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A Single Gene May Encode Differentially Localized Ca^{2+}-ATPases in Tomato

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Previously, a partial-length cDNA and a complete genomic clone encoding a putative sarcoplasmic reticulum-type Ca^{2+}-ATPase (LCA, *Lycopersicon* Ca^{2+}-ATPase) were isolated from tomato. To determine the subcellular localization of this Ca^{2+}-ATPase, specific polyclonal antibodies raised against a fusion protein encoding a portion of the LCA polypeptide were generated. Based on hybridization of the LCA cDNA and of the nucleotide sequence encoding the fusion protein to genomic DNA, it appears that LCA and the fusion protein domain are encoded by a single gene in tomato. Antibodies raised against the LCA domain fusion protein reacted specifically with two polypeptides of 116 and 120 kD that are localized in the vacuolar and plasma membranes, respectively. The distribution of vanadate-sensitive ATP-dependent Ca^{2+} transport activities in sucrose gradients coincided with the distribution of the immunodetected proteins. The ATP-dependent Ca^{2+} transport activities associated with tonoplast and plasma membrane fractions shared similar properties, because both fractions were inhibited by vanadate but insensitive to carbonyl cyanide *m*-chlorophenylhydrazone, nitrate, and calmodulin. Moreover, antibodies raised against the LCA domain fusion protein inhibited ATP-dependent Ca^{2+} uptake activity associated with both the tonoplast and plasma membrane fractions. These data suggest that a single gene (*LCA*) may encode two P-type Ca^{2+}-ATPase isoforms that are differentially localized in the tonoplast and plasma membrane of tomato roots.

INTRODUCTION

Calcium is widely recognized as an important second messenger in eukaryotic organisms (Hepler and Wayne, 1985; Bush, 1993; Poovaiah and Reddy, 1993). Calcium fluxes have been associated with major changes in metabolism in plants and animals (Hepler and Wayne, 1985; Hochachka, 1986), and it has been proposed that transient fluctuations in cytoplasmic Ca^{2+} concentrations may play a central role in both chilling and salt stress of plant cells (Minorsky, 1985; Rengel, 1992). Maintaining a low, free cytoplasmic concentration of Ca^{2+} is necessary for its function as a second messenger, and these low concentrations of cytosolic calcium are maintained by the action of active Ca^{2+} transport systems. In plant cells, two types of transporters have been identified: high-affinity Ca^{2+}-ATPases localized in the endoplasmic reticulum (ER), plasma membrane (PM), and tonoplast (TN), and a lower affinity Ca^{2+}/H^+ antiport localized in the vacuolar membranes (Evans et al., 1991; Chanson, 1993). Although the plant cell vacuolar Ca^{2+}/H^+ antiport is likely to play an important role in high-capacity intracellular storage, high-affinity Ca^{2+}-ATPase–driven Ca^{2+} transport has been proposed to play the most significant role in modulating cytosolic Ca^{2+} levels in the physiological range (Carafoli, 1987; Briskin, 1990).

Plant Ca^{2+}-ATPases belong to the evolutionarily related family of P-type ATPases that includes the PM H^+-ATPase of fungi and plants, the Na^+/K^+-ATPase of animals, and Ca^{2+}-ATPases of plants and animals (Serrano, 1989). In animals, Ca^{2+}-ATPases on both the sarcoplasmic reticulum (SER) and PM have been well characterized and can be distinguished from each other by several criteria. The SER Ca^{2+}-ATPase has a molecular mass of 110 kD, and the PM Ca^{2+}-ATPase has a molecular mass of 140 kD. The PM Ca^{2+}-ATPase is stimulated by Ca^{2+}/calmodulin, whereas the SER Ca^{2+}-ATPase is not (Carafoli, 1987, 1991; Schatzmann, 1989).

Plant ER and PM Ca^{2+}-ATPases have been characterized extensively by measuring Ca^{2+} uptake and calcium-dependent phosphoenzyme formation. However, the differences between Ca^{2+}-ATPases of the PM and ER have not yet been well established. Both Ca^{2+}-ATPases are inhibited by vanadate, have an affinity constant for Ca^{2+} of <1 μM, require Mg^{2+}, and have a high affinity for Mg-ATP, although the PM Ca^{2+}-ATPase can also use other Mg-nucleotide triphosphates (Chanson, 1993; Evans, 1994). The reported molecular masses for the PM and ER Ca^{2+}-ATPases from different plant species vary widely. For example, the PM Ca^{2+}-ATPases from maize have a reported molecular mass of 140 kD (Briars and Evans, 1989), whereas values of 120 and 100 kD were reported for the PM Ca^{2+}-ATPase from carrot cells (Hsieh et al., 1991) and red beets (Briskin, 1990), respectively. Also, the molecular mass of the ER Ca^{2+}-ATPase was estimated as 120 kD in carrot cells (Chen et al., 1993), whereas the ER Ca^{2+}-ATPases in red

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beet roots (Briskin, 1990) and cauliflower inflorescences (Thomson et al., 1993) were 100 and 115 kD, respectively. Moreover, there are conflicting reports concerning the calmodulin sensitivity of Ca^{2+}-ATPases in plant ER and PM preparations (Evans, 1994), indicating the possibility of biochemical differences between species and/or organs. Reports characterizing plant vacuolar Ca^{2+}-ATPases are less abundant, but their presence has been demonstrated in several plants (Malatialy et al., 1988; Zocchi, 1988; Dupont et al., 1990; Gavin et al., 1993).

To gain insight into the structure, localization, abundance, and function of plant Ca^{2+}-ATPases, we isolated a partial-length cDNA clone (LCA1, for *Lycopersicon* Ca^{2+}-ATPase) and a complete genomic clone (gLCA13) encoding a Ca^{2+}-ATPase in tomato with extensive sequence similarity to the animal SER Ca^{2+}-ATPases (Wimmers et al., 1992). A similar cDNA clone was isolated that encodes a partial sequence of a putative ER Ca^{2+}-ATPase of tobacco (Perez-Prat et al., 1992). In tomato, the deduced amino acid sequence specifies a protein with a molecular mass of ~116 kD, eight possible transmembrane domains, all of the highly conserved functional domains common to P-type cation translocating ATPases, and three domains that are conserved between mammalian SER Ca^{2+}-ATPases. Genomic DNA gel blot analysis indicated that the tomato Ca^{2+}-ATPase is encoded by a single gene, whereas RNA gel blot analysis indicated the presence of three transcript sizes in roots and a single one in leaves (Wimmers et al., 1992).

