At-ACA8 Encodes a Plasma Membrane-Localized Calcium-ATPase of Arabidopsis with a Calmodulin-Binding Domain at the N Terminus[1]

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A Ca^{2+}-ATPase was purified from plasma membranes (PM) isolated from Arabidopsis cultured cells by calmodulin (CaM)-affinity chromatography. Three tryptic fragments from the protein were microsequenced and the corresponding cDNA was amplified by polymerase chain reaction using primers designed from the microsequences of the tryptic fragments. *At*-ACA8 (Arabidopsis-autoinhibited Ca^{2+}-ATPase, isoform 8, accession no. AJ249352) encodes a 1,074 amino acid protein with 10 putative transmembrane domains, which contains all of the characteristic motifs of Ca^{2+}-transporting P-type Ca^{2+}-ATPases. The identity of *At*-ACA8p as the PM Ca^{2+}-ATPase was confirmed by immunodetection with an antiserum raised against a sequence (valine-17 through threonine-31) that is not found in other plant CaM-stimulated Ca^{2+}-ATPases. Confocal fluorescence microscopy of protoplasts immunodecorated with the same antiserum confirmed the PM localization of *At*-ACA8. *At*-ACA8 is the first plant PM localized Ca^{2+}-ATPase to be cloned and is clearly distinct from animal PM Ca^{2+}-ATPases due to the localization of its CaM-binding domain. CaM overlay assays localized the CaM-binding domain of *At*-ACA8p to a region of the N terminus of the enzyme around tryptophan-47, in contrast to a C-terminal localization for its animal counterparts. Comparison between the sequence of *At*-ACA8p and those of endomembrane-localized type IIB Ca^{2+}-ATPases of plants suggests that *At*-ACA8 is a representative of a new subfamily of plant type IIB Ca^{2+}-ATPases.

Ca^{2+} plays a crucial role in plant physiology by acting as a second messenger of a number of endogenous and environmental signals. Growing evidence indicates that signal specificity is given by the amplitude and frequency of waves of cytosolic Ca^{2+} concentration. These waves of cytosolic Ca^{2+} are due to both opening of Ca^{2+} channels in the plasma membrane (PM) or endomembranes and to Ca^{2+} extrusion into the apoplast or intracellular stores catalyzed by active Ca^{2+} transporters. The active Ca^{2+} transporters of endomembranes, such as the tonoplast and endoplasmic reticulum (ER), and of the PM are also responsible for re-establishing the resting cytosolic Ca^{2+} concentration after a stimulus-induced increase in Ca^{2+} concentration (Sanders et al., 1999; Trewavas, 1999). Since the capacity of internal stores is intrinsically limited and a very high electrochemical gradient favors Ca^{2+} influx across the PM, the active Ca^{2+} transport systems localized at the PM are likely to play a crucial role in cytosolic Ca^{2+} homeostasis, at least upon stimulus-induced opening of PM-localized Ca^{2+} channels (Miller et al., 1990; De Michelis et al., 1992). This has been shown to be the case for example in the response to abscisic acid of *Egeria densa* leaves, which involves the activation of the PM Ca^{2+}-ATPase (Beffagna et al., 2000).

Active Ca^{2+} transporters identified so far in plant membranes can be classified into two main groups: high affinity Ca^{2+}-ATPases and low affinity H^+/Ca^{2+} antiporters. Members of the former group belong to two phylogenetic types: (a) Type IIA Ca^{2+}-ATPases similar to animal Ca^{2+}-ATPases of the sarcoplasmic or ER; and (b) type IIB Ca^{2+}-ATPases similar to animal calmodulin (CaM)-stimulated Ca^{2+}-ATPases found in the PM (Askerlund and Sommarin, 1996; Axelsen and Palmgren, 1998; Evans and Williams, 1998; Sanders et al., 1999; Geisler et al., 2000). In plant cells type IIA and type IIB Ca^{2+}-ATPases are found both in endomembranes and in the PM and can co-exist in the same membrane system (Evans, 1994; Askerlund and Sommarin, 1996; Evans and Williams, 1998; Sanders et al., 1999; Geisler et al., 2000). This distribution is in contrast to that in animal cells, where type IIA and type IIB Ca^{2+}-ATPases are found exclusively in inner membranes and in the PM, respectively (Brandt and Vanaman, 1998).

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Biochemical characteristics of the type IIB Ca^{2+}-ATPases of endomembranes (tonoplast, ER, and possibly chloroplast envelope) and of the PM are quite similar; the PM Ca^{2+}-ATPase has a slightly higher MW, as determined from SDS-PAGE analysis, and perhaps a higher sensitivity to derivatives of fluorescein, but the differences are too small to be used as discriminating tools (Askerlund and Evans, 1993; Thomson et al., 1993, 1994; Bush and Wang, 1995; Askerlund, 1996; Askerlund and Sommarin 1996; Dainese et al., 1997; Hwang et al., 1997; Olbe et al., 1997; Geisler et al., 2000). While stimulation of tonoplast or ER Ca^{2+}-ATPase activity by exogenous CaM can be easily observed in membrane vesicles, the PM Ca^{2+}-ATPase is not stimulated by exogenous CaM unless the PM has been extensively washed with strong Ca^{2+} chelators, suggesting that the PM enzyme has a higher affinity for CaM than those in other membranes (Robinson et al., 1988; Williams et al., 1990; Evans et al., 1992; Rasi-Caldogno et al., 1993; Kurosaki and Kaburaki, 1994; Dainese et al., 1997; Olbe et al., 1997).

A consequence of this situation is that although the first claim to identification of a PM-localized CaM-stimulated Ca^{2+}-ATPase goes back to the early 1980s (Dieter and Marmè, 1981) and several laboratory searches after such an ATPase since then, identification of a PM-localized CaM-stimulated Ca^{2+}-ATPase at the molecular level has been achieved only relatively recently (Askerlund and Evans, 1993; Rasi-Caldogno et al., 1995; Dainese et al., 1997; Hwang et al., 1997; Olbe et al., 1997; Olbe and Sommarin, 1998).

To date molecular cloning of type IIB Ca^{2+}-ATPases has been achieved only for endomembrane-localized isoforms (Huang et al., 1993, 1994; Malmström et al., 1997; Harper et al., 1998; M. Geisler and M.G. Palmgren, unpublished results). Analysis of the deduced amino acid sequence has shown that these isoforms share an unusually long cytosolic N-terminal stretch, which has been demonstrated to contain an autoinhibitory CaM-binding domain (Malmström et al., 1997, 2000; Harper et al., 1998; Hwang et al., 2000; M. Geisler and M.G. Palmgren, unpublished results).

