Molecular properties of a novel, hydrophilic cation-binding protein associated with the plasma membrane

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Abstract

A new type of protein was found in Arabidopsis thaliana, PCaP1, which is rich in glutamate and lysine residues. The protein bound $^{45}\text{Ca}^{2+}$ even in the presence of a high concentration of Mg$^{2+}$. Real-time polymerase chain reaction and histochemical analysis of promoter–β-glucuronidase fusions revealed that PCaP1 was expressed in most organs. The PCaP1 protein was detected immunochemically in these organs. Treatment of Arabidopsis seedlings with Cu$^{2+}$, sorbitol, or flagellin oligopeptide enhanced the transcription. On the other hand, other sugars, abscisic acid, gibberellic acid, dehydration, and low temperature had little or no effect on PCaP1 transcript abundance. The transient expression of PCaP1 fused to green fluorescent protein in Arabidopsis cells and the subcellular fractionation of tissue homogenate showed that PCaP1 protein is localized to the plasma membrane, although PCaP1 has no predicted transmembrane domain. PCaP1 was associated with the plasma membrane under natural conditions and was released from the membrane at high concentrations of Ca$^{2+}$ or Mg$^{2+}$ in vitro. These results suggest that the hydrophilic protein PCaP1 binds Ca$^{2+}$ and other cations and is stably associated with the plasma membrane.

Key words: Arabidopsis thaliana, calcium, cation, membrane-associated protein, myristoylation, plasma membrane.

Introduction

Calcium ions have been demonstrated to control a variety of cellular processes with a high degree of spatial and temporal precision. In cells of all organisms, a complicated mechanism exists to control Ca$^{2+}$ in a localized fashion. Ca$^{2+}$-ATPases, Ca$^{2+}$/H$^+$ antiporters, Ca$^{2+}$/Na$^+$ antiporters, and Ca$^{2+}$ channels are involved in regulation of cytosolic Ca$^{2+}$ concentration (Sze et al., 2000; Sanders et al., 2002). Several other kinds of calcium signalling components have also been identified in plants (Reddy and Reddy, 2004). For example, calcium-dependent, but calmodulin-independent, protein kinases serve as one of the largest families of Ca$^{2+}$ sensor-transducer proteins in plants (Dammann et al., 2003; Chehab et al., 2004). In addition to these elements, several kinds of calcium-binding proteins (CaBPs) are thought to mediate Ca$^{2+}$ signal transduction (Mackrill, 1999), some with a low affinity for Ca$^{2+}$, binding it only at millimolar concentrations, and some with a high affinity, binding in the nanomolar to micromolar range. In the cytosol, resting free Ca$^{2+}$ levels are kept extremely low, and small changes in Ca$^{2+}$ concentration function as a second messenger in cooperation with CaBPs and membrane transport systems for Ca$^{2+}$. Several CaBPs in the cytosol and organelles function as Ca$^{2+}$ sensors.

Plants have various families of CaBPs, such as the EF-hand protein family, the endoplasmic reticulum (ER) luminal CaBP group, and the annexin family. The EF-hand family is the largest group of CaBPs, and its members function as ‘Ca$^{2+}$ buffer’ proteins or as ‘modulator’ proteins. Calmodulin, which contains EF-hand motifs, functions as a Ca$^{2+}$-dependent modulator protein involved in transducing a variety of signals. Plant cells have multiple calmodulin isoforms (Zielinski, 1998; Snedden and Fromm, 2001; Yamakawa et al., 2001), including a newly found vacuolar luminal member (Yamaguchi et al., 2005). Calreticulin is a typical ER luminal CaBP and is functionally involved in Ca$^{2+}$ storage and signalling, and

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Abbreviations: AHA, Arabidopsis plasma membrane H$^+$-ATPase; Blp, binding protein; CaBP, calcium-binding protein; DTT, dithiotheritol; ER, endoplasmic reticulum; GFP, green fluorescent protein; GUS, β-glucuronidase.

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regulation of gene expression (Krause and Michalak, 1997). Annexins have a characteristic ability to bind acidic phospholipid in a Ca\(^{2+}\)-dependent manner and play a role in essential cellular processes such as membrane trafficking (Seaton and Dedman, 1998). Seven annexin genes have been identified in *Arabidopsis* (Clark et al., 2001).

In addition to these CaBP families, a novel acidic CaBP [radish (*Raphanus sativus*) vacuole Ca\(^{2+}\)-binding protein (RVCaB)] was identified in radish taproots (Yuasa and Maeshima, 2000, 2001). RVCaB was characterized as having high capacity and low affinity for Ca\(^{2+}\), and may function as a Ca\(^{2+}\) buffer and/or Ca\(^{2+}\)-sequestering protein in the vacuole (Yuasa and Maeshima, 2000, 2002). No orthologues of RVCaB have been found in the genome databases of various organisms.

During the present study, a predicted acidic protein with some sequence similarity to RVCaB was found in a search of the *Arabidopsis* protein database. The protein contains glutamate-rich motifs, such as VEE\(_x\)K, which are also found in RVCaB. Tissue- and cell-specific expression patterns, response of gene