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A calcium pump at the higher plant nuclear envelope?

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Abstract Evidence for a Ca^{2+}-pump at the nuclear envelope (NE) in plant cells has been obtained using confocal and electron microscope immunocytochemistry and antibodies raised to a plant homologue of the mammalian SERCA pump. This is the first evidence suggesting an NE Ca^{2+}-pump in plants. In addition to being localised with the NE in interphase, the antigen was localised to membrane derived from the NE and associated ER during mitosis, correlating with known Ca^{2+}-pools. The work suggests that a SERCA pump is present at the NE of plant as well as animal cells.

Key words: *Lycopersicon esculentum*; Calcium; Higher plant; Nuclear envelope; SERCA pump

1. Introduction

The role of the nuclear envelope (NE) in animal cell signalling has been well documented [1]; however, to our knowledge, no attempts have been made to investigate equivalent functions in the NE of higher plants. In the work described here, we present immunocytochemical evidence for a similar Ca^{2+}-pump at a higher plant NE.

Calcium signalling in eukaryotic cells involves the generation of spatially localised Ca^{2+}-transients within the cell. These localised increases in Ca^{2+}-concentration act upon Ca^{2+}-response elements to evoke a variety of cellular responses. Calcium transients have been observed in both plant [2] and animal [1] cells, and great progress has been made in identifying Ca^{2+}-response elements and the mechanisms by which the signals are generated within the cytosol [1]. The location, magnitude and duration of Ca^{2+}-transients are known to be important in determining the nature of the cellular response evoked [1]. Plant cells also show such localised Ca^{2+}-transients, for instance in the closure of stomata [3], in fertilisation in the alga *Fucus* [2] and when leaves are stimulated by cold or by mechanical stimulation [4].

In animal cells, presence of a Ca^{2+}-signalling pool at the NE, which is linked with that at the endoplasmic reticulum (ER), has been demonstrated [5]. Immunological evidence indicates the presence of a SERCA homologue at the NE [6], while presence of Ca^{2+}-binding proteins and IP_3 receptors at the NE all indicate a role in intracellular signalling [7,8]. Calcium transport at the nucleus has been implicated inter alia in apoptosis [9], nuclear envelope breakdown and mitosis [10].

Plant cells also have Ca^{2+}-pumps homologous to mammalian [11]. In the absence of immunocytochemical data, localisation of plant Ca^{2+}-pumps has largely relied on membrane fractionation. Plant SERCA homologues were initially suggested to be located at the ER (e.g. [12,13]); more recently, studies using membrane fractionation and the segregation of marker enzymes in tomato indicated tonoplast and plasma membrane locations [14], while a SERCA homologue (ECA1) in *Arabidopsis* has been assigned an ER location [15]. The plant NE has been neglected in such studies, because of difficulties in obtaining purified membranes and the lack of appropriate membrane markers. This study builds on previous work [12,14] on a 116 kDa putative SERCA homologue, LCA in tomato. LCA has 50% amino acid identity with animal SERCAs and contains the conserved functional domains of P-type ATPases and the essential residues for Ca^{2+}-transport. Five of the transmembrane domains identified showed $>60\%$ amino acid identity with equivalent regions in mammalian SERCAs [12].

The application of immunocytochemical techniques to the study of the NE provides a specific and sensitive tool with which to localise Ca^{2+}-pumps. In this study, an anti-peptide serum (315) to the extreme C-terminus of the tomato Ca^{2+}-pump LCA [14], together with an antibody raised to a fusion protein from a central region of the same protein (LCA; [14]) has been used to suggest the presence of a homologue of the SERCA Ca^{2+}-pump at the NE of tomato root cells.

2. Materials and methods

All chemicals and immunological reagents were obtained from Sigma (Poole, Dorset, UK) unless otherwise indicated. Anti-peptide serum 315 was generated to the C-terminus of the published sequence of a tomato SERCA homologue, LCA (Genbank accession number M96324; [12]; sequence VILIDEVLKFVGRRRRTKLKAA; see Table 1). Peptide was made on a Novasyn Crystal peptide synthesiser (NovaBiochem) and purified by reverse phase HPLC before it was immunised into New Zealand White rabbits using Imject Alum as adjuvant (Pierce and Warriner, Chester, UK). Animals were kept and handled in accordance with local and national welfare requirements. IgG purification of the antiserum was carried out using a protein-A column (Pierce and Warriner) and antibody affinity and specificity confirmed by ELISA, Western blotting against fractions containing NE and other membranes, and by immunodepletion of the antisera onto agarose-conjugated peptide (data not shown). Antiserum LCA [14] was the kind gift of Professor Alan Bennett, University of California at Davis, and had been generated to a fusion protein including a non-conserved hydrophilic domain (amino acids 714–944; [14]) of LCA (see above) in rabbits.

