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ECA1 complements yeast mutants defective in Ca^{2+} pumps and encodes an endoplasmic reticulum-type Ca^{2+}-ATPase in *Arabidopsis thaliana*

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ABSTRACT **To understand the structure, role, and regulation of individual Ca^{2+} pumps in plants, we have used yeast as a heterologous expression system to test the function of a gene from *Arabidopsis thaliana* (ECA1). ECA1 encoded a 116-kDa polypeptide that has all the conserved domains common to P-type Ca^{2+} pumps (EC 3.6.1.38). The amino acid sequence shared more identity with sarcoplasmic/endoplasmic reticulum (53%) than with plasma membrane (32%) Ca^{2+} pumps. Yeast mutants defective in a Golgi Ca^{2+} pump (*pmr1*) or both Golgi and vacuolar Ca^{2+} pumps (*pmr1 pmc1 cnb1*) were sensitive to growth on medium containing 10 mM EGTA or 3 mM Mn^{2+}. Expression of ECA1 restored growth of either mutant on EGTA. Membranes were isolated from the *pmr1 pmc1 cnb1* mutant transformed with ECA1 to determine if the ECA1 polypeptide (ECA1p) could be phosphorylated as intermediates of the reaction cycle of Ca^{2+}-pumping ATPases. In the presence of [γ-^{32}P]ATP, ECA1p formed a Ca^{2+}-dependent [^{32}P]phosphoprotein of 106 kDa that was sensitive to hydroxylamine. Cyclopiazonic acid, a blocker of animal sarcoplasmic/endoplasmic reticulum Ca^{2+} pumps, inhibited the formation of the phosphoprotein, whereas thapsigargin did not. Immunoblotting with an antibody against the carboxyl tail showed that ECA1p was associated mainly with the endoplasmic reticulum membranes isolated from *Arabidopsis* plants. The results support the model that ECA1 encodes an endoplasmic reticulum-type Ca^{2+} pump in *Arabidopsis*. The ability of ECA1p to restore growth of mutant *pmr1* on medium containing Mn^{2+}, and the formation of a Mn^{2+}-dependent phosphoprotein suggested that ECA1p may also regulate Mn^{2+} homeostasis by pumping Mn^{2+} into endomembrane compartments of plants.**

Calcium is not only an important intracellular signal for many stimuli-induced responses in eukaryotes (1), it is also essential for the functioning of the secretory system. A variety of signals can trigger the opening of Ca^{2+}-specific channels on the plasma membrane and endomembranes, causing massive Ca^{2+} influx and accumulation in the cytoplasm. The fluctuation in cytosolic Ca^{2+} directly elicits responses by altering the function of Ca^{2+}-binding proteins and their targets. The increase in cytosolic Ca^{2+} is transient as Ca^{2+} pumps and antiporters at the plasma membrane (PM) or internal membranes become activated and restore cytosolic Ca^{2+} to basal levels. In addition to a role of Ca^{2+} in signaling, lumenal Ca^{2+} concentration ($[Ca^{2+}]$) is emerging as an important player in the secretory system. For example, *pmr1* mutant defective in a Golgi Ca^{2+} pump secretes proteins that in wild-type cells are retained in the endoplasmic reticulum (ER) (2, 3). In mammalian cells, the correct folding and assembly of proteins depend on chaperones, like a Ca^{2+}-binding protein, calnexin (4). Thus intralumenal Ca^{2+} supplied by pumps would be required for the normal operation of the secretory system in plants (5). Although Ca^{2+} pumps play an important role in both signaling and secretion, the properties and regulation of individual Ca^{2+} pumps that fine tune cytosolic $[Ca^{2+}]$ and supply lumenal Ca^{2+} are not well understood in plants (1).

The literature illustrates the diversity of Ca^{2+} pumps on the plasma membrane and endomembranes from a variety of plants; however, biochemical distinction among the pumps has been difficult for several reasons. The pumps share many similarities as P-type ATPases, and there is a lack of distinguishing features such as specific inhibitor sensitivity. Furthermore, each pump type is not necessarily restricted to one particular organelle or membrane. A few biochemical traits are useful in discriminating between two major types of Ca^{2+} pumps in plants. The PM-type pump is energized by GTP or ATP and is stimulated by calmodulin. This type of pump can be located on endomembranes, like the vacuole (6, 7), as well as the plasma membrane (7, 8). Another pump is associated mainly with the ER. This ER-type pump hydrolyzed ATP preferentially and was inhibited by cyclopiazonic acid (CPA), but not stimulated by calmodulin (7). Although PM-type Ca^{2+}-ATPases have been partially purified by calmodulin-affinity chromatography, several related calmodulin-stimulated Ca^{2+} pumps could be copurified simultaneously. Thus, a molecular approach to study individual pumps is necessary.

In spite of the multiplicity of Ca^{2+} pumps, none of the genes encoding Ca^{2+} pump homologs isolated so far have been functionally characterized. For example, Wimmers *et al.* (9) isolated a gene (LCA) encoding a protein that shares ≈50% identity with animal sarcoplasmic/endoplasmic reticulum Ca^{2+} ATPase (SERCA). The deduced polypeptide of LCA has 1048 amino acids (116 kDa), 8 transmembrane (TM) domains, and all of the highly conserved domains of P-type translocating ATPases. Another gene (PEA1) isolated from *Arabidopsis thaliana* encoded a polypeptide that is 40–44% identical to various mammalian PM-type Ca^{2+}-ATPase (10). PEA1p (plastid envelope ATPase) was located to the chloroplast inner envelope, although its function is unclear. Recently, a related gene was identified in cauliflower. Based on sequence identity with tryptic peptides, BCA1 appeared to encode a calmodu-

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Abbreviations: ECA1, an ER-type Ca^{2+}-ATPase gene; ECA1p, ECA1 polypeptide; TM, transmembrane; PMSF, phenylmethylsulfonyl fluoride; BTP, 1,3-bis[Tris (hydroxymethyl)methylamino]propane; CPA, cyclopiazonic acid; SERCA, sarcoplasmic/endoplasmic reticulum Ca^{2+} ATPase; ER, endoplasmic reticulum; PE, phosphoenzyme; SC-URA, synthetic complete medium minus uracil; TCA, trichloroacetic acid; PM, plasma membrane.

