Identification of a Calmodulin-Regulated Soybean Ca\(^{2+}\)-ATPase (SCA1) That Is Located in the Plasma Membrane

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Ca\(^{2+}\)-ATPases are key regulators of Ca\(^{2+}\) ion efflux in all eukaryotes. Animal cells have two distinct families of Ca\(^{2+}\) pumps, with calmodulin-stimulated pumps (type IIB pumps) found exclusively at the plasma membrane. In plants, no equivalent type IIB pump located at the plasma membrane has been identified at the molecular level, although related isoforms have been identified in non-plasma membrane locations. Here, we identify a plant cDNA, designated SCA1 (for soybean Ca\(^{2+}\)-ATPase 1), that encodes Ca\(^{2+}\)-ATPase and is located at the plasma membrane. The plasma membrane localization was determined by sucrose gradient and aqueous two-phase membrane fractionations and was confirmed by the localization of SCA1p tagged with a green fluorescent protein. The Ca\(^{2+}\)-ATPase activity of the SCA1p was increased approximately sixfold by calmodulin (\(K_{\text{d}} = \sim 10\) nM). Two calmodulin binding sequences were identified in the N-terminal domain. An N-terminal truncation mutant that deletes sequence through the two calmodulin binding sites was able to complement a yeast mutant (K616) that was deficient in two endogenous Ca\(^{2+}\) pumps. Our results indicate that SCA1p is structurally distinct from the plasma membrane-localized Ca\(^{2+}\) pump in animal cells, belonging instead to a novel family of plant type IIB pumps found in multiple subcellular locations. In plant cells from soybean, expression of this plasma membrane pump was highly and rapidly induced by salt (NaCl) stress and a fungal elicitor but not by osmotic stress.

INTRODUCTION

Ca\(^{2+}\) plays a central role as a second messenger in signal transduction of all eukaryotes (Bootman and Berrige, 1995; Clapham, 1995). The transient increase of cytosolic free Ca\(^{2+}\) concentration, \([\text{Ca}^{2+}]_{\text{cyt}}\), is correlated with a variety of external signals such as touch, temperature shift, abscisic acid, auxin, red light, fungal elicitors, salinity/drought, anoxia, gravity, gibberellic acid, cytokinin, oxidative stress, and hypoxia (Bush, 1995; Sanders et al., 1999). In turn, the increased \([\text{Ca}^{2+}]_{\text{cyt}}\) triggers many signal transduction pathways, including the regulation of enzyme activity, ion channel activity, and gene expression, which results in diverse cellular responses (Bush, 1995). In addition to its role as a second messenger, Ca\(^{2+}\) also is important in regulating the processing of proteins in secretory pathways (Rudolph et al., 1989). Thus, cells require carefully regulated transport systems to control \([\text{Ca}^{2+}]\) in the cytoplasm and endomembrane compartments. Influx of Ca\(^{2+}\) to the cytosol occurs as a “downhill” transport through Ca\(^{2+}\) channels (Sanders et al., 1999). In contrast, the efflux of Ca\(^{2+}\) from the cytosol is mediated by two active Ca\(^{2+}\) transporters—Ca\(^{2+}\) pumps and Ca\(^{2+}\)/H\(^{+}\) antiporters—which are powered by ATP hydrolysis and proton motive force, respectively (Bush, 1995). In plants, Ca\(^{2+}\)-ATPases are believed to be a major Ca\(^{2+}\) transporter for the endoplasmic reticulum (ER), Golgi apparatus, vacuole, plastid inner membrane, and plasma membrane (Sanders et al., 1999).

Plant and animal cells utilize two distinct types of Ca\(^{2+}\) pumps, identified as type IIA and type IIB, based on their protein sequences (Axelson and Palmgren, 1998). Type IIA Ca\(^{2+}\) pumps include sarcoplasmic reticulum-type or ER-type Ca\(^{2+}\)-ATPases. None of these pumps appears to be directly activated by calmodulin. Evidence suggests that members of this family in plants may be located at the tonoplast and plasma membrane (Ferrol and Bennett, 1996; Hwang et al., 1997) as well as at the ER (Liang et al., 1997).

Type IIB Ca\(^{2+}\) pumps include the plasma membrane-type Ca\(^{2+}\)-ATPases, first characterized in animals (Carafoli, 1994), and a subgroup of novel Ca\(^{2+}\) pumps recently identified in...
plants, including ACA2p (Harper et al., 1998), which was identified from the model plant system Arabidopsis, and BCA1p (Malmstrom et al., 1997), identified from cauliflower. In contrast to type IIA Ca\(^{2+}\) pumps, type IIB Ca\(^{2+}\) pumps are stimulated by binding calmodulin.

Two features distinguish the currently known plant type IIB pumps from those found in animals. First, the plant isoforms have regulatory domains located at the N terminus instead of the C terminus of the pump and show little sequence identity to the regulatory domain of an animal pump. Second, unlike type IIB pumps from animals, which are thought to be located exclusively in the plasma membrane, the plant isoforms have been found only in non–plasma membrane locations, such as ACA2p in the ER membrane (Hong et al., 1995), BCA1p in the tonoplast (Malmstrom et al., 1997), and ACA1p, which is thought to reside in the chloroplast inner membrane (Huang et al., 1993). Despite the biochemical evidence for a calmodulin-regulated Ca\(^{2+}\)-ATPase in the plant cell plasma membrane (Evans, 1994; Askerlund, 1997; Dainese et al., 1997; Hwang et al., 1997; Bonza et al., 1998), the corresponding gene for this has not been cloned, and it is not known whether the anticipated pump is more similar to an animal type IIB pump or to the calmodulin-regulated pumps found in plant cell endomembranes.

In this article, we report the isolation and characterization of a gene, SCA1 (for soybean Ca\(^{2+}\)-ATPase 1), encoding a calmodulin-regulated Ca\(^{2+}\)-ATPase that is located at the plasma membrane in soybean cells. Both biochemical and genetic evidence demonstrate that SCA1p has an N-terminal calmodulin-regulated autoinhibitor. This indicates that SCA1p is structurally distinct from the analogous plasma membrane–localized Ca\(^{2+}\) pump in animal cells and belongs instead to a novel family of plant type IIB pumps previously found in non–plasma membrane locations. We also report that the expression of SCA1 mRNA is rapidly induced by salt (NaCl) stress or by a fungal elicitor but not by osmotic stresses, suggesting that the activity of this pump may be involved in the response to specific biotic and abiotic stresses.