Based on its substantial sequence similarity to SER Ca^{2+}-ATPases, the LCA Ca^{2+}-ATPase was proposed to be ER localized. The goal of our research has been to test this proposal and to determine the precise subcellular localization of the LCA Ca^{2+}-ATPase. To this end, we generated specific polyclonal antiserum raised against an LCA polypeptide domain and localized the LCA Ca^{2+}-ATPase by immunodetection of the protein after fractionation of cell membranes.

RESULTS

LCA Fusion Protein Domain Antiserum

The LCA domain selected for expression as a fusion protein for antibody production is indicated by the boxed region in the hydropathic profile of the deduced LCA amino acid sequence shown in Figure 1A. This domain corresponds to a hydrophilic and antigenic region that does not overlap with any of the highly conserved P-type ATPase functional domains, which would be expected to be found in multiple cellular P-type ATPases. A comparison of the LCA domain used for antibody production with the deduced amino acid sequences from the GenBank and EMBL DNA sequence data base revealed high sequence similarity only to animal Ca^{2+}-transporting ATPases and did not reveal high sequence similarity to any other plant protein sequence. To determine the specificity of the antibodies result-

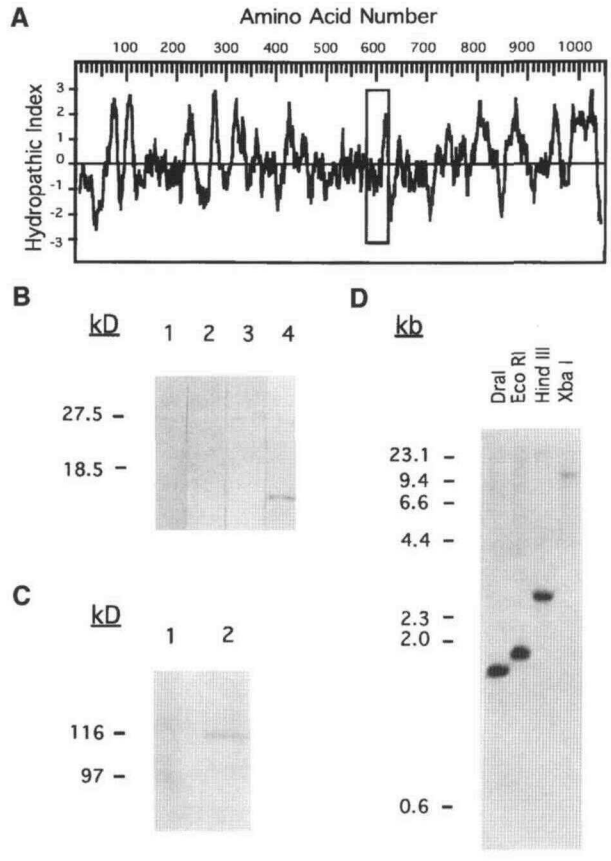

Figure 1. Antibodies to a Hydrophilic Domain of the LCA Ca^{2+}-ATPase Correspond to a Unique Sequence in Tomato Genomic DNA.

(A) Hydropathic profile of the deduced LCA amino acid sequence from Wimmers et al. (1992). The boxed region shows the LCA domain used for expression as fusion protein for antibody production and to make a cDNA probe for genomic DNA gel blot analysis.

(B) Characterization of LCA antibodies. IPTG-induced (lanes 2 and 4) and uninduced (lanes 1 and 3) extracts from *E. coli* were separated by SDS-PAGE (15% acrylamide), blotted, and incubated with the preimmune (lanes 1 and 2) or immune (lanes 3 and 4) serum at 1:10,000 dilution. Numbers at left indicate the sizes of low molecular mass markers.

(C) Characterization of LCA antibodies used to probe microsomal proteins. Tomato root microsomal proteins were separated on SDS gels, blotted, and incubated with the preimmune (lane 1) or immune (lane 2) serum at 1:1000 dilution. Numbers at left indicate the sizes of high molecular mass markers.

(D) Genomic DNA gel blot hybridization analysis of the LCA domain used for antibody production. Each lane was loaded with 10 μg of tomato genomic DNA digested with the indicated restriction enzymes, and the blot was hybridized at low stringency to nucleotides 1740 to 1872 of the LCA cDNA. Numbers at left indicate the lengths of DNA markers.

ing from immunization of rabbits with the LCA fusion protein, immunoblot analysis of isopropyl β-D-thiogalactopyranoside (IPTG)–induced and uninduced extracts from *Escherichia coli* and crude microsomal proteins from tomato roots was carried out. Figure 1B shows that the antiserum reacted specifically with the IPTG-induced fusion protein made by *E. coli*. Furthermore, Figure 1C shows that the antiserum reacted specifically with a protein of 116 kD in tomato root microsomes, in good agreement with the molecular mass predicted by the cDNA sequence.

To assess whether the LCA domain that was used for antibody production might also reside in other tomato proteins, the same domain also was used to probe gel blots of tomato genomic DNA. Figure 1D shows that at low stringency (40°C below the melting temperature), the probe hybridized to a single genomic restriction fragment in each digest. This confirmed previous results indicating that LCA is encoded by a single gene in tomato (Wimmers et al., 1992) and further indicated that the LCA domain used for antibody production is found exclusively in the *LCA* gene.

Distribution of LCA Ca^{2+}-ATPase in Membrane Fractions

To assess the subcellular distribution of the LCA protein, tomato root membranes were separated on continuous sucrose gradients in the presence or absence of Mg^{2+}, and the LCA fusion domain antibodies were used to detect the LCA protein by immunoblot analysis of membrane fractions. Figures 2A and 2B show that in fractionated membranes, the antibodies specifically detected two proteins of 116 and 120 kD that were present in distinct subcellular membranes. The 120-kD polypeptide was less abundant than the 116-kD polypeptide, and this may account for why it was not detected in crude microsomes (Figure 1C).

To identify the specific subcellular localization of these immunodetected LCA polypeptides, membrane fractions were characterized using enzyme markers as well as specific antibodies to proteins associated with different subcellular membranes. Figures 2 and 3 show that when Mg^{2+} was present in the homogenization and gradient buffers, BiP (Figure 2) and the NADH–cytochrome *c* reductase activity (Figure 3B) were shifted to higher densities, which is expected for the behavior for ER markers (Quail, 1979). In the presence of Mg^{2+}, the TN markers (58-kD subunit of the vacuolar ATPase or V-ATPase, in Figure 2B, or nitrate-sensitive ATPase activity, in Figure 3D) were separated from the ER markers, but the PM marker (H^+-ATPase, in Figure 2 or Figure 3C) cofractionated with the ER. In contrast, in the absence of Mg^{2+}, the ER markers cofractionated with the TN markers but were clearly separated from the PM. Thus, the characteristic Mg^{2+} density shift of the ER allowed us to assess whether the LCA protein was ER localized.