The plant PM Ca^{2+}-ATPase has an autoinhibitory CaM-binding domain, which is localized in a terminal region, since the fully activated Ca^{2+}-ATPase released by controlled proteolysis, which is unable to bind CaM, is only about 10 kD smaller than the native enzyme (Rasi-Caldogno et al., 1995; Olbe and Sommarin, 1998). However, attempts to better localize the autoinhibitory domain by means of *N*- or *C*-peptidases have been unfruitful (M.C. Bonza, unpublished results). In mammalian type IIB Ca^{2+}-ATPases the autoinhibitory CaM-binding domain is localized at the C terminus (Carafoli, 1991; Brandt and Vanaman, 1998).

Given the similarity between the PM Ca^{2+}-ATPase of plant cells and type IIB Ca^{2+}-ATPases of endomembranes indicated by biochemical analysis (Askerlund and Evans, 1993; Thomson et al., 1993, 1994; Bush and Wang, 1995; Askerlund, 1996; Askerlund and Sommarin, 1996; Dainese et al., 1997; Hwang et al., 1997; Olbe et al., 1997; Geisler et al., 2000), homologous probes are probably required to clone the PM Ca^{2+}-ATPase cDNA(s). To this end we applied the CaM-affinity purification procedure developed for the PM Ca^{2+}-ATPase of radish seedlings (Bonza et al., 1998) to highly purified PM isolated from Arabidopsis cultured cells and then microsequenced the purified enzyme. Using the sequence information obtained we have cloned by PCR the first cDNA coding for a PM-localized type IIB Ca^{2+}-ATPase, which we called *At*-ACA8. Like the endomembrane-localized type IIB Ca^{2+}-ATPases of plant cells, *At*-ACA8p has a CaM-binding domain localized at the N terminus. Comparison with the available cDNA and genomic DNA sequences points to *At*-ACA8 as the first member of a new subfamily of plant type IIB Ca^{2+}-ATPases.

RESULTS

Purification and Microsequencing of the PM Ca^{2+}-ATPase of Arabidopsis Cultured Cells

A highly purified PM fraction obtained from Arabidopsis cultured cells by the two-phase partitioning technique (Larsson et al., 1987) and then extensively washed with EDTA (Rasi-Caldogno et al., 1993) was used to purify the Ca^{2+}-ATPase by CaM-affinity chromatography with the batch procedure developed for purifying the enzyme from the PM of germinating radish seeds (Bonza et al., 1998). A typical purification procedure is shown in Table I. About 80% PM Ca^{2+}-ATPase activity was solubilized with *n*-dodecyl β-D-maltoside (4:4, milligram protein:milligram detergent) and applied to a CaM-agarose matrix. The bulk of PM protein did not bind to the matrix upon overnight incubation, including a large part of the Ca^{2+}-ATPase activity virtually insensitive to CaM. After several washing steps with decreasing free [Ca^{2+}], 15% to 20% of the loaded PM Ca^{2+}-ATPase activity was eluted by washing the column with 5 mM EDTA. Based on the increase of the specific activity of the Ca^{2+}-ATPase in the EDTA-eluted fraction, purification was only 10-fold. However, this value is likely an underestimation due to loss of enzyme activity during the purification procedure. In fact silver staining of the SDS-PAGE gel containing the EDTA-eluted fraction (Fig. 1, lane 1) showed a prominent band with an apparent molecular mass of 123 kD—barely detectable in native PM (data not shown).

The 123-kD band in the EDTA-eluted fraction was identified as the PM Ca^{2+}-ATPase (Rasi-Caldogno et al., 1995; Bonza et al., 1998) by labeling with fluorescein isothiocyanate (FITC) under stringent conditions (Fig. 1, lane 2) and by its ability to bind CaM in an overlay assay (Fig. 1, lane 3).

Table I. *Purification of the PM Ca^{2+}-ATPase by CaM-agarose affinity chromatography*

PM proteins were solubilized with *n*-dodecyl β-D-maltoside (4:4, mg detergent mL^{-1}:mg protein mL^{-1}) and purified by CaM-agarose affinity chromatography as described in the "Materials and Methods." The first wash was performed in the presence of 100 μM $CaCl_2$ and 100 μM $MgSO_4$; the second one in the absence of added divalent cations. Ca^{2+}-ATPase activity was measured as Ca^{2+}-dependent ITPase activity plus or minus 20 μg mL^{-1} CaM; ITPase activity measured in the absence of Ca^{2+} was about 100 nmol P_i min^{-1} in the native and solubilized PM and in the fraction which did not bind to CaM-agarose and barely detectable (1–3 nmol P_i min^{-1}) in the EDTA eluted fraction. Results are from one experiment, representative of more than 10. ND, Not determined.

Fraction	Protein	Ca^{2+}-ATPase Activity			
		Total		Specific	
		−CaM	+CaM	−CaM	+CaM
	mg	*nmol P_i min^{-1}*		*μmol min^{-1} mg^{-1} protein*	
PM	4	191	337	0.05	0.08
Solubilized PM	3.4	172	255	0.05	0.07
CaM-agarose:					
Unbound	2.3	96	103	0.04	0.04
I Wash	ND	26	31		
II Wash	ND	5	5		
1 mM EGTA, I eluate	ND	7	9		
1 mM EGTA, II eluate	ND	5	5		
5 mM EDTA eluate	0.05	24	44	0.43	0.82

Figure 1 (lane 4) also shows that, like the enzyme purified from radish seeds (Bonza et al., 1998), the PM Ca^{2+}-ATPase purified from Arabidopsis cultured cells cross-reacted with an antiserum raised against a portion of *At*-ACA1p (amino acids 637–871), mainly corresponding to the large cytosolic loop (Huang et al., 1993). However, the purified PM Ca^{2+}-ATPase was not recognized by an antiserum against a portion of the N terminus (amino acids 119–161) of *At*-ACA2p (Fig. 1, lane 6), a type IIB Ca^{2+}-ATPase of the ER (Harper et al., 1998; Hong et al., 1999); the antiserum against *At*-ACA2p clearly identified a 119-kD band in a lane loaded with a microsomal fraction containing similar Ca^{2+}-ATPase activity (Fig. 1, lane 5).

Taken together these data show that the 123-kD band in the EDTA-eluted fraction represents a Ca^{2+}-ATPase of the PM. To obtain information on its sequence, the EDTA-eluted fraction was concentrated by methanol precipitation (see "Materials and Methods"), subjected to SDS-PAGE, and the excised 123-kD band was microsequenced. Since the N terminus of the enzyme was blocked, the band was extensively cleaved with trypsin; sequences were obtained for three tryptic fragments of 19, 8, and 7 amino acids, respectively (Table II).

