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Review

Molecular aspects of higher plant P-type Ca^{2+}-ATPases

Markus Geisler [1], Kristian B. Axelsen [2], Jeffrey F. Harper [3], Michael G. Palmgren *

Department of Plant Biology, Royal Veterinary and Agricultural University, Thorvaldsensvej 40, DK-1871 Frederiksberg C, Denmark

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Abstract

Recent genomic data in the model plant *Arabidopsis thaliana* reveal the existence of at least 11 Ca^{2+}-ATPase genes, and an analysis of expressed sequence tags suggests that the number of calcium pumps in this organism might be even higher. A phylogenetic analysis shows that 11 Ca^{2+}-ATPases clearly form distinct groups, type IIA (or ECA for ER-type Ca^{2+}-ATPase) and type IIB (ACA for autoinhibited Ca^{2+}-ATPase). While plant IIB calcium pumps characterized so far are localized to internal membranes, their animal homologues are exclusively found in the plasma membrane. However, *Arabidopsis* type IIB calcium pump isoforms *ACA8*, *ACA9* and *ACA10* form a separate outgroup and, based on the high molecular masses of the encoded proteins, are good candidates for plasma membrane bound Ca^{2+}-ATPases. All known plant type IIB calcium ATPases seem to employ an N-terminal calmodulin-binding autoinhibitor. Therefore it appears that the activity of type IIB Ca^{2+}-ATPases in plants and animals is controlled by N-terminal and C-terminal autoinhibitory domains, respectively. Possible functions of plant calcium pumps are described and – beside second messenger functions directly linked to calcium homeostasis – new data on a putative involvement in secretory and salt stress functions are discussed.

Keywords: Ca^{2+}-ATPase; Calcium signaling; CaM-stimulated Ca^{2+}-ATPase; Calcium pump in sarcoplasmic or endoplasmic reticulum; Calmodulin; Plant; Phospholamban

1. Introduction

In the last two decades, research on higher plant calcium pumps – both at the biochemical and molecular level – has revealed many similarities to animal calcium transporters in terms of their structure-function relationship and physiological role [1–4]. In animals, and in fungi as well as in higher plants, Ca^{2+}-ATPases are thought to function mainly in a fine-regulation of low cytosolic calcium concentrations [2]. Reports about higher plant calcium ATPases have been dominated by the essential attempt to assign the intracellular location of individual pumps (see [1,2,5] for reviews). As a result, differences between plant and animal homologues have been revealed. For example, while in animals calmodulin (CaM) regulated Ca^{2+}-ATPases are exclusively found in the plasma membrane [6], plant homologues are also present in endomembrane systems [1,4,5]. In

* Corresponding author. Fax: +45 35283333; E-mail: palmgren@biobase.dk

[1] Present address: Institut de Botanique, Université de Neuchâtel, Rue Emil-Argand 13, CH-2007 Neuchâtel, Switzerland. Fax: +41 (32) 7182271; E-mail: markus.geisler@bota.unine.ch

[2] Present address: Swiss Institute of Bioinformatics, University of Geneva, 1, rue Michel Servet, CH-1211 Geneva 4, Switzerland. Fax: +41 (22) 7025502; E-mail: kristian.axelsen@isb-sib.ch

[3] Present address: Scripps Research Institute, Department of Cell Biology, North Torrey Pines Road, La Jolla, CA 92037, USA.

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Table 1
Features of cloned higher plant Ca^{2+}-ATPase isoforms

Gene product name (synonym)	Accession No., cDNA	Accession No., genomic clone	Type IIA (P_{2A}) or IIB (P_{2B})	Molecular mass (kDa)	Calcium specificity	CaM stimulation	Membrane location	Ref.
A. thaliana								
ACA1 (PEA1)	L08468	–	B	111	n.d.	n.d.	chloroplast inner envelope	[15,32]
ACA2	AF025842	AL035605	B	110	yes[a]	yes[a]	ER[b]	[7,14]
ACA3 (ECA1)	U93845/ U96455	–	A	116	yes[c]	no[c]	ER	[16,21]
ACA4	AF200739	AC002510	B	113	yes[d]	yes[d]	endomembrane	
ACA5 (ECA2)	AJ132387	AF013294	A	116	n.d.	n.d.	n.d.	
ACA6 (ECA3)	AJ132388/ AF117296	AC004122[e]	A	109	n.d.	n.d.	n.d.	
ACA7	–	AC004786	B	111	n.d.	n.d.	n.d.	
ACA8	–	AB023042	B	(118)[f]	n.d.	n.d.	n.d.	
ACA9	–	AB023045	B	(119)[f]	n.d.	n.d.	n.d.	
ACA10 (ORF5)	T43417[g]	AL050352	B	117	n.d.	n.d.	n.d.	
ECA4	AF117125[h]	–	A	–	n.d.	n.d.	n.d.	
Brassica oleracea								
BCA1	X99972	–	B	112	yes[i]	yes[i]	tonoplast	[17,34]
Lycopersicon esculentum								
LCA	M96324	–	A	116	n.d.	n.d.	tonoplast and plasma membrane	[19,20]
Oryza sativa								
OsCa-ATPase	U82966	–	A	115	n.d.	n.d.	n.d.	[22]

[a]Shown genetically by yeast complementation and calcium transport studies (I. Hwang, J. Harper, H. Sze, pers. comm.).
[b]Fluorescence of the GFP-tagged ACA2 was also detected around the nuclei of mature epidermal cells.
[c]Shown by calcium transport studies. ECA1 was discussed also to harbor a Mn^{2+} transport activity.
[d]Shown genetically by yeast complementation studies (M. Geisler, M.G. Palmgren, unpublished results).
[e]The C-terminus of ECA3 is also encoded by the genomic clone AC005489.
[f]The amino acid sequence has been assembled by comparing related Ca^{2+}-ATPases to the genomic clone and manually deducing the splice sites. The assembled sequence does not end with a stop codon. The molecular mass is therefore likely to be higher.
[g]The accession number only contains a partial sequence (an expressed sequence tag).
[h]The cDNA is only partial.
[i]Shown by calcium transport studies on the purified enzyme.

addition, at least some of the plant homologues have a unique structural organization with a CaM-regulated autoinhibitor located in an N-terminal domain (M. Geisler, M.G. Palmgren, unpublished results) [7]. Previously, only C-terminal autoinhibitory domains have been identified in P-type ATPases, such as the CaM-stimulated Ca^{2+} pumps in animals [6,8,9] and the plasma membrane H^+-ATPases in plants [10].

While recent reviews on plant Ca^{2+} pumps [1,5,11] concentrated on biochemical data, we will focus in this article mainly on molecular and genetic aspects of these transporters. Emphasis will be placed on a novel subfamily of N-terminal CaM-regulated Ca^{2+}-ATPases [7].

2. Molecular features of plant Ca^{2+}-ATPases

Plant Ca^{2+}-ATPases belong to the superfamily of P-type ATPases forming a phospho-aspartate (hence P) enzyme intermediate during the reaction cycle (see [12] for review). Plant genes have been identified from all five families of P-type ATPases [13].

Plant calcium ATPases belong to two phylogenetic subgroups, type IIA and type IIB Ca^{2+}-ATPases [12,13] (in shorthand P_{2A}- and P_{2B}-ATPases). Type IIA Ca^{2+}-ATPases show similarity to animal calcium pumps found in the sarcoplasmic or endoplasmic reticulum (SERCA). Type IIB Ca^{2+}-ATPases show similarity to animal CaM-stimulated Ca^{2+}-ATPases

Table 2
Proposed nomenclature for higher plant Ca^{2+}-ATPase isoforms

Gene product name (synonym)	New name	Accession No.	Type IIA (P_{2A}) or IIB (P_{2B})
A. thaliana			
ACA3/ECA1	*At*-ECA1	U93845	A
ACA5/ECA2	*At*-ECA2	AJ132387/AF013294	A
ACA6/ECA3	*At*-ECA3	AJ132388/AC04122	A
ECA4	*At*-ECA4	AF117125	A
ACA1/PEA1	*At*-ACA1	L08468	B
ACA2	*At*-ACA2	AF025842/AL035605	B
ACA4	*At*-ACA4	AF200739/AC002510	B
ACA7	*At*-ACA7	AC004786	B
ACA8	*At*-ACA8	AB023042	B
ACA9	*At*-ACA9	AB023045	B
ACA10/ORF5	*At*-ACA10	T43417/AL050352	B
B. oleracea			
BCA1	*Bo*-ACA1	X99972	B
L. esculentum			
LCA	*Le*-ECA1	M96324	A
O. sativa			
OsCa-ATPase	*Os*-ECA1	U82966	A

(PMCA) found in the plasma membrane. In the following, we will use the type IIA/IIB nomenclature throughout.

2.1. Cloning of putative plant Ca^{2+}-ATPases

Molecular cloning has allowed genetic approaches to characterize several higher plant Ca^{2+}-ATPase isoforms at the molecular level [7,14–17]. Several type IIA and IIB Ca^{2+}-ATPases have been heterologously expressed in yeast [7,14,16] and the results obtained have broadened our knowledge significantly. Further, we have included recent genomic DNA data obtained from the *Arabidopsis thaliana* genome project. For some of those sequences, no translated protein sequence is provided; in these cases exons have been assembled following sequence alignments with known Ca^{2+}-ATPase cDNAs.

In the model plant *Arabidopsis*, at least 11 Ca^{2+}-ATPases have been identified so far (Tables 1 and 2). In addition, analysis on expressed sequence tags (ESTs [18]) provides a method to investigate the number and nature of expressed genes in *Arabidopsis*. A current search in EMBL and GenBank databases reveals six and 14 ESTs of distinct mRNA origin with reasonable sequence similarity to type IIA and type IIB Ca^{2+}-ATPases, respectively (Table 3). Taking into account uncertainties due to poor sequence quality in the EST database and the fact that far from all *Arabidopsis* genes are represented by an EST [18], the number of putative type IIA and type IIB Ca^{2+}-ATPases can be estimated to be

Table 3
Expressed sequence tags (ESTs) in *A. thaliana* showing significant similarity to type IIA and type IIB Ca^{2+}-ATPases

Ca^{2+}-ATPase	Type IIA (P_{2A}) or IIB (P_{2B})	EST[a]
ECA1	A	AA042787, T43830
ECA2	A	–
ECA3	A	AA598128, R64945, Z37285, Z37289
ECA4	A	–
ACA1	B	AA042627, W43599
ACA2	B	N96705, T41649, T41650
ACA4	B	AA597439, N65090, R65015, T46834
ACA7	B	–
ACA8	B	–
ACA9	B	–
ACA10	B	N38404, R84090, T43417, Z25618
–[b]	B	H37540

[a]The indicated ESTs are identical to the corresponding Ca^{2+}-ATPase.
[b]The EST represents an additional type IIB Ca^{2+}-ATPase.

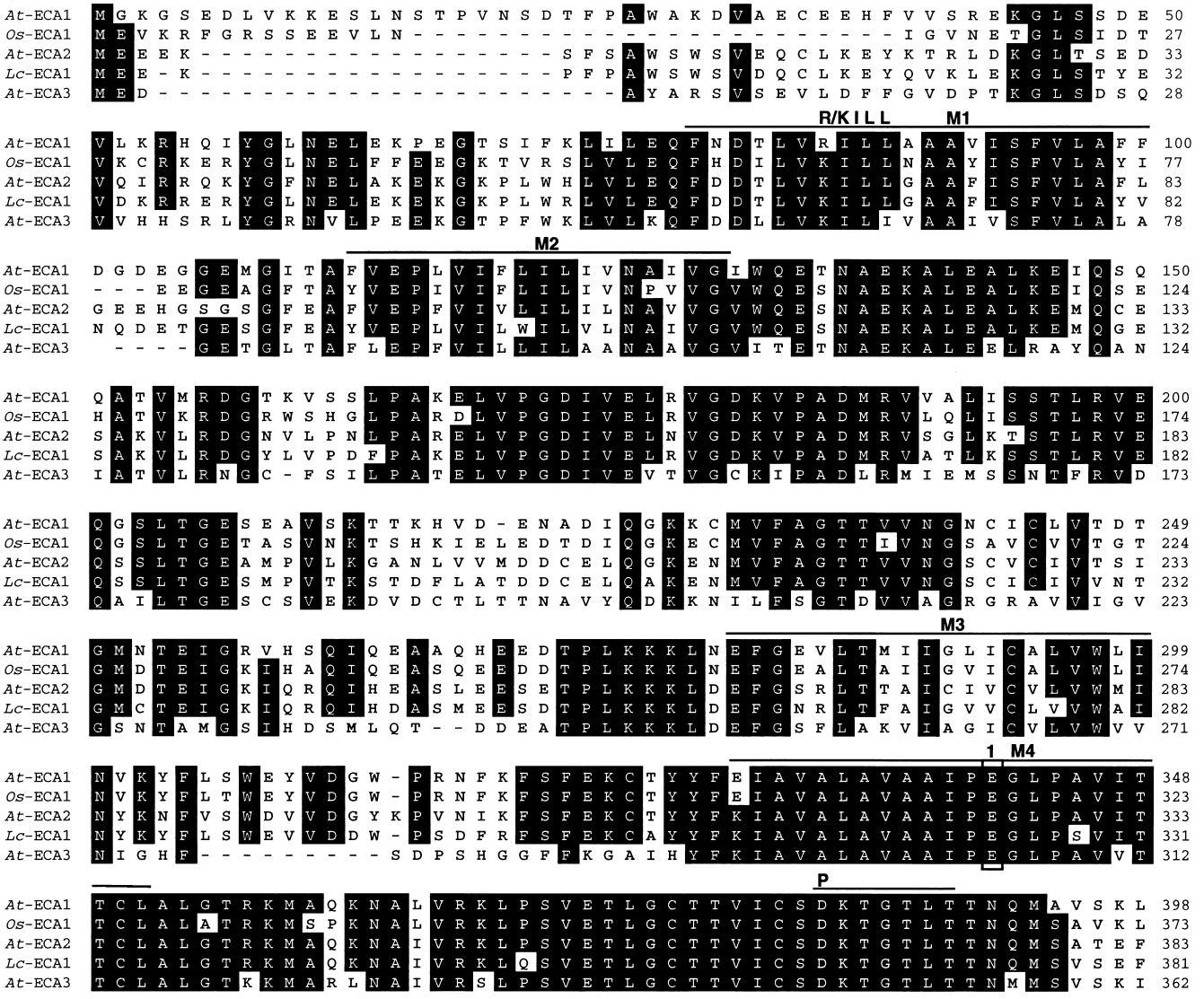


Fig. 1. Multiple amino acid sequence alignment of higher plant type IIA Ca^{2+}-ATPases. Deduced amino acid sequences were aligned by the Clustal method (gap penalty 20, gap length penalty 10) of the MEGALIGN program (DNAstar, Madison, WI). Residues identical to the consensus (present in at least four of the five proteins) are shaded, gaps introduced to maximize alignment scores are denoted by hyphens. The ten putative transmembrane domains (M1–10) are overlined. The positions were determined from the alignment of type IIA ATPases in the P-type ATPase database (http://biobase.dk/~axe/Patbase.html). The potential phosphorylation site is marked with P. Six amino acid residues thought to be essential for calcium ion transport [29–31] are boxed and numbered. The position of putative ER retention signals with the consensus motif (K/X)(K/X)KXX-stop [87,89] and R/KILL [88] are indicated with KXKXX and R/KILL, respectively.