Data deposition: The sequence reported in this paper has been deposited in the GenBank database (accession no. U96455).

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lin-stimulated Ca^{2+} pump previously purified from endomembranes (11).

Here we demonstrate that a gene from *A. thaliana* (ECA1) complemented yeast mutants defective in Ca^{2+} pumps by restoring their growth on EGTA. The protein encoded by the ECA1 gene formed a phosphoprotein that has characteristics of a phosphorylated intermediate of Ca^{2+}-pumping ATPases. Thus we demonstrate a plant gene encoding a functional ER-type Ca^{2+}-ATPase.

MATERIALS AND METHODS

Yeast Strains and Their Growth Media. *Saccharomyces cereviciae* strains W303–1A (*MAT***a**, *leu2, his3, ade2, trp1, ura3*), *pmr1* AA542 (*MAT***a**, *pmr1*::*HIS3, leu2, ade2, trp1, ura3*), *pmr2* K633 (*MAT***a** *pmr2*::*HIS3, leu2, ade2, trp1,ura3*), triple mutant K616 (*MAT***a** *pmr1*::*HIS3 pmc1*::*TRP1 cnb1*::*LEU2, ura3*) were used (12). Wild-type and mutant strains were grown for 24 h in standard YPD medium (yeast extract/peptone/dextrose) for transformation except for strains AA542 and K616 which were supplemented with 10 mM $CaCl_2$. Transformants were selected on synthetic complete medium minus uracil (SC-URA). The medium consisted of 6.7 g/liter yeast nitrogen base without amino acids, 2 g/liter of drop-out mix without uracil and 2% glucose as a carbon source (13).

DNA Manipulations. *Screening cDNA libraries and DNA sequencing.* An *A. thaliana* (L. cv. Columbia) leaf cDNA library in λZAP (14) was screened with a partial cDNA clone encoding a putative Ca^{2+}-ATPase from tobacco (15). A 1.9-kb clone was isolated and used to probe a size-fractionated cDNA library (3–6 kb) prepared from hypocotyls (16). Five positive clones were isolated and *in vivo* excised into pBluescript. Restriction mapping and partial sequencing of these clones showed that they fell into two groups, named ECA1 (3.3 kb) and ECA2 (2.5 kb). ECA1 cDNA was restricted and subcloned into pUC18 and pUC19. Both strands were sequenced by the dideoxynucleotide chain-termination method (17) with T7 polymerase (Pharmacia). The DNA sequences were analyzed using the Wisconsin Package GCG program as well as Mac DNASIS.

Construction of expression plasmid. Taking advantage of an *Eco*RI site 6 bp before the initiation codon, ATG, the entire open reading frame of ECA1 plus a partial 3′-untranslated region was excised with *Eco*RI from pBluescript. The 3.2-kb fragment was subcloned into a yeast expression vector, p426Gal1, at the *Eco*RI site between the yeast galactokinase gene (Gal1) promoter and CYC1 termination sequence (18). The orientation and junctions of the construct, designated as pECA1, were verified by sequencing.

Yeast Transformation and Growth. The wild-type and mutants strains of *S. cereviciae* were transformed with p426 vector alone or with pECA1 by the LiOAc/PEG methods (19) and selected for uracil prototrophy by plating on SC-URA medium. The Ura^+ colonies were picked and grown for 2–3 days in SC-URA medium for complementation studies.

Isolation of Yeast Membranes. Transformants were inoculated into 20 ml of SC-URA medium and incubated overnight. The culture was diluted 10-fold into SC-URA/Gal medium and grown overnight to an OD_{600} of 1–1.8. The cells were pelleted at 4,000 × *g* for 1 min, washed with 10 ml of distilled water, and pelleted. Membranes were isolated using the glass bead method with some modification (20). Cells were suspended in 10 ml of glass beads buffer (GBB; 10% sucrose/25 mM Hepes-KOH, pH 7.5/3 mM EGTA/2 mM DTT) and repelleted. Typically 1–2 ml cells were resuspended in 1 volume of GBB plus 1 mM phenylmethylsulfonyl fluoride (PMSF), 10 mM benzamidine, 5 μg/ml pepstatin and leupeptin, and 0.5 mg/ml BSA. An equal volume of glass beads (Sigma) was added and the mixture was vortex mixed for 2 min at maximum speed. The lysate was centrifuged at 5,000 × *g* for 5 min and the supernatant was saved. The pellet was suspended in 1 volume of GBB plus protease inhibitors, vortex mixed, and centrifuged as above. The supernatants were pooled and layered onto a 25/45% sucrose step gradient containing 20 mM Hepes-KOH (pH 7.0), 1 mM DTT, 0.2 mM PMSF, and 5 mM benzamidine and centrifuged at 108,000 × *g* for 2 h. Membranes at the 25/45% sucrose interface were collected and stored at −80°C. Protein concentration was determined with the Bio-Rad reagent.