Cloning of the PM Ca^{2+}-ATPase cDNA

The peptide sequences obtained from the trypsin-cleaved purified enzyme were used to design primers to amplify the PM Ca^{2+}-ATPase cDNA by PCR. Unfortunately, the genetic code for most of the amino acids in the sequences obtained is highly degenerate and all attempts to use degenerate or inosine-containing primers were unsuccessful, although a range of cDNA libraries was screened (data not shown).

Searching databases with the longest of the sequences obtained was also unsuccessful until the sequence of a genomic clone of Arabidopsis (clone AB023042, pertaining to chromosome 5) was released

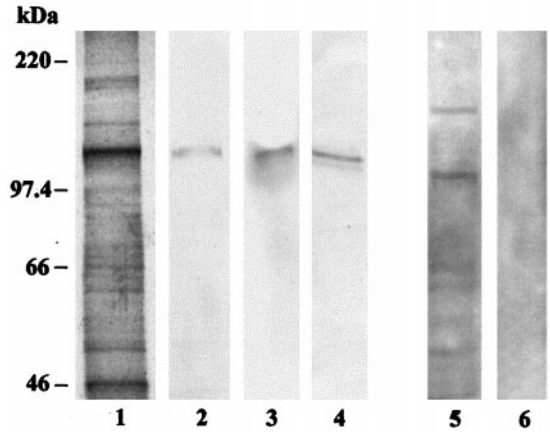

Figure 1. Identification of PM Ca^{2+}-ATPase in the EDTA-eluted fraction of the purification procedure. The EDTA-eluted fraction was separated by SDS-PAGE (7.5% [w/v] polyacrylamide) and stained with silver impregnation method (lane 1) or blotted and probed with one of the following: anti-FITC antiserum (lane 2), ^{125}I-CaM overlay (lane 3), anti-*At*-ACA1 antiserum (lane 4), or anti-*At*-ACA2 antiserum (lane 6). The sample in lane 2 had been pretreated with 5 μM FITC as described in "Materials and Methods." Lane 5 was loaded with proteins from the microsomal fraction and immunodecorated with anti-*At*-ACA2 antiserum. All lanes were loaded with the same Ca^{2+}-ATPase activity (approximately 0.3 nmol Pi min^{-1}). Numbers at the left indicate the size of molecular mass markers.

Table II. *Sequences of tryptic fragments obtained from purified PM Ca^{2+}-ATPase and localization of their coding sequence in the genomic clone AB023042 (minus strand)*

Slash indicates the presence of one intron.

	Amino Acid Sequence	Position in Genomic Clone
		bp
Peptide 1	TGPATPAGDFGITPEQLVI	22,292–22,236
Peptide 2	IHLE/VLR	21,408–21,397/ 21,304–21,296
Peptide 3	LLLVQSLR	17,819–17,795

to the databases. This clone coded for all of the three peptides of the purified PM Ca^{2+}-ATPase that had been sequenced (Table II); moreover, analysis of the genomic clone with different gene identification programs strongly suggested that it could code for a Ca^{2+}-ATPase. Thus, we used primers matching the nucleotide sequences of the genomic clone AB023042 coding for the tryptic peptides of the PM Ca^{2+}-ATPase to amplify the corresponding cDNA by PCR (see "Materials and Methods"). As template, a cDNA library of Arabidopsis seedlings (Minet et al., 1992) was used.

The cDNA of about 3,600 bp obtained contained a 3,225-bp open reading frame preceded by a 123-bp leader (Fig. 2A; accession no. AJ249352). According to the nomenclature suggested for plant Ca^{2+}-ATPases by Geisler et al. (2000), we named this cDNA *At*-ACA8. Alignment of *At*-ACA8 with the genomic clone AB023042 revealed the presence of 33 introns spread throughout the open reading frame (Fig. 2A).

Figure 2B also shows the deduced amino acid sequence of *At*-ACA8p, which is a 1,074-amino acid protein containing the sequences of the three peptides sequenced from the purified protein. *At*-ACA8p contains all of the characteristic motifs of type II P-type ATPases (Møller et al., 1996; Axelsen and Palmgren, 1998). Analysis of hydropathy indicates the presence of 10 transmembrane domains. The calculated molecular mass of 116,174 D is fairly close to that estimated from the SDS-PAGE of the purified enzyme and about 5 kD higher than that of the other CaM-regulated Ca^{2+}-ATPases identified so far in plants (Huang et al., 1993, 1994; Malmström et al., 1997; Harper et al., 1998; M. Geisler and M.G. Palmgren, unpublished results).

Table III shows a comparison between the sequence of *At*-ACA8p and those of various Ca^{2+}-ATPases. The amino acid sequence of *At*-ACA8p is most similar (about 70% identity) to those of *At*-ACA9p and *At*-ACA10p, two putative Ca^{2+}-ATPases encoded by the genomic clones AB023045 (pertaining to chromosome 3) and AL050352 (pertaining to chromosome 4), respectively. *At*-ACA8p is 45% to 47% identical to the other type IIB Ca^{2+}-ATPases identified so far in plants and 35% identical to PMCA1b, a mammalian type IIB Ca^{2+}-ATPase (Kumar et al., 1993). Identity with *At*-ECA1p, a representative of plant type IIA Ca^{2+}-ATPases (Liang et al., 1997), is only 27%. It is worth noting that *At*-