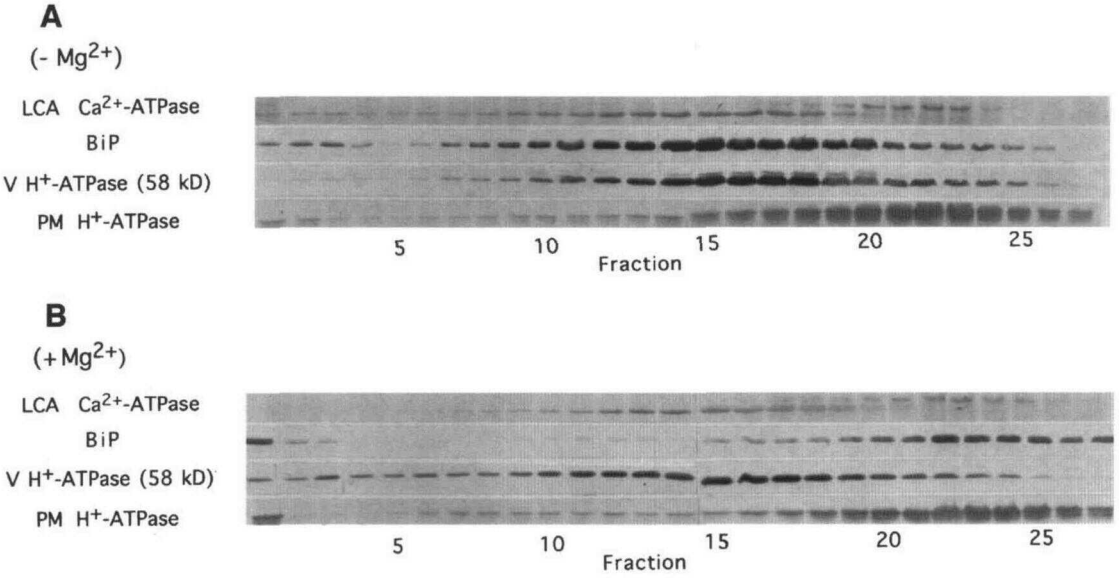

Figure 2. Protein Gel Blot of Membrane Fractions from Tomato Roots Probed with LCA Ca^{2+}-ATPase, BiP, PM H^+-ATPase, and V-ATPase Antibodies.

(A) Membranes fractionated in the presence of 2 mM EDTA.

(B) Membranes fractionated in the presence of 2 mM EDTA and 5 mM $MgCl_2$.

Vesicles (20 to 50 μg) were separated by SDS-PAGE (7.5% acrylamide), blotted, and probed with anti–Ca^{2+}-ATPase antibody (1:1000 dilution), anti-BiP antibody (1:1000 dilution), anti–PM H^+-ATPase antibody (1:5000 dilution), or anti–58-kD subunit of V-ATPase antibody (1:2000 dilution). Results are from one experiment but representative of two experiments.