Light microscope immunocytochemistry was performed as described in [16]. Briefly, seeds of tomato (*Lycopersicon esculentum* L. *cv*. Alicante) were germinated on damp filter paper at 22°C in the dark. Root tips (2–3 mm) were excised under fixative (4% paraformaldehyde in 0.1 M PIPES buffer pH 6.9) and fixed for 1 h at room temperature. All subsequent media were made up in 0.1 M PIPES buffer, pH 6.9. Roots were digested for 8–10 min in a cellulase (1%; Onozuka; Unwin, Welwyn Garden City, UK)/pectinase (0.17%)/bovine serum albumin (2%) mixture and cells were released by gently squashing onto Vectabond coated slides (Vector Laboratories, Peterborough, UK). Cells were further permeabilised in 0.5% Triton X-100 for 10 min, blocked with 1% BSA for 15 min and incubated in

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Table 1
Comparison of C-termini of SERCA ATPase homologues

		***	**	**	***		* *
LCA (tomato)	ILLSAP	VILIDE	VLKFVG	RRRRRT	——	—	KLKAA
Arabidopsis	VLAVSL	VILIDE	VLKFVG	RCTSGY	RYSPRT	LST	KQKEE
Rabbit	LKISLP	VILMDE	TLKFVA	RNYLEP	AILE-	—	—-
Peptide		*VILIDE*	VLKFVG	*RRRRRT*			*KLKAA*

Tomato (LCA; [34]); *Arabidopsis* (ECA1-P; [15]); rabbit [34].

primary antibody (diluted 1:700 315, 1:1000 LCA) for 1 h at room temperature. Control cells were incubated in pre-immune serum (diluted as primary antibody) or secondary antibody alone. Finally, after extensive washing in PIPES buffer containing 1% fish gelatin, cells were incubated for 1 h with a fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit secondary antibody diluted 1/40 in PIPES buffer, washed extensively and mounted in Citifluor antifade (Agar Scientific Ltd., UK). Sections were viewed using a Zeiss LSM 410 laser scanning confocal microscope (Carl Zeiss, Germany) using a 488 nm argon laser and FITC filter sets. Images were recorded on optical disc and printed using a Tetronix Phaser 440 dye sublimation printer (Tetronix, Wilsonville, OR, USA).

Root tips were prepared for electron microscopy using the progressive lowering of temperature (PLT) technique [17]. Roots were fixed for 1 h in 1% paraformaldehyde/1% glutaraldehyde in 0.1 M PIPES buffer (pH 6.9), washed and dehydrated in a graded ethanol series during which the temperature was reduced to −20°C over 4 h. Dehydrated tissue was infiltrated with LR White resin in ethanol (Agar Scientific Ltd, UK) containing 0.5% benzyl methyl ether, with decreasing proportions of ethanol over 3 h followed by repeated changes of pure resin over 5 days at −20°C; finally, the resin was polymerised under ultra-violet light at −20°C for 24 h and at 0°C for 24 h. Sections were cut using a Reichart Ultracut E ultramicrotome and collected on Formvar coated nickel grids. Sections were blocked in 10 mM phosphate buffered saline, 1% BSA (Fraction V), 0.5% Tween-20 (PBST) for 20 min at room temperature, washed and incubated in primary antibody (1:1500 in PBST) on a vibrating platform for 1 h. Sections were then washed and incubated in 5 nm or 10 nm gold-conjugated goat anti-rabbit secondary antibody (British Biocell, Cardiff, UK) diluted 1:20 with PBST. Sections were examined directly, or the signal intensified using a silver enhancement kit (British Biocell) according to the manufacturer's instructions, before viewing using a JOEL 1200 EXII transmission electron microscope, where appropriate sections were post-stained using uranyl acetate and lead citrate [13] before examination.

