant Ca2+ pumps: insights from expression in yeast. Annu Rev Plant Physiol Plant Mol Biol 51: 433–462
Toyoshima C, Nakasako M, Nomura H, Ogawa H (2000) Crystal structure of the calcium pump of sarcoplasmic reticulum at 2.6A resolution. Nature 405: 647–655
van de Mortel JE, Villanueva LA, Schat H, Kewkeboom J, Coughlan S, Moerland PD, van Themaat EVL, Koornneef M, Aarts MGM (2006) Large expression differences in genes for iron and zinc homeostasis, stress response, and lignin biosynthesis distinguish roots of Arabidopsis thaliana and the related metal hyperaccumulator Thlaspi caerulescens. Plant Physiol 119: 1127–1147
van der Zaal BJ, Neuteboom LW, Pinas JE, Chardonnens AN, Schat H, Verkleij JAC, Hooykaas PJ (1999) Overexpression of a novel Arabidopsis gene related to putative zinc-transporter genes from animals can lead to enhanced zinc resistance and accumulation. Plant Physiol 119: 1047–1055
Vert G, Briat JF, Curie C (2001) Arabidopsis IRT2 gene encodes a root-periphery iron transporter. Plant J 26: 181–189
Verwoerd TC, Dekker BM, Hoekema A (1989) A small scale procedure for the rapid isolation of plant RNAs. Nucleic Acids Res 17: 783–791
White AR, Xin Y, Pezeshk V (1993) Xyloglucan glucosyltransferase in Pisum sativum Golgi membranes from Pisum sativum (pea). Biochem J 294: 231–238
White PJ (2002) Calcium channels in higher plants. Biochim Biophys Acta 1465: 171–189
Williams LE, Mills RF (2005) P1p-ATPases—an ancient family of transition metal pumps with diverse functions in plants. Trends Plant Sci 10: 491–502
Williams LE, Pittman J, Hall JL (2000) Emerging mechanisms for heavy metal transport in higher plants. Biochim Biophys Acta 1465: 104–126
Wintz H, Fox T, Wu YY, Feng V, Chen WQ, Chang HS, Zhu T, Vulpe C (2003) Expression profiles of Arabidopsis thaliana in mineral deficiencies reveal novel transporters involved in metal homeostasis. J Biol Chem 278: 47644–47653
Wu ZY, Liang F, Hong BM, Young JC, Sussman MR, Harper JF, Sze H (2002) An endoplasmic reticulum-bound Ca2+/Mn2+ pump, ECA1, supports plant growth and confers tolerance to Mn2+ stress. Plant Physiol 130: 128–137
Yadav J, Muen S, Zhang Y, Rao R (2007) A phenoics approach tin yeast links proton and calcium pump function in the Golgi. Mol Biol Cell 18: 1480–1489
Zimmermann P, Hirsch-Hoffmann M, Hennig L, Grussow W (2004) GENEVESTIGATOR: Arabidopsis Microarray Database and Analysis Toolbox. Plant Physiol 136: 2621–2632