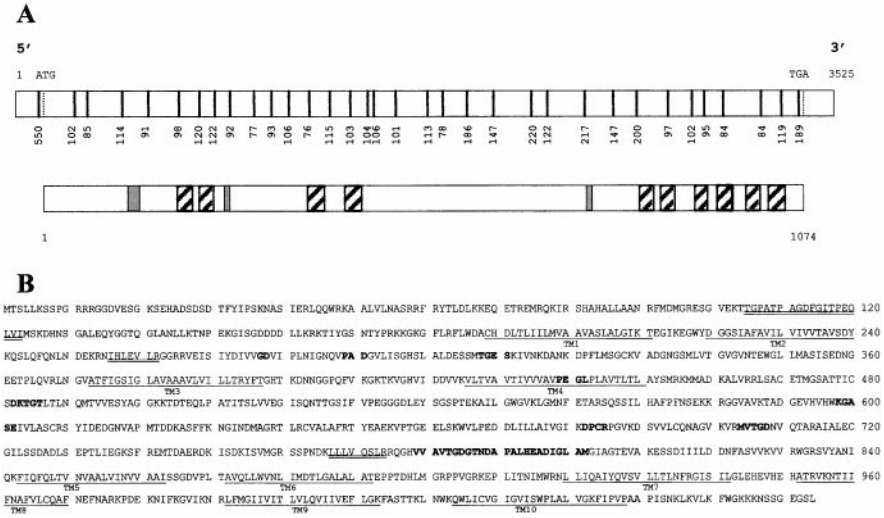

Figure 2. Structure of *At*-ACA8 gene and protein. A, Schematic representation of the alignment of *At*-ACA8 cDNA with the genomic clone AB023042 (bp 23,578–15,601) and of the protein structure. In the top bar (cDNA), the region limited by dashed lines represents the open reading frame. Black lines show the position of the introns deduced from alignment with the genomic clone; the length of each intron is indicated by numbers. In the lower bar (protein), gray boxes indicate the positions of the three peptides obtained from trypsin cleavage of the purified enzyme and hatched boxes indicate the putative transmembrane domains. B, Primary structure of *At*-ACA8 protein as deduced from the nucleotide sequence of the cDNA. Peptide sequences obtained from the purified proteins and used to design oligonucleotide primers are underlined by double lines; putative transmembrane domains (TM) are underlined and numbered consecutively; conserved amino acids in all type II P-type ATPases are in bold. The complete nucleotide sequence, including 5′ and 3′-untranslated regions, has been deposited at EMBL, accession number AJ249352.

Table III. *Percentage of amino acid identity among sequences of different P-type Ca^{2+}-ATPases*

	At-ACA9	*At*-ACA10	*At*-ACA1	*At*-ACA2	*At*-ACA4	*At*-ACA7	*Bo*-ACA1	PMCA1b	[d]*At*-ECA1
At-ACA8[a]	67.4	70.4	47.0	46.1	46.3	46.7	46.8	34.6	27.0
At-ACA9[a]		64.5	45.1	45.8	46.2	45.7	45.5	34.0	27.5
At-ACA10[a]			45.2	46.5	45.9	46.8	46.1	32.0	26.6
At-ACA1[a]				77.3	61.4	76.7	60.4	34.0	27.4
At-ACA2[a]					61.7	92.9	61.1	33.6	26.5
At-ACA4[a]						61.8	84.4	34.7	27.5
At-ACA7[a]							61.3	33.8	27.0
Bo-ACA1[b]								35.0	27.4
PMCA1b[c]									24.3

[a] Arabidopsis type IIB Ca^{2+}-ATPases: *At*-ACA8, *At*-ACA9 (amino acid sequence manually extracted from genomic clone AB023045), *At*-ACA10 (putative Ca^{2+}-ATPase CAB43665.1 encoded by genomic clone AL050352), *At*-ACA1 (L08468; Huang et al., 1993, 1994), *At*-ACA2 (AF025842; Harper et al., 1998), *At*-ACA4 (AC002510; M. Geisler and M.G. Palmgren, unpublished results), and *At*-ACA7 (putative Ca^{2+}-ATPase AAC3244.1 encoded by genomic clone AC004786). [b] Cauliflower type IIB Ca^{2+}-ATPase: *Bo*-ACA1 (X99972; Malmström et al., 1997). [c] Human IIB type Ca^{2+}-ATPase: PMCA1b (P20020; Kumar et al., 1993). [d] Arabidopsis type IIA Ca^{2+}-ATPase: *At*-ECA1 (U93845; Liang et al., 1997).

ACA8p, *At*-ACA9p, and *At*-ACA10p are more similar to each other than to any of the other type IIB Ca^{2+}-ATPases of plants; conversely, *At*-ACA1p (Huang et al., 1993), *At*-ACA2p (Harper et al., 1998), *At*-ACA4p (M. Geisler and M.G. Palmgren, unpublished results), *At*-ACA7p (a putative Ca^{2+}-ATPase codified by the genomic clone AC004786), and *Bo*-ACA1p (Malmström et al., 1997) are more similar to each other than to *At*-ACA8p, *At*-ACA9p, and *At*-ACA10p.

At-ACA8p shares an unusually long N-terminal domain with the other plant type IIB Ca^{2+}-ATPases. Figure 3 shows the alignment of the N terminus of plant type IIB Ca^{2+}-ATPases; the N terminus of *At*-ACA8p, *At*-ACA9p, and *At*-ACA10p is even more extended than that of other Ca^{2+}-ATPases. Moreover, amino acid identity between *At*-ACA8p and the other type IIB Ca^{2+}-ATPases is low in the N terminus.

Immunolocalization of *At*-ACA8p

To obtain highly specific polyclonal antibodies against *At*-ACA8p, a peptide corresponding to the sequence Val-17 through Thr-31 of *At*-ACA8p (underlined in Fig. 3), which is absent or highly variable in the other plant type IIB Ca^{2+}-ATPases, was conjugated to ovalbumin and used to inoculate rabbits. Figure 4 (lane 1) shows that the antibody obtained strongly reacted with a fusion protein between glutathione *S*-transferase (GST) and the first 122 amino acids of *At*-ACA8p (see below) and identified a major band of 123 kD in the EDTA-eluted fraction of the Ca^{2+}-ATPase purification procedure (lane 2).

Figure 5 shows the confocal microscopy analysis of Arabidopsis protoplasts immunodecorated with the polyclonal antibody and a FITC-conjugated secondary antibody; labeling is clearly restricted to the protoplast outer layer, consistent with a PM localization of the antigen. This conclusion was strengthened by the immunodecoration of western blots of equal amounts of proteins of different membrane fractions. Figure 4 shows that the polyclonal antibody obtained identified a 123-kD band in all fractions, and that this protein was highly enriched in the PM fraction (lane 5) with respect to the starting microsomes (lane 3) and barely detectable in the endomembrane enriched fraction (lane 4).

Localization of the CaM-Binding Domain of *At*-ACA8p

The CaM-binding autoinhibitory domains of *At*-ACA2p, *At*-ACA4p, and *Bo*-ACA1p have been demonstrated to be located at the N terminus (Malmström et al., 1997, 2000; Harper et al., 1998; Hwang et al., 2000; M. Geisler and M.G. Palmgren, unpublished results). In contrast, the CaM-binding domain of the animal PM Ca^{2+}-ATPase is localized in the extended C-terminal domain (Carafoli, 1991, 1994; Brandt and Vanaman, 1998). The finding that *At*-ACA8p has an extended N terminus and a short C terminus sug-