around four and eight, respectively. In the current nomenclature, *Arabidopsis* Ca^{2+}-ATPases are named ACAx and no distinction is made between these two subfamilies of Ca^{2+}-ATPases. For clarity we suggest that in the future, *Arabidopsis* type IIA and type IIB Ca^{2+}-ATPases are designated *At*-ECAx and *At*-ACAx, respectively (Table 2). In this context, ECA stands for ER-type Ca^{2+}-ATPase whereas ACA indicates autoinhibited Ca^{2+}-ATPase.

2.1.1. Type IIA Ca^{2+}-ATPases

A plant type IIA Ca^{2+}-ATPase, *LCA*, was first cloned from tomato [19] (Fig. 1). A partial cDNA clone and a full-length genomic clone were obtained

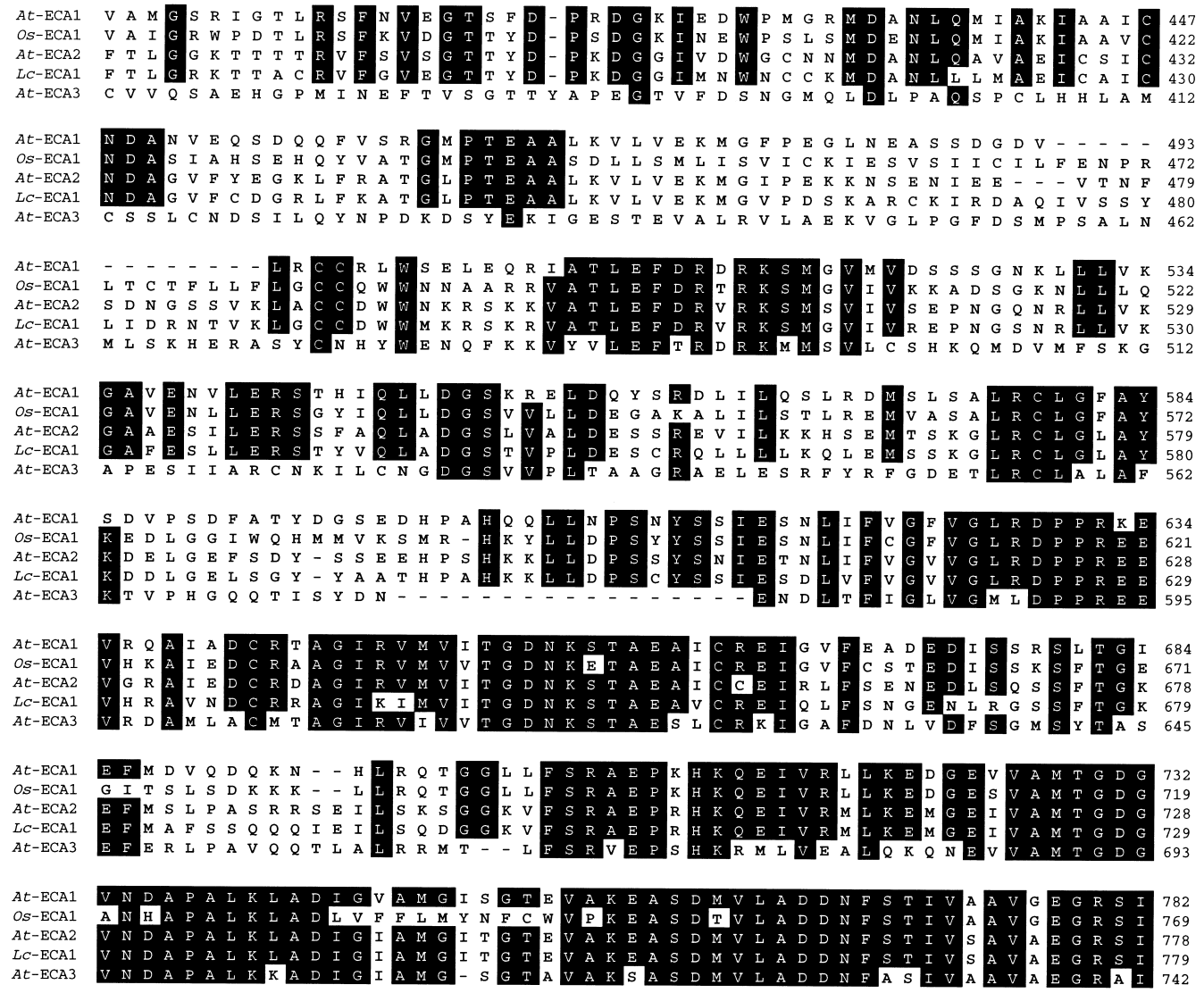
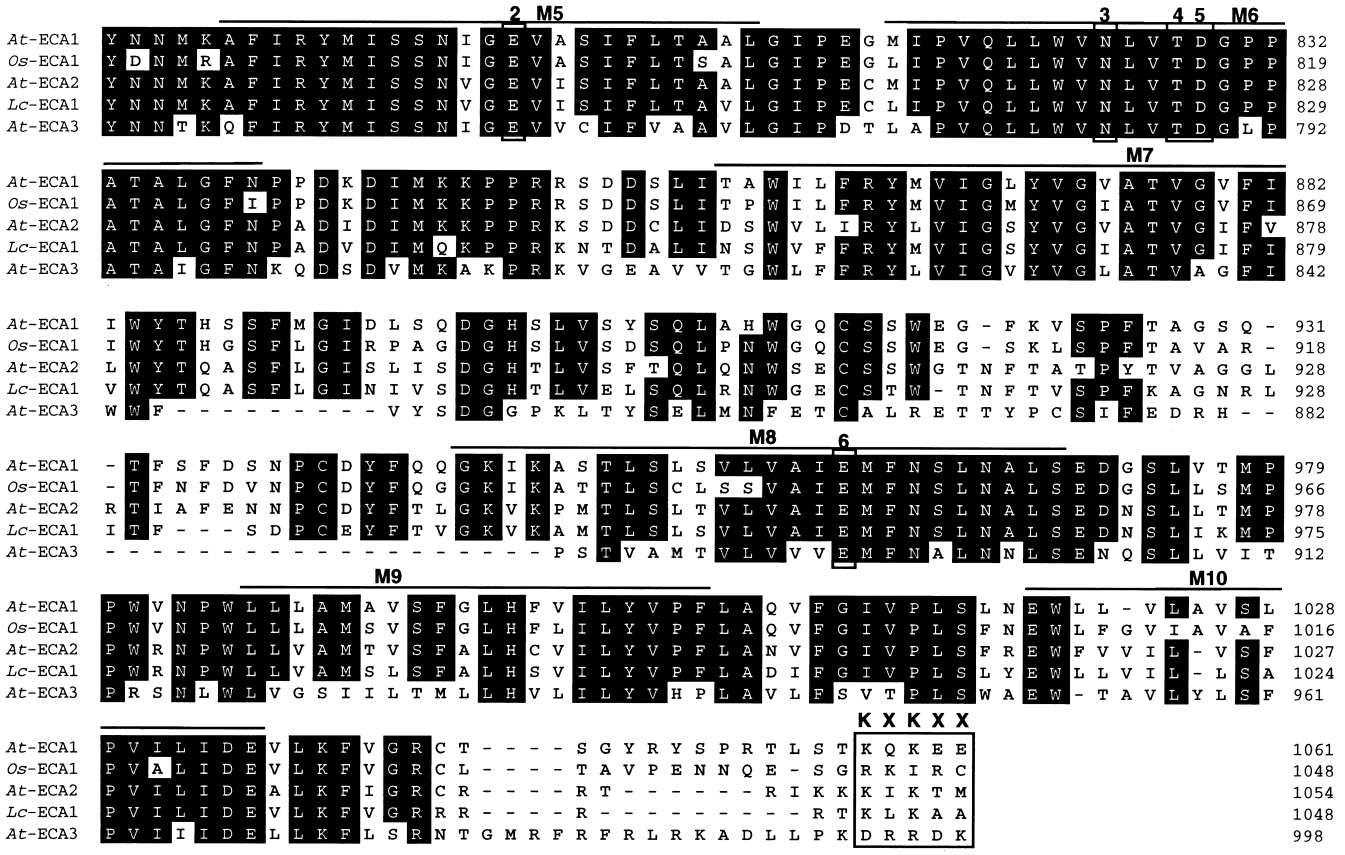


Fig. 1 (*continued*).

by screening a tomato cDNA library using a probe covering a region highly conserved in all P-type ATPases and consecutive screening of a genomic library using the partial cDNA clone. The deduced amino acid sequence specifies a protein of 116 kDa with extensive sequence similarity to animal type IIA Ca^{2+}-ATPases (SERCA pumps). Genomic DNA blot analysis indicated that tomato LCA is encoded by a single gene while Northern blot analysis indicated the presence of three transcript sizes in roots and a single transcript in leaves [19]. Antibodies raised against an LCA domain fusion protein reacted with two polypeptides of 116 and 120 kDa in the vacuolar and in the plasma membrane, respectively [20]. The immunological data correlated with Ca^{2+} transport activities of tonoplast and plasma membrane fractions, and the authors suggested that a single LCA gene encodes two Ca^{2+}-ATPase isoforms differently localized in the tonoplast and plasma membrane.

There is genetic and biochemical evidence for the calcium specificity for *Arabidopsis* ECA1/ACA3, encoding a protein of 116 kDa. Thus, ECA1 has been shown to pump Ca^{2+} in vitro and *ECA1* complements a disruption of yeast endogenous calcium pumps ([16], see Section 4). Like mammalian type



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IIA Ca^{2+}-ATPases, ECA1 has been immunolocalized predominantly to ER membranes using polyclonal antisera raised against its C-terminus [16]. The calcium transport activity of ECA1 is not stimulated by CaM, but the enzyme is insensitive to thapsigargin, a potent inhibitor of mammalian type IIA Ca^{2+}-ATPases [21]. The ability of ECA1 to restore the Mn^{2+} dependence of the yeast *pmr1* mutant and the formation of Mn^{2+}-dependent phosphointermediates also suggests an involvement in Mn^{2+} homeostasis [16].

ECA1 is closely related to OsCa-ATPase, a type IIA Ca^{2+}-ATPase from rice revealing 71% sequence identity. OsCa-ATPase has been cloned using differential display in order to identify gibberellic acid-responsive genes [22]. Overexpression of the Ca^{2+}-ATPase in rice bypassed the requirement for gibberellic acid for expression of the α-amylase gene, a major target gene. It therefore seems likely that OsCa-ATPase gene expression plays an important role in the gibberellic acid signal transduction pathway (see Section 6.3).

To date, three other type IIA Ca^{2+}-ATPases of plant origin have been cloned [23]. These are *Arabidopsis* ECA2/ACA5 (AJ132387; AF013294), ECA3/ACA6 (AJ132388/AF117296; AC004122) and ECA4 (AF117125). Thus, several genes seem to encode type IIA Ca^{2+}-ATPases homologues in *A. thaliana* consistent with the EST analysis. Plant type IIA Ca^{2+}-ATPases cloned so far display molecular masses very similar to those predicted from their amino acid sequence (115–116 kDa), although ECA3 seems to be smaller with 109 kDa. Mammalian type IIA Ca^{2+}-ATPases are in a similar range varying between 100 and 110 kDa [24].