**RESULTS**

**Primary Structure of SCA1p**

A full-length cDNA clone encoding a putative type IIB Ca\(^{2+}\)-ATPase was isolated from a soybean hypocotyl cDNA library (Hong et al., 1995) by using a partial cDNA clone (F955) that encodes another putative Ca\(^{2+}\) pump from Brassica (Lim et al., 1996). For this gene, named SCA1, the cDNA sequence showed an open reading frame of 3042 bp that encodes a polypeptide (SCA1p) with a molecular mass of 110.4 kD (Figure 1). Ten transmembrane domains were predicted by hydropathy analysis and comparisons with other P-type ATPases. This predicted topology suggested that SCA1p has a long N-terminal regulatory domain similar to other type IIB pumps in plants. Overall, SCA1p showed the greatest sequence identity to the plant type IIB Ca\(^{2+}\)-ATPases ACA2p (80%) and ACA1p (78%) from Arabidopsis and BCA1p (63%) from Brassica (Figure 1). In contrast, SCA1p showed only 45% identity to a typical mammalian plasma membrane–type Ca\(^{2+}\)-ATPase and even less identity (<30%) to typical ER-type (type IIA) Ca\(^{2+}\)-ATPases, such as ECA1p from Arabidopsis (Liang et al., 1997) or SERCA1p from rabbit (Brandl et al., 1986).

**Tissue-Specific Expression of SCA1 mRNA**

To determine whether SCA1 has tissue-specific expression, we subjected the RNA isolated from various soybean tissues to RNA gel blot analysis. The transcript was highly abundant in apical hypocotyl, elongating hypocotyl, mature hypocotyl, plumule, and root tissues and much less abundant in cotyledon and leaf tissues (Figure 2). The message was almost undetectable in immature seeds.

**SCA1p Is Located at the Plasma Membrane**

To determine the localization of SCA1p, we fractionated soybean microsomal membranes by using two procedures. First, centrifugation through a continuous sucrose gradient was used to compare the distribution of SCA1p with that of markers for the plasma membrane, ER, and Golgi. As shown in Figure 3, SCA1p was most abundant in fractions with sucrose content between 34 and 45%, similar to the H\(^{+}\)-ATPase commonly used as a marker for the plasma membrane (DeWitt et al., 1996; Liang et al., 1997). In contrast, peak fractions containing an ER marker (homolog of the ER-residence immunoglobulin heavy chain binding protein [BIP]; Haas, 1994) or a Golgi marker (latent UDPase activity; Nagahashi and Kane, 1982) were found in lighter (less sucrose) fractions.

To corroborate a cofractionation of SCA1p with the plasma membrane, we used a second fractionation procedure to purify plasma membrane by an aqueous two-phase partitioning method (Figure 4). In this procedure, SCA1p was found to be enriched in the upper phase along with a plasma membrane marker (H\(^{+}\)-ATPase), which is consistent with SCA1p being located at the plasma membrane. Controls indicated that endomembranes were fractionated to the lower phase, as indicated by the enrichment there of the ER marker (BIP) (Figure 4) and chlorophyll (not shown).

To provide cytological confirmation of the membrane fractionation result, we expressed a green fluorescent protein (GFP)-tagged version of SCA1p (encoded by construct p3S5-SCA1-GFP) in tobacco protoplast (Watanabe et al., 1987), and the spatial distribution of the GFP fluorescence was observed by confocal laser scanning microscopy. Without optically sectioning the cells, we could see that the fluorescent signal appeared to cover the entire surface of the cell (data not shown). Optical sectioning through the center...
Figure 1. Amino Acid Sequence Alignment Showing a High Degree of Identity between SCA1p, ACA2p, and BCA1p.

The sequence used as an antigen to generate an anti-SCA1p antibody is underlined.
of the cell, however, verified that the fluorescent signal was localized to the cell periphery, consistent with a plasma membrane location (Figures 5B and 5C). This pattern was distinct from controls that showed non-plasma membrane locations such as a GFPp-tagged ER-localized pump (ACA2-GFPp; Hong et al., 1999) (Figure 5D) or a soluble mutant proteins, nt means N-terminal truncation, and the number indicates the number of truncated residues.) These results suggest that the N-terminal region of SCA1p contains two Ca\textsuperscript{2+}-dependent calmodulin binding sequences, the first (CaMBD1) lying within the first 40 residues and the second (CaMBD2) between residues 52 and 71. Analysis of the sequence of the first 40 residues identified a likely candidate for CaMBD1 as a potential amphipathic basic helix, characteristic of a typical calmodulin binding domain (CaMBD) (Figures 7A and 7B). Most CaMBDs are stretches of 16 to 35 amino acids that show the segregation of having basic and polar residues on one side and hydrophobic amino acids on the others (Ames et al., 1995). An analysis of sequence downstream of position 52 revealed a potential modified IQ motif (Penniston and Enyedi, 1998), as shown in Figure 7C by the alignment of CaMBD2 with characterized modified IQ motifs that exhibit Ca\textsuperscript{2+}-dependent binding with calmodulin.

**N-Terminal-Truncated Mutant Pump (SCA1p nt85) Complement Disruption of Yeast Ca\textsuperscript{2+} Pump**

To determine if SCA1p functions as a Ca\textsuperscript{2+} pump in vivo, we expressed a wild-type (SCA1p) and an N-terminal–truncated mutant (SCA1 nt85) protein in a yeast strain (K616) harboring a disruption of three genes encoding proteins involved in Ca\textsuperscript{2+} homeostasis: PMR1p, PMC1p, and CNB1p (Cunningham and Fink, 1994). PMR1 and PMC1 encode Ca\textsuperscript{2+}-ATPases in the vacuole and Golgi, respectively. CNB1 encodes the B subunit of calcineurin, protein phosphatase 2B, which regulates a H\textsuperscript{+}/Ca\textsuperscript{2+} exchanger. Because of the disruption of the yeast Ca\textsuperscript{2+}-ATPases, the K616 strain shows poor growth on Ca\textsuperscript{2+}-depleted media (i.e., containing 10 mM EGTA). Both SCA1 and SCA1 nt85 cDNAs were cloned in a yeast expression vector, pYES2 (Invitrogen, Carlsbad, CA), to express proteins under the control of the galactose promoter. The constructs were introduced into K616, and the transformed yeasts were grown in Ca\textsuperscript{2+}-depleted media containing glucose or galactose. As controls, the growth patterns of the wild type and of K616 transformed with the vector alone were compared. Only K616 expressing SCA1p nt85 was able to grow on 10 mM EGTA-galactose medium (Figure 8A). This result suggests that SCA1p nt85, an N-terminal-truncation mutant of SCA1p, functions as an active Ca\textsuperscript{2+}-ATPase in yeast. To confirm the expression of SCA1p and SCA1p nt85, we fractionated microsomal membranes from yeasts over continuous sucrose gradients from 20 to 60%. Protein gel blot analyses of these gradients indicated that in yeast, SCA1p and SCA1p nt85 were most abundant in the endomembrane fractions rather than in the plasma membrane (Figure 8B).