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At-ACA8p   MTSLLKS----SPGRRRGGDVESG---KSEHADSD------SDTFYIPS-KNASTERLQQWRKAALVLNASRRFRYTLDLKKEQETREMRQ----KIRSHAHALLAANRFMDMG-RESG 100
At-ACA9p   .STSSSNGLLLTSMSG.HD.M.A.SAKTE..S.HEELQHDPD.P.D.DNT...SV.S.RR..Q.................NKEEHYDNRRR----M..A..QVIR-------------- 101
At-ACA10p  .SGQFNN----.P-.GEDK.V.A.---T.SFTEYE------DSP.D.A.T...PV...RR..QA.................REEDKKQMLR----.M.A..Q.IR..HL.KAAAS.VT. 101
At-ACA1p   ----------------------------MESYLNEN----------.GDVKP..S.D.A.QR...LCWIVKNPK.RFRFTANLSKRSEAEAIRRSNQEKFRV.VLVSQ.ALQFINSLKL.S  83
At-ACA2p   ----------------------------MESYLNEN----------.-DVKA.HS.E.V.EK..NLCG.VKNPK.RFRFTANLSKRYEAAAMRRTNQEKLRI.VLVSK.AFQFISGVSP.-  81
At-ACA4p   ----------------------------M.NLLRD----------.-EVEA.NP.L.ARQR..SSVSIVKNRT.RFRNIRDLDKLADYENKKHQIQEKIRV.FFVQK.ALHFIDAAARP-  80
At-ACA7p   ----------------------------MESYLN.N----------.-DVKA.HS.E.V.EK..NLCS.VKNPK.RFRFTANLSKRYEAAAMRRTNQEKLRI.VLVSK.AFQFISGVSP.-  81
Bo-ACA1p   ----------------------------M.NLLKD----------.-QVEA..P.L.ARQR..SSVSIVKNRA.RFRMISNLEKLAENEKKRCQIQEKIRV.FYVQK.ALQFIDAGTRR-  80
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Figure 3. Alignment of the N-terminal domain of type IIB Ca^{2+}-ATPases of plants. The amino acid sequence of *At*-ACA8p used to generate isoform specific polyclonal antibody is underlined. The sequence of the putative CaM-binding domain of *At*-ACA8p is boxed. The CaM-binding domains identified in other type IIB Ca^{2+}-ATPases are in bold. Dashes represent insertions to optimize alignments; dots represent amino acids identical to those of *At*-ACA8p.

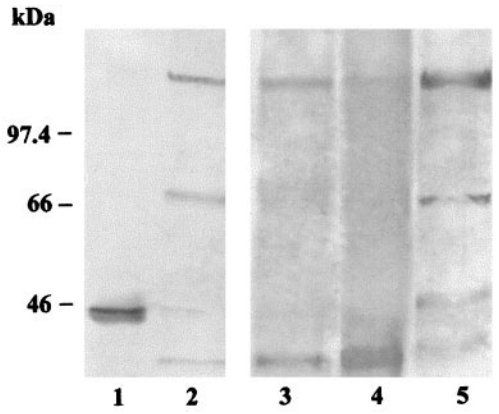

Figure 4. Western-blot analysis of various membrane fractions with anti-*At*-ACA8p polyclonal antibody. The fusion protein between GST and the first 122 amino acids of *At*-ACA8p (lane 1, 0.2 μg), the EDTA-eluted fraction of the Ca^{2+}-ATPase purification procedure (lane 2, 1 μg), and proteins (50 μg) from various membrane fractions of the two-phase partitioning (lane 3, microsomal fraction; lane 4, first lower phase; lane 5, second upper phase) were separated by SDS-PAGE and blotted onto 0.45 μm of nitrocellulose. Immunodecoration was performed with an antiserum raised against *At*-ACA8 (Val-17–Thr-31) as described in "Materials and Methods." No signal was detected when the preimmune serum was used.

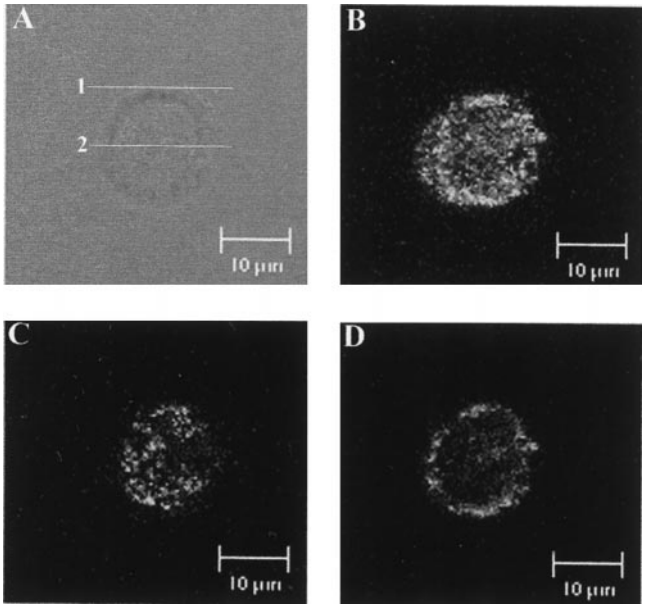

Figure 5. Confocal microscopy of Arabidopsis protoplasts labeled with the anti-*At*-ACA8p polyclonal antibody. Ethanol-fixed protoplasts were incubated with anti-*At*-ACA8p polyclonal antibody and FITC-conjugated secondary antibody as described in "Materials and Methods." A, Phase contrast image; B, reconstituted fluorescence image; C, top section image (1 in A); D, central section (6 μm deep, 2 in A) image. No fluorescence was detectable in control samples treated only with the secondary antibody, whereas the preimmune serum originated only a light diffuse fluorescence.

gested that its CaM-binding domain might be localized at the N terminus. To test this hypothesis a fusion protein between GST and the first 122 amino acids of *At*-ACA8p was constructed, expressed in *Escherichia coli*, and purified by GSH-affinity chromatography. After SDS-PAGE and western blotting, its ability to bind CaM was assayed by ^{125}I-CaM overlay. Figure 6A shows that the fusion protein (lane 3) bound CaM in a Ca^{2+}-dependent manner; binding was at the *At*-ACA8p N terminus, since neither GST alone (lane 1) nor a fusion between GST and the last 102 amino acids of isoform 1 of the Arabidopsis PM H^+-ATPase (AHA1, lane 2) bound CaM.