Biochemical evidence suggests the presence of ten transmembrane domains in mammalian type IIA Ca^{2+}-ATPases [12,25,26]. Hydrophobicity analysis of the primary structure has in some cases resulted in the prediction of eight transmembrane domains in plant Ca^{2+}-ATPases [19,22] but these conflicting results might be explained by differences in the algorithms and parameters used. The presence of ten

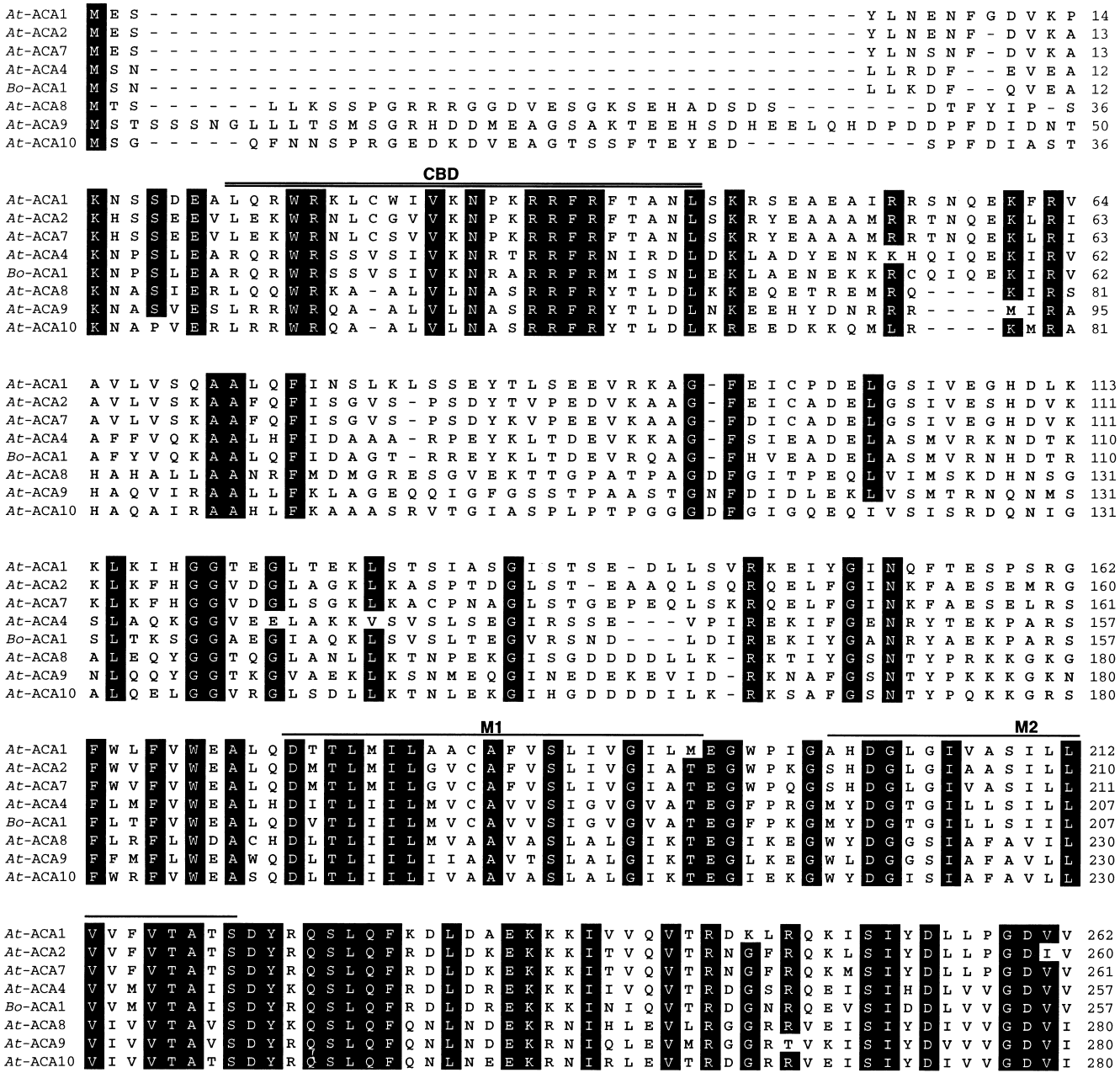

Fig. 2. Multiple amino acid sequence alignment of higher plant type IIB Ca^{2+}-ATPases. Deduced amino acid sequences were aligned by the Clustal method (gap penalty 20, gap length penalty 10) of the MEGALIGN program (DNAstar, Madison, WI). Residues identical to the consensus (present in at least seven of the eight proteins) are shaded, gaps introduced to maximize alignment scores are denoted by hyphens. The ten putative transmembrane domains (M1–10) are overlined. The positions were determined from the alignment of type IIB ATPases in the P-type ATPase database (http://biobase.dk/~axe/Patbase.html). The potential phosphorylation site is marked with P. A putative CaM-binding motif is indicated by CBD. The assembled sequences of ACA8 and ACA9 do not end with a stop codon and the C-termini of these enzymes are therefore likely to be longer.

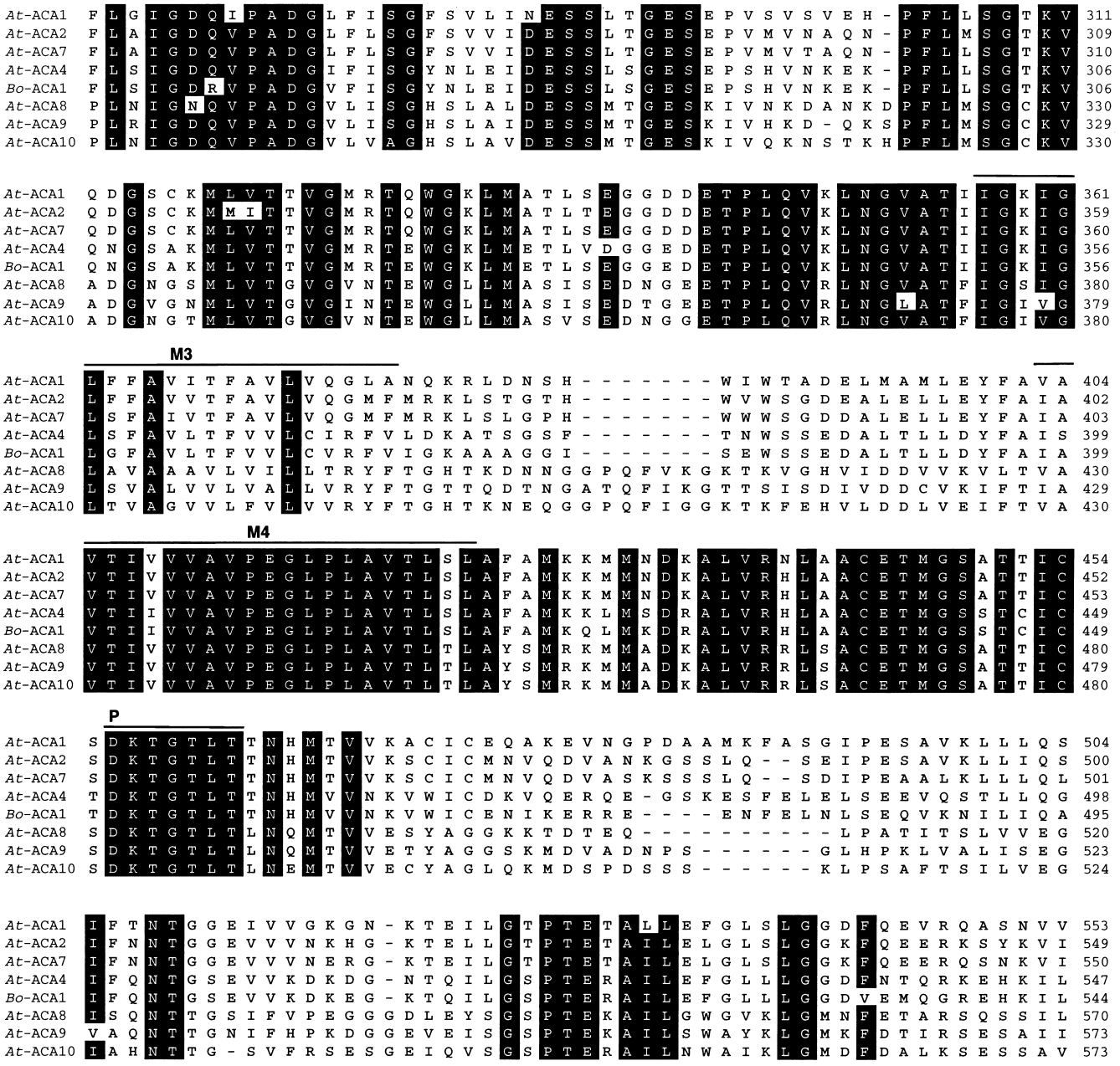

Fig. 2 (*continued*).

transmembrane helices is in line with structures obtained recently from crystals of a mammalian type IIA Ca^{2+}-ATPase at 8 Å resolution [27].

Deriving the ion specificity from analysis of the primary structure has its pitfalls. Yeast PMR2 shows high similarity to Ca^{2+}-ATPases [26] but genetic evidence indicates that this pump transports sodium [28]. In mammalian type IIA Ca^{2+}-ATPases, six amino acid residues are conserved in transmembrane helices M4, M5, M6 and M8, and it has been suggested that they form a high-affinity calcium-binding site [29–31]. Thus, the presence of these residues in other pumps has been used as a fingerprint for a calcium specificity. However, this is only an indirect indication and, remarkably, four out of these six amino acid residues are also conserved in

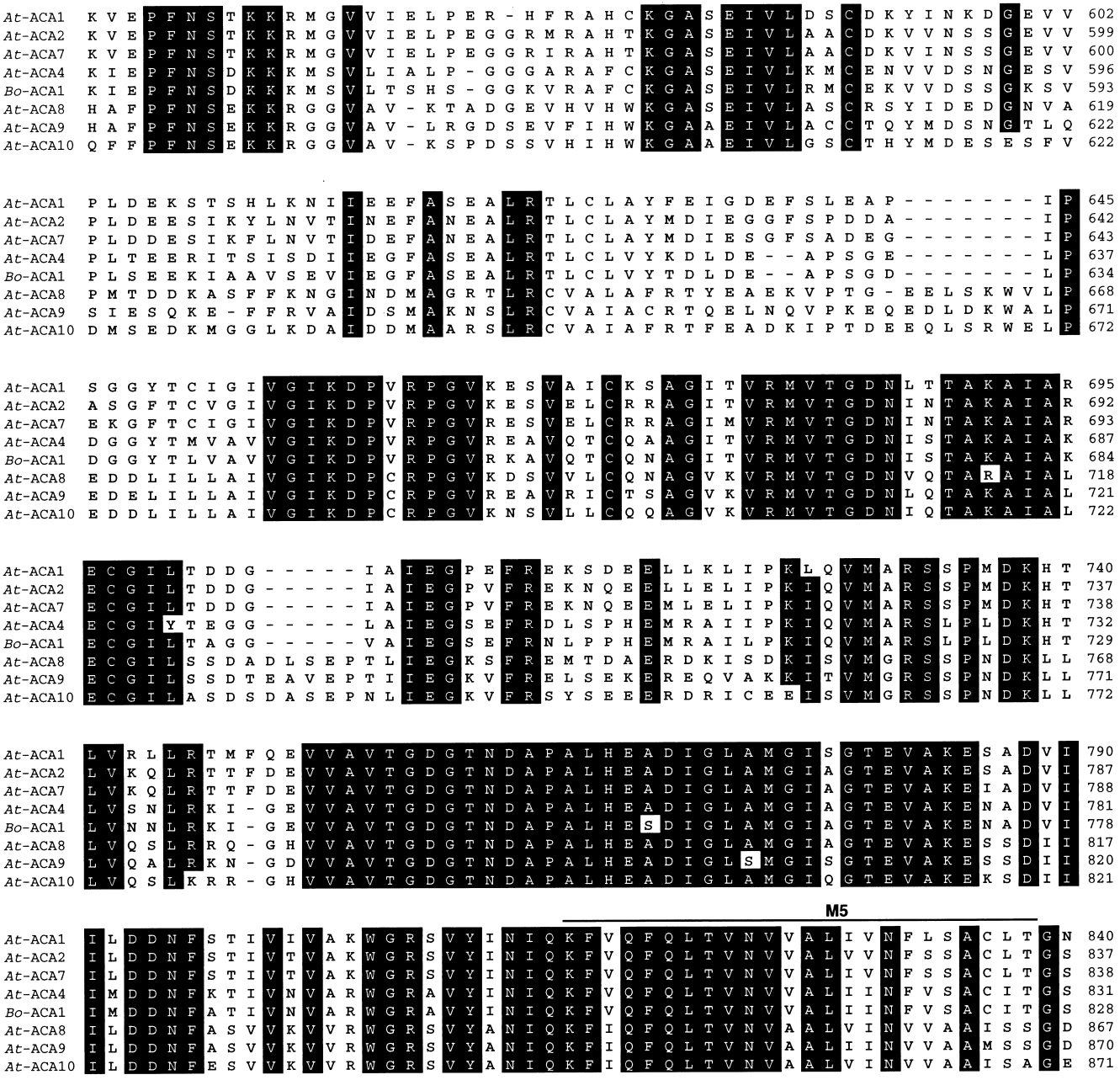

Fig. 2 (*continued*).

(Na$^+$+K$^+$)-ATPases and (H$^+$+K$^+$)-ATPases. In type IIB Ca^{2+}-ATPases, only three of the above mentioned residues are conserved (Fig. 2).