Formation of Phosphoenzyme (PE). [^{32}P]Phosphoprotein formation was assayed according to Chen *et al.* (21) with some modification. The reaction mixture (200 μl) contained 25 mM Hepes-1,3-bis[Tris (hydroxymethyl)methylamino]propane (BTP) (pH7.0), 100 mM KCl, and 5–20 μg membrane protein. To test cation dependence, EGTA was added to a final concentration of 500 μM to reduce the free Ca^{2+} concentration to <10 nM (22). Then various divalent salt was added to a total concentration of 1 mM $MgSO_4$, 540 μM $CaCl_2$, or 500 μM $MnCl_2$ so that the free concentration was 989 μM, 45 μM, or 3 μM, respectively, as computed with the MAXCHELATOR program (23). The reaction was started by adding [γ-^{32}P]ATP (1–2 mCi/reaction; 1 Ci = 37 GBq) (Amersham; 3,000 Ci $mmol^{-1}$) to a final concentration of 2 or 100 nM, and terminated after 15 or 120 sec by adding 0.2 ml of a stop solution containing 50 mM NaH_2PO_4, 2 mM ATP and 20% trichloroacetic acid (TCA). The TCA precipitate was pelleted, washed with a solution containing 10% TCA, 25 mM NaH_2PO_4, and 1 mM ATP, and pelleted again. The pellet was suspended in 30 μl of sample buffer and subjected to SDS/PAGE (pH 6.0) and autoradiography (21).

Fractionation of Membranes from *Arabidopsis*. Five grams of *A. thaliana* L. cv. Columbia plants grown in B5 liquid culture for 4 weeks was homogenized with a mortar and pestle in 50 mM Hepes-BTP (pH 7.4), 250 mM sorbitol, 3 mM EGTA, 2 mM EDTA, 1 mM DTT, 0.1 mM PMSF, and 0.5% BSA. The homogenate was centrifuged at 12,000 × *g* for 10 min, and the resulting supernatant was centrifuged at 110,000 × *g* for 50 min. The microsomal pellet was resuspended in the above solution without BSA and layered onto a 12-step gradient with 1.2 ml each of 12, 15, 18, 21, 24, 27, 30, 33, 36, 39, 42, and 45% sucrose. The sucrose solutions contained 25 mM BTP (pH 7.0), 2 mM EDTA, 1 mM DTT, 0.1 mM PMSF, and 2 mM benzamidine. After centrifugation at 110,000 × *g* for 16 h (Beckman SW28), 0.75-ml fractions were collected and stored at −80°C.

Immunostain. Membranes from yeast or from the sucrose gradient fractions (25 μl) of *Arabidopsis* were mixed with an equal vol. of a 2× sample buffer. The proteins were separated on a 7% SDS/polyacrylamide gel, blotted onto Immobilon-P and probed with several antibodies (24). Polyclonal antibody (anti-ACA3 1374) was generated against a glutathione *S*-transferase fusion protein containing the last 27 residues of ECA1 polypeptide (ECA1p). Monoclonal antibody 11A1 recognized calnexin (X. Li and H.S., unpublished data).

Chemicals. Erythrosin B and CPA were obtained from Sigma, and thapsigargin was purchased from LC Service (Woburn, MA). All other chemicals were reagent grade.

RESULTS

ECA1 Encodes a P-type Cation Pump Homolog. *Arabidopsis* cDNA libraries were screened with a partial cDNA encoding a putative Ca^{2+}-ATPase from tobacco (15). Five positive clones were obtained and sequence analysis showed that a 3.3-kb clone, named ECA1, was nearly full-length and identical to ACA3 (J.F.H., unpublished data). The translation initiation codon of ECA1 was identified at position 72, as this ATG was preceded by a stop codon and was contiguous to a consensus sequence (CAUGG) of translation initiation sites (25). ECA1 contained an open reading frame of 1,061 amino acids. The deduced polypeptide had a molecular mass of 116,359 Da, and

all the highly conserved domains characteristic of P-type cation pumps (Fig. 1). Ten TM domains were predicted by hydrophobic moment analysis (26) and by analogy to SERCA pumps (27). ECA1p shared 67% and 53% identity to a tomato Ca^{2+}-ATPase, LCA1p (9), and to a mammalian muscle SERCA 2a pump (27), respectively; but less than 32% identity to PEA1, a putative Ca^{2+}-ATPase on the chloroplast envelope (10). A hydrophilic domain between transmembrane domain 4 (TM4) and TM5 of ECA1p included a potential aspartyl phosphorylation site within the motif CS**D**K, and ATP-binding domains conserved in P-type ion pumping ATPases (Fig. 1). TM5 and -6 of ECA1p shared 82% and 88% identity to the corresponding TM domains of the rabbit SERCA pump. Significantly, six amino acids within TM4, -5, -6, and -8 (see Fig. 1) required for high-affinity Ca^{2+} transport in animal SERCA pumps (28) are all conserved in ECA1p. Sequence analyses therefore strongly suggested ECA1 encoded a P-type Ca^{2+} pump (EC 3.6.1.38).

Complementation of Yeast Mutants Defective in Ca^{2+} Pumps by pECA1. To test the function of ECA1p in yeast mutants defective in either Na^+ pumps or Ca^{2+} pumps, the 3.2-kb ECA1 cDNA was subcloned into a yeast expression vector, p426Gal1, under the control of a galactose-inducible promoter. Wild-type yeast transformed with vector (p426) alone grew in a medium containing 3 mM Mn^{2+} (Fig. 2*A*) or 10 mM EGTA (Fig. 3*A*). However, *pmr1* mutants in which a Golgi Ca^{2+} pump was disrupted were sensitive to Mn^{2+} toxicity (Fig. 2*A*) and to EGTA (Fig. 3*A*) as shown before (2, 29). When *pmr1* mutants were transformed with pECA1, growth on SC-URA medium containing either Mn^{2+} (Fig. 2*A*) or EGTA (Fig. 3*A*) was restored in the presence of galactose,