**SCA1p Has Ca\textsuperscript{2+}/Calmodulin-Dependent Ca\textsuperscript{2+}-ATPase Activity**

To determine the enzymatic activity of SCA1p as a Ca\textsuperscript{2+}-ATPase, we produced the full-length enzyme (SCA1p) and N-terminal truncation mutant proteins (SCA1p nt52 and SCA1p nt85) in yeast. The mutant pumps, SCA1p nt52 and SCA1p nt85, have deletions of the first calmodulin binding domain (CaMBD1) or of both calmodulin binding domains (CaMBD1 and CaMBD2), respectively. These enzymes were partially expressed in yeast.
Calmodulin-Regulated Ca\(^{2+}\)-ATPase purified as an endomembrane-enriched fraction obtained by sucrose gradient fractionation of total microsomal membranes (Figure 8B). Enzymes present in the lighter sucrose fractions (e.g., fraction 5) were used to avoid the high background H\(^{+}\)-ATPase activity in the denser plasma membrane fractions. As a control, the equivalent fraction was analyzed from microsomal membranes from a yeast transformed with the vector only, pYES2, which were processed in parallel. To test these pumps for calmodulin-stimulated activity, we assayed samples from fraction 5 (~36% sucrose) for ATPase activity with increasing concentrations of calmodulin at a relatively high, fixed concentration of Ca\(^{2+}\) (5 \(\mu\)M). To compare the specific activities of a full-length pump and two mutant pumps, we corrected each activity for expression level, as determined by a scanned protein gel blot probed with affinity-purified anti-SCA1p antibody.

In the absence of calmodulin, the basal activity of SCA1p was very low; the activity was stimulated approximately six-fold by calmodulin in a dose-dependent manner (Figure 9). In contrast, the specific activities of SCA1p nt52 and SCA1p nt85 were ~280 and 440%, respectively, of the basal activity of SCA1p in the absence of calmodulin. SCA1p nt52 was further stimulated ~1.7-fold by calmodulin, but neither SCA1p nt85 nor the vector control was. Calmodulin activated SCA1p and SCA1p nt52 half-maximally at ~10 and 20 nM, respectively.

The Ca\(^{2+}\) dependence of these enzymes also was determined in the absence or presence of 100 nM calmodulin (Figures 10A and 10B). Samples from fraction 5 of each gradient were used for ATPase activity at different concentrations of free Ca\(^{2+}\). In the absence of calmodulin, SCA1p,

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**Figure 3.** SCA1p Cofractionates with a Plasma Membrane Marker in Sucrose Gradient Fractionation of Plant Membranes.

**A** Protein gel blot analyses of fractions after sucrose gradient fractionation of microsomal membrane from soybean. Soybean microsomal membranes were fractionated over continuous sucrose gradients of 15 to 45% (w/w), and 0.8-mL fractions were collected from the top of the gradients. Samples (10 \(\mu\)L) from each fraction were separated by 8% SDS-PAGE, transferred to an Immobilon-P membrane (Millipore), and probed with affinity-purified anti-SCA1p antibody (0.25 \(\mu\)g/mL), anti-H\(^{+}\)-ATPase antibody (DeWitt et al., 1996; 1:5000 dilution), and anti-BIP antibody (Denecke et al., 1991; Hofte and Chrispeels, 1992; 1:3000 dilution). Bands were detected by electrochemiluminescence and exposed to x-ray film.

**B** Marker enzyme assay using 10 \(\mu\)L of samples from membrane fractions for Triton-stimulated UDPase activity as a Golgi marker (Nagahashi and Kane, 1982).

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**Figure 4.** SCA1p Cofractionates with a Plasma Membrane Marker in Aqueous Two-Phase Partitioning of Plant Membranes.

Total proteins from 2-day-old soybean seedlings were fractionated into the soluble fraction (S) and the total microsomal membranes (M). Total microsomal membranes were fractionated into an upper phase (U) and a lower phase (L), corresponding to plasma membrane and endomembrane-enriched fractions, respectively. Samples (10 \(\mu\)g) from each fraction were separated by 8% SDS-PAGE and transferred to Immobilon-P membranes. Parallel protein gel blot analyses were performed by the procedure described in Figure 3.
SCA1p nt52, and SCA1p nt85 were half-maximally activated at \( z = 15, 0.6, \) and 0.2 to 0.3 \( \mu \text{M} \) free \( \text{Ca}^{2+} \), respectively. The equivalent fraction from the vector control had little ATPase activity (results not shown). When calmodulin was added, the \( K_{\text{1/2}} \) for \( \text{Ca}^{2+} \) of SCA1p was shifted from 15 to 1.5 \( \mu \text{M} \). However, the \( K_{\text{1/2}} \) values for \( \text{Ca}^{2+} \) of SCA1p nt52 and SCA1p nt85 were not substantially affected.

**Figure 5.** Confocal Microscopy Showing a Plasma Membrane Location for SCA1p Tagged with GFPp.

(A) Confocal microscopy images of green fluorescence from protoplasts expressing a GFPp-only control.
(B) Optical sectioning microscopy image of green fluorescence from protoplasts expressing SCA1-GFPp.
(C) and (D) The fluorescence image of SCA1-GFPp (C) compared with that of ACA2-GFPp (D), which is located at the ER membrane (Hong et al., 1999). Fluorescence images of SCA1-GFPp and ACA2-GFPp in protoplasts were detected under the same conditions by optical sectioning microscopy. Arrows indicate plasma membrane (PM). Bar in (B) = 0.5 \( \mu \text{m} \) for (A) and (B).