2.1.2. Type IIB Ca^{2+}-ATPases

Plant type IIB Ca^{2+}-ATPase sequences are aligned in Fig. 2. Putative chloroplast inner envelope *ACA1/PEA1* from *Arabidopsis* was the first plant type IIB Ca^{2+}-ATPase to be cloned [15]. The initial paper [15] reporting a size of 946 amino acids has been corrected to a size of 1020 residues giving a molecular mass of about 111 kDa [32]. ACA1 is lacking an obvious C-terminal CaM-binding domain present in mammalian homologues. Instead it reveals a long N-terminal stretch containing a 70–80 amino acid region showing some similarity to the transit peptide of

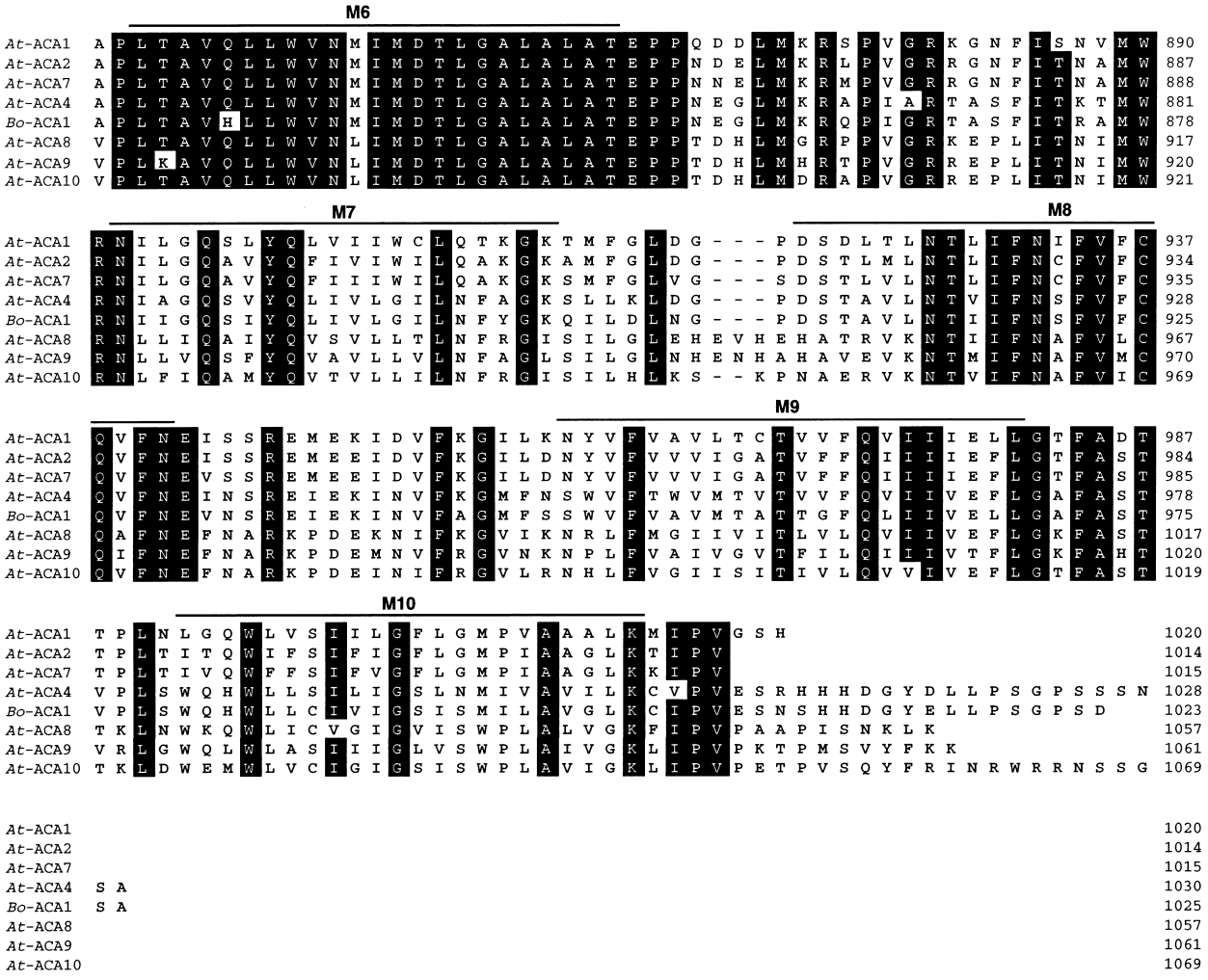

Fig. 2 (*continued*).

the plastid envelope phosphate translocator [15]. Targeting to the plastid was supported by immunological detection of a 90–95 kDa polypeptide in chloroplasts isolated from pea leaves using polyclonal antisera raised against a part of *ACA1*. This part of the enzyme (Glu^{714}-Ser^{950}), however, is well conserved between related isoforms. Biochemical evidence for the substrate specificity of *ACA1* is still lacking and attempts to identify a Ca^{2+}-ATPase activity on plastid envelopes has failed so far [33].

More recently, cauliflower *BCA1* [17] and *Arabidopsis ACA2* [7] were cloned and biochemical evidence was provided that they actually encode CaM-regulated calcium pumps [7,17,34]. The cDNA of cauliflower *BCA1* was cloned using a PCR approach based on tryptic peptide sequence information [17] derived from a CaM-stimulated Ca^{2+}-ATPase that was purified by CaM-affinity chromatography from cauliflower vacuolar membranes [34]. BCA1 was shown to contain a putative N-terminal CaM-binding domain (Ala^{19}-Leu^{43}) but CaM binding to peptides covering these regions was calcium independent.

Arabidopsis ACA2 was functionally expressed in yeast and shown to have a Ca^{2+}/CaM-stimulated ATPase activity [7]. Further, the CaM-binding domain was mapped to the first 36 residues of the N-terminus as shown by CaM-overlay experiments on fusion proteins. The function of the N-terminal domain in autoinhibition was verified by genetic and biochemical analysis of the N-terminal deleted (Δ2–80 residues) enzyme (see Section 3.2).

In a similar study (M. Geisler, M.G. Palmgren, unpublished results), an *Arabidopsis* sequence with very high similarity to cauliflower BCA1, ACA4, was identified as an EST and subsequently cloned as a full-length cDNA using a PCR approach. Following expression in yeast, Ca^{2+}-ATPase activity by ACA4 is activated by CaM. CaM binding to the expressed N-terminus of ACA4 was demonstrated in overlay assays, and interaction between the ACA4 N-terminus and CaM was confirmed by fluorescence experiments using dansylated CaM. Fluorescence of dansylated CaM was enhanced upon titration with the N-terminus of ACA4 in a calcium-dependent manner. ACA4 was strongly inhibited by vanadate (IC_{50} around 6 μM) and FITC, and could be energized by GTP and ITP as alternative substrates; the latter two are typical features for plant CaM-stimulated Ca^{2+}-ATPases. ACA4 was localized to endomembranes as shown by immunodetection after two-phase partitioning of *Arabidopsis* root microsomal membranes. ACA4 is the first member of this subfamily for which a physiological function could be assigned; there is evidence that *ACA4* might function in calcium signaling upon salt stress since modification of gene expression results in altered salt sensitivity (see Section 6.2).

It has been suggested that BCA1 and ACA2-like enzymes form a novel subfamily of CaM-regulated Ca^{2+}-ATPases, distinguished by their N-terminal CaM-binding autoinhibitor and their non-plasma membrane localization [7,17]. All cloned members seem to belong to this novel subfamily; they all feature similar molecular mass (111–116 kDa) and there seems to be consensus on the distribution of ten transmembrane domains [7,17]. At present all characterized members of this subfamily appear to be targeted to endomembranes (i.e. not the plasma membrane). So far, endomembranes found to harbor CaM-regulated Ca^{2+}-ATPases are the tonoplast [35] and the ER [14].

The *Arabidopsis* genome project has recently released the genomic sequence of a close homologue of ACA2, namely ACA7 (AC004786). This pump shows 93% sequence identity to ACA2 and contains an N-terminal candidate CaM-binding domain. A genomic clone also exists for ACA2 (AL035605) proving that they are distinct proteins. The genome project has released further three type IIB ATPases ACA8 (AB023042), ACA9 (AB023045) and ACA10 (AL050352), which are slightly larger than ACA2, ACA4 and ACA7. The gene structures of the former two proteins have been reconstructed based on similarity to the known type IIB ATPases, but the stop codons have not been identified. The proteins are, therefore, slightly larger than the current 1082 amino acids. The third genomic clone AL050352 is identical to ORF5, an 5′ truncated cDNA clone encoding 985 amino acids obtained by PCR extension of EST clone T43417 (M. Geisler, M.G. Palmgren, unpublished results). The full length protein is 1069 amino acids long.

2.2. Phylogenetic relationships of plant Ca^{2+}-ATPases

In a recent attempt to investigate the evolution of the P-type ATPase superfamily, analysis of 159 P-type ATPases has revealed the existence of five apparently monophyletic families [13]. The five branches in the phylogenetic tree are type I ATPases (heavy metal pumps), type II ATPases (Ca^{2+}-ATPases, (Na^{+}+K^{+})-ATPases and (H^{+}+K^{+})-ATPases), type III (H^{+}-ATPases and Mg^{2+}-ATPases), type IV ATPases (phospholipid ATPases) and type V (a group of eukaryotic ATPases without assigned specificity).

A phylogenetic tree constructed using the deduced amino acid sequences of higher plant Ca^{2+}-ATPases and selected other P-type ATPases (Fig. 3) shows that type IIA or type IIB Ca^{2+}-ATPases form clearly distinct groups. Type IIA Ca^{2+}-ATPases are found in bacteria, archaea and eukarya, and must have evolved early [36]. Type IIB Ca^{2+}-ATPases are found only in eukaryotes and might be considered as having evolved later. Plant Ca^{2+}-ATPases identified so far group together with either type IIA or type IIB Ca^{2+}-ATPases.

In the phylogenetic tree, *Arabidopsis* type IIA Ca^{2+}-ATPase isoforms are ECA1, ECA2 and ECA3. ECA3 forms an outgroup in this subfamily. *Arabidopsis* type IIB Ca^{2+}-ATPase isoforms ACA1, ACA2, ACA4 and ACA7 branch together with cauliflower BCA1. ACA8, ACA9 and ACA10 form a separate outgroup in type IIB Ca^{2+}-ATPases but have not yet been characterized functionally. However, based on their relatively high molecular masses

(Table 1), they are likely to encode plasma membrane bound Ca^{2+}-ATPases (see Section 5).

It still remains unclear whether N-terminal and C-terminal regulatory domains of plant and animal Ca^{2+}-ATPases are evolutionarily related. The question is whether these domains evolved independently or diverged after domain swapping in a common ancestor. Animal type IIA Ca^{2+}-ATPases are thought to be regulated by the membrane-bound regulatory protein phospholamban [37,38] and interestingly, there is functional analogy between the intramolecular autoinhibitor of type IIB Ca^{2+}-ATPases and the extramolecular inhibitor phospholamban ([39] see Section 3). Therefore, it has been speculated that these autoinhibitory moieties have a common evolutionary origin and that in some cases they fused to the protein they regulate [36], either to the C- or to the N-terminus.

Vacuolar BCA1 and plastid ACA1 Ca^{2+}-ATPase isoforms type IIB have been suggested to be phylogenetically close to vacuolar Ca^{2+}-ATPases PAT1 [40,41] and PMC1 [42] from *Dictyostelium discoideum* and *Saccharomyces cerevisiae*, respectively, based mainly on the lack of a C-terminal regulatory domain [4,17]. However, unlike BCA1 and ACA1, it has not been possible to identify a putative CaM-binding motif in the N-termini of PAT1 and PMC1. Further, ACA1 was suggested to be related to cyanobacterial Ca^{2+}-ATPases [15], since ACA1 contains a putative N-terminal plastid targeting sequence. In light of newer results, a phylogenetic relationship seems to be very unlikely as the genome