2.1. Western blotting

Tomato (cv. Alicante) roots (50–100 g fresh weight) were homogenised in ice cold homogenisation buffer (2 ml/g fresh weight) (50 mM MOPS-BTP pH 7.5, 0.33 M sucrose, 0.6% (w/v) polyvinylpyrrolidone, 5 mM EDTA, 1 mM Benzamidine-HCl, 5 mM DTT, 0.2% BSA (w/v), plus 2 mM PMSF, 20 μg/ml chymostatin and 40 μM leupeptin). Homogenate was filtered through six layers of muslin and centrifuged (Sorvall F-28/50 rotor) for 10 min at $10\,000\times g$ (microsomal membranes). The supernatant was centrifuged for 45 min at $100\,000\times g$ in the same rotor. Pellets enriched in nuclei were obtained by low-speed centrifugation; tomato homogenate obtained as described above was filtered through six layers of muslin and centrifuged (Sorvall F-28/50 rotor) for 10 min at $1000\times g$. The supernatant was then centrifuged again in the same rotor for 10 min at $3000\times g$ and finally the supernatant was centrifuged at $10\,000\times g$ for 20 min. TCA-precipitated pellets were solubilised in SDS sample buffer for 30–45 min at room temperature and run on 4–20% gradient SDS-PAGE gels (mini PROTEAN Ready Made Gels, Biorad, UK), alongside both prestained (Sigma 7B) and biotinylated broad-range (Biorad, UK) SDS-PAGE standards. Proteins were electrophoretically transferred to nitrocellulose (BA 65, Scleicher and Schuell, Anderman and Co Ltd, UK) using a Biorad mini-blot apparatus. Antiserum 315 was detected using a donkey secondary anti-rabbit antibody conjugated to horseradish peroxidase (Amersham Life Sciences plc; UK), diluted 1/5000 and an ECL detection system (Amersham Life Science plc, Amersham, UK).

3. Results and discussion

In this study, two independently generated antibodies to the plant SERCA homologue LCA were used, one, LCA, having been previously independently characterised by Western blotting [14] against both expressed protein and tomato root membranes, in which it was shown to recognise a polypeptide of appropriate M_r (116 kDa) in tomato microsomal membranes. The second, antibody 315, was raised to a peptide at the C-terminus of LCA ([12]; and see Table 1). Its specificity was confirmed by a variety of techniques. Firstly, the antibody was shown to recognise the peptide on ELISA (data not shown) and to bind to an affinity column prepared by coupling the entire peptide through its amino groups to NHS-activated sepharose (Pharmacia HiTrap). Elution of active antibody after extensive column washing was achieved using buffer of pH 2.0. Antibody pre-absorbed with immobilised peptide was shown to be inactive using several immunocytochemical techniques (data not shown). Western blotting antibody 315 against tomato root membranes resulted in two bands, estimated to be 105 and 116 kDa (Fig. 1, lanes 1, 2). This pattern is similar to the known mobilities of plant SERCA homologues LCA [14] and ECA1p [15]. The 105 kDa polypeptide may be a result of proteolysis; however, ECA1p has also been detected as a 105 kDa polypeptide, located at the ER [15]. Antiserum 315 therefore recognised the peptide to which it was raised and polypeptides of appropriate M_r. Furthermore, the staining obtained was shown to be eliminated by pre-absorption with peptide. The antiserum also stained polypeptides of similar relative molecular mass in fractions obtained by low-speed centrifugation containing nuclei and depleted in endomembranes (Fig. 1, lanes 3, 4).

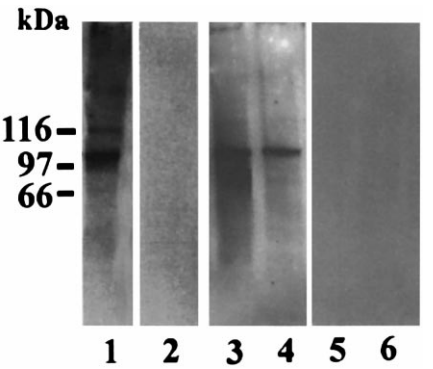

Fig. 1. Western blot of antibody 315 (lanes 1, 3, 4) and control (pre-immune serum; lanes 2, 5, 6) binding to tomato root microsomal membranes (lane 1) and 1–$3\,000\times g$ (lane 3) and 3000–$10\,000\times g$ (lane 4) pellets. Intact nuclei would be expected to sediment in the latter two fractions, while stripped nuclear envelope and associated ER would sediment with the microsomal membranes.