In an attempt to better localize the CaM-binding domain in the N terminus of *At*-ACA8p, a peptide

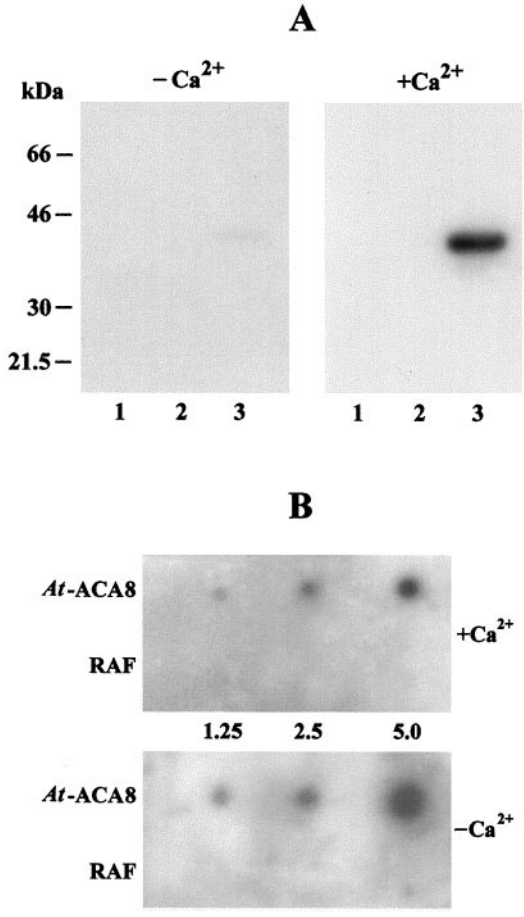

Figure 6. Localization of the CaM-binding domain of *At*-ACA8p at the N terminus by[^{125}I]CaM overlay. [^{125}I]CaM overlay was performed in the presence or absence of 0.2 mM Ca^{2+} as described in "Materials and Methods." A, Western blot of the fusion protein between GST and the first 122 amino acids of *At*-ACA8p (lane 3). As negative controls, GST alone (lane 1) or a fusion protein between GST and the last 102 amino acids of AHA1 (lane 2) were used. After SDS-PAGE (4%–20% [w/v] polyacrylamide), proteins were blotted onto 0.2 μm nitrocellulose. B, Dot spot of a synthetic peptide corresponding to residues 41 to 55 of *At*-ACA8p. As a control, a peptide of 15 amino acids derived from the protein RAF (residues 613–627) was used. Numbers indicate the amount of loaded peptide in micrograms.

corresponding to the sequence Ile-41 through Asn-55 (boxed in Fig. 3) was synthesized; this sequence is predicted to form an amphipathic α-helix with essential aromatic clusters characteristic of CaM-binding motifs (Ikura et al., 1992; Brandt and Vanaman, 1998) and largely overlaps the CaM-binding domains of *At*-ACA2p, *At*-ACA4p, and *Bo*-ACA1p (bold in Fig. 3). Figure 6B shows that the peptide was strongly labeled by overlay with ^{125}I-CaM; binding was specific, since a control peptide corresponding to amino acids 613 through 627 of the oncoprotein RAF (Muslin et al., 1996) gave no signal. Although the GST-N terminus fusion protein (Fig. 6A) and the native protein (Rasi-Caldogno et al., 1995) bind CaM in a strictly Ca^{2+}-dependent manner, the *At*-ACA8p peptide bound ^{125}I-CaM also in the absence of Ca^{2+} (Fig. 6B). Such a discrepancy has been observed before and it has been suggested that it may reflect a higher affinity of the synthetic peptide for CaM due to the lack of surrounding residues (James et al., 1988; Malmström et al., 1997).

DISCUSSION

Identification of a cDNA coding for a PM-localized Ca^{2+}-ATPase has been long sought after by several laboratories, due to the crucial role that PM Ca^{2+}-ATPases are thought to play in the control of cytosolic Ca^{2+} homeostasis, as well as in determining the amplitude and frequency of Ca^{2+} waves, which confer specificity to Ca^{2+}-transmitted signals (Miller et al., 1990; De Michelis et al., 1992; Sanders et al., 1999; Trewavas, 1999; Beffagna et al., 2000).

Here we report about the isolation of *At*-ACA8, the first cDNA coding for a PM-localized type IIB Ca^{2+}-ATPase in plants. This was made possible by integrating protein sequence information obtained from the enzyme partially purified by CaM-affinity chromatography from high purity PM isolated from Arabidopsis cultured cells with information arising from the Arabidopsis sequencing project. Three lines of evidence indicate that *At*-ACA8p is the PM Ca^{2+}-ATPase: (a) *At*-ACA8 is the only DNA coding for all the three microsequences identified in the enzyme purified from Arabidopsis PM (Fig. 2); (b) a polyclonal antibody raised against a sequence that is not conserved in the plant type IIB Ca^{2+}-ATPases identified so far selectively labels the PM (Figs. 4 and 5); and (c) the same antibody recognizes the partially purified PM Ca^{2+}-ATPase (Fig. 4).

The *At*-ACA8 gene is part of chromosome 5 and is contained in a fully sequenced bacteria artificial chromosome clone (AB023042). Alignment of *At*-ACA8 cDNA with the genomic clone AB023042 reveals the presence of 33 introns within the open reading frame (Fig. 2). This is a very high number if compared with *At*-ACA1, *At*-ACA2, and *At*-ACA4 genes, which all contain only six introns. It is interesting that *At*-ACA9 and *At*-ACA10, of which only the genomic clones are available, also contain about 30 introns. The genes coding for the mammalian type IIB Ca^{2+}-ATPase (PMCA), which have been shown to be subjected to alternative splicing, also contain a very high number of introns (22 or 23, Brandt and Vanaman, 1998). In PMCA genes one of the splice junctions is localized within the CaM-binding domain, and the isoforms arising from alternative splicing have been shown to have different CaM-binding affinities (Carafoli, 1994; Brandt and Vanaman, 1998). Although a correlation between the presence of introns and the occurrence of alternative splicing has not been demonstrated, it is tempting to speculate that alternative splicing may also occur for *At*-ACA8. It is worth noting in this context that the first intron within the coding region of the *At*-ACA8 DNA occurs between Trp-47 and Arg-48, i.e. roughly in the middle of the CaM-binding domain (see below).

At-ACA8 codes for a protein of 1,074 amino acids, with all of the features characteristic of type II P-type ATPases (Fig. 2). *At*-ACA8p is more similar to *At*-ACA9p and *At*-ACA10p (about 70% identity) than to the other type IIB Ca^{2+}-ATPases identified so far in plants (less than 50% identity). Like the other plant type IIB Ca^{2+}-ATPases, *At*-ACA8p is more similar to a mammalian type IIB Ca^{2+}-ATPase than to a type IIA Ca^{2+}-ATPase of Arabidopsis (Table III). Taken together these results clearly indicate that *At*-ACA8p is a type IIB Ca^{2+}-ATPase.