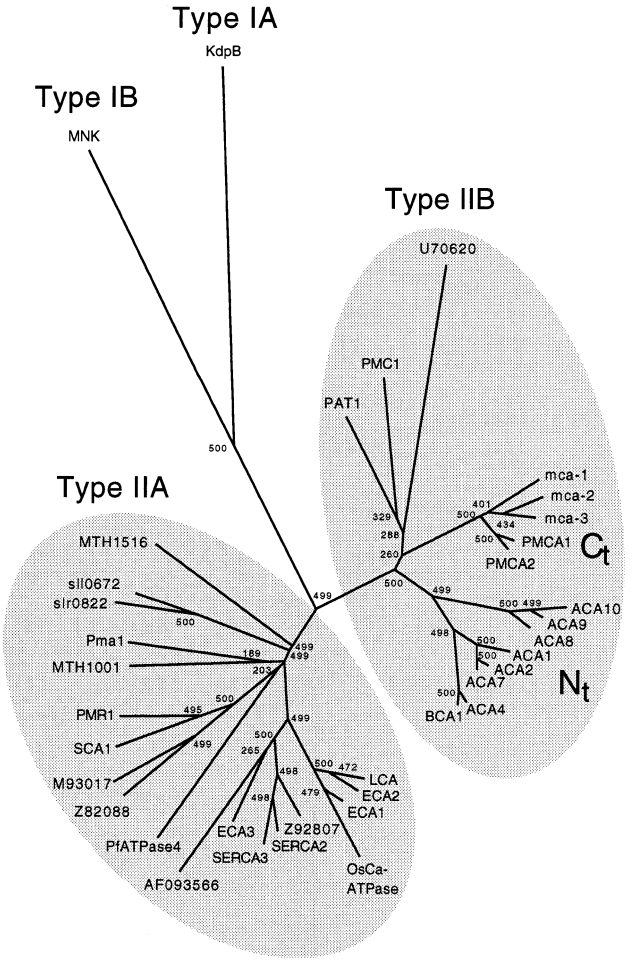

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Fig. 3. Phylogenetic tree of selected P-type ATPases including all known plant type IIA and type IIB ATPases. The tree was constructed using the neighbor joining method. Sequence alignments were based on conserved core sequences ([13]; 265 amino acid residues in total) extracted from the full-length sequences. The trees were bootstrapped 500 times. The numbers at the node points represent the number of times that particular node was present in the replicas. The sequences represented in the tree mentioned clockwise are from the following organisms (accession numbers are in brackets): Type IB: MNK: *Homo sapiens* (Q04656). Type IA: KdpB: *E. coli* (P03960). Type IIB: PAT1: *D. discoideum* (P54678); PMC1: *S. cerevisiae* (P38929); U70620: *Trypanosoma cruzi* (U70620); mca-1: *Caenorhabditis elegans* (AJ223616); mca-2: *C. elegans* (AJ010708); mca-3: *C. elegans* (AJ010646); PMCA2: *H. sapiens* (Q01814); PMCA1: *H. sapiens* (P20020); ACA10: *A. thaliana* (T43417/AL050352); ACA8: *A. thaliana* (AB023042); ACA9: *A. thaliana* (AB023045); ACA1: *A. thaliana* (L08468); ACA2: *A. thaliana* (AF025842); ACA7: *A. thaliana* (AC004786); ACA4: *A. thaliana* (AC002510); BCA1: *Brassica oleracea* (X99972). Type IIA: LCA: *L. esculentum* (M96324); ECA2: *A. thaliana* (AJ132387); ECA1: *A. thaliana* (U93845); OsCa-ATPase: *O. sativa* (U82966); Z92807: *C. elegans* (Z92807); SERCA2: *H. sapiens* (P16615); SERCA3: *H. sapiens* (Q93084); ECA3: *A. thaliana* (AJ132388); AF093566: *T. cruzi* (AF093566); PfATPase4: *Plasmodium falciparum* (U39298); Z82088: *C. elegans* (Z82088); M93017: *Rattus norvegicus* (M93017); SCA1: *Yarrowia lipolytica* (O43108); PMR1: *S. cerevisiae* (P13586); MTH1001: *Methanobacterium thermoautotrophicum* (AE000873); Pma1: *Synechocystis* PCC6803 (P37367); slr0822: *Synechocystis* PCC6803 (D90911); sll0672: *Synechocystis* PCC6803 (D64005); MTH1516: *M. thermoautotrophicum* (AE000912). C_t, ATPases with a CaM-binding site in the C-terminus; N_t, ATPases with a CaM-binding site in the N-terminus.

analysis of the cyanobacterium *Synechocystis* 6803 has revealed the existence of four putative type IIA calcium pumps, whereas no type IIB Ca^{2+}-ATPases were detected [36,43].

3. Regulation of plant Ca^{2+}-ATPases

The current paradigms for models on the regulation of Ca^{2+}-ATPases come from mammalian systems. In these systems, type IIA and type IIB Ca^{2+}-ATPases are regulated by different mechanisms, but a closer look reveals that their regulation is analogous (Fig. 5). Type IIB Ca^{2+}-ATPases are inhibited by an autoinhibitor, an inhibitory sequence within the pump molecule, while type IIA Ca^{2+}-ATPase are inhibited by a protein separated from the pump itself. In both types of Ca^{2+}-ATPases, releasing the (auto)inhibitor from its molecular interaction results in an increased affinity for Ca^{2+} and an increased maximal velocity of the enzyme.

Inhibition is generally thought to function either by a direct steric block of substrate *access* to the active site or by indirect structural locking of the enzyme in an inactive conformation [7]. As the mechanisms of inhibition are still poorly understood, it is not possible to predict whether both subfamilies of pumps employ similar mechanisms of inhibition.

The homopentameric integral membrane protein phospholamban acts as an external inhibitory domain recognizing animal type IIA Ca^{2+}-ATPases (SERCAs). The binding site is close to the aspartyl phosphate-forming active site, and as a result of the interaction at least some of the SERCA pump isoforms are inhibited [38]. The use of chimeric SERCA2/SERCA3 molecules has revealed a phospholamban-binding sequence in SERCA2 isoforms directly following the phosphorylation domain [43]. Mutation analysis has identified the interaction site between Lys^{397} and Val^{402} in SERCA2, and most probably also in SERCA1 [9]. Inhibition is released by phosphorylation of phospholamban, causing dissociation of phospholamban from the pump. Either CaM-regulated (CaMK) or cAMP-dependent protein kinases (PKA) are known to mediate phospholamban phosphorylation [44], thereby coupling the regulation of the pump to calcium and cAMP signals. Data suggesting that phospholamban may also function as a calcium-selective ion channel [37] have been contradicted by others [45].

C-terminal CaM-binding/autoinhibitory domains regulate animal type IIB Ca^{2+}-ATPases through a fine balance between internal autoinhibition and direct CaM binding which causes activation. Release of autoinhibition can also be achieved by phosphorylation of the CaM-binding/autoinhibitory domain by protein kinase A and/or C resulting in a drop in affinity of the domain for the active site. Irreversible activation of the Ca^{2+}-ATPase employs the function of proteases like calpain [46,47] cleaving off the CaM-binding/autoinhibitory domain.

Most of the CaM-binding domains characterized so far are stretches of 16–35 residues and they are poorly conserved in their primary structure (Fig. 4). But, in the α-helical wheel presentation, they show a segregation of basic and polar residues on one side and hydrophobic amino acids on the other [8,48,49]. Many CaM targets are characterized by an aromatic residue at positions 1 and 15 followed by hydrophobic residues at positions 5 and 8 [49]. However, the conformation of the CaM-binding stretch in the native protein remains an open question. Inhibitory and CaM-binding domains are not synonymous but can be overlapping. In case of the human type IIB Ca^{2+}-ATPases, there are two autoinhibitory regions: one region is overlapping and another region slightly further C-terminal is separated from the CaM-binding domain [50].

In mammalian type IIB Ca^{2+}-ATPases, the C-terminal autoinhibitor(s) is thought to interact with multiple regions of the pump. Cross-linking experiments revealed the central and the small cytoplasmic loops as possible candidates [51].

Interestingly, analogies between the internal autoinhibitor of type IIB Ca^{2+}-ATPases and the external inhibitor phospholamban [39,44] have been obtained. A 45 amino acid peptide (A45), corresponding to the phospholamban-binding domain of the sarcoplasmic reticulum ATPase, was shown to induce a conformational change in the plasma membrane Ca^{2+}-ATPase as shown by circular dichroism. Another peptide (C28W; 28 residues), corresponding to an autoinhibitory domain of a type IIB Ca^{2+}-ATPase, induced a similar conformational change in the pump molecule. C28W is about 50% similar to the cytosolic domain of phospholamban, the hydrophilic portion of which

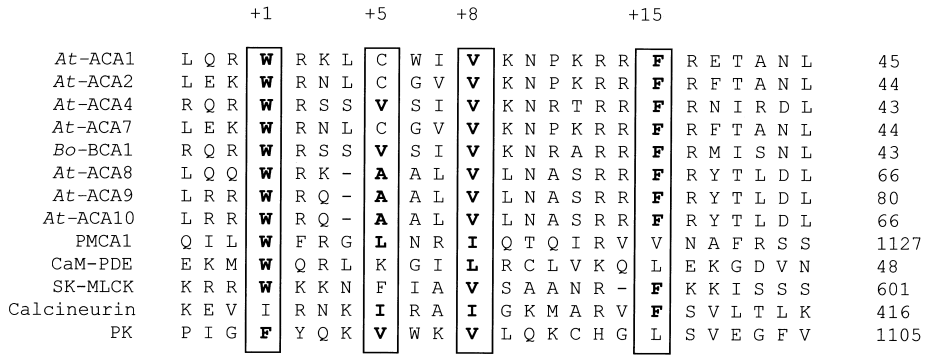

Fig. 4. Multiple amino acid sequence alignment of selected known and putative CaM-binding domains. Putative CaM-binding domains of cloned higher plant type IIB Ca^{2+}-ATPases were aligned with mammalian CaM-binding motifs. The aromatic residues at positions 1 and 15 and the hydrophobic residues at positions 5 and 8 [49] involved in CaM binding are boxed; conserved residues are printed in bold. The sequences and references are as follows: *At*-ACA1 (residues 22–45 [15,32]), *At*-ACA2 (21–44 [7]), *At*-ACA4 (20–43, M. Geisler, M.G. Palmgren, unpublished results), *At*-ACA7 (21–44), *Bo*-ACA1 (20–43 [17]), *At*-ACA8 (44–66), *At*-ACA9 (58–80), *At*-ACA10 (44–66), PMCA1 (plasma membrane Ca^{2+}-ATPase, 1104–1127 [143]), CaM PDE (calcium/CaM-dependent 3′,5′-cyclic nucleotide phosphodiesterase, 25–48 [144]), SK-MLCK (skeletal muscle myosin light chain kinase, 580–601 [145]), calcineurin (catalytic subunit, α isoform 1, 393–416 [146]), PK (phosphorylase kinase, α regulatory chain, 1082–1105 [27]). Numbering of amino acid residues in *At*-ACA8 and *At*-ACA9 was based on putative cDNA sequences extracted from genomic clones AB023042 and AB023045, respectively.

was found to interact directly with CaM. Thus, the extramolecular inhibitor of the sarcoplasmic reticulum Ca^{2+}-ATPase, phospholamban, and the intramolecular inhibitor of the plasma membrane Ca^{2+}-ATPase, peptide C28W, are functionally and structurally analogous [39].

Furthermore, peptides corresponding to the autoinhibitory domain of the plasma membrane Ca^{2+}-ATPase were synthesized and found to inhibit the sarcoplasmic reticulum Ca^{2+}-ATPase. C28W, corresponding to the entire autoinhibitory domain, was the most potent inhibitor. However, while CaM reversed the inhibition of the SR ATPase by C28W, it failed to reverse that produced by non-phosphorylated phospholamban [44].

3.1. Plant type IIA Ca^{2+}-ATPases

It is not known how plant type IIA Ca^{2+}-ATPases are regulated. A plant homologue of phospholamban has not been identified, and attempts to screen for a related gene using a PCR approach with degenerate primers have failed so far (Y. Wang, J.F. Harper, unpublished results). In addition, the putative phospholamban-binding motif identified in mammalian SERCA2 isoforms [9,43] is not present in plant type IIA Ca^{2+}-ATPases.

Careful search in the N- and C-termini of all cloned putative plant type IIA calcium pumps has not resulted in identification of putative CaM-binding domains. The search is made more difficult by the fact that there exists no consensus motif for CaM-binding domains [49] as conservation is more related to its secondary structure (see above). However, a potential to form an α-helix with a fairly similar segregation of residues as above described could also be found in the N-termini of plant type IIA Ca^{2+}-ATPases. On the other hand, the Ca^{2+} transport activity of ECA1, a plant type IIA Ca^{2+}-ATPase has been shown to be independent of CaM and the enzyme does not bind CaM [21]. In the light of this, direct CaM activation of ECA1 is unlikely. It should be kept in mind that among animal type IIB Ca^{2+}-ATPases pumps, the strength of CaM interaction is known to be isoform specific [8]. Therefore, a role for CaM in regulation of plant type IIA Ca^{2+}-ATPases will need to be tested for in all isoforms.

Beside phospholamban regulation, the activity of animal Ca^{2+}-ATPases might also be influenced by their lipid environment, temperature-dependent fluctuations and oligomerization [52]; whether those parameters also influence plant members of this subfamily is not known.

3.2. Plant type IIB Ca^{2+}-ATPases

Remarkably, all plant type IIB Ca^{2+}-ATPases cloned so far seem to employ an N-terminal CaM-binding domain. Fig. 4 shows a multiple alignment of putative CaM-binding stretches of cloned members of this subfamily with different CaM-binding domains from various sources. The aromatic residue in position 1 as well as the two hydrophobic residues in the +5 and +8 positions are conserved in all putative members of this subfamily, while an aromatic residue conserved in mammalian enzymes at position 14 is found in position 15. Biochemical proof for CaM binding to *Arabidopsis* isoforms ACA1, ACA7, ACA8 and ACA9 is lacking, but BCA1 displayed a Ca^{2+}-independent CaM interaction using a synthetic peptide covering Ala^{19}-Leu^{43}. Binding of native BCA1 to CaM is strictly dependent on calcium [35], but short CaM peptide targets are well known to exhibit higher CaM affinities compared to the corresponding sequences in their native environment [48].

Harper et al. [7] mapped a CaM-binding domain of *Arabidopsis* ACA2 to a region including the stretch between residues 19 and 36 in the sequence. The existence of an N-terminal autoinhibitor was confirmed genetically and biochemically by analysis of ACA2 expressed as full-length and N-terminal truncated enzyme (Δ80-ACA2) in yeast [7]. Only Δ80-ACA2 was able to complement a disruption of endogenous yeast Ca^{2+}-ATPases when grown on low calcium and basal Ca^{2+}-ATPase activity of the full-length enzyme was stimulated by CaM, while the truncated enzyme was fully active and insensitive to CaM.

In a similar study, an N-terminal CaM/autoinhibitory domain of ACA4 was verified by CaM-overlay and fluorescence experiments (M. Geisler, M.G. Palmgren, unpublished results). Both the autoinhibitor and CaM-binding sequence appear to be located in the first 44 residues, and the affinities toward CaM are in the low-nanomolar range. Mammalian type IIB Ca^{2+}-ATPase isoforms differ in their regulator sensitivity most likely due to alternative splicing at the mRNA level [53]. It has been suggested that these highly divergent sequences could reflect regulatory and functional specialization of the enzyme to meet physiological needs [54]. It is not known whether such splicing events take place in plants.