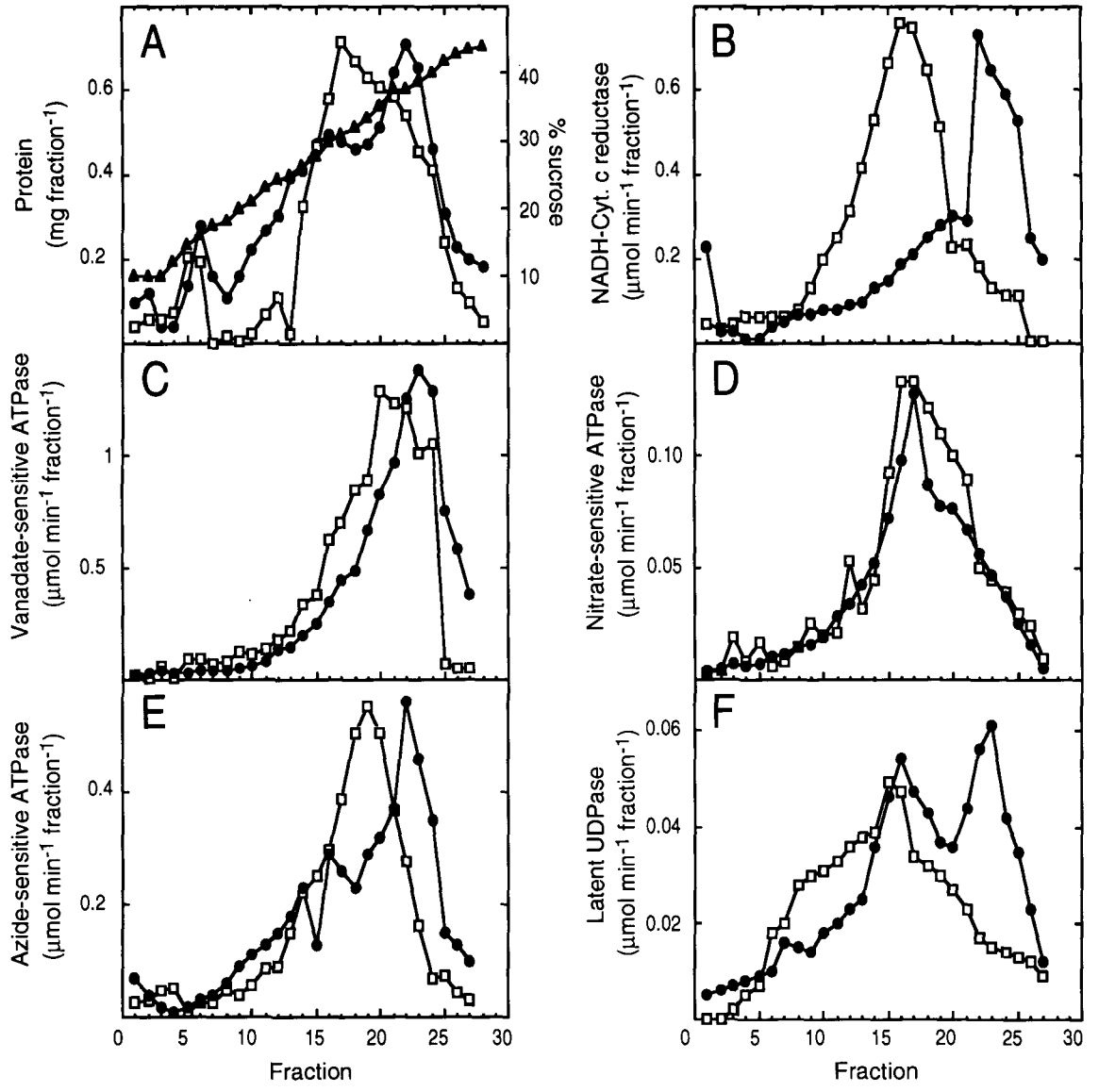

Figure 3. Distribution of Marker Enzymes Associated with Tomato Root Microsomal Membranes on Continuous Sucrose Gradient (15 to 45% [w/w]) in the Presence (●) or Absence (□) of Mg^{2+}.

(A) Percentage of sucrose (▲) and protein distribution.
(B) NADH–cytochrome *c* (NADH–Cyt. c) reductase activity (ER).
(C) Vanadate-sensitive ATPase activity (PM).
(D) Nitrate-sensitive ATPase activity (TN).
(E) Azide-sensitive ATPase activity (mitochondria).
(F) Latent UDPase (Golgi apparatus).

Figure 2 shows that the distribution of the proteins detected by the LCA antibodies was not shifted by Mg^{2+} in the gradient, indicating that the LCA Ca^{2+}-ATPase was not localized in the rough ER in tomato root cells. However, because there is some NADH–cytochrome *c* reductase activity associated with the TN-enriched fraction in the Mg^{2+} gradient, it is possible that the 116-kD protein is also present in the smooth ER. In the presence of Mg^{2+}, the azide-sensitive ATPase activity was shifted slightly to a higher density (Figure 3E), and there is a second peak of UDPase activity (Figure 3F), which indicates that the LCA Ca^{2+}-ATPase was neither mitochondria nor Golgi localized. The distribution of the 116-kD LCA polypeptide coin-

cides in both gradients with the observed distribution for the V-ATPase (Figure 2) and the nitrate-sensitive ATPase activity (Figure 3D), whereas the 120-kD polypeptide cofractionated with the PM markers (Figure 2 or Figure 3C). These data strongly suggest that the LCA antibodies react specifically with a TN-localized protein with an apparent molecular mass of 116 kD and with a less abundant PM-localized protein of 120 kD.

To analyze further the specificity of the antibodies, blocking experiments with the LCA fusion protein were performed. Figure 4 shows that incubating the crude antiserum with the fusion protein before its use for immunoblots strongly reduced the signal intensity of the 116- and 120-kD immunoreactive polypeptides in the TN- and PM-enriched fractions, respectively. These results indicate that the immunoreactivity of both the 116- and 120-kD polypeptides observed in Figures 1C and 2 is due to the Ca^{2+}-ATPase epitope defined by the LCA fusion protein.

Distribution of ATP-Dependent Ca^{2+} Uptake in Membrane Fractions

To determine whether there is a Ca^{2+}-ATPase associated with the TN and with the PM in tomato roots and the relationship of such an activity with the LCA protein, we monitored the distribution of ATP-dependent Ca^{2+} transport activity on continuous sucrose gradients. Transport activity was measured in the presence of 5 μM carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) to collapse the H^+ electrochemical gradient established by H^+-ATPases and therefore inhibit the activity of Ca^{2+}/H^+ antiporters that also may reside in these membranes. Figures 5A and 5B show that two peaks of ATP-dependent $^{45}Ca^{2+}$ transport were found in sucrose gradients prepared in both the presence and absence of Mg^{2+}. In the absence of Mg^{2+}, the high-density peak of Ca^{2+} transport cofractionated with the PM fraction and was well resolved from the ER markers. In the presence of Mg^{2+}, the low-density peak of Ca^{2+} transport coincided with the peak of V-ATPase and was well separated from the PM and ER markers.

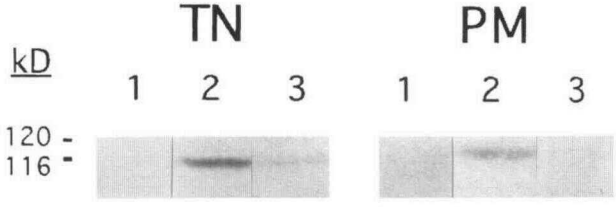

Figure 4. Blocking of LCA Antibodies with the LCA Fusion Protein.

Fifty micrograms of TN and PM proteins was separated on SDS gels, blotted, and incubated overnight with the preimmune antiserum (lane 1), crude immune antiserum (lane 2), or "blocked" immune antiserum that was preincubated with LCA fusion protein (lane 3), as described in Methods. Antiserum dilution was 1:5000. Numbers to left of figure refer to protein molecular mass in kilodaltons (kD), as determined by *molecular mass markers*.

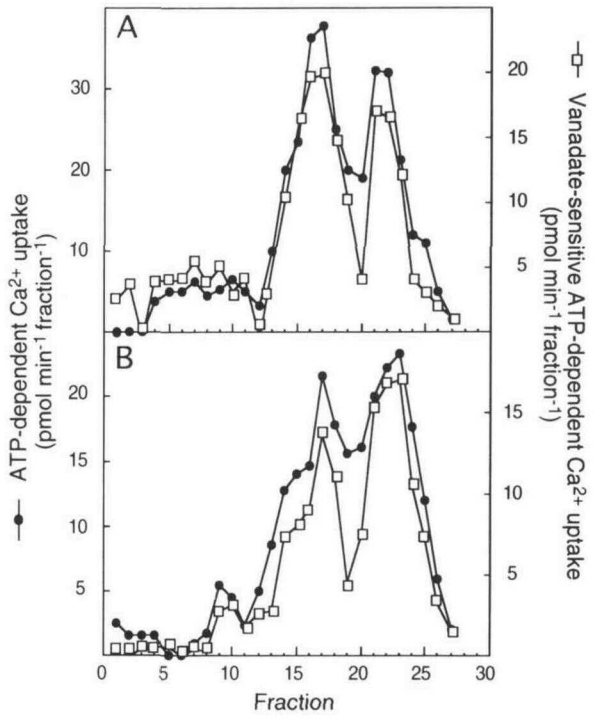

Figure 5. Distribution of ATP-Dependent Calcium Uptake Activity on a Continuous Sucrose Gradient in the Presence or Absence of Mg^{2+}.