Both antisera to LCA (LCA and 315) stained NE of tomato root cells (Fig. 2a, b). NE staining using 315 was evident using both confocal light microscopy (Fig. 2a) and by electron microscopy, using immunogold staining (Fig. 3a) and immunogold staining followed by silver intensification (Fig. 3b). Control sections (no primary antibody, primary antibody pre-absorbed against immobilised peptide and pre-immune serum) did not stain (data not shown). Immunogold labelling without silver intensification (data not shown) suggested the epitope to be associated with the NE, as suggested for the Ca^{2+}-pump of rat liver nuclei [6], suggesting the presence of a pump capable of transport of Ca^{2+} from the cytoplasm to form a signalling pool in the NE. It was not possible to discern whether labelling was exclusively at the outer NE or at both membranes. Identical staining patterns were observed with antiserum purified by immunoabsorption to a column to which the peptide '(C)KLKAA' was coupled through its cysteine side chain to amino-hexyl sepharose 4B (Pharmacia) that had been activated with the *N*-hydroxysuccimide ester of B-maleimido propionic acid (Sigma). Antibody LCA showed staining of the NE at the light microscope level (Fig. 2b); the antibody was less suitable for microscopy and particularly electron microscopy than 315, but gave occasional staining of NE at the electron microscope level (data not shown).

The NE in plant cells breaks down during mitosis and forms tubular and cisternal structures (comprising part of the ER) which surround the spindle poles and are closely associated with the chromosomes; these membranes are known to accumulate Ca^{2+} during metaphase which is released at the onset of anaphase [18–22]. Microscopy revealed 315 antigen distributed with the membranes associated with the metaphase spindle poles (Fig. 3a). The distribution closely parallels that described for the cisternal membranes shown to accumulate Ca^{2+} (Fig. 3a, b; reviewed by [18]). Later, the antigen remains with this membrane as it distributes around the daughter chromatids (Fig. 3c, d) before reforming the new telophase NE of the daughter cells (Fig. 3e, f). Our results therefore suggest that the Ca^{2+}-pump may be associated with the formation of the Ca^{2+}-signalling pool involved in the regulation of chromosome separation in mitosis. Differences in the intensity and concentration of signal between confocal light micrographs and electron micrographs (e.g. Fig. 3a, b) almost certainly result from the difference between immunostaining a permeabilised whole cell mount (Fig. 3a) and the surface of a thin section (Fig. 3b).

Biochemical studies and molecular cloning have resulted in the identification of several classes of Ca^{2+}-pump in plants [11,12,15,23–28]. Ferrol and Bennet [14] detected only one gene encoding the tomato SERCA homologue (LCA) used in this study. LCA was assigned locations at the tonoplast and plasma membrane [14] based on Western blotting membrane fractions (see Fig. 2 in [14]). Given the incomplete resolution of sucrose density gradients and the presence of some LCA staining in fractions of intermediate density between tonoplast and PM, other locations were not ruled out. Absence of tonoplast staining in the present study by either LCA or 315 may be the result of the fact that cells from the meristematic and just post meristematic region were used for microscopy, as opposed to whole root extracts used for membrane fractionation by Ferrol and Bennett [14], or from incomplete preservation of a tonoplast epitope. There is some suggestion of PM staining by LCA, not evident for