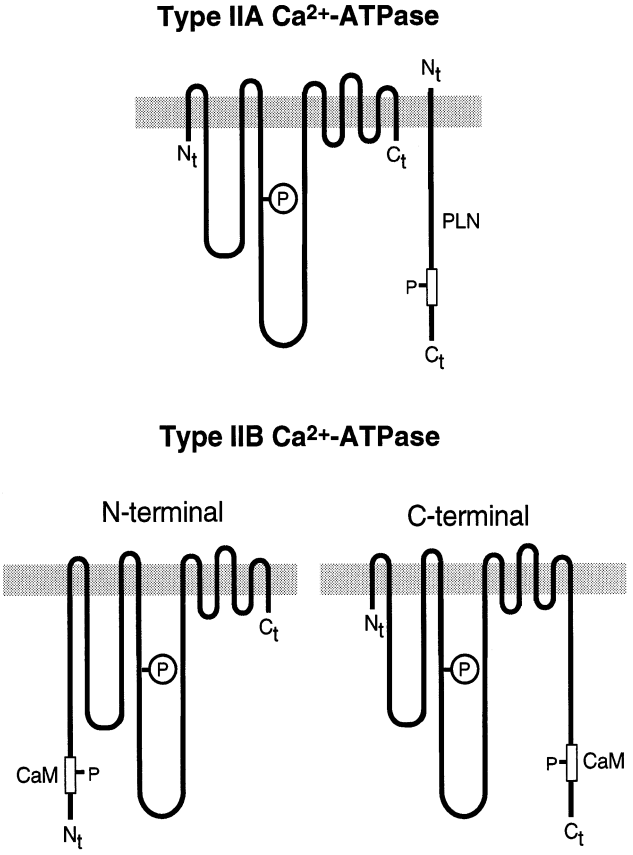

Fig. 5. Model indicating the analogous regulation of type IIA (top) and type IIB Ca^{2+}-ATPases (bottom). The membrane is presented as light grey bar; the position of the phosphorylated aspartate residue is marked (encircled P). Inhibitory regions are boxed. In many type IIB ATPases, CaM binds to an autoinhibitor which can be situated in either the N-terminus (plants) or in the C-terminus (animals). Phosphorylation of regulatory domains/subunits as a likely mechanism for regulation is indicated. C-terminal, C-terminal regulatory domain; N-terminal, N-terminal regulatory domain; P, phosphate; N_t, N-terminus; C_t, C-terminus, PLN, phospholamban.

In summary, it appears that in plant and animals, respectively, the activity of type IIB Ca^{2+}-ATPases can be controlled by either N-terminal or C-terminal autoinhibitors.

An important question to be answered is therefore: Do all plant members of this subfamily contain an N-terminal autoinhibitor? Or is an N-terminal autoinhibitor a feature of endomembrane type IIB Ca^{2+}-ATPases? Accumulative evidence indicates functional differences between Ca^{2+}-ATPases in the

plasma membrane and type IIB Ca^{2+}-ATPases in endomembranes. Although both are clearly CaM stimulated, the plasma membrane Ca^{2+}-ATPases seem to have a higher molecular mass (130 kDa compared to around 116 kDa for the latter) and higher affinities for CaM [33,55,56]. With plasma membrane Ca^{2+}-ATPases, stimulation by exogenous CaM was low unless endogenous CaM was stripped by extensive washing [55,57,58]. Here, CaM could be functioning as a bound subunit of the plant enzyme as has been suggested [59].

A comparative investigation (e.g. employing domain swapping) between N- and C-terminally regulated type IIB Ca^{2+}-ATPases will permit more insightful comparison on their potentially different mechanisms of regulation. Thus, domain swapping has been used to demonstrate that the C-terminal autoinhibitory domain of a human plasma membrane Ca^{2+}-ATPase can be relocated to the N-terminus without loss of function [60]. In addition, 3D crystals of sufficient quality allowing high resolution X-ray crystallography at a resolution of 3 Å or below would be extremely helpful in elucidating the structure-function relationships of CaM binding and autoinhibition. So far, 8 Å structures have been achieved for two P-type ATPases: *Neurospora* H^+-ATPase PMA1 [61] and mammalian type IIA Ca^{2+}-ATPase SERCA1 [27].

In addition to CaM, other factors are known to regulate the activity of mammalian type IIB Ca^{2+}-ATPases. These pumps are known targets for protein kinases being reversibly regulated by protein kinases A [59,62] and C [51,62]. Protein kinase A phosphorylates the enzyme at a Ser at the very C-terminus, while a Thr and Ser, both targets of PKC phosphorylation, are found in the middle of the CaM-binding domain and close carboxy-terminal to this [51]. Phosphorylation of the CaM-binding domain has been shown to prevent CaM binding [8], on the other hand, CaM binding reduces the amount of phosphorylation [51]. Interestingly, protein kinase C recognition sites are also conserved in the N-terminus of plant type IIB Ca^{2+}-ATPases (M. Geisler, M.G. Palmgren, unpublished results).

Mammalian type IIA Ca^{2+}-ATPases are thought to be regulated by the activity of acidic phospholipids and long-chain polyunsaturated fatty acids; these are known to interact with a phospholipid-binding domain close to the phosphorylation site [63]. In the absence of CaM binding results in a decrease of the K_m for calcium and in an increase in the V_{max} [64]. Calcium transport by a type IIA Ca^{2+}-ATPase in carrot vesicles was shown to be stimulated by phosphatidylserine and phosphatidylinositol [65]. However, it is not clear whether the stimulatory effect was the result of a direct interaction between the pump and lipid, or whether the lipids were stimulating a kinase which activated the pump.

Further, plant Ca^{2+}-ATPases might be stimulated by the fungal toxin fusicoccin [66] and by diverse plant hormones (including gibberellic acid, abscisic acid, and auxin and cytokinin derivatives) [67]. For at least some of these interactions, however, it is not clear whether they are product of a direct regulation of the pump or whether they reflect indirect events.

4. *S. cerevisiae* as a model to study plant Ca^{2+}-ATPases

In recent years, the yeast *S. cerevisiae* has turned out to be the ideal host for functional expression of heterologous P-type ATPases [68]. Thus, *S. cerevisiae* has provided us with a model in which to functionally characterize plant type IIA and IIB Ca^{2+}-ATPases and in which to make comparisons.

Heterologously expressed membrane proteins have in many cases proven toxic in prokaryotic systems [69], and with one known exception [70], *Escherichia coli* failed so far as suitable host. Compared to other expression systems, like baculovirus or mammalian cell cultures, yeast has many advantages in respect to its easy handling, short reproduction time and low costs. Appropriate amounts of heterologous expressed ATPase typically accumulate in internal membranes and/or the plasma membrane, both of which can be easily harvested. A set of yeast mutants deficient in one or more enzymes directly or indirectly involved in calcium homeostasis have been engineered by Cunningham and coworkers [42] thus facilitating the characterization of heterologously expressed Ca^{2+}-ATPases.

Triple mutant yeast strain *K616* (*pmr1*, *pmc1*, *cnb1*) has been used for heterologous expression of plant Ca^{2+}-ATPases in a number of studies [7,14,16,21] and is now well established as a genetic

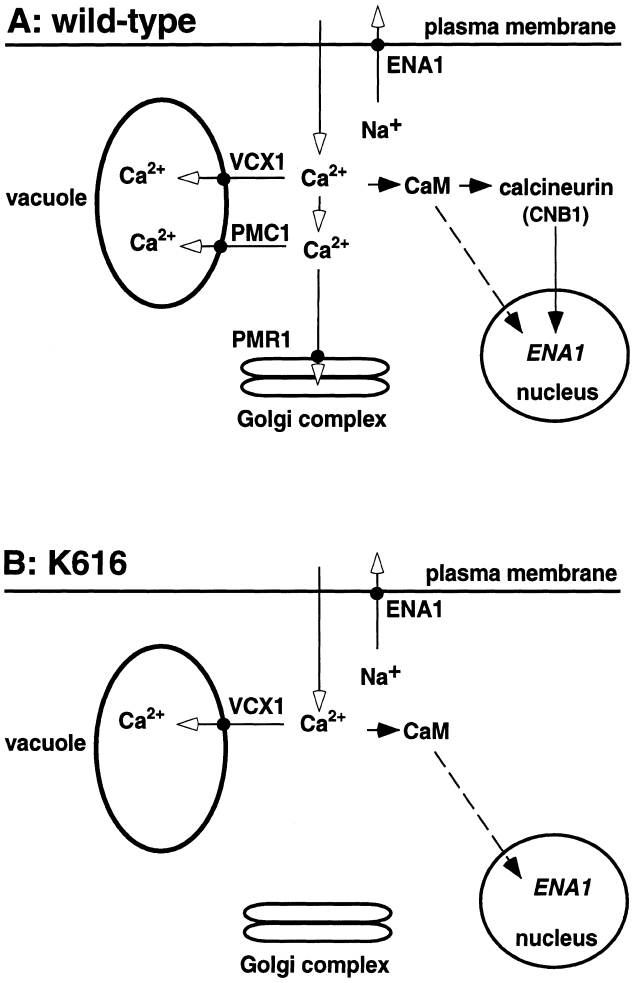

Fig. 6. Model showing putative involvement of enzymes in regulation of cytoplasmic calcium and NaCl concentrations in wild-type (A) and triple mutant yeast strain *K616* (B). In wild-type yeast, calcium uptake in the yeast vacuole is mainly achieved by the low affinity high capacity H^+/Ca^{2+} antiporter VCX1 [42]. At low calcium concentrations calcium is sequestered into the vacuole by the function of the high affinity PM-type Ca^{2+}-ATPase PMC1 [12] and – in the absence of PMC1 – by PMR1 [42]. In the triple mutant *K616* (*pmr1*, *pmc1*, *cnb1*) – a well established genetic tool for the demonstration of calcium pumping activity [7,14,16,21] – both endogenous Ca^{2+}-ATPases are deleted causing very low growth on calcium depleted medium most likely due to insufficient filling of Golgi stores. Open arrowheads indicate transport activities, solid arrowheads show activating interactions. The figure is adapted from [67].

tool for the demonstration of calcium pumping activity. In wild-type yeast (Fig. 6A) calcium uptake in the yeast vacuole – shown to be the main calcium store [67] – is mainly achieved by the low affinity high capacity H^+/Ca^{2+} antiporter VCX1 [71]. At low cytoplasmic calcium concentrations calcium is sequestered into the vacuole by virtue of the high affinity type IIB Ca^{2+}-ATPase PMC1 [42]. In the absence of PMC1, the residual calcium sequestration is achieved by PMR1 [42], a type IIA Ca^{2+}-ATPase in the Golgi complex known to support a variety of secretory functions [26]. In the triple mutant *K616* (Fig. 6B), both endogenous Ca^{2+}-ATPases are deleted.

Suppression of calcineurin function by disruption of the regulatory subunit B (*CNB1*) is essential for *pmr1*, *pmc1* knockout strains to be viable. One function of calcineurin is to inhibit the vacuolar H^+/Ca^{2+} antiporter VCX1 [71], implying that in *pmr1*, *pmc1* double mutants, there is no system to fill vacuolar Ca^{2+} stores. Accordingly, the *pmr1*, *pmc1* double mutant is lethal, whereas the *pmr1*, *pmc1*, *cnb1* triple mutant is perfectly viable. Wild-type yeast is able to grow at very low external Ca^{2+}, e.g. at 20 mM EGTA in the medium which equalizes an estimated 300 nM free Ca^{2+} depending on the Ca^{2+} content of the medium [21]. Under these circumstances, the yeast triple mutant cannot grow. This is probably because cytoplasmic Ca^{2+} remains too low to activate VCX1.

The triple mutant strain has so far been used to demonstrate that *Arabidopsis* Ca^{2+}-ATPase isoforms ECA1, ACA2 and ACA4 can function as high affinity endomembrane calcium pumps. Expression of the full-length enzyme of ECA1 provided growth on low calcium comparable to wild-type [16], while in case of ACA2 [7] and ACA4 (M. Geisler, M.G. Palmgren, unpublished results) only N-terminal truncated – activated – enzymes were successful in complementing PMR1 function. This provided the first evidence that the N-terminal domain of these enzymes contains an autoinhibitor. Complementation of PMR1 in the two latter cases is remarkable, as ACA2 and ACA4 are closer to PMC1 than to the type IIA Ca^{2+}-ATPase PMR1. Lack of complementation for the full-length enzyme also indicates that activation of ACA2 and ACA4 by endogenous yeast CaM isoforms did not occur. This might be due to calcium concentrations in the yeast cytosol being too low.

Besides its usage as an in vivo assay for functional identification of active calcium pumps, the triple mutant, being devoid of background Ca^{2+} pump activity, can be used as a powerful expression host [16].

Strategies to separate heterologous calcium pump activity from the endogenous vacuolar H^+/Ca^{2+} antiporter activity have been described [21]; these include use of ionophores like gramicidin and the V-ATPase inhibitor bafilomycin. In addition, a C-terminal 6×His-tagged version of ACA4 could be functionally expressed in this strain allowing for purification of the enzyme by affinity chromatography and functional reconstitution into liposomes (M. Geisler, M.G. Palmgren, unpublished results).