(A) EDTA (2 mM) in the homogenization and in the gradient buffers. **(B)** EDTA (2 mM) and $MgCl_2$ (5 mM) in both the homogenization and gradient buffers.

The calcium transport was assayed in the presence of 5 μM CCCP. Gradient fractions (20 to 50 μg) were assayed for uptake of $^{45}Ca^{2+}$ in the absence of ATP and in the presence of ATP or ATP plus 100 μM vanadate. ATP-dependent Ca^{2+} uptake (●) was calculated as the difference in activity measured in the presence and absence of ATP. Vanadate-sensitive ATP-dependent uptake (□) was calculated as the difference in activity measured in the presence or absence of vanadate. All assays were done in duplicate and repeated.

Moreover, the distribution of these peaks of Ca^{2+} transport is similar to the distribution of the 116- and 120-kD polypeptides recognized by the LCA antibodies, supporting the identification of these proteins as Ca^{2+}-ATPases.

Ca^{2+} uptake was also assayed in the presence of vanadate, an inhibitor of P-type ATPases. When vanadate was present in the assay medium, Ca^{2+} uptake was inhibited. Figures 5A and 5B show the distribution of the vanadate-sensitive ATP-dependent Ca^{2+} uptake in the EDTA and in the Mg^{2+} gradients, respectively. These experiments provide further support for the hypothesis that P-type ATP-dependent Ca^{2+} pumps are present in tomato root membranes and that these pumps cofractionate with the 116- and 120-kD polypeptides recognized by the LCA antibodies. However, a small proportion of vanadate-sensitive ATP-dependent Ca^{2+} uptake activity does shift from the low-density to high-density peak in the Mg^{2+} gradient. This result suggests that a small proportion of the total Ca^{2+}-

ATPase activity in tomato root microsomes is ER localized. Because the immunodetected 116- and 120-kD polypeptides recognized by the LCA antibodies do not exhibit a similar density shift, we conclude that the ER-localized Ca^{2+}-ATPase does not cross-react with the LCA antibodies.

Characterization of Ca^{2+}-ATPase Activity in TN and PM Fractions

To characterize further the Ca^{2+} transport associated with tomato TN and PM vesicles, inhibitor sensitivity of ATP-dependent Ca^{2+} uptake was determined. TN- or PM-enriched fractions were prepared by pooling fractions 16 to 19 from the Mg^{2+} gradient or fractions 21 to 24 from the gradient without Mg^{2+}, respectively. Table 1 shows that Ca^{2+} uptake associated with the TN fraction was highly inhibited by nitrate, whereas the Ca^{2+} uptake activity associated with the PM fraction was nitrate insensitive. Inhibition by vanadate and *N,N'*-dicyclohexylcarbodiimide was greater in the PM fraction than in the TN fraction. Nigericin and CCCP inhibited $^{45}Ca^{2+}$ uptake in the TN vesicles by ~70 and 30% in the PM vesicles. In the presence of CCCP, nitrate did not affect the Ca^{2+} uptake activity associated with the vacuolar membranes. These data show that the principal Ca^{2+} transport activity in the TN is that of a Ca^{2+}/H^+ antiporter driven by the nitrate-sensitive V-ATPase or the pyrophosphatase; there was also a considerable Ca^{2+} uptake driven by a primary Ca^{2+}- ATPase. However, in the PM vesicles, a Ca^{2+}-ATPase is primarily responsible for Ca^{2+} transport activity, and the antiport activity associated with this fraction is likely to be due to mitochondrial contamination. The uptake of Ca^{2+} into the TN and PM vesicles was released fully upon addition of the Ca^{2+} ionophore A23187, and it was calmodulin insensitive.

Table 1. Effect of Inhibitors on Ca^{2+} Uptake in Tomato TN- and PM-Enriched Vesicles

	Ca^{2+} Uptake (% of Control)[a]	
Inhibitor	TN	PM
Control	100	100
50 mM nitrate	32 ± 2	96 ± 3
200 μM azide	87 ± 2	72 ± 4
100 μM vanadate	69 ± 1	28 ± 2
100 μM *N,N'*-dicyclohexylcarbodiimide	67 ± 3	13 ± 1
1 μM calmodulin	98 ± 6	104 ± 4
1 μM nigericin	35 ± 4	76 ± 3
5 μM CCCP	39 ± 0	63 ± 5
5 μM CCCP + 50 mM nitrate	38 ± 1	65 ± 2
5 μM CCCP + 100 μM vanadate	15 ± 5	2 ± 1
5 μM CCCP + 1 μM calmodulin	41 ± 2	69 ± 4
5 μM A23817	2 ± 1	7 ± 3

[a] Calcium uptake was determined, in the presence of the indicated inhibitors, as indicated in Methods. Data are the means and standard deviations for duplicate assays from two independent experiments.

Effect of LCA Fusion Protein Antibodies on Ca^{2+} Uptake by the TN and PM

To confirm that the immunodetected proteins correspond to Ca^{2+}-ATPases, we assessed the effect of the antibodies on the ATP-dependent Ca^{2+} uptake in the TN and the PM fractions. Membranes were incubated for 30 min at 37°C with increasing amounts of preimmune and immune serum, and Ca^{2+} transport activity was assayed in the presence of 5 μM CCCP to abolish transport associated with Ca^{2+}/H^+ antiporter activity. Figures 6A and 6B show that under these conditions, the immune serum (150 μg) inhibited Ca^{2+} uptake by ~65% in the TN-enriched fraction and by 80% in the PM-enriched fraction.

DISCUSSION

In this study, we show that antibodies raised against a polypeptide of a putative Ca^{2+}-ATPase (LCA) react specifically with a 116-kD protein localized in the TN and with a less abundant 120-kD polypeptide localized in the PM. The finding that these antibodies also inhibit ATP-dependent Ca^{2+} uptake activity associated with these membranes suggests that these proteins are Ca^{2+}-ATPases that are immunologically related. Our results also indicate the presence of an ER-localized vanadate-sensitive Ca^{2+}-ATPase, which was relatively less abundant than the PM and TN forms and did not cross-react with the LCA antibodies.