SCA1 Gene Expression Is Induced by Environmental Stresses

To examine if SCA1 gene expression can be altered in response to abiotic and biotic stress, we treated soybean suspension cells (SB-P cells; Horn et al., 1989) with 100 mM NaCl, 100 mM KCl, 200 mM mannitol, or 50 \( \mu \text{g/mL} \) of total reducing sugar prepared from Fusarium solani. Gel blots of total RNA isolated from treated suspension cells were probed for SCA1 mRNA (Figure 11). NaCl stress and fungal elicitor produced a marked 20-fold increase in SCA1 mRNA within 30 min. In controls using KCl and mannitol to produce an osmotic stress equivalent to 100 mM NaCl, we observed no increase in SCA1 mRNA. This result indicated that the accumulation of SCA1 mRNA was increased in response to a sodium ion stress rather than a general osmotic stress. In response to the NaCl stress treatment, the SCA1 mRNA was sustained at high quantities for 1 hr, then returned to nearly its basal value by 6 hr, despite the continued presence of 100 mM NaCl. In contrast, fungal elicitor-treated cells sustained high quantities of SCA1 mRNA for the first 6 hr after treatment before declining \( \sim 12 \) hr after treatment.

**DISCUSSION**

Biochemical evidence previously suggested the presence of a calmodulin-regulated \( \text{Ca}^{2+} \) pump in the plant cell plasma membrane (Evans, 1994; Bonza et al., 1998), but the corresponding gene for this was not identified until now. In this study, both membrane fractionation and cytology indicated that SCA1p was located in the plasma membrane, and biochemical and genetic evidence demonstrated that SCA1p was a calmodulin-stimulated \( \text{Ca}^{2+} \) pump.

The localization of SCA1p in the plasma membrane was determined by two different membrane fractionation protocols, buoyant density sucrose gradients and aqueous two-phase partitioning. In both protocols, SCA1p cofractionated with a typical plasma membrane marker, \( \text{H}^{+}\text{-ATPase} \) (Figures 3 and 4), rather than with control markers for endomembranes, such as the ER, chloroplasts, tonoplasts, and Golgi. This plasma membrane location was corroborated by cytological imaging of SCA1p tagged with a GFPp. In contrast to the fluorescent signal from a similarly tagged \( \text{Ca}^{2+} \) pump ACA2p, which showed a reticulate-like endomembrane localization (Hong et al., 1999), SCA1-GFPp showed a peripheral pattern of fluorescence, consistent with a plasma membrane location (Figure 5). This evidence from a GFPp-tagged pump was important because it provided an isoform-specific analysis coupled to a cytological verification.

Biochemical evidence that SCA1p was a calmodulin-stimulated ATPase was demonstrated by enzyme assays. Calmodulin stimulated ATPase activity by approximately sixfold in a dose-dependent manner, showing a half-maximum activation at \( \sim 10 \text{nM} \) for ScCAM-1 (Figure 9) or bovine calmodulin (not shown). This value represents a higher \( K_{\text{1/2}} \) than that reported for ACA2p (\( K_{\text{1/2}} = 30 \text{nM} \) bovine calmodulin), in agreement with prior findings on the biochemical properties of enzymes purified from plant tissues, that is, that \( \text{Ca}^{2+} \) pumps from the plasma membrane have a stronger binding affinity for calmodulin than do pumps from the tonoplast (Askerlund, 1996, 1997) or other endomembranes (Dainese et al., 1997; Bonza et al., 1998). The greater calmodulin binding affinity of SCA1p also might be attributable to the
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The presence of two CaMBDs, delineated here as CaMBD1 and CaMBD2 (Figure 6).

The presence of an N-terminal regulatory domain in SCA1p was demonstrated by an N-terminal deletion that resulted in a calmodulin-independent (i.e., deregulated) pump with kinetic properties similar to those of a full-length pump stimulated by calmodulin. Calmodulin stimulation of a full-length pump showed a five- to sixfold increase in $V_{\text{max}}$ (Figure 10A) and a 10-fold increase in Ca\textsuperscript{2+} affinity (from $K_{1/2} = 15$ \textmu M to $K_{1/2} = 1.5$ \textmu M) (Figure 10B), like a typical plasma membrane-type Ca\textsuperscript{2+} pump (Enyedi et al., 1987). In comparison, even in the absence of calmodulin, the $V_{\text{max}}$ of a truncated pump SCA1p nt85 was similar to that of a calmodulin-stimulated wild-type pump. This high constitutive activity of SCA1p nt85 was accompanied by a high affinity for Ca\textsuperscript{2+} ($K_{1/2} = 0.2$ to 0.3 \textmu M), regardless of whether calmodulin was present. Compared with ACA2p, a calmodulin-regulated Ca\textsuperscript{2+} pump in the ER membrane, SCA1p showed much lower affinity for Ca\textsuperscript{2+} (0.67 \textmu M versus 15 \textmu M for ACA2p) in the absence of calmodulin. When calmodulin was added, the $K_{1/2}$ of SCA1p for Ca\textsuperscript{2+} was shifted by $\sim$10-fold, but that of ACA2p was shifted only 1.5-fold (Hwang et al., 2000).

Genetic complementation of yeast mutant K616 by SCA1p nt85 confirmed that SCA1p can function as a Ca\textsuperscript{2+}
pump in vivo. Both full-length and truncated mutants were expressed in yeast K616, in which not only its two endogenous Ca\(^{2+}\) pumps (PMR1p and PMC1p) but also a Ca\(^{2+}\)/calmodulin-dependent protein phosphatase, calcineurin (CNB1p), are disrupted (Cunningham and Fink, 1994). An N-terminally truncated mutant (SCA1p nt85) allowed the yeast K616 to grow on Ca\(^{2+}\)-depleted media, whereas full-length SCA1p did not (Figure 8A). This ability of the truncated but not the full-length enzyme to provide complementation is probably the result of a higher constitutive Ca\(^{2+}\)-ATPase activity of the truncated enzyme. This hypothesis is consistent with in vitro ATPase assays, which show that the truncated enzyme has 4.4-fold more activity than the basal activity of the full-length enzyme (Figure 10). Under normal cytosolic conditions, the full-length SCA1p is expected to remain in a basal state because calmodulin activation requires above-normal calcium concentrations. Activation of the full-length enzyme is even less likely in cells grown on Ca\(^{2+}\)-depleted media (i.e., starved for calcium). However, we cannot rule out an alternative explanation that complementation by the truncated SCA1p is related to its relatively greater accumulation in a particular endomembrane compartment (Figure 8B). The unique ability of a truncated version of SCA1p to provide complementation also was seen with a truncated version of ACA2p (Harper et al., 1998). Although SCA1p is a plasma membrane pump in plants, its capacity to provide complementation in yeast probably resulted from an artifactual accumulation in the yeast secretory pathway (Figure 8B). Such mistargeting of heterologous proteins in yeast is common. For example, when a plant plasma membrane H\(^{+}\)-ATPase was expressed in yeast, most of the enzyme was retained in the ER membrane (Villalba et al., 1992). For complementation in K616 to occur, a Ca\(^{2+}\) pump is presumed to function in the ER–Golgi to scavenge enough Ca\(^{2+}\) from the cytosol for proper functioning of the secretory pathway.