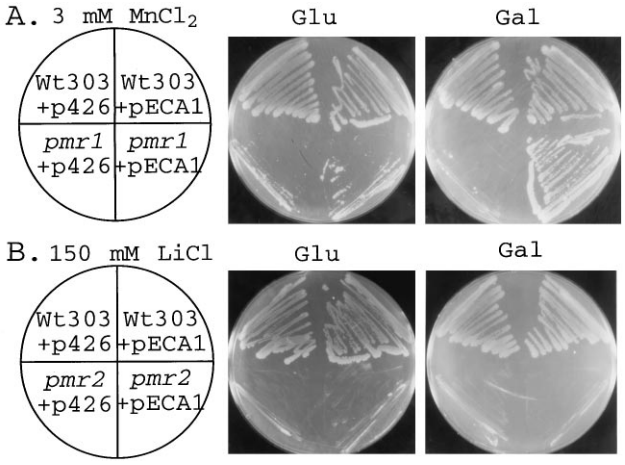

FIG. 2. pECA1 restored the growth of yeast *pmr1* mutant on medium with Mn^{2+} but not of *pmr2* mutant on Li-containing medium. Wild-type (W303), *pmr1* (AA542), and *pmr2* (K633) cells were transformed with either a high-copy plasmid vector (p426) alone or the vector containing the *Arabidopsis* ECA1 (pECA1). The cells were streaked on SC-URA plates containing glucose (Glu) or galactose (Gal) plus either 3 mM $MnCl_2$ (*A*) or 150 mM LiCl (*B*) and incubated for 4 days at 30°C.

but not of glucose. In contrast, pECA1 was unable to restore growth of *pmr2* mutant on 150 mM LiCl (Fig. 2*B*) or 1 M NaCl (data not shown). PMR2 gene cluster encodes putative Na^+ and Li^+ efflux pumps on the plasma membrane that are required for tolerance of these ions (30). Thus ECA1p was

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                                                                                                                    TM1
ECA MGKGSEDLVKKESLNSTPVNSDTFPAWAKDVAECEEHFVVSREKGLSSDEVLKRHQIYGLNELEKPEGTSIFKLILEQFNDTLVRILLAAAVISFVLAFF 100
                                 | .|.|.|. :||.|. ..||| ::| | .: :| |||. .||..::.|::|||:| |||||| ||.||||||:|
SER                          MENAHTKTVEEVLGHFGVNESTGLSLEQVKKLKERWGSNELPAEEGKTLLELVIEQFEDLLVRILLLAACISFVLAWF 78
                                                TM2
ECA DGDEGGEMGITAFVEPLVIFLILIVNAIVGIWQETNAEKALEALKEIQSQQATVMRDGTK.VSSLPAKELVPGDIVELRVGDKVPADMRVVALISSTLRV 199
    |:|| .|||||||:||:|||:.||||||:||| |||.|:||||||.:.::.| |:: |  | .:.|::|:||||||: ||||||||:|:..: |.|||
SER ...EEGEETITAFVEPFVILLILVANAIVGVWQERNAENAIEALKEYEPEMGKVYRQDRKSVQRIKAKDIVPGDIVEIAVGDKVPADIRLTSIKSTTLRV 175
                                                                                                        TM3
ECA EQGSLTGESEAVSKTTKHV.DENADIQGKKCMVFAGTTVVNGNCICLVTDTGMNTEIGRVHSQIQEAAQHEEDTPLKKKLNEFGEVLTMIIGLICALVWL 298
    :|: ||||| .| | |..| |...|  |:|| |:|.||.:..|...:.|..||:||||||:::.::  .| .:| |||..||:|||| |..:|:||| ||:
SER DQSILTGESVSVIKHTDPVPDPRAVNQDKKNMLFSGTNIAAGKAMGVVVATGVNTEIGKIRDEM..VATEQERTPLQQKLDEFGEQLSKVISLICIAVWI 273
                                  TM4                                                    phosphorylation
ECA INVKYFLSWEYVDGWPRNFKFSFEKCTYYFEIAVALAVAAIPEGLPAVITTCLALGTRKMAQKNALVRKLPSVETLGCTTVICSDKTGTLTTNQMAVSKL 398
    ||: .| .  ..::| |.       ..|||.||||||||||||||||||||||||||:||.|||:||.||||||||||.|||||||||||||.|:::
SER INIGHFNDPVHGGSWIRG.......AIYYFKIAVALAVAAIPEGLPAVITTCLALGTRRMAKKNAIVRSLPSVETLGCTSVICSDKTGTLTTNQMSVCRM 366
                                                  *
ECA VAMGSRIGTLRSFN...VEGTSFDPRDG..KIEDWPMGRMDANLQMIAKIAAICNDANVE..QSDQQFVSRGMPTEAALKVLVEKMG.FPEGLNEASSDG 490
    . ::.  |.  |:|   :.|..:.| ::  | :. ...:  ..|  :|.|.|:|||..::  :... : . | :||.||..|||||. |..:|.: |. :
SER FILDKVDGDTCSLNEFTITGSTYAPIGEVHKDDKPVKCHQYDGLVELATICALCNDSALDYNEAKGVYEKVGEATETALTCLVEKMNVFDTELKGLSKIE 466
                                          ATP-Binding
ECA DVLRCCRLWSELEQRIATLEFDRDRKSMGVMVDSSSGNKL....LLVKGAVENVLERSTHIQLLDGSKRELDQYSRDLILQSLRDMSLS..ALRCLGFAY 584
    .  | .: .:| .:  ||||.||||||:| .......:     ::||||.|.|::|:|||. :::.| .:..  :: |:  :|: : :  .||||::|
SER RANACNSVIKQLMKKEFTLEFSRDRKSMSVYCTPNKPSRTSMSKMFVKGAPEGVIDRCTHIR.VGSTKVPMTAGVKQKIMSVIREWGSGSDTLRCLALAT 565