Recently, antibodies against a peptide that is conserved between the animal SER Ca^{2+}-ATPase isoform 1 and the deduced amino acid sequences of tomato (LCA) and tobacco Ca^{2+}-ATPases have been raised. In maize shoots, these antibodies identified two proteins of 91 and 102 kD that are localized in different subcellular membranes (Logan and Venis, 1995). This suggests that a family of plant Ca^{2+}-ATPases exists that is expressed in a tissue-specific manner and has a heterogeneity of size and localization. The presence of a single hybridizing restriction fragment on genomic DNA gel blots of tomato DNA probed at very low stringency with the LCA domain used to make the antibodies further suggests the possibility that a single gene product may be processed to give rise to two different polypeptides that are differentially localized. Because multiple mRNA transcripts corresponding to the Ca^{2+}-ATPase have been detected in tomato roots (Wimmers et al., 1992), it is possible that differential mRNA processing gives rise to distinct Ca^{2+}-ATPase isoforms, as has been observed for the animal SER Ca^{2+}-ATPases (Lytton and MacLennan, 1988; Dormer et al., 1993; Van den Bosch et al.,

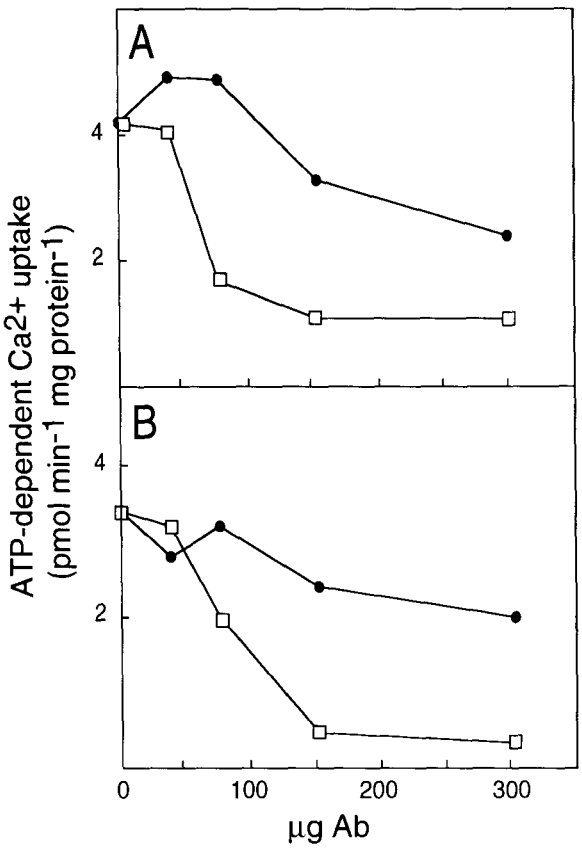

Figure 6. Inhibition of ATP-Dependent, CCCP-Insensitive Ca^{2+} Uptake into Vesicles of TN- or PM-Enriched Fractions by Antibodies Raised against Tomato Ca^{2+}-ATPase.

(A) TN-enriched fraction. Vesicles (20 µg) from the TN-enriched fraction of a continuous sucrose gradient were preincubated with immune serum to tomato Ca^{2+}-ATPase (□) or preimmune serum (●) for 30 min at 37°C in the assay medium without ATP, and uptake was initiated by the addition of ATP. Each point is the mean of triplicate measurements.

(B) PM-enriched fraction. Vesicles (20 µg) from the PM-enriched fraction of a continuous sucrose gradient were preincubated with immune serum to tomato Ca^{2+}-ATPase (□) or preimmune serum (●) for 30 min at 37°C in the assay medium without ATP, and uptake was initiated by the addition of ATP. Each point is the mean of triplicate measurements.

1994). However, because an antibody may recognize an epitope of only a few amino acids, we cannot rule out the possibility that the LCA antibody cross-reacts with other, as yet unidentified Ca^{2+}-ATPases.

Based on the high degree of sequence similarity between the coding region of LCA and the SER Ca^{2+}-ATPases (Wimmers et al., 1992), it was expected that the LCA protein would be ER localized. However, the immunodetected Ca^{2+}-ATPases in tomato roots were localized in the TN and PM. In this regard, there is evidence showing that some proteins are targeted to a different subcellular location from what was anticipated by amino acid sequence similarity to animal proteins. For example, the *PMC1* (plasma membrane Ca^{2+}-ATPase) gene of yeast, a homolog of PM Ca^{2+}-ATPases of mammalian cells, encodes a vacuolar Ca^{2+}-ATPase (Cunningham and Fink, 1994).

Although the mechanisms by which the subcellular distribution of Ca^{2+}-ATPases is established are not known, the localization of proteins in intracellular membranes is postulated to be an active process with proteins being sorted by specific targeting sequences, which may function through interaction with receptors (Pelham, 1989). Targeting sequences have been identified in both plant and animal soluble ER proteins (Pelham, 1989; Napier et al., 1992) and in animal and yeast transmembrane ER proteins (Jackson et al., 1990). It has recently been demonstrated that a lysine-rich (KKXX or KXKXX) motif at the cytoplasmically exposed C terminus of multispanning proteins can act as a consensus motif for retention of membrane proteins in the ER (Jackson et al., 1990; Townsley and Pelham, 1994). Interestingly, the coding sequence of LCA contains this consensus motif (KLKAA) at its extreme C terminus (Wimmers et al., 1992), although the data presented here indicate that the proteins are not retained in the ER in tomato root cells, and indeed it is not known whether the extreme C-terminal amino acids of LCA are retained in the mature protein. Gabathuler and Kvist (1990) have suggested that the ER membrane retention signal is not a linear sequence but a more complicated structure involving the majority of the cytoplasmic tail. Moreover, it has been shown that the SER Ca^{2+}-ATPase SERCA 1A (Karin and Settle, 1992) and the PM isoform 4CI (Zvaritch et al., 1995) both contain ER targeting information. Further research is clearly needed to elucidate the mechanisms by which the subcellular distribution of Ca^{2+}-ATPases is established.