Calmodulin binding overlay assays demonstrated that SCA1p contains two separable Ca\(^{2+}\)-dependent calmodulin binding sites in its N-terminal domain (Figure 6). The first calmodulin binding site (CaMBD1) is located in the first 40 residues and is probably a putative amphipathic basic helix between Leu\(^{21}\) and Phe\(^{38}\). The second (CaMBD2) includes the residues between positions 52 and 71 is probably a modified IQ motif between Ile\(^{57}\) and Val\(^{67}\). The modified IQ motif is similar to the CaMBD of an L-type Ca\(^{2+}\) channel (Zuhlke et al., 1999). Whether other related plant pumps also contain

**Figure 7.** Comparison of the Two Predicted Calmodulin Binding Domains (CaMBD1 and CaMBD2) with Motifs of Other Calmodulin Binding Proteins.

(A) Amino acid sequences of the first calmodulin binding domain (CaMBD1; 21 to 40 residues) were compared with other CaMBDs. Asterisks indicate the positions of SCA1p key residues Trp\(^{24}\), Cys\(^{28}\), Val\(^{31}\), and Phe\(^{38}\), which are believed to be important for binding to calmodulin. Conserved amino acid residues of CaMBD1 and other CaMBDs were aligned with these important positions. The other CaMBDs were ACA2p (Harper et al., 1998), BCA1p (Malmstrom et al., 1997), plasma membrane Ca\(^{2+}\) pump (PMCA; Kataoka et al., 1991), calcineurin (Kincaid et al., 1988), and calmodulin (CaM) kinase II (Meader et al., 1993).

(B) Amino acid residues 21 to 38 of SCA1p, plotted as an α-helical wheel. A basic hydrophilic face and a hydrophobic face are underlined with corresponding notes.

(C) A modified IQ motif of SCA1p (CaMBD2; 53 to 70 residues) aligned with other modified IQ motifs from rPMCA1b (Penniston and Enyedi, 1998), L-type Ca\(^{2+}\) channel (Zuhlke et al., 1999), P/Q-type Ca\(^{2+}\) channel, and R-type Ca\(^{2+}\) channel (Peterson et al., 1999). The consensus sequence for an IQ motif is shown at the top (Rhoads and Friedberg, 1997).
a second binding site functionally equivalent to this modified
IQ motif is unclear, given that similar deletion analyses have
not been done. However, the modified IQ motif in SCA1p
(CaMBD2) appears to facilitate a regulatory binding interac-
tion, as shown by the ability of calmodulin to stimulate a
truncation mutant (SCA1p nt52) in which the first site has
been deleted but the second remains intact. In an example
from an animal plasma membrane–type Ca2+ pump (hPMCA
4a), the presence of a second CaMBD was noted (Kessler et
al., 1992; Verma et al., 1996). Whether a two-binding-site
organization is common to all of the plant type IIB pumps or
instead endows specific isoforms with a different mode of
regulation awaits further investigation.

The abundance of SCA1 mRNA was rapidly and highly in-
duced by NaCl stress and fungal elicitor (Figure 11). This in-
crease was specific, given that a control transcript from
ScAM-1 did not show a similar response. NaCl stress, os-
motic stress, and a fungal elicitor have been shown to
cause increases in [Ca2+]cyt in plant cells (Knight et al., 1997;
Sanders et al., 1999), providing a basis for speculating that a
calcium signaling pathway upregulates SCA1 transcription.
At the same osmolality, however, KCl and mannitol did not
increase the SCA1 mRNA. These results suggest that cal-
cium signals specific to a NaCl stress or elicitor treatment
evoke an increase in SCA1 transcription or mRNA stability.
Although there are no prior reports of stress inducing the

Figure 8. Complementation of Yeast Mutant K616 through Expression of SCA1p nt85.

(A) Complementation of yeast Ca2+-ATPases mutant by an N-terminal-truncated mutant pump (SCA1p nt85). Wild-type (W303-1A) and triple
mutant (K616) cells were transformed with a vector (pYES2) alone, or with pYES2-SCA1 and pYES2-SCA1 nt85. The cells were streaked onto
SC-URA plates containing 10 mM EGTA, pH 6.0, and either glucose (Glc) or galactose (Gal); the cells were incubated for 4 days at 30°C. A dia-
agram indicates yeast strains and transformed vectors. Full-length and ΔN indicate SCA1p and SCA1p nt85, respectively.
(B) Sucrose gradient fractionation of SCA1p and SCA1p nt85. Microsomal membranes isolated from yeasts transformed with pYES2-SCA1 and
pYES2-SCA1 nt85 were fractionated over a continuous sucrose gradient of 20 to 60% (w/w), and 1-mL fractions were collected from the top of
each gradient. Samples (10 μL) from each gradient fraction were subjected to 8% SDS-PAGE and transferred to Immobilon-P membranes. The
gel blots were probed with purified anti-SCA1 antibody. Immunoreactive bands were detected by electrochemiluminescence and exposure to
x-ray film. Asterisks indicate the peak fractions for plasma membrane fractions, as determined by assays for vanadate-sensitive (calcium-inde-
dependent) H+-ATPase activity.
gene expression of a plant type IIB Ca\(^{2+}\) pump, NaCl stress has been reported to induce the expression of type IIA Ca\(^{2+}\) pump genes in tobacco and tomato (Perez-Prat et al., 1992; Wimmers et al., 1992). However, the induction reported here for SCA1 was at least 20-fold faster than in those studies. Interestingly, for SCA1 the NaCl stress and fungal elicitor produced different patterns of transcription activation, with the induction sustained twice as long by the elicitor. The consequences of upregulating the Ca\(^{2+}\) pump in response to these abiotic and biotic stresses is not understood, but we speculate it provides an adaptive response such that a stimulated cell would acquire an enhanced efflux capacity capable of substantially decreasing the magnitude or duration of a calcium release in response to further exposure to a given stimulus.