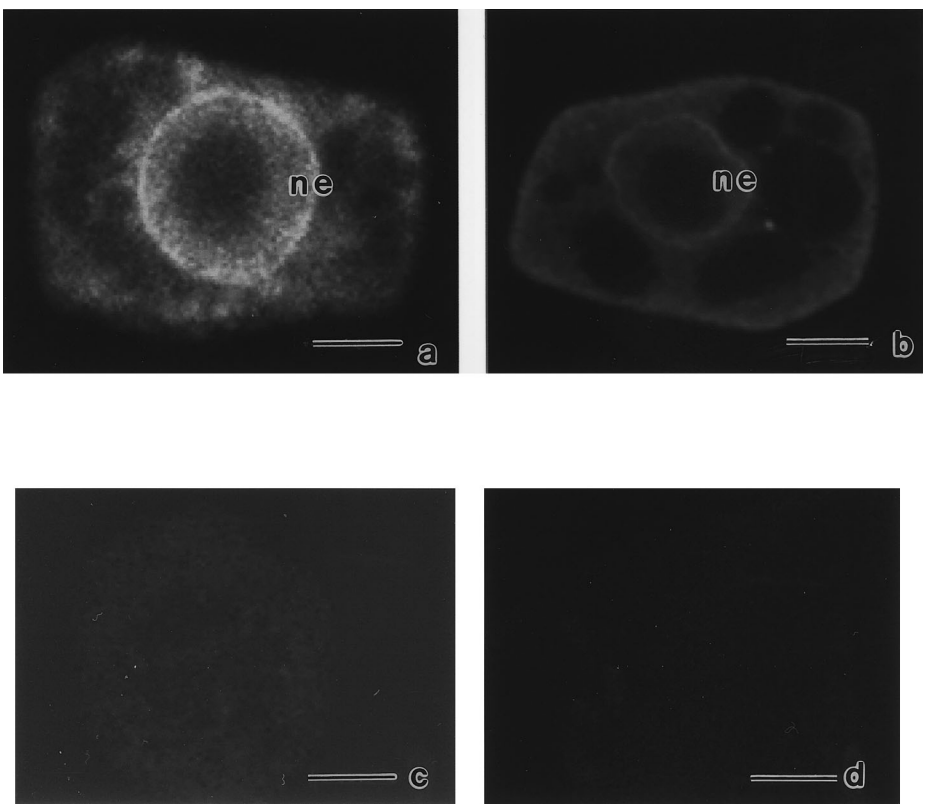

Fig. 2. Confocal micrographs of anti-peptide antibody 315 (a) and anti-fusion protein antibody LCA (b) staining nuclear envelope (ne) in tomato root cells in interphase (scale bar 5 mm). Pre-immune serum (c); no primary antibody (d).