The inhibitor sensitivity of Ca^{2+} uptake by TN vesicles suggested the presence of two Ca^{2+} transport systems operating in this membrane: one is the LCA Ca^{2+}-ATPase, and the other is a Ca^{2+}/H^{+} antiporter. The presence of both transporters in the same membrane has been reported previously in maize leaf PM (Kasai and Muto, 1990) and in the TN of barley roots (Dupont et al., 1990) and spinach leaves (Malatialy et al., 1988). The functional significance of several transporters on a single membrane or cell is not yet known. It has been proposed that Ca^{2+}-ATPases are primarily responsible for setting resting levels of cytosolic calcium (Evans et al., 1991), whereas the lower affinity $Ca^{2+}/H+$ antiporter may be more important during periods or locations of increased cytosolic Ca^{2+}. Although a TN Ca^{2+}-ATPase has been reported previously (Dupont et al., 1990; Gavin et al., 1993), most of the studies and discussions about the role of higher plant Ca^{2+}-ATPases in cellular Ca^{2+} homeostasis have focused on only the ER and PM Ca^{2+}-ATPases. However, the presence in the TN of a primary Ca^{2+}-ATPase may be of particular significance in

the regulation of cytoplasmic Ca^{2+} levels. In this regard, it has been demonstrated that the vacuolar Ca^{2+}-ATPase in yeast (PMC1) is essential to deplete the cytosol of Ca^{2+} ions which, at elevated concentrations, inhibit growth (Cunningham and Fink, 1994). Thus, it is likely that the three Ca^{2+} pumps identified in higher plants (PM, ER, and TN) function together to regulate cellular Ca^{2+} homeostasis and growth and development.

The data presented in this paper indicate that neither the PM nor the TN LCA Ca^{2+}-ATPases are stimulated by calmodulin. These data are consistent with the lack of a calmodulin binding domain in the deduced LCA amino acid sequence (Wimmers et al., 1992). In previous studies, calmodulin sensitivity was reported for the PM Ca^{2+}-ATPases from maize (Robinson et al., 1988; Briars and Evans, 1989), red beet roots (Williams et al., 1990), carrot cell cultures (Hsieh et al., 1991), and radish seedlings (Rasi-Caldogno et al., 1992, 1993). In contrast, calmodulin had no effect on the PM Ca^{2+}-ATPases from pea roots (Butcher and Evans, 1987) and *Commelina communis* leaves (Siebers et al., 1990). Studies regarding calmodulin sensitivity of TN Ca^{2+}-ATPases are less abundant, but it has been reported that the TN Ca^{2+}-ATPases from spinach leaves (Malatialy et al., 1988) and maize roots (Gavin et al., 1993) are stimulated by calmodulin. The calmodulin sensitivity of the ER Ca^{2+} pump is also a matter of debate (Butcher and Evans, 1987; Hsieh et al., 1991; Askerlund and Evans, 1992; Chen et al., 1993). The discrepancies reported in sensitivity to calmodulin among Ca^{2+}-ATPases suggest that the calmodulin-stimulated ATPases are present only in certain tissues or species or that calmodulin sensitivity may be masked by high levels of endogenous calmodulin in isolated membranes. The fact that the two cloned Ca^{2+}-ATPases from higher plants lack a calmodulin binding domain (Perez-Prat et al., 1992; Wimmers et al., 1992) might suggest that plant Ca^{2+}-ATPases could be activated indirectly by a calmodulin-dependent protein kinase. On the other hand, calmodulin-affinity purification of a cauliflower Ca^{2+}-ATPase (Askerlund and Evans, 1992) supports the hypothesis of direct stimulation of a class of plant Ca^{2+}-ATPases by calmodulin. Thus, there may be two types of Ca^{2+}-ATPases in plants, as detected in other eukaryotes (Carafoli, 1987; Evans, 1994).

The identification of differentially localized Ca^{2+}-ATPase isoforms, apparently derived from a single gene, raises a number of questions regarding the mechanisms by which Ca^{2+}-ATPases are targeted to their subcellular sites. We speculate that the different size and localization of the two isoforms result from either post-transcriptional or post-translational processing of the mRNA or protein, respectively. Because of the very low abundance of the LCA Ca^{2+}-ATPases in plant cells, it has not yet been possible to determine its protein sequence directly. A major current research goal is to identify differences in sequences of each isoform to identify the domains potentially responsible for differential subcellular localization and to verify that the same gene gives rise to both forms of the Ca^{2+}-ATPase.

METHODS

Generation of Polyclonal Antibodies

To raise specific antibodies against tomato (*Lycopersicon esculentum*) Ca^{2+}-ATPase, a 132-bp hydrophilic and nonconserved P-type ATPase domain of a tomato Ca^{2+}-ATPase (LCA, for *Lycopersicon* Ca^{2+}-ATPase) was subcloned in the pRSET expression vector (Invitrogen, San Diego, CA) by using polymerase chain reaction primers to specify the domain precisely. This produced a fusion of a poly(H) domain with the coding region of LCA for amino acids 580 to 624 (Wimmers et al., 1992) when expressed in *Escherichia coli*. The recombinant plasmid was introduced into *E. coli* BL21pLysS, and production of the recombinant protein was induced by addition of isopropyl β-D-thiogalactosidase (IPTG) to the culture medium (Studier et al., 1990). The fusion protein was purified from the insoluble cell fraction by affinity chromatography based on the selective interaction between the histidine hexapeptide present in the recombinant protein and a metal chelating matrix charged with Ni^{2+} ions (Hochuli et al., 1988). The recombinant protein was purified further by preparative SDS-PAGE on 12% polyacrylamide gels. Antibodies to the LCA polypeptide were raised in female New Zealand rabbits by subcutaneous injection of purified protein (1.0 mg) in Freund's complete adjuvant, followed 4 weeks later by injection of 0.5 mg in Freund's incomplete adjuvant. The blood was collected 7 days after the final immunization.

Plant Material

Tomato plants were grown hydroponically in aerated 1 × Hoagland solution under fluorescent lighting. Roots were harvested from 7-day-old seedlings.

Membrane Preparation

Microsomal membranes from tomato roots were prepared as described by Joyce et al. (1988), with minor modifications. All isolation procedures were conducted at 4°C. Roots were chopped with a razor blade and homogenized in buffer (4 mL/g fresh weight) containing 250 mM sucrose, 50 mM Tris-HCl, pH 8.0, 2 mM EDTA, 4 mM DTT, 2 mM phenylmethylsulfonyl fluoride (PMSF), 20 μg/mL chymostatin, 40 μM leupeptin, 0.1% (w/v) BSA, and 0.2% (w/w) polyvinylpolypyrrolidone, using a mortar and pestle. For the Mg^{2+}-sucrose gradients, 5 mM $MgCl_2$ was present in the homogenization and gradient buffers. After filtration through four layers of cheesecloth, the homogenate was centrifuged at 10,000*g* for 15 min. The supernatant was loaded on top of a cushion of gradient buffer (10 mM Tris-HCl, pH 7.0, 2 mM EDTA, 2 mM DTT) plus 58% (w/w) sucrose and centrifuged in a swinging bucket rotor (SW-28; Beckman, Palo Alto, CA) at 80,000*g* for 30 min. The membranes at the interface were collected and mixed with two volumes of gradient buffer plus 2 mM PMSF, 40 μM leupeptin, and 20 μg/mL chymostatin. The diluted membranes were applied to a continuous 15 to 45% (w/w) sucrose gradient, with or without Mg^{2+}, and were centrifuged for 2 hr at 80,000*g*. Fractions (1 mL) were collected, frozen in liquid nitrogen, and stored at −80°C until assayed.