In addition, analogous mechanisms that function in calcium homeostasis and salt stress adaptation make yeast the ideal host to study plant calcium ATPases. In both plants and yeast, the vacuole is thought to represent the major calcium store [2]. NaCl stress perception and tolerance in yeast as well as in plants involves calcium-dependent signaling events [72,73], consistent with a role for calcium signaling in the salt adaptive response of both plant and yeast (for reviews see [74,75]). The yeast calcineurin B subunit and the corresponding *Arabidopsis* gene *SOS3* [74] appear to link salt tolerance and calcium signaling. NaCl hypersensitivity of the *pmr1*, *pmc1*, *cnb1* triple mutant results mainly from loss of calcineurin induced expression of the ENA1/PMR2 Na^+ pump [67].

5. Subcellular distribution of plant Ca^{2+}-ATPases

Recent reviews document that plant Ca^{2+}-ATPases are present in multiple membrane systems [1,4,5,11]. There seems to be a general consensus on the model that plant cells contain both types of calcium pumps – type IIA and type IIB – in varying proportions as has been suggested by Hwang et al. [56]. Type IIA and type IIB Ca^{2+}-ATPases have been named after their intracellular locations in animal cells where their distribution is limited to sarcoplasmic or endoplasmic reticulum or the plasma membrane, respectively. Fungal and plant cells provide the first examples where these rules seem to have been broken: type IIA Ca^{2+}-ATPases are found also in the yeast Golgi apparatus [76], in the vacuole and in the plant plasma membrane [20], and type IIB ATPases have been identified in an increasing number of intracellular membranes such as the vacuole [17,40,42], the chloroplast inner envelope [15] and the ER [14].

The following section aims at summarizing the literature on the distribution of Ca^{2+}-ATPases within the plant cell. Readers interested in a detailed overview on the properties and localization of individual pumps are referred to the recent review by Evans and Williams [4].

5.1. Biochemical and immunological approaches

A general problem in the distinction between the different calcium pump isoforms and the two functional types is in part caused by the usage of different plant materials, organs and tissues from various developmental stages. Thus, some tissues are enriched for a certain calcium pump isoform [35,55,56]. In addition, difficulties in isolating pure plant membranes [33] have added to uncertainties of their intracellular origin.

A number of biochemical tools have been used to discriminate between type IIA and type IIB Ca^{2+}-ATPases. Ca^{2+} transport and phosphoenzyme formation of individual ATPase isoforms were investigated mainly with respect to their sensitivity toward several inhibitors. The fluorescein derivative erythrosin B seems to act more as a general inhibitor of (plant) Ca^{2+}-ATPases [4,5] and can be used successfully to discriminate between Ca^{2+} and H^+ transport [77]. Diagnostic inhibitors of animal type IIA Ca^{2+}-ATPases [24,38,78] like the indole tetrameric mycotoxin cyclopiazonic acid (CPA), thapsigargin or benzohydroquinone derivatives (like AHQ or BHQ) have been used intensively only in a few studies [21,56,79]. CPA is an efficient inhibitor of calcium transport and/or Ca^{2+}-dependent ATP hydrolysis in red beet [77], carrot [56] and with *Arabidopsis* type IIA Ca^{2+}-ATPase isoform ECA1 expressed in yeast (IC_{50} of 3 nmol/mg membrane protein; 100 nmol/mg is needed to block 90% of the activity) [21].

The sesquiterpene thapsigargin, a widely used ubiquitous mammalian type IIA calcium pump inhibitor [78], is known to inhibit calcium binding and has been proposed to interact directly with the third transmembrane domain [80]. Unfortunately, this inhibitor seems not to affect plant calcium pumps [12,21]; in the micromolar range – typical concentrations used for efficient inhibition of mam-

malian type IIA calcium pumps – thapsigargin had no effect on red beet calcium pumps [79] or on *Arabidopsis* ECA1 expressed in yeast tested up to 3 μM [21]. It has been speculated that the plant pumps may have evolved as thapsigargin resistant enzymes since the compound was originally obtained from a plant source [21].

Unfortunately, in most localization studies, antibodies raised against Ca^{2+}-ATPases were not shown to be isoform-specific. It is important to keep in mind that rigorous isoform specificity of antibodies can first be determined after the completion of the genome and subsequent identification of all isoforms in a given species. *Arabidopsis* ACA1 has been localized to the chloroplast inner envelope, based on membrane fractionation and immunodetection using a polyclonal antibody raised against a relative large portion of the enzyme (Glu^{714}-Ser^{950}). However, this region of ACA1 is well conserved between related isoforms and also other P-type ATPases. Accordingly, anti-ACA1 antibodies have been shown to cross-react with a plasma membrane-bound Ca^{2+}-ATPase from radish seedlings purified by CaM-affinity chromatography [55].

Tomato type IIA Ca^{2+}-ATPase LCA [19] was localized not to the ER but to the plasma membrane and the tonoplast, using polyclonal antisera raised against a fusion protein of LCA. The existence of signals of two different sizes (116 and 120 kDa, respectively) was explained by splicing events [20]. Although an ER retention signal KXKXX is well conserved in the very C-terminus of LCA [4] (see below), the immunological data correlated with Ca^{2+} transport activities of tonoplast and plasma membrane fractions. However, in light of recent genomic sequencing data, it cannot be ruled out that the antibodies have cross-reacted with a type IIB Ca^{2+}-ATPase in the plasma membrane.

An interesting alternative approach to categorize Ca^{2+}-ATPase activities was performed by Sze and coworkers. Instead of separating pumps by their membrane association, they differentiated Ca^{2+}-ATPases by their biochemical characteristics [56]. Following this idea, Table 4 provides a summary of biochemical studies from several laboratories of the last two decades giving a picture outlined in the following.

Roughly, type IIA Ca^{2+}-ATPases are characterized by a strong specificity for ATP as a substrate and are not stimulated by CaM.

Plant type IIB Ca^{2+}-ATPases can be partially energized also by GTP and ITP (to a certain extent) and are CaM-stimulated. The low nucleotide specificity has been successfully used in a number of studies to differentiate between the calcium pump activity and other ATPase activities [55,81]. Moreover, type IIB Ca^{2+}-ATPases have been shown to be highly sensitive toward FITC [55] (M. Geisler, M.G. Palmgren, unpublished results), which acts as a competitive inhibitor with the nucleotide [82].

Plant Ca^{2+}-ATPases localized in the plasma membrane are typically above 124 kDa and thus seem to have a somewhat higher molecular mass than their endomembrane homologues [4,34,35,55,79]. Further, they are shown to have a higher sensitivity towards CaM. In fact, CaM stimulation by exogenous CaM can be masked due to a very tight complex formation with endogenous CaM [55,81]. In contrast, stimula-

Table 4
Subcellular location and molecular mass of type IIA and type IIB Ca^{2+}-ATPase activities[a] from various plant material

Plant material	Type IIA Ca^{2+}-ATPase activity	Type IIB Ca^{2+}-ATPase activity	Ref.
Barley leaves		ER: 116 kDa (PE); PM: 130 kDa (PE)	[33]
Carrot suspension cells	ER: 120 kDa (PE)	Endomembrane: 120 kDa (PE); PM: 127 kDa	[56,65,139,140]
Cauliflower florets		Endomembrane: 111 kDa; PM: 116 kDa	[34]
Maize coleoptiles		ER: 140 kDa; ER: 102 kDa	[5,141]
Radish seedlings		PM: 124–133 kDa	[55,57,142]
Red beet storage root	ER: 119 kDa (PE)	PM: 124 kDa (PE)	[79]
Tomato fruit	Tonoplast: 116 kDa; plasma membrane: 120 kDa		[20]

[a]CaM stimulation was chosen as criterion for the categorization of type IIA and IIB Ca^{2+}-ATPase activities. Only activities with known molecular masses determined either by Western analysis or phosphoenzyme formation (PE) are listed.

tion by exogenous CaM can be easily detected with endomembrane type IIB Ca^{2+}-ATPases [7,35,56,77].

In this context, it is worth recalling that plant plasma membrane type IIB Ca^{2+}-ATPases have been shown to be extremely sensitive toward proteolytic loss of the CaM-binding domain [55]. This might have been caused by a lack of CaM stimulation in some studies and confusion concerning the precise molecular mass of individual pumps.

However, some conflicting observations require further investigation. For example, in maize shoots a 140 kDa CaM-stimulated Ca^{2+}-ATPase has been found that is most probably localized to the ER as deduced from immunocytochemical data using independent monoclonal antisera [83].

It has only in a few cases been possible to purify and reconstitute a single Ca^{2+}-ATPase isoform from plant sources without cross-contamination by other ATPases. Since the first purification of a CaM-stimulated Ca^{2+}-ATPase by Dieter and Marmé [84], CaM-affinity chromatography on solubilized microsomal membrane fractions has become the method of choice to purify calcium pumps that bind CaM. From tobacco cell cultures two active CaM-binding pumps of 116 kDa and 130 kDa [33] and from carrot suspension-cultured cells a 120 kDa endomembrane Ca^{2+}-ATPase [56] have been purified using a CaM resin.

Only the vacuolar Ca^{2+}-ATPase from cauliflower florets [34] and the mentioned putative ER-localized 140 kDa Ca^{2+}-ATPase from maize [83] have been functionally reconstituted. The cauliflower Ca^{2+}-ATPase, originally assigned a molecular mass of 115 kDa upon phosphoenzyme formation [85], is most likely identical to the 111 kDa pump purified from cauliflower by affinity chromatography of solubilized light (vacuolar) microsomes [34,35]. Overlapping peptide information has resulted in cloning of the corresponding gene *BCA1* [17] and the subsequent production of antibodies directed against portions of this enzyme. Recent immunofluorescence microscopy data confirm the vacuolar location (P. Askerlund, pers. comm.).

The group of De Michelis succeeded recently in the purification by CaM-affinity chromatography of Ca^{2+}-ATPases from plasma membranes of radish [55] and *Arabidopsis* (C. Bonza, M.I. De Michelis, pers. comm.).

5.2. Molecular approaches

It is not well understood how Ca^{2+}-ATPases are targeted to individual membrane systems [86]. In animal type IIA Ca^{2+}-ATPase two different ER-targeting or retention signals have been shown to be responsible for ER association of these pumps [87,88]. The C-terminal ER retention signal for type I proteins [89] with the consensus motif (K/X)(K/X)KXX-stop [87] is well conserved in *Arabidopsis* type IIA isoforms ECA1 and ECA2, as well as in tomato LCA, but not in the rice OsCa-ATPase and ECA3 (see Fig. 1). The motif KXKXX seems to be recognized also in yeast [90] which has led to the proposal that this retention signal is conserved between phylogenetically distant organisms [14].

Data obtained from chimeric molecules of mammalian type IIA and IIB Ca^{2+}-ATPases have shown that transmembrane domain(s) contain an ER retention signal but that also other motifs [91] contribute to a proper ER localization. The targeting motif R/KILL has been suggested to be conserved in the first transmembrane domain of animal type IIA Ca^{2+}-ATPases but not in type IIB Ca^{2+}-ATPases [88] and is also well conserved in most plant homologues (see Fig. 1). In *Arabidopsis* isoforms ECA1 and ECA2, both putative targeting sequences are perfectly conserved and for the former the intramembrane location is in accordance with that [16].

Recent results have shown that the above described localization problems can be overcome using genetic approaches like epitope- and green fluorescent protein (GFP)-tagging techniques [14,92]. These have allowed precise intracellular localization of a H^+-ATPase and *Arabidopsis* ACA2 *in planta*. In the first case [92], the 10-residue c-*myc* epitope, derived from the animal oncogene [93], was inserted into the C-terminus of H^+-ATPase isoform AHA3 expressed in transgenic *Arabidopsis* under the control of the endogenous AHA3 promoter. Immunofluorescence studies with tissue sections of transgenic plants revealed that tagged AHA3 is restricted to the plasma membranes of phloem companion cells. C-*myc* was shown to be an appropriate epitope tag for *Arabidopsis* immunocytochemistry as there was little or no background immunoreactivity in wild-type plants. Immunogold labeling was successfully used for an

independent quantification of AHA3 in plasma membranes.

In a similar study [14], the type IIB Ca^{2+}-ATPase PMC1 could be localized to the vacuolar membrane in yeast by immunofluorescence experiments using a HA-epitope tagged enzyme [42]. A 96 bp fragment encoding three tandem repeats of the HA-epitope was cloned into the N-terminus of PMC1 and the tagged enzyme was overexpressed in yeast. In one study, the HA-epitope was tested in *Arabidopsis* and was found to be associated with large background signals [92]. Epitope tagging in general provides a major advantage that highly specific monoclonal antisera against the short epitopes are commercially available reducing background and eliminating the possibility that cross-reaction with related isoforms occurs.

Another tagging strategy has been used to determine the intracellular location of ACA2 [14]. A variant of the jellyfish *Aequorea victoria* GFP [94] was fused to the C-terminus of ACA2 and the fusion protein was expressed under the control of the constitutive CAMV-35S promoter. The C-terminus was chosen in order to avoid interference with the N-terminal regulatory domain [14]. Employing confocal and computational optical-sectioning microscopy, ACA2-GFP was detected in the ER of live root cells and additionally, strong fluorescence was also detected around the nuclei of mature epidermal cells. ER residence was confirmed by buoyant density sucrose gradient experiments where ACA2 was shown to cofractionate with the ER marker enzyme BiP and ER-localized ECA1 [16]. A GFP-tagged ACA2 was shown to be functionally unaltered by complementation studies using the triple mutant yeast strain *K616* [14].