ECA SDVPSDFATYDGSEDHPAHQQLLNPSNYSSIESNLIFVGFVGLRDPPRKEVRQAIADCRTAGIRVMVITGDNKSTAEAICREIGVFEADEDISSRSLTGI 684
     | |           :... :| :..|: ..|.||.||| ||: |||| ||  .:  || ||||||:|||||||:|| |||| ||:|:.:||:..:.:||
SER HDNPL.........RREEMHLKDSANFIKYETNLTFVGCVGMLDPPRIEVASSVKLCRQAGIRVIMITGDNKGTAVAICRRIGIFGQEEDVTAKAFTGR 655
                                          ATP-Binding
ECA EFMDVQDQKNHLRQTGGLLFSRAEPKHKQEIVRLLKEDGEVVAMTGDGVNDAPALKLADIGVAMGISGTEVAKEASDMVLADDNFSTIVAAVGEGRSIYN 784
    || ::..  .:    .:  |.|.||.|| .|| :|.. :|:.TTTTTTTTTTTTTTT |:||:||| |||.|||.||:|||||||||||||||:|||.|||
SER EFDELNPSAQRDACLNARCFARVEPSHKSKIVEFLQSFDEITAMTGDGVNDAPALKKAEIGIAMG.SGTAVAKTASEMVLADDNFSTIVAAVEEGRAIYN 754
                  TM5                  TM6                                    TM7
ECA NMKAFIRYMISSNIGEVASIFLTAALGIPEGMIPVQLLWVNLVTDGPPATALGFNPPDKDIMKKPPRRSDDSLITAWILFRYMVIGLYVGVATVGVFIIW 884
    |||.||||:||||:|||.:|||||||:||::|||||||||||| ||||||||||| |||.||||...:.||.:|::|||:.|| |||.||||.   |
SER NMKQFIRYLISSNVGEVVCIFLTAALGFPEALIPVQLLWVNLVTDGLPATALGFNPPDLDIMNKPPRNPKEPLISGWLFFRYLAIGCYVGAATVGAAAWW 854
                  *                    * **                                  TM8
ECA YTHSSFMGIDLSQDGHSLVSYSQLAHWGQCSSWEGFKVSPFTAGSQTFSFDSNPCDYFQQGKIKASTLSLSVLVAIEMFNSLNALSEDGSLVTMPPWVNP 984
    :          :..|| . ||: ||.|: ||.              .:. .|:: .|..|: .   : |:.|||||.||| |.||.|||:.||: |||| |
SER F.........IAADGGPRVSFYQLSHFLQCK.............EDNPDFEGVDCAIFESP..YPMTMALSVLVTIEMCNALNSLSENQSLLRMPPWENI 930
          TM9                  TM10                                  *
ECA WLLLAMAVSFGLHFVILYVPFLAQVFGIVPLSLNEWLLVLAVSLPVILIDEVLKFVGRCTSGYRYSPRTLSTKQKEE 1061
    ||: .:.:|::|||:||||. |: :|.|.||.:.:||:|| :||||||:||.||||:|   ..| .|.
SER WLVGSICLSMSLHFLILYVEPLPLIFQITPLNVTQWLMVLKISLPVILMDETLKFVARNY....LEPAILE        997
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FIG. 1. Alignment of the deduced amino acid sequences of ECA1 from *Arabidopsis* and of rabbit SERCA 2a (SER) (27) illustrates the conserved domains of P-type Ca^{2+}-ATPases. The potential phosphorylation site (Asp-383) and two regions (533–536 and 727–740) that form the putative ATP-binding domain (underlined in bold) are located in the central hydrophilic loop. Each TM region is indicated with a line above the ECA1 sequence and a line below the corresponding SERCA TM domain. Potential Ca^{2+}-binding sites (E^{341}, E^{800}, N^{825}, T^{828}, D^{829}, E^{961}) within predicted TM4, -5, -6, and -8 in ECA1 correspond to residues (marked with an asterisk below the residue) required for Ca^{2+} transport in rabbit SERCA pump (28). Residues sharing identity or similarity are denoted by | and :, respectively. Spaces shown as periods were introduced to maximize the alignment. The alignment was performed with GAP in the Wisconsin Package GCG program.

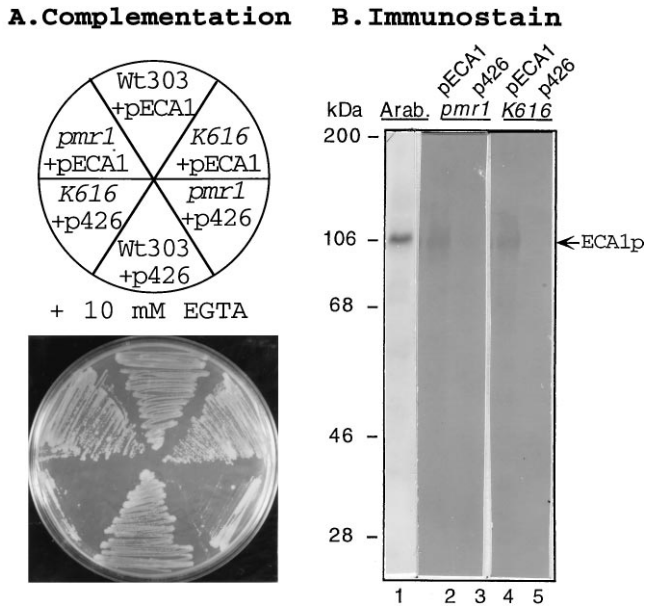

FIG. 3. Complementation of growth on EGTA by pECA1 is accompanied by expression of the ECA1 polypeptide in *pmr1* and *pmr1 pmc1 cnb1* mutants. Wild-type yeast (W303), *pmr1* (AA542), and *pmr1 pmc1 cnb1* (K616) were transformed with control vector (p426) or pECA1 as in Fig. 2. (*A*) The cells were streaked on SC-URA plates containing galactose and 10 mM EGTA at pH 6.2 and incubated for 3 days at 30°C. (*B*) To detect the ECA1 polypeptide, microsomal membranes were isolated from *pmr1* and *pmr1 pmc1 cnb1* mutants transformed with p426 alone (lanes 3 and 5) or pECA1 (lanes 2 and 4). Protein (5 μg) was separated by SDS/PAGE, blotted, and immunostained with antibody against ECA1p (1:50,000). Lane 1 was loaded with microsomal membranes isolated from *Arabidopsis* (1:500).

more effective as a Ca^{2+} pump than as a Li^+ or Na^+ efflux pump under these experimental conditions.