SDS-PAGE and Protein Blotting

SDS-PAGE was performed according to the method of Laemmli (1970). All gels were 7.5% polyacrylamide. Samples were precipitated with trichloroacetic acid, and pellets were resuspended in SDS-PAGE buffer containing 50 mM Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 5% mercaptoethanol, 2 mM PMSF, 40 μM leupeptin, and 100 μg/mL chymostatin and incubated for 30 min at room temperature before electrophoresis. Proteins were transferred from gels to polyvinylidene fluoride membranes at 1.0 A for 1.5 hr in a transfer cell (model TE45; Hoefer, San Francisco, CA) with a buffer consisting of 10 mM 3-cyclohexylamino-1-propane sulfonic acid-NaOH, pH 11.0, and 10% (v/v) methanol (Moos, 1992).

Immunodetection of the Ca^{2+}-ATPase on Protein Blots

Immunodetection was performed according to the method of Birkett et al. (1985). Following transfer, the membrane was rinsed for 5 min with water, for 5 min with TBS (20 mM Tris-HCl, pH 7.5, 0.5 M NaCl), and for 5 min with TBS containing 0.1% Tween-20 (TBST). The membrane was incubated overnight with the polyclonal antibody at a 1:1000 dilution in HST (20 mM Tris-HCl, pH 7.5, 1 M NaCl, 0.5% Tween-20). The membrane was then rinsed twice for 5 min each in TBST, once for 5 min in HST, and twice for 5 min each in TBST before incubation for 2 hr with goat anti-rabbit IgG conjugated to alkaline phosphatase at a 1:3000 dilution in HST. The membrane was then rinsed three times for 5 min each in TBST, once for 15 min in HST, three times for 5 min each in TBST, and briefly with 200 mM Tris-HCl, pH 8.8. The reaction of alkaline phosphatase was developed with nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate (Blake et al., 1984).

"Blocked" antiserum was obtained by preincubating 10 μL of crude antiserum overnight at 1:100 dilution in TBST with 500 μg of the blotted LCA fusion protein. After preincubation, the blocked antiserum was used to probe protein gel blots of tonoplast (TN) and plasma membrane (PM) fractions at 1:5000 dilution in HST, as described above.

Membrane Analysis and Protein Determination

The membrane fractions were identified using marker enzymes and specific antibodies to proteins associated with the different subcellular membranes. The activities of different marker enzymes were determined as described by Joyce et al. (1988). Vanadate-sensitive ATPase and a PM H^+-ATPase antibody (kindly provided by Dr. R. Serrano, Universidad Politécnica de Valencia, Spain) were used as PM markers. Nitrate-sensitive ATPase and an antibody to the 58-kD subunit of the vacuolar ATPase (V-ATPase) were used as TN markers; antimycin A–insensitive NADH–cytochrome *c* reductase and an antibody to BiP (D.J. Meyer and A.B. Bennett, University of California at Davis) were used as endoplasmic reticulum (ER) markers; azide-sensitive ATPase was used for mitochondria and latent UDPase for the Golgi membrane marker.

Protein determinations were performed using the method of Bradford (1976), with BSA as a reference standard.

Calcium Transport Assays

ATP-dependent $^{45}Ca^{2+}$ uptake into membrane vesicles was measured by the filtration method (Bush and Sze, 1986). Assays were conducted at 22°C in 200-μL volume containing 250 mM sucrose, 25 mM 1,3-bis[tris(hydroxylmethyl)-methylamino]propane-2-[N-morpholino]ethanesulfonic acid (BTP-MES), pH 7.2, 3 mM $MgSO_4$, 50 mM KCl, 50 μM Na_2MoO_4, 3 mM ATP-BTP, pH 7.2, and 1 μM $CaCl_2$ (containing 2 to 3 μCi/mL ^{45}Ca). The water used in all solutions was double deionized. Uptake was initiated by the addition of membrane protein. After 15 min, 180 μL of the reaction mixture was removed and filtered through presoaked 0.45-μm Whatman No. 1 cellulose nitrate filters. The filters were washed with 4 mL of a chilled solution of 250 mM sucrose, 25 mM BTP-MES, pH 7.2, and 1 mM $CaCl_2$, and radioactivity associated with the filters was measured by using a scintillation counter. Background values resulting from incubations with no ATP were subtracted from corresponding values in the presence of ATP. The addition of inhibitors and ionophores to the assay mixture are indicated in the text or legends to Figures 5 and 6 and in Table 1. To determine the effect of the antibodies on Ca^{2+} uptake into TN and PM vesicles, membranes were incubated with the immune or the preimmune serum for 30 min at 37°C in the assay mixture without ATP and the uptake was initiated by the addition of ATP.

Genomic DNA Gel Blot Hybridization Analysis

Tomato genomic DNA (10 μg) was digested with the indicated restriction enzymes, fractionated by agarose gel electrophoresis, and blotted to nylon membranes, as described by Southern (1975). The membrane was hybridized with the LCA cDNA sequence corresponding to the domain shown in Figure 1A (nucleotides 1740 to 1872; GenBank accession number M96324), labeled with α^{32}-P-dATP by random priming (Feinberg and Vogelstein, 1983). Hybridization was performed at 55°C in 0.5% (w/v) SDS, 6 × SSPE (1 × SSPE is 150 mM NaCl, 10 mM NaH_2PO_4, and 1 mM EDTA), 5 × Denhardt's solution (1 × Denhardt's solution is 0.2 g/L Ficoll 400 [Pharmacia], 0.2 g/L PVP, and 0.2 g/L BSA), and 100 μg/mL denatured salmon sperm DNA with the labeled probe added at 6.5 × 10^6 cpm/mL. Final washes were performed in 6 × SSC (1 × SSC is 0.15 M NaCl, 0.015 M sodium citrate), 0.1% (w/v) SDS at room temperature for 30 min and at 55°C for 30 min. Autoradiography was performed at −80°C with Kodak XAR-5 film for 48 hr.

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