In summary, the discovery of SCA1p identifies a pathway for calmodulin-stimulated Ca\(^{2+}\) efflux across the plasma membrane in plant cells. In contrast to the analogous pump in animal cells, this plant pump belongs to a structurally unique subfamily of calmodulin-stimulated pumps harboring an N-terminal instead of a C-terminal regulatory domain. Members of this family have now been identified in four different subcellular locations in plant cells, providing a dramatic contrast to animal cells, in which calmodulin-regulated pumps appear to be used exclusively in the plasma membrane. It is not clear how members of the plant type IIB pumps are targeted to their different locations or what their specific functions may be.

**METHODS**

**Plant Material and Yeast and Escherichia coli Strains**

Soybean (Glycine max cv Williams) and soybean suspension cells (SB-P: Horn et al., 1989) were used for plant materials. Saccharomyces cerevisiae strains used for complementation studies, protein expression, and ATPase assays were W303-1A (MAT\(_a\), leu2, his3, ade2, ura3) and K616 (MAT\(_a\) pmr1::HIS3 pmc1::TRP1 cnb1::LEU2, ura3) (Cunningham and Fink, 1994). For DNA cloning, we used the E. coli strains XL1-Blue (Stratagene) and DH5\(_a\) (Stratagene). The expression of fusion protein was performed in E. coli BL21 (pLysS) DE3.

**Isolation of Ca\(^{2+}\)-ATPase cDNA from Soybean cDNA Library and 5' Rapid Amplification of cDNA Ends**

As a probe, a \(^{32}\)P-labeled F955 cDNA was used to screen a \(\lambda\) ZAPII (Stratagene)-based cDNA library constructed from half-apical and half-elongating regions of hypocotyls (0.3- to 1.3-cm-long sections below cotyledon tissues) of 4-day-old etiolated soybean seedlings (Hong et al., 1995). The F955 clone was a partial cDNA that encoded a putative plasma membrane-type Ca\(^{2+}\)-ATPase isolated from Brasicca flower bud cDNA (Lim et al., 1996). Positive plaques from the primary screening were purified by two additional rounds of plaque hybridizations. To isolate cDNA inserts from phages, we excised the purified positive plaques in vivo with a helper phage R408 (Stratagene).

To obtain 5' regions of a SCA1 cDNA, we performed 5' rapid amplification of cDNA ends (RACE) with a Marathon cDNA amplification kit according to the manufacturer’s instructions (Clontech, Palo Alto, CA). cDNA was synthesized from 1 \(\mu\)g of mature hypocotyl poly(A)\(^+\) RNA by using oligo(dT) primer (5'-TTTTTGN-N3') and Moloney Murine Leukemia Virus (MMLV) reverse transcriptase, and a unique adapter (Clontech) was ligated to both of the cDNA ends. The oligonucleotides used for the 5' RACE were a SCA1-specific oligonucleotide (5'-CATCCCCGGTCCAGTCCGTAAGC-3') and an adapter primer from Clontech. For polymerase chain reaction (PCR) amplification of 5' cDNA ends, a 1.250 portion of the adapter-ligated cDNA and the expand long polymerase (Boehringer Mannheim) were used in reactions with primer combinations subjected to the following amplification cycles: 30 cycles at 95°C for 2 min and at 68°C for 5 min and a final extension step at 68°C for 10 min. The amplified product was subcloned into the Smal site of pBluescript SK+ (Stratagene). A full-length cDNA clone, obtained by subcloning the 5' RACE product of SCA1 into the 5' regions of the SCA1 partial cDNA clone, was named pSCA1.

**Constructs**

We used standard PCR and subcloning procedures to modify SCA1 sequences into the clones described below. Standard molecular
techniques were used (Sambrook et al., 1989). PCR was performed using Ampli-Taq (Perkin-Elmer) or the expand long template PCR system (Boehringer Mannheim). All PCR-produced sequences were sequenced to verify the absence of PCR mistakes.

pSCA1-wt, which encodes a full-length SCA1p, was derived from pSCA1 by deleting the 5' and 3' untranslated regions. The following oligomers were synthesized: start primer S, 5'-AAAACTCGAGGAATCAGTGGGAGTTATTAATAGAAT-3'; and end primer AS, 5'-TTTTGTACCTCGGTCGTAGACCAGCAACAGAGAATCTTCTTTTAAGCCAGG-3'. Start primer S corresponded to the cDNA sequence encoding SCA1p and introduced XhoI and BamHI sites. End primer AS corresponded to the complement sequence of the coding strand and introduced a XbaI site. PCR was performed with pSCA1 as a template, and the fragment obtained was subcloned into the BamHI and XbaI sites of pBluescript SK-

pYES-SCA1 encodes the SCA1p full-length protein and was subcloned from a BamHI-XbaI fragment of pSCA1-wt into the BamHI-XbaI site of pYES2 (Invitrogen). pYES2-SCA1 nt17, pYES2-SCA1 nt41, pYES2-SCA1 nt52, pYES2-SCA1 nt71, and pYES2-SCA1 nt85 encode SCA1p nt17, SCA1p nt41, SCA1p nt52, SCA1p nt71, and SCA1p nt85, respectively. The following oligonucleotides were synthesized: SCA1 nt17S, 5'-AAAGGATCCATGGAAAGGTTCTTCAACGATGG-3'; SCA1 nt41S, 5'-AAAGGATCCATGGCACAATCCTTCAGAAGGAGCC-3'; SCA1 nt52S, 5'-AAAGGATCCATGGCGTCGTACCATTCAGGAG-3'; SCA1 nt71S, 5'-AAAGGATCCATGGCACAATCCTTCAGAAGGAGGC-3'; and SCA1 nt85S, 5'-AAAGGATCCATGGCACAATCCTTCAGAAGGAGGC-3'. These oligonucleotides corresponded to the coding strands and introduced BamHI sites; the ATG start codons are underlined. PCR was performed with pSCA1 as a template and by using each primer mentioned and end primer AS, and the BamHI-XbaI fragments were subcloned into the BamHI-XbaI sites of pYES2.