ECA1 expression also complemented the Mn^{2+} and EGTA sensitivity of a triple mutant (*K616*) which lacked both endogenous Ca^{2+} pumps (PMR1 and PMC1) and calcineurin (CNB1) function. PMC1 encodes a vacuolar Ca^{2+} pump required by cells for growth on medium containing high [Ca^{2+}] (12). Disruption of the gene encoding the B subunit of the calcineurin gene (*cnb1*) was necessary as the *pmr1 pmc1* double mutant is inviable unless calcineurin inhibition of a vacuolar H^+/Ca^{2+} antiporter (Vcx1p) was relieved (29). Like the *pmr1* mutant, the triple mutant transformed with the vector alone grew poorly on a medium containing Mn^{2+} (data not shown) or very low Ca^{2+} (+10 mM EGTA) (Fig. 3*A*). However, the triple mutant transformed with pECA1 became tolerant of both EGTA (Fig. 3*A*) and 3 mM Mn^{2+} (data not shown) supporting the idea that ECA1 encoded a functional Ca^{2+} pump.

Complementation of growth on EGTA-containing media was accompanied by the expression of ECA1p in transformants. A polyclonal antibody against the carboxyl tail of ECA1p recognized a 106-kDa polypeptide in microsomal membranes isolated from pECA1-transformants (Fig. 3*B*, lanes 2 and 4), but not from yeast transformed with a control vector (Fig. 3*B*, lanes 3 and 5). The antibody was highly specific, as it recognized one major polypeptide in microsomal membranes from *Arabidopsis* plants (Fig. 3*B*, lane 1). The similar size of proteins expressed in *Arabidopsis* plants and in the pECA1-transformed mutants indicated that the coding region in ECA1 was full-length, although the deduced polypeptide was about 10 kDa larger than its apparent size estimated by SDS/PAGE.

ECA1p Forms a Ca^{2+}-Dependent PE. If ECA1p is a P-type Ca^{2+}-pumping ATPase, it should form a phosphorylated intermediate as a part of the reaction cycle (31). To test this, microsomes isolated from pECA1-transformed or p426-transformed *K616* were incubated with [γ-^{32}P]ATP under various conditions. One major phosphoprotein of 106 kDa was formed in membranes isolated from pECA1-transformed *K616* (Fig. 4*A Lower*), but not from p426-transformed *K616* (Fig. 4*A*). Phosphorylation was dependent on the presence of Ca^{2+}, but not Mg^{2+}. La^{3+}enhanced the steady-state level of the phosphoprotein several fold (Fig. 4*A*, lanes 5 and 6). The denatured PE was sensitive to hydroxylamine (Fig. 4*B*), indicating the hydrolysis of an acyl phosphate bond (32) probably to Asp-383 (Fig. 1). Together, these results provide compelling evidence that ECA1p is a P-type Ca^{2+}-dependent ATPase.

It is interesting that CPA, a blocker of animal SERCA pumps, inhibited the phosphorylation of ECA1p, whereas another SERCA ATPase inhibitor, tharpsigargin (33), had little or no effect (Fig. 4*C*). CPA decreased the initial rate of PE formation by 50% at concentrations (10 μM) that block the activity of animal SERCA-type ATPase (34). The initial rate of PE formation was inhibited 90% by 1 μM erythrosin B (Fig. 4*C*, lane 4), a halogenated derivative of fluorescein that binds to nucleotide-binding sites with high affinity and specificity (35). Erythrosin B is a potent inhibitor of both PM-type and ER-type Ca^{2+}-ATPases from plants (36). These results suggested ECA1 encoded a Ca^{2+} pump that was related to the ER-type.

The ability of pECA1 to restore growth of *pmr1* or *K616* mutants on Mn^{2+}-containing medium (Fig. 2) suggested the possibility that this ATPase might pump Mn^{2+} in addition to Ca^{2+}. If so, binding of Mn^{2+} to the ATPase should stimulate the formation of a phosphorylated intermediate as seen above with Ca^{2+}. To test this idea, EGTA was added to the reaction mixture to chelate divalent cations. When Mn^{2+} was added to