However, *At*-ACA8 differs from the type IIB Ca^{2+}-ATPases cloned so far from plants, both at the gene and at the protein level; as mentioned above, the *At*-ACA8 gene contains a much higher number of introns than *At*-ACA1, *At*-ACA2, and *At*-ACA4. Moreover, the *At*-ACA8 protein is slightly bigger than *At*-ACA1p, *At*-ACA2p, *At*-ACA4p, and *Bo*-ACA1p. Finally, whereas the latter Ca^{2+}-ATPases have all been localized to endomembranes (Huang et al., 1993, 1994; Malmström et al., 1997; Harper et al., 1998; Hong et al., 1999; M. Geisler and M.G. Palmgren, unpublished results), *At*-ACA8p is localized at the PM. Given the strong similarity of *At*-ACA9 and *At*-ACA10 to *At*-ACA8, both at the protein and at the gene level, we propose that these three genes are members of one of two subfamilies of plant type IIB Ca^{2+}-ATPases. The other subfamily would then comprise *At*-ACA1, *At*-ACA2, *At*-ACA4, *At*-ACA7, and *Bo*-ACA1.

Similarity between type IIB Ca^{2+}-ATPases of plants is very low in the N terminus (Fig. 3). Accordingly, antisera raised against this portion of the protein show high specificity; antisera against an N-terminal domain of endomembrane-localized Ca^{2+}-ATPases such as *At*-ACA2p or *Bo*-ACA1p do not cross react with the PM Ca^{2+}-ATPase (Fig. 1; Harper et al., 1998; Malmström et al., 2000), and the antiserum against an N-terminal domain of *At*-ACA8p does not cross react with endomembrane-localized Ca^{2+}-ATPases (Fig. 4). Although variable, the N terminus of plant type IIB are all extended and contain the CaM-binding

domain. In *At*-ACA8p, the CaM-binding domain has been localized to the region around Trp-47 (Fig. 6); it contains the recognized CaM-binding motif consisting of an amphipathic α-helix with essential aromatic clusters (Ikura et al., 1992; Brandt and Vanaman, 1998).

Localization of the autoinhibitory CaM-binding domain at the N terminus clearly differentiates *At*-ACA8p from its mammalian counterparts. In fact the mammalian PM Ca^{2+}-ATPase has the autoinhibitory CaM-binding domain at the extended C terminus (Carafoli, 1991, 1994; Brandt and Vanaman, 1998). In addition the autoinhibitory domain of the H^+-ATPase of plant and yeast PM, which plays an important role in regulation of the enzyme activity, is localized at an extended C terminus (Palmgren, 1998). Thus, localization of the CaM-binding domain at the N terminus is a peculiar feature of type IIB Ca^{2+}-ATPases of plants, independent of their membrane localization.

MATERIALS AND METHODS

Plant Material and Isolation of PM Vesicles

Cell suspension cultures of Arabidopsis ecotype Landsberg were grown as described in Curti et al. (1993). Arabidopsis cells, harvested from 6-d-old subcultures, were homogenized in ice-cold extraction medium (2 mL^{-1} fresh weight), and a microsomal fraction was obtained as previously described (Rasi-Caldogno et al., 1995). A highly purified PM fraction was obtained by a two-step aqueous two-phase partitioning system containing 6.2% (w/w) Dextran T500 (Pharmacia Biotech, Uppsala, Sweden), 6.2% (w/w) polyethylene glycol (P3350, Sigma, St Louis), 11% (w/w) Suc, 5 mM potassium phosphate buffer, pH 7.8, and 1 or 5 mM KCl in the first and second phase system, respectively. The second upper phase (PM fraction) was treated with 30 mM EDTA as in Bonza et al. (1998) to strip endogenous CaM, diluted with five volumes of ice-cold washing medium (10% [v/v] glycerol, 3 mM dithiothreitol, 0.1 mg mL^{-1} polyoxyethylene(20)cetyl ether [Brij 58], 1 mM phenylmethylsulfonil fluoride, and 1 mM 1,3-bis[tris(hydroxymethyl) methylamino]-propane)-4-(2-hydroxymethyl)-1-piperazine-ethanesulfonic acid, pH 7) and collected by centrifugation at 48,000*g* for 35 min at 4°C. The pellets were resuspended in resuspension medium (10% [v/v] glycerol, 0.5 mM dithiothreitol, 1 mM 3-[*N*-morpholino]propane sulfonic acid-KOH, pH 7, and 5 μM leupeptin) at about 7 to 10 mg of membrane proteins per milliliter, immediately frozen, and kept at −80°C until use. When needed, the first lower phase was also collected in the same way.

The purity of the PM fraction was tested by assaying the activities of marker enzymes (vanadate-sensitive H^+-ATPase, oligomycin-sensitive ATPase, H^+-pyrophosphatase, and latent IDPase) as in De Michelis et al. (1991), or by immunodetection with polyclonal antibodies, namely anti-PM H^+-ATPase (Papini and De Michelis, 1997) and anti-BiP (Denecke et al., 1991). The second upper phase contained about 25% PM present in the starting microsomal fraction, with a 5-fold higher specific activity. Mitochondrial, ER, and Golgi membranes were barely detectable (less than 1% of the microsomal content) and tonoplast contamination was very low (less than 5% of the microsomal content).

CaM-Affinity Purification of PM Ca^{2+}-ATPase and Microsequencing of the Enzyme

PM Ca^{2+}-ATPase was solubilized and purified by CaM-agarose affinity chromatography as described in Bonza et al. (1998). The eluted fractions were immediately used for assay of Ca^{2+}-ATPase activity or frozen in aliquots and kept at −80°C.

EDTA-eluted fractions from different purification procedures were pooled, diluted 3-fold with methanol, and vortexed vigorously; after an incubation of 2 h at −80°C, proteins were recovered by centrifugation at 9,000*g* for 10 min at 4°C. About 30 μg of protein was loaded onto a 7.5% (w/v) polyacrylamide gel as described below. The 123-kD prominent band (see Fig. 1) was cut out from the gel. Trypsin digestion and sequencing by Edman degradation of the resulting peptides resolved by HPLC were carried out by Eurosequence (Groningen, The Netherlands). Three sequences were obtained: TGPATPAGDFGITPEQLVI, IHLEVLR, and LLLVQSLR.

Assays

The hydrolytic activity of the PM Ca^{2+}-ATPase was measured as Ca^{2+}-dependent MgITP hydrolysis; this procedure allows precise determination of the hydrolytic activity of the PM Ca^{2+}-ATPase also in native PM vesicles, since the much more abundant H^+-ATPase cannot use inosine 5′-triphosphate as an alternative substrate (Carnelli et al., 1992). The assay medium contained 40 mM 1,3-bis[tris(hydroxymethyl) methylamino]-propane)-4-(2-hydroxymethyl)-1-piperazine-ethanesulfonic acid, pH 7, 50 mM KCl, 3 mM $MgSO_4$, 0.1 mM ammonium molybdate, 1 mM ITP, 5 μM A_{23187}, 1 μg mL^{-1} oligomycin, 5 mM $(NH_4)_2SO_4$, 0.1 mg mL^{-1} Brij 58, and 1 mM EGTA ± $CaCl_2$ to give a free Ca^{2+} concentration of 50 μM (De Michelis et al., 1993). Bovine brain CaM was supplied at 20 μg mL^{-1}; incubation was performed at 25°C for 60 min. Ca^{2+}-ATPase activity was determined as the difference between the activity measured in the presence and absence of Ca^{2+}.