pGST-SCA1Ag encodes GST-SCA1Ag, which contains residues K468 to E 567 of SCA1p as a fusion with glutathione S-transferase (GST) at the N terminus. The primer SCA1Ag-S (5'-AAACCATGGGATCCAAAACATGCTTTTGCATGAACAGC-3') was synthesized. The SCA1Ag-S oligonucleotide corresponded to the cDNA encoding SCA1 and introduced NcoI and BamHI sites. PCR was performed using pSCA1 as a template and SCA1Ag-S primer and end primer AS. The pGST-SCA1Ag was derived from subcloning the NcoI-Ecl136II PCR fragment into the NcoI-SmaI sites of pAK-SS, a modified pGEX-2T vector (Amersham Pharmacia Biotech).

pGST-SCA1p (1-40)-GFP encodes the first 40 residues of SCA1p as a fusion protein with GST at the N terminus and a green fluorescent protein (GFP) at the C terminus. It was constructed by subcloning a PCR fragment into the Ncol-EcoRI site of pAK-SS. The sequence at the fusion site between SCA1 and GFP is TTCCCGGGAACCATG, where

Figure 10. Ca2⁺-Dependent Activation of Full-Length and N-Terminal Deletion Pumps.

Vanadate-sensitive ATPase activities of SCA1p (squares), SCA1p nt52 (diamonds), and SCA1p nt85 (circles) were determined at different concentrations of free Ca2⁺. Free Ca2⁺ concentration was determined using fluo-3, pentapotassium salt, and the Calcium Calibration Concentrated Buffer Kit, according to the manufacturer’s instructions (Molecular Probes). Representative data from one of three similar experiments are shown.

(A) ATPase activities measured in the absence of calmodulin (open symbols).

(B) ATPase activities measured in the presence of 100 nM ScaM-1 (filled symbols).
Figure 11. NaCl Stress and a Fungal Elicitor Increase SCA1 mRNA.

Each lane was loaded with 10 μg of total RNA isolated from SB-P cells treated with NaCl (100 mM), KCl (100 mM), mannitol (200 mM), or fungal elicitor for the times indicated. The gel blots were hybridized with a 32P-labeled SCA1 gene-specific probe and washed at high stringency at 60°C in 0.1 x SSC (see Methods). The parallel RNA gel blot analysis of SCaM-1 mRNA provides a control, showing the expression of gene that did not respond to any of the stress treatments. Equal loading of total RNA was verified by using ethidium bromide staining to detect rRNA (not shown).

DNA Sequencing and Data Analysis

Nucleotide sequences were determined from both strands of cDNAs by using a Taq dye primer sequencing kit on an automated DNA sequencer (model 373A; Applied Biosystems, Foster City, CA). Primary analyses of nucleotide and deduced amino acid sequences were performed using MacVector program (IBI Kodak, Rochester, NY). Transmembrane domains of Ca2+ -ATPases were predicted using TMpred and the PROSITE program at the ExPASy website.

RNA Gel Blot Analyses

Tissues and organs from various parts of 4-day-old etiolated soybean seedlings and 5-week-old mature soybean plants were divided into sections as described (Hong et al., 1989). Isolation of total RNA and RNA gel blot hybridization were performed as described (Lee et al., 1995). SB-P cell culture was maintained in KN-1 medium as described (Lee et al., 1995) with minor modifications. Immunized serum was precipitated with 50% (w/v) ammonium sulfate and resuspended in PBS. Polyclonal antiserum to SCA1Ag was purified to remove any remaining minor cross-reactivities by antigen affinity chromatographies with SCA1Ag peptide-Sepharose columns. The column was prepared by conjugating SCA1Ag peptide (residues K468 to E567) to cyanogen bromide-activated Sepharose 4B according to the procedure recommended by the manufacturer (Amersham Pharmacia Biotechnology). SCA1Ag peptide (residues K468 to E567) was obtained by thrombin digestion of purified GST-SCA1Ag fusion protein. Briefly, anti-SCA1Ag antiserum was bound to a SCA1Ag peptide-Sepharose column and washed with PBS. Anti-SCA1Ag antibody was eluted with 0.1 M glycine, pH 2.7, immediately neutralized with 0.1 M Tris-HCl, pH 8.0, and dialyzed against PBS in a Centricon (Millipore Corp., Bedford, MA) dialyzer. Protein gel blots were performed as described (Harper et al., 1998).

Preparation of Soybean Membrane Fractions

Microsomal membrane from 2-day-old etiolated soybean seedlings was prepared as described (Hong et al., 1999). All procedures were performed on ice or in a cold room (4°C) with prechilled buffers. Two-day-old etiolated soybean seedlings were homogenized with a mortar and pestle under a homogenization buffer of 20% (w/w) sucrose, 50 mM Tris-HCl, pH 8.0, 2 mM EDTA, 4 mM DTT, 2 mM phenylmethylsulfonyl fluoride, 20 μg/mL chymostatin, 20 μg/mL pepstatin, and 40 μM leupeptin. After filtration through two layers of Miracloth (Calbiochem), the homogenate was centrifuged at 12,000 g for 10 min to remove intact organelles and cell walls. The supernatant was centrifuged in a Type SW28 rotor (Beckman) at 100,000 g for 1 hr. The resulting supernatants were used as the soluble protein fraction. Membrane pellets were resuspended in homogenizing buffer (1 mL/10 g starting material) by using glass homogenizers. Microsomes (1 mL) were applied onto a continuous sucrose gradient from 15 to 45% (w/w) in a centrifugation buffer (10 mM Tris-HCl, pH 7.5, 1 mM
EDTA, 1 mM DTT, 2 mM phenylmethylsulfonyl fluoride, 20 μg/mL chymostatin, and 40 μM leupeptin). Gradients were centrifuged in a type SW41 rotor (Beckman Instruments) at 110,000g for 18 hr. Fractions (0.8 mL) were collected from the top, frozen in liquid nitrogen, and stored at −80°C until used. A refractometer was used to determine the sucrose concentration of each fraction.