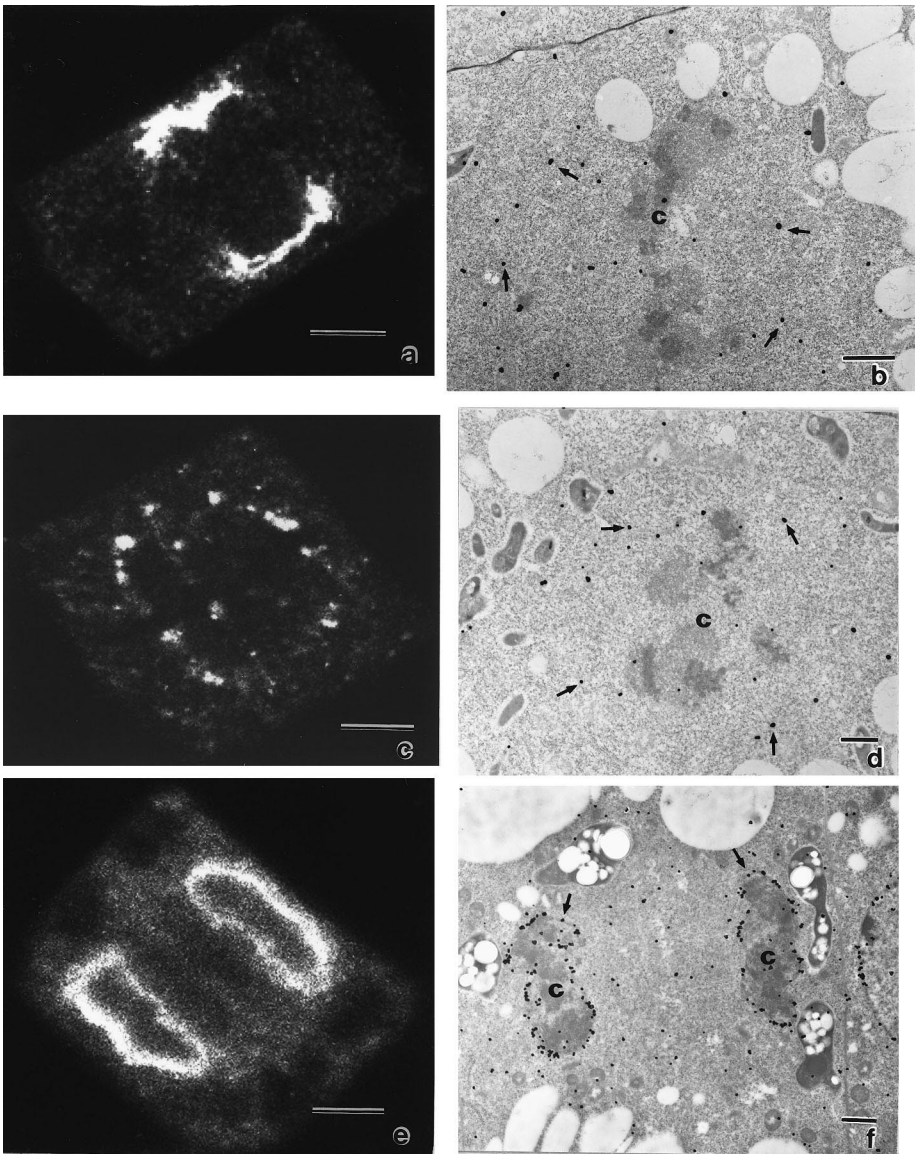

Fig. 3. Confocal light (a, c, e) and electron (b, d, f) micrographs of antibody 315 staining tomato root cells at metaphase (a, b), anaphase (c, d) and telophase (e, f). Note staining associated with intact NE in cells in interphase (Figs. 1 and 2) now associated with NE-derived membrane at the spindle poles during metaphase (a, b) and anaphase (c, d) and reforming with the developing NE during late telophase (e, f). Bar = 5 mm (a, c, e), 1 mm (b, d, f). Arrows indicate silver-intensified gold particles; c, chromosomal material; ne, nuclear envelope.

315 (see Fig. 2a, b) which may suggest that the internal epitope recognised by LCA is available for antibody staining at the PM, while the terminal 315 epitope is cleaved, modified or masked in some way from LCA at the PM. We therefore suggest that our work adds a further location to those suggested by Ferrol and Bennett [14], the NE. Ferrol and Bennett [14] suggested that the several locations are the result of differential splicing of the gene product as Southern hybridization revealed only one LCA gene. Several genes which could encode SERCA homologues have, however, been identified in *Arabidopsis* (see [25] for detail); an antibody to one of these, ECA1p, co-migrates with ER markers [15]. This suggests that there may be at least three isoforms of SERCA homologues in plants, located at the NE/ER (as well as other ER domains), PM and tonoplast, perhaps of different abundances in different tissues. We are unable to comment as to whether these result from differential splicing or are the products of different genes (as in *Arabidopsis*); however, it is evident that the situation in plants may be similar to that in mammals, where three isoforms of SERCA pumps have [29] been described as well as a complex intron organisation resulting in splice variants, particularly at the C-terminus (see [30]).

The outer NE is likely to be a functional continuum with the ER. One question raised by this study is how the Ca^{2+}-pump is targeted to the NE. The motif KKXX or KXKXX has been suggested to be an ER retrieval signal in animal cells [29,31,32] and is present (as KLKAA) in the C-terminus of LCA. Calreticulin in mammalian cells also bears a nuclear retention signal (KIKDPD) as well as an ER KDEL salvage sequence and has been observed to be localised at the nucleus as well as at the ER, its nuclear location probably being due to its interaction with glucocorticoid receptors [33]. The mammalian SERCA pumps shown to be located at the NE do not show a KXKXX/KKXX motif. On the other hand, such a

motif is present in another plant SERCA homologue, from *Arabidopsis thaliana* [34] (KQKEE; see Table 1). The three sequences shown to be responsible for targeting to the nucleoplasm in plants (the bipartite domain, the SV40-like domain and the MATa-2-like domain [35]) are all rich in arginine and lysine and so is the LCA C-terminus. The SV40-like domain, for instance, RKRREKL resembles the LCA sequence RRRRTKL. However, there is no evidence at present concerning NE-targeting in plants and further studies are required.

The work described opens up for investigation one of the least studied plant membranes, in terms of ion transport and signalling properties, the NE. It also indicates that it is likely that all eukaryotes have a SERCA homologue Ca^{2+}-pump at the NE. Given the rapid increase in interest in, and understanding of, signalling at the mammalian NE it is surprising that studies on plant cell signalling have largely overlooked this membrane. If a substantial Ca^{2+}-signalling pool exists at the NE in plants, investigations into the presence of Ca^{2+}-release channels, the presence of Ca^{2+}-binding and -response elements and the relationship between the NE and ER Ca^{2+}-pools in plants should be undertaken.

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