The major advantage in using GFP as a reporter molecule lies in the fact that GFP allows a non-invasive investigation on live cells. Several GFP variants have been engineered [95] with respect to more suitable codon usage, removal of alternative splice sites, increased protein stability, and higher fluorescence [96]. For example, Harper et al. used a modified version of the GFP gene (S65T*GFP*) which is well suited for confocal and standard epi-fluorescence microscopy in plant cells [14].

With new tools in our hands, epitope tagging of plant Ca^{2+}-ATPases allows for many interesting approaches. Beside deeper knowledge about tissue and developmental expression patterns and the intracellular locations of all these calcium pump isoforms, one should expect closer insights into the molecular mechanism of intracellular targeting. Very little is known about the targeting of integral membrane proteins in plant cells [97], and how mechanisms might differ from paradigms being established in animal and yeast systems. However, a unique research opportunity is presented by the multiple membrane locations of the type IIB calcium pumps in plants. Members of this family of pumps, which are exclusively located in the plasma membrane of animal cells, target to multiple locations in plant cells, for example ACA2 is located in the ER, whereas BCA1 is located in the tonoplast. Thus, structural differences between these pumps may give important clues as to the information used to target closely related enzymes to different membrane systems.

6. Higher plant Ca^{2+}-ATPase functions

As with H^+-ATPases, where more than ten isoforms have been found so far [98], it is not known why there are so many isoforms of plant Ca^{2+}-ATPases. At least three non-exclusive hypotheses have been proposed [99]. First, individual isoforms are functionally distinct and harbor unique biochemical properties. Second, the greater variety of promoters allows for enhanced control at the gene level, e.g. enzymatically equivalent isoforms are tissue-specifically expressed. Third, functionally redundant isoforms have evolved by gene duplication events. It is still too early to say whether one or more of these hypotheses are correct.

Recent data document a critical involvement of Ca^{2+}-ATPases in plant calcium homeostasis required for (i) maintaining Ca^{2+} second messenger functions and (ii) for the stability and activity of many protein functions. Further, we have included data pointing to a putative involvement in protein processing and salt stress adaptation.

6.1. Involvement in Ca^{2+} second messenger functions

Calcium is now established as a second messenger coupling a wide range of extracellular stimuli to re-

sponses in animal [100] and in higher plant cells [2]. Transient and fast changes in cytoplasmic calcium concentrations occur upon extracellular signals like hormones [101], light [102], touch [103], cold [104], drought [105] and salinity [74,105,106]. Recent evidence suggests that plants – like animals – produce graded responses resulting in Ca^{2+} signatures like oscillations and waves [2,4,107–109]. In the vacuolar membrane of guard cells, the best understood system so far, voltage-gated Ca^{2+} release channels and inositol 1,4,5-trisphosphate and cADP-ribose-sensitive calcium release pathways have been identified as part of the system encoding calcium signatures [110]. The signaling machinery downstream from calcium is unknown; however, CaM isoforms or CaM-like Ca^{2+}-binding proteins [111,112] are good candidates that could function in decoding calcium signals [109,113].

Low cytoplasmic calcium levels in plants (typically < 100 nM) – a prerequisite for second messenger function – are achieved by the function of high-affinity primary Ca^{2+}-ATPases of the P-type and low-affinity H^+/Ca^{2+} antiporters [1,2,4]. These energy-dependent transporters extrude calcium either to the external medium or into internal stores, like the ER and the vacuole [1,2]. The latter has been shown to constitute the dominant plant calcium pool [1,110]. Because of the higher affinity of the Ca^{2+}-ATPases toward calcium [1,2,4,21], Ca^{2+}-ATPases are thought to be responsible for a fine tuning of calcium concentrations [56].

An interesting work has described the rapid increase of Ca^{2+}-ATPase activity in the tendrils of *Bryonica dioica* [114] during the early stages of tendril coiling. Cells were shown to have both a CaM-stimulated Ca^{2+}-ATPase in the ER and a CaM-insensitive calcium pump in the plasma membrane. Recent studies employing calcium indicator dyes have demonstrated that perception of a nodulation signal results in calcium waves and oscillations in root hairs of alfalfa [107].

In animal cells, Ca^{2+}-ATPases are thought to play a crucial role in the maintenance and regulation of waves and oscillations [115,116]. In frog oocytes, the frequency of inositol 1,4,5-trisphosphate induced repetitive calcium waves increased in cells over-expressing a type IIA Ca^{2+}-ATPase [115]. Further, calcium extrusion across the plasma membrane seems to be essential for spike recovery [117]. So far, a direct involvement of Ca^{2+}-ATPase activities in the control or recovery of calcium oscillations has not been established in a plant system.

Modern bio-imaging techniques like fluorescence resonance energy transfer (FRET) can be used to study protein interaction [118,119]. Advantages of FRET are that it is not destructive and is observable by fluorescence microscopes in intact cells and tissues. Beside protein-protein interaction, a new developed pair of GFP variants has recently been employed as fluorescent indicator for Ca^{2+}, named cameleon [120]; such a FRET pair fused to a calcium pump might upon expression in transgenic plants allow monitoring of calcium concentrations in the direct vicinity of the individual Ca^{2+}-ATPase.

A type IIA (ECA1 [16,21]) and a type IIB (ACA2 [7,14]) Ca^{2+}-ATPase have been shown to be co-localized in the ER of *Arabidopsis*. In this context, it is worth asking why there are two Ca^{2+}-ATPases of different types in certain organelles. Harper et al. offered the attractive hypothesis that since only one of the two pumps is directly regulated by CaM, the two distinct pumps may function in concert or unilaterally to produce different cytoplasmic calcium signals (or changes in lumenal ER calcium levels) in response to different stimuli [14].

6.2. Involvement in salt stress functions

The ability of plants to adjust to high salt environments correlates with the activation of a signal transduction system involving calcium [74]. Although NaCl causes a rapid increase in cytosolic calcium, it is still not clear whether this increase mediates salt adaptation or acts as a general stress signal [121]. An increase in external calcium ameliorates the inhibitory effect of salt [106,122]. The discovery of drought and salt induced expression of calcium-dependent protein kinases [123,124], calcium-binding proteins [112,125] and putative Ca^{2+}-ATPases [19,126] gives indirect evidence of the importance of calcium in these processes.

Insight into the underlying mechanism has been provided by the recent reports from Zhu and coworkers [106,127]. These workers identified an *Arabidopsis sos3* mutant that is hypersensitive to NaCl and LiCl, whereas millimolar levels of calcium sup-

pressed the mutant phenotype [127]. *SOS3* encodes a protein that shares significant sequence similarity with the calcineurin B subunit from yeast and neuronal calcium sensors from animals [106]. Therefore, it seems likely that intracellular calcium signaling through a calcineurin-like pathway helps mediate the calcium effect on salt tolerance.

Two lines of evidence suggest that plant Ca^{2+}-ATPases are involved in salt stress adaptation. First, Ca^{2+}-ATPase transcripts have been shown to accumulate upon NaCl treatment in tomato [19], tobacco [126] and *Arabidopsis* (M. Geisler, M.G. Palmgren, unpublished results), which suggests that the increase in cytosolic calcium that follows NaCl exposure may be lowered by increased capacity of calcium pumps [121]. Second, *Arabidopsis* seedlings overexpressing an N-terminally truncated form of ACA4 show increased tolerance toward NaCl exposure compared to wild-type plants (M. Geisler, M.G. Palmgren, unpublished results).

6.3. Involvement in secretory functions

Related to the involvement of Ca^{2+}-ATPases in Ca^{2+} second messenger functions, plant Ca^{2+}-ATPases have also been proposed to function in protein processing in the secretory pathway [76]. The correct folding and assembly of proteins is dependent on chaperones in the ER [128,129] that require calcium for activity. This model is supported by studies on yeast *PMR1* mutants that secrete proteins that are retained in wild-type cells [76]. It is well known that calcium is an important component of vesicle fusion and trafficking in plants and therefore, calcium pumps have been predicted to reside in the plasma membrane, tonoplast, ER, Golgi or other secretory vesicles [56]. Thus, endolumenal calcium supplied by Ca^{2+}-ATPases on the ER, Golgi and secretory vesicles could affect processing and sorting of proteins [76].

Successful secretion of the calcium-binding metalloprotein α-amylase in aleurone cells most likely requires the pumping activity of an ER-localized CaM-stimulated Ca^{2+}-ATPase [130]. The activity of this pump was higher in cells treated with gibberellic acid in the presence of calcium; gibberellic acid could be substituted by external CaM [131]. Interestingly, overexpression of the recently cloned Ca^{2+}-ATPase from rice aleurone cells, OsCa-ATPase, was shown to bypass the need for gibberellic acid induction of the pump [22]. Another well understood secretory system in plants is the influence of calcium on pollen tube tip growth [132].

6.4. Additional functions

The ability of *Arabidopsis* ECA1 to restore growth of a PMR1 yeast mutant on high Mn^{2+}, as well as the formation of Mn^{2+}-dependent phosphoenzyme formation, has suggested that this enzyme may also function in Mn^{2+} transport [16]. Recently PMR1 has been implicated in supplying Mn^{2+} (beside Ca^{2+}) into the Golgi store [71]. Manganese is known to be required in the Golgi in order to activate Mn^{2+}-dependent enzymes involved in protein processing and secretion. Manganese is an essential plant nutrient and may also function in intracellular signaling [16]. Therefore, ECA1 may have a dual function in secretory pathways, regulating both manganese and calcium levels. This dual function may be a general property of type IIA Ca^{2+}-ATPases since a rabbit SERCA pump has also been shown to transport Mn^{2+} into the ER [133].

Interestingly, there is also evidence for two Ca^{2+}-ATPases around the nuclear envelope and/or in the ER surrounding the nucleus of *Arabidopsis* [14] and tomatoes [134]. A GFP-tagged ACA2 (type IIB pump) has been found around the nuclei [14] and a type IIA related Ca^{2+}-ATPase has been immunolocalized by confocal and electron immunocytochemistry using antibodies raised against a plant homologue of the mammalian SERCA pump [134]. Although the specific functions of these putative nuclear envelope pumps are unclear, a mechanism for calcium homeostasis is clearly needed for calcium signaling and nuclear functions. For example, cisternal calcium controls nuclear pore permeability and thereby regulates transport across the nuclear envelope [135].

Huang et al. have discussed the possible role of a plastid envelope Ca^{2+}-ATPase that, like ACA1, has been immunolocalized to the chloroplast inner envelope [15]. They suggested an export of calcium into the cytosol, that might be relevant for signaling between the organelle and the cytoplasm (see [136] for review). Further, an involvement in the energy-de-

pendent import of proteins into the plastid stroma has been proposed [137]. On the other hand, ACA1 transcript levels were reported to be higher in roots than in shoot tissues, suggesting that this isoform is also located in other plastids, including the amyloplast which functions in gravitropism [15]. Further investigation is necessary to establish the physiological function of these Ca^{2+}-ATPases localized in different organelles.

7. Concluding remarks

Molecular analysis of higher plant P-type Ca^{2+}-ATPases has in the last couple of years substantially helped to increase our knowledge about the structure and function of individual calcium pumps. The localization of CaM-stimulated (or type IIB) Ca^{2+}-ATPases to endomembrane locations of the plant cell, has now been conclusively documented. In fact, all cloned members of type IIB Ca^{2+}-ATPases seem to be localized to endomembranes. Contrary to these findings, animal cell type IIB ATPases seem to be exclusively localized in the plasma membrane. Another striking difference is that all cloned members of plant type IIB Ca^{2+}-ATPases seem to be regulated by an N-terminal autoinhibitory domain. It will be interesting to find out whether this is a typical feature of plant endomembrane or plant type IIB ATPases in general. The regulation mechanism by N-terminal autoinhibition of plant calcium pumps is a major task to be solved. Additional tasks for the future are cloning of plant plasma membrane Ca^{2+}-ATPases and identification of mechanisms involved in regulation of type IIA Ca^{2+}-ATPases in plants. In this context, the identification of putative interacting proteins (like phospholamban) should have high priority.

On the other hand, recent molecular results have underlined the complexity of type IIA and type IIB Ca^{2+}-ATPases at each of the plant cellular membrane systems. We speculate that a multiplicity of Ca^{2+}-ATPases reflects the complexity of calcium signaling in plant cells. For example, in the ER and probably also in the nuclear envelope, a Ca^{2+}-ATPase of each type seems to co-reside. In order to assign the physiological function and the redundancy of individual calcium pumps, the intracellular location and/or tissue- or even species-dependent expression pattern of Ca^{2+}-ATPases have to be determined.

Beside functioning as a major component in regulating the calcium homeostat in order to provide second-messenger functions, plant Ca^{2+}-ATPases are most likely directly or indirectly involved in other functions such as processing of proteins in the secretory pathway, transport of Mn^{2+} and adaptation to salt stress. Future work solving the various physiological function of individual Ca^{2+}-ATPases should therefore yield interesting results. This task can only be addressed in the plant organism itself. Multiple knockouts, obtained from identified T-DNA inserted lines or targeted disruptions [138], will help elucidate the in vivo function of all these calcium pumps. Bioimaging techniques combined with GFP-tagging, fluorescence resonance energy transfer and fluorescent calcium indicator techniques can be used to study not only the intracellular targeting but also protein-protein interactions and to monitor calcium concentrations in the direct vicinity of the individual Ca^{2+}-ATPases upon various stimuli. Further, yeast provides an excellent model system in which to investigate individual biochemical differences between the isoforms by heterologous expression. Strategies to maintain calcium second messenger function in yeast and plants are markedly similar.

Once the function of individual calcium pumps is assigned, the knowledge obtained may allow us to engineer crop plants to be more productive and nutritious through modifications of calcium signaling and homeostatic pathways.

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