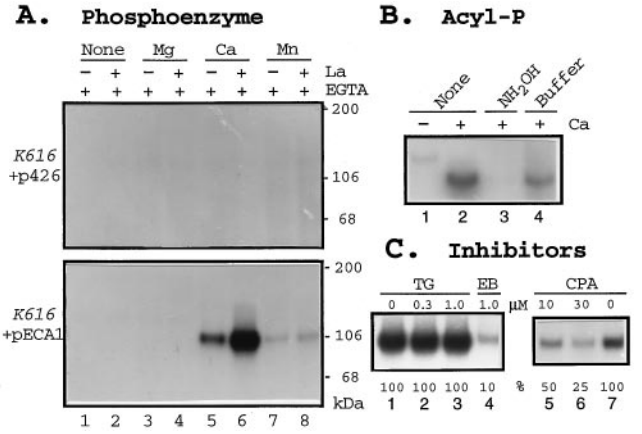

FIG. 4. Formation of a Ca^{2+}-dependent phosphoprotein is inhibited by CPA. (*A*) Ca^{2+}-dependent PE. Membranes were isolated from triple mutants, K616, transformed with either pECA1 (*Lower*) or with vector alone (*Upper*). Membranes were incubated with 2 nM [^{32}P]ATP for 2 min with 0.5 mM EGTA alone (none), or with added divalent cations to give a final free concentration of 990 μM Mg^{2+}, 45 μM Ca^{2+}, or 3 μM Mn^{2+} in the presence (+) or absence (−) of 50 μM La^{3+}. The reaction was stopped with TCA, and the proteins were analyzed by SDS/PAGE and autoradiography. (*B*) Sensitivity of PE to hydroxylamine. Membranes were isolated from pECA1-transformed triple mutants. PE formed without La^{3+} was terminated with TCA (as in *A*). The TCA pellet was either untreated (lanes 1 and 2) or incubated with 1 ml of 0.5M hydroxylamine (NH_2OH) in 100 mM Mes-KOH buffer at pH 6.0 (lane 3) or with buffer alone (lane 4) for 15 min. Protein was precipitated with 0.2 ml 50% TCA. (*C*) Inhibition of PE formation by erythrosin B (EB) and CPA, but not by thapsigargin (TG). Membranes isolated from triple mutant transformed with pECA1 were incubated with DMSO (lanes 1 and 7) or inhibitors for 20 min before assay. PE formation was assayed with 45 μM Ca^{2+}, and 100 nM ATP for 15 sec without La^{3+}. PE was quantitated by PhosphorImager.

give a final free concentration of about 3 μM, a phosphoprotein of 106 kDa was formed (Fig. 4*A*, lanes 7 and 8). La^{3+} slightly enhanced the steady-state levels of Mn^{2+}-dependent phosphoprotein. The results supported the idea that ECA1p is a divalent cation pump with specificity for Mn^{2+} as well as Ca^{2+}.

ECA1p Is Mainly Localized on ER Membranes. To determine the subcellular location of ECA1p, membranes were isolated from *Arabidopsis* plants and fractionated by a sucrose density gradient. ECA1p migrated to a density of 33–35% sucrose, similar to that of calnexin, an ER chaperone (Fig. 5) (4). The peak of ECA1p was separated from the PM (39–41%) and the vacuolar membrane (26–30%), which were detected by immunoblotting with antibodies to the PM H^+-ATPase and to the V-ATPase subunit B, respectively. When Mg^{2+} was present in the homogenization buffer and in the sucrose gradient, the peak of ECA1p was shifted to 38% sucrose in parallel with calnexin (data not shown). These results demonstrated that ECA1p was mainly localized in the ER. Because ECA1p was distributed in membranes at a density higher than that of calnexin, ECA1p could also be localized on other organelles, such as the Golgi.

DISCUSSION

A Plant Gene, ECA1, Encodes a P-Type Ca^{2+}-ATPase. Using yeast as a heterologous expression system (38), we provide the first direct evidence that a cloned plant gene encodes a functional Ca^{2+}-ATPase. Overexpression of an *Arabidopsis* gene, ECA1, restored the growth of yeast mutants (*pmr1* or *K616*) defective in Ca^{2+} pumps on a medium containing submicromolar levels of Ca^{2+} (Fig. 3). Wild-type yeast contains two Ca^{2+} pumps. Pmr1p pumps Ca^{2+} (39) into the Golgi compartment (2, 3) to support a variety of secretory functions required for normal growth rates. Pmc1p is required for efficient Ca^{2+} sequestration into the vacuole and is necessary for tolerance of high external [Ca^{2+}] (12). Wild-type yeast grows well on a medium with submicromolar concentrations of Ca^{2+}, apparently because the two Ca^{2+} pumps can sequester Ca^{2+} and thus increase [Ca^{2+}] in endomembrane compartments. Growth of *pmr1* or triple mutants on medium with 10 mM EGTA was poor, probably because [Ca^{2+}] in the Golgi and secretory vesicles was insufficient to support normal growth rates (2, 12). Restoration of growth of pECA1-transformed triple mutant or *pmr1* on EGTA medium (Fig. 3) would suggest that endolumenal [Ca^{2+}] has been sufficiently increased to support normal growth rates. Thus the function of ECA1p in yeast resembled closely the function of the Golgi Ca^{2+} pump, Pmr1p, although the two proteins shared only 37% identity. The results suggested that pECA-transformed cells expressed an active Ca^{2+} pump on the endomembranes of either *pmr1* or the triple mutant. This idea is supported by *in vitro* studies. As Ca^{2+} pumping by ECA1p was masked by the activity from the vacuolar H^+/Ca^{2+} antiporter (data not shown), we determined whether ECA1p could be phosphorylated in a manner similar to PE intermediates formed during the reaction cycle of Ca^{2+}-pumping ATPases.

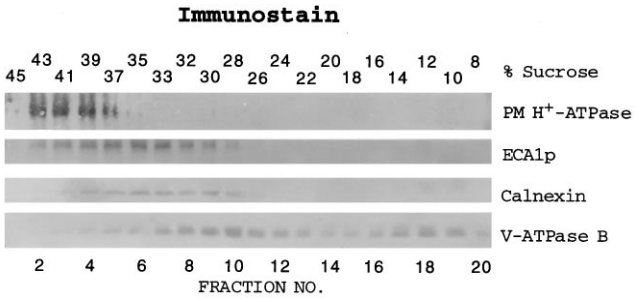

FIG. 5. ECA1p was mainly localized on the ER of *Arabidopsis* plants. Membrane (2–6 μg protein) fractionated by a sucrose gradient was separated by SDS/PAGE, blotted, and probed with either polyclonal antibodies against ECA1p (1:500 dilution), anti-CTF2 (37) against the C-terminal end of PM H^+-ATPase, AHA2 (1:10,000), or monoclonal antibodies 11A1 against oat calnexin (1:100) and 2E7 (24) against the B subunit of oat V-ATPase (1:500). One experiment is representative of two experiments.