### Aqueous Two-Phase Partitioning

Plant microsomal membranes were prepared as described above. After ultracentrifugation, the membrane pellet was resuspended in resuspension buffer (0.33 M sucrose, 4 mM KCl, and 5 mM KH₂PO₄, pH 7.8). Plasma membranes were partially purified by aqueous two-phase partitioning as described by Harper et al. (1998).

### Preparation and Transformation of Protoplast

Protoplasts of the tobacco cell line BY-2 were prepared and transformed by electroporation as previously described (Watanabe et al., 1987). Approximately 10⁶ protoplasts were transformed by electroporation with 15 μg of p35S-SCA1-GFP plasmid. After transformation, the protoplasts were resuspended in 10 mL of medium and cultured in 35-mm-diameter Petri dishes in the dark at 28°C for 24 hr before examination under a confocal microscope. Confocal laser scanning micrographs of a transformed protoplast were recorded using a Zeiss microscope (Axivert 100; Zeiss, Inc., Thornwood, NY) equipped with a laser scanning unit (LSM 510). Focal planes were scanned at 488 nm with an argon krypton laser, using a 550-nm barrier filter and a plan-Apo 63×, 1.4 NA oil-immersion objective lens. Optical sections were made using a software program provided by the manufacturer.

### Calmodulin Binding Overlay Assays

Calmodulin gel overlay was performed as described (Lee et al., 1997) with minor modifications. Samples (1 to 5 μg) mixed with SDS sample buffer were separated on 8% or 10% SDS–polyacrylamide gels. After the proteins were transferred onto an Immobilon-P membrane (Millipore), the gel blot was blocked by incubating for 1 hr with TBS-T (Tris-buffered saline [25 mM Tris-HCl, pH 7.4, 137 mM NaCl, 27 mM KCl] plus 0.1% [w/v] Tween 20) containing 6% (w/v) nonfat dry milk. After being washed three times in TBS-T for 5 min, the gel blot was equilibrated in buffer G (50 mM imidazole, pH 7.5, and 150 mM NaCl) for 1 hr. The gel blot was further blocked for another 2 hr in buffer G containing 9% (w/v) gelatin from cold-water fish skin (Sigma), 0.5% (w/v) Tween 20, and 1 mM CaC₂. The horseradish peroxidase (HRP)-conjugated SCAm-1 (Lee et al., 1997) used as a probe was added to the gelatin-containing buffer G at a final concentration of 0.2 μg/mL (~12 nM). After incubation for 2 hr with the probe, the gel blot was sequentially washed five times with each of the following (5-min period) washes: buffer W (TBS-T containing 1 mM CaC₂ and 50 mM imidazole, pH 7.5), buffer W containing 0.2 M KCl, and finally 20 mM Tris-HCl, pH 7.5, containing 1 mM CaC₂, 0.1% (w/v) Tween 20, and 1 mM MgCl₂. After the washing, bound SCAm-1 conjugated with HRP was detected using the ECL detection kit (Amersham Pharmacia Biotechnology). As a control, the same procedure was repeated in the presence of 5 mM EGTA instead of 1 mM CaC₂.

### Yeast Transformation

For transformations, W303-1A and K616 were grown in standard yeast extract peptone dextrose (1% [w/w] yeast extract, 2% [w/w] peptone, 2% [w/w] dextrose) (YPD) media supplemented with 10 mM CaC₂. Yeast was transformed with the pYES2 vector (Invitrogen) and with pYES2-SCA1, pYES2-SCA1 nt17, pYES2-SCA1 nt41, pYES2-SCA1 nt52, pYES2-SCA1 nt71, and pYES2-SCA1 nt85 by using the lithium acetate/polyethylene glycol methods (Ebbe, 1992). Transformants were selected for uracil prototrophy by plating on synthetic complete medium minus uracil (SC-URA) supplemented with 2% (w/v) glucose as a carbon source and 2% (w/v) agar. For complementation studies, colonies were streaked on SC-URA agar plates containing 2% (w/v) glucose or galactose and 10 mM EGTA, pH 6.0, and incubated for 4 days at 30°C.

### Isolation of Yeast Membranes

Yeast cells were grown to late log phase with shaking at 30°C in 500 mL of SC-URA containing 10 mM CaC₂, harvested, and washed with cold water. Yeast cells were homogenized and fractionated on a continuous gradient of 20 to 60% (w/v) sucrose (Harper et al., 1998). One-milliliter fractions were collected from the top, frozen in liquid N₂, and stored at −70°C until used. A refractometer was used to determine the sucrose concentration of each fraction.

### ATPase Assays

ATPase assays were performed as described by Lanzetta et al. (1979). All samples (10 μL) were assayed at 25°C in a buffer of 20 mM MOPS, 8 mM MgSO₄, 50 mM KNO₃, 0.25 mM K₂MoO₄, 3 mM ATP, 5 mM NaN₃, and 1 mM EGTA in the presence or absence of 0.1 mM sodium vanadate. Liberated free phosphate was measured using malachite green (Lanzetta et al., 1979). ATPase activity was calculated as the amount of vanadate-sensitive phosphate released. Calmodulin stimulation was tested by the addition of SCAm-1 or bovine calmodulin at ~5 μM free [Ca²⁺]. To measure Ca²⁺ affinity, we measured the activities at several different concentrations of free Ca²⁺ in the presence or absence of SCAm-1. SCAm-1 was purified as described (Lee et al., 1995), and bovine brain calmodulin was obtained from Sigma. Free [Ca²⁺] was determined by the use of fluo-3, pentapotassium salt, and the Calcium Calibration Concentrated Buffer Kit (Molecular Probes, Eugene, OR), according to the manufacturer’s instructions. In brief, a 1 mM fluo-3 stock was made in distilled water, aliquoted, and stored in the dark at −20°C. Using a final 1-μM concentration of fluo-3, we constructed a calibration curve based on known free [Ca²⁺] by exciting the sample at 425 nm and reading the emission at 520 nm in a spectrofluorometer. The emission at 520 nm of the 425-nm excited samples containing the ATPase reaction mix and various free Ca²⁺ concentrations was determined, and free [Ca²⁺] was calculated from the calibration curve.

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Identification of a Calmodulin-Regulated Soybean Ca\textsuperscript{2+}-ATPase (SCA1) That Is Located in the Plasma Membrane

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