Protein concentration was determined according to Markwell et al. (1978) after methanol precipitation of the EDTA-eluted fraction as described above. All the assays were performed with three replicates; the SE of the mean did not exceed 4%.

Amplification of Ca^{2+}-ATPase cDNA

A cDNA library from complete young Arabidopsis seedlings (Minet et al., 1992) was screened by PCR. A primer hybridizing to vector sequences and a reverse primer matching the sequence coding for part of the tryptic fragment GITPEQLVI were used to amplify the 5′ end of the

cDNA; conversely, a forward primer matching the sequence coding for the tryptic fragment LLLVQSLR and a reverse primer hybridizing the cDNA vector arm were used to amplify the 3′ end of the cDNA. Partial cDNAs coding for internal parts of the protein were also amplified using primers matching two of the tryptic fragments and validated by nested PCR. The complete open reading frame was amplified using primers hybridizing its 5′ and 3′ ends (forward and reverse, respectively). All PCR reactions were performed in a Robocycler Gradient 40 (Stratagene, La Jolla, CA) using Advantage cDNA Polymerase mix (CLONTECH, Palo Alto, CA). The full-length cDNA obtained from at least two PCR reactions, the identity of which was confirmed by nested PCR with internal primers, was sequenced on both strands.

Peptide Synthesis

Both the peptides from *At*-ACA8p (I_{41}ERLQQWRK-AALVLN$_{55}$) and from RAF protein (L_{613}PKINRSASE-PSLHR$_{627}$) were synthesized by Primm (Milano, Italy). The identity and purity of the peptides were tested by HPLC and mass spectrometry.

Generation of Polyclonal Antibodies

Anti-*At*-ACA8p rabbit polyclonal antibodies were produced by Primm. Antiserum was raised against a 15-amino acid synthetic peptide of *At*-ACA8p (V_{17}ESGKSEHAD-SDSDT$_{31}$) conjugated to ovalbumin. The serum was treated at 4°C overnight with 1% (w/v) ovalbumin and then partially purified by ammonium sulfate fractionation; the fraction precipitating between 33% and 50% saturation $(NH_4)_2SO_4$ was resuspended in Tris-buffered saline.

Protoplasts Immunofluorescence

Protoplasts isolated from Arabidopsis cultured cells by enzymatic digestion of the cell wall as described by Colombo and Cerana (1991) were partially purified by centrifugation on a discontinuous Suc gradient (0.5–2 M) for 5 min at 300*g*. The upper phase was diluted 1:1 with the same medium used for the enzymatic digestion and protoplasts were collected by centrifugation for 5 min at 300*g*. Protoplast were fixed by incubation with 70% (v/v) ethanol for 1 h at 0°C, followed by centrifugation at 150*g* for 5 min.

Fixed protoplasts were smeared on a microscopy slide, allowed to dry by incubation at 25°C for 12 h, and rehydrated in saline medium (100 mM Tris, 100 mM NaCl, and 10 mM EDTA) for 5 min. For immunodecoration, protoplasts were incubated for 2 h with the anti-*At*-ACA8p polyclonal antibody diluted 1:10 with saline medium. After several washings with saline medium, protoplasts were incubated for 20 min with FITC-conjugated secondary antibody diluted 1:400 with saline medium. Immunofluorescence localization was performed by confocal microscopy.

SDS-PAGE and Western Blotting

SDS-PAGE was performed according to Laemmli (1970). Proteins from the various fractions were pretreated as described in Bonza et al. (1998) and loaded (0.2–50 μg per lane) on to Tris Gly polyacrylamide pre-made gels (7.5% Tris-Gly gel with 4% stacking gel, or 4%–20% linear gradient; Invitrogen, Carlsbad, CA). After running, the gel was stained using the silver impregnation method (Sigma) or blotted as in Rasi-Caldogno et al. (1995). Immunodetection of FITC-labeled proteins required pretreatment of the EDTA-eluted fraction with 5 μM FITC conducted as described in Bonza et al. (1998). [^{125}I]CaM overlay was performed as in Rasi-Caldogno et al. (1995) in the presence of 0.2 mM $CaCl_2$ or 10 mM EDTA to ensure absence of Ca^{2+}. Immunodetection with an antiserum against a portion of *At*-ACA1p and with an antiserum against a portion of the N terminus of *At*-ACA2p were as described by Huang et al. (1993) and by Harper et al. (1998), respectively. For the immunodecoration with anti-*At*-ACA8p, the blot, blocked as described in Rasi-Caldogno et al. (1995), was incubated for 2 h at 25°C with the antiserum diluted 1:2,000 in 3% (w/v) bovine serum albumin, 0.1% (w/v) polyoxyethylene(20)sorbitan monolaurate, 0.15 M NaCl, and 20 mM Tris-HCl, pH 7.4. After several washes, signal detection was performed with a second antibody coupled to alkaline phosphatase (Sigma).

Constructs and Purification of Fusion Proteins

Standard PCR reactions were used to amplify the first 122 amino acids at the N terminus of *At*-ACA8p using specific oligonucleotides corresponding to the *At*-ACA8 regions Met-1 through Ser-7 (forward) and Gly-115 through Val-122 (reverse). Both primers contained *Bam*HI restriction sites at the 5′ end. PCR was performed in a Robocycler Gradient 40 (Stratagene) using Advantage cDNA Polymerase mix (CLONTECH) for 25 cycles as follows: 94°C for 40 s, 58°C for 1 min, and 72°C for 1 min. The sequencing of a single clone (Primm) ensured the absence of mistakes in the first 116 amino acids.

The PCR product was first cloned into the vector pCR2.1 and then moved into a pGEX-2TK for the construction of a fusion protein with GST at the N-terminal end (insertion at the *Bam*HI site). As a negative control, a construct with the C-terminal 102 amino acids of isoform 1 of Arabidopsis PM H^+-ATPase (AHA1) was obtained using the same vector. Fusion proteins were overexpressed in *Escherichia coli* BL21 (DE3) p LysS. Cells were grown at 37°C until an OD_{595} of 0.6 was reached, then isopropyl β-D-thiogalactopyranoside was added (1 mM final concentration) and the culture grown for 2 h. Purification of fusion proteins was as described in Frangioni and Neel (1993).

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