The mechanism of P-type ion pumping ATPases involves a conformational change of two alternate phosphorylated intermediates (E_1E_2) as part of the reaction cycle. The reaction cycle is essential for coupling ATP hydrolysis to ion transport. Briefly, the sequence of reactions for a SERCA pump (31) is as follows: (*i*) two Ca^{2+} bind to the high-affinity binding sites (K_m = 0.2–2 μM) on the cytoplasmic face of E_1 thus activating the ATP-binding site; (*ii*) ATP binds and transfers its terminal phosphate to an aspartyl residue to form an acyl phosphate bond (E_1~P); (*iii*) a conformational change (E_2-P) occurs so that the bound Ca^{2+} ions face the exoplasmic surface; (*iv*) Ca^{2+} are released to the exterior as the K_m of the Ca^{2+} binding site in E_2P is 1–3 mM; and (*v*) the acyl phosphate is hydrolyzed to regenerate E_1. The sensitivity to hydroxylamine indicated that the phosphate was linked to the ECA1 protein by an acyl phosphate linkage (Fig. 4*B*). The phosphoprotein was identified as a Ca^{2+}-ATPase based on its dependence on Ca^{2+}, and the increased PE level in the presence of La^{3+} (Fig. 4*A*). La^{3+} can substitute for Ca^{2+} in activating enzyme phosphorylation by binding to the high-affinity Ca^{2+} binding sites of a SERCA pump (40). The resulting PE undergoes very slow hydrolytic cleavage. The slow turnover of the Ca^{2+} pump results in an increase in PE levels when La^{3+} is present under appropriate conditions. Thus the properties of the phosphorylated ECA1p are characteristic of the phosphorylated intermediate of E_1E_2 Ca^{2+}-pumping ATPases.

ECA1p Is an ER-Type Ca^{2+}-ATPase. Several lines of evidence indicate that ECA1 encodes an ER-type Ca^{2+} pump: (*i*) its amino acid sequence shares 53% identity to SERCA pump (27) and less than 32% identity to PM-type Ca^{2+} ATPase (10); (*ii*) the phosphoprotein formed by ECA1p is sensitive to CPA, a specific SERCA inhibitor (Fig. 4); and (*iii*) ECA1 encodes a 116-kDa polypeptide that is mainly localized on the ER of *Arabidopsis* plants (Fig. 5). Thus ECA1p resembled a carrot ER-associated Ca^{2+} pump that was partially inhibited by CPA and insensitive to calmodulin (7). Preliminary results showed that ECA1p did not bind to calmodulin. It is interesting that the carboxyl tail of ECA1p contained a motif, KXKXX (Fig. 1), that functions as an ER-retention sequence of type I proteins (41). To distinguish between multiple genes encoding ER-type and PM-type Ca pumps in plants, we have named the first gene encoding a functional ER-type Ca-ATPase from *Arabidopsis* as ECA1.

Surprisingly, the localization of ECA1p on the ER membranes in *Arabidopsis* differs from that of a putative Ca^{2+}-ATPase (LCAp) from tomato (9). ECA1 and LCA encode related homologs of one another as the two polypeptides share 67% identity and 80% similarity. Immunoblotting with antibodies against a region (580–624) of the LCAp hydrophilic domain showed that this polypeptide was localized on both the PM and the vacuolar membrane, but not the ER. Although alternative splicing of a single gene is a possibility in tomato (42), several genes encode ER-type Ca^{2+} pump homologs in *A. thaliana* (F.L. and H.S., unpublished data).

Role in Mn^{2+} Transport? The ability of ECA1p to restore the growth of *pmr1* mutant in Mn-containing medium suggested that this Ca^{2+} pump may also catalyze Mn^{2+} transport (Fig. 2). Recently, Pmr1p has been implicated in supplying Mn^{2+}, in addition to Ca^{2+}, into the Golgi compartment (43). *pmr1* mutants contain 3–4 times more Mn^{2+} than did wild-type cells. The high level of Mn^{2+} is associated with an increased sensitivity to Mn added in the growth medium. Thus Pmr1p could affect cellular Mn^{2+} homeostasis by pumping Mn^{2+} into

the Golgi apparatus and by extruding excess Mn^{2+} by vesicular transport out of the cell or to the vacuole. Mn^{2+} is required in the Golgi to activate Mn^{2+}-dependent enzymes involved in protein processing and secretion (44), and Mn^{2+} may also function in intracellular signaling (22). Although direct evidence for Mn^{2+} transport has not been demonstrated with Pmr1p, a rabbit SERCA ATPase does catalyze active $^{54}Mn^{2+}$ transport into sarcoplasmic reticulum vesicles (45). In plants, manganese is an essential nutrient that is required by the oxygen-evolving complex in photosynthesis and for activating many enzyme-catalyzed reactions (46). Because the free cytoplasmic [Mn^{2+}] of 0.2 μM (47) is less than the average [Mn^{2+}] of 100 μM in plant cells (46), Mn^{2+} must be actively sequestered into endomembrane compartments. The Mn^{2+}-dependent formation of a PE would support the idea that ECA1p could translocate Mn^{2+}, as well as Ca^{2+}, into the lumen of the ER or the Golgi. Recently, a mutant in which the ACA3 gene (GenBank accession no. U93845; identical to ECA1) was disrupted by transferred DNA (T-DNA) insertion has been isolated from *Arabidopsis* (48). It will be interesting to test whether this mutant is sensitive to high levels of Mn^{2+}.

Summary. We have demonstrated that a yeast mutant, *K616*, defective in two Ca^{2+} pumps provides an expression system to study individual Ca^{2+} pumps from plants and perhaps other sources. The triple mutant shows no background activity of Ca^{2+}-dependent PE formation (Fig. 3*A*), indicating the cell was devoid of other P-type Ca^{2+} pumps. This expression system will allow us to identify functionally and characterize putative Ca^{2+} or divalent cation pumps that are encoded by multiple genes from *Arabidopsis*, and thus to understand better the biological role of each pump in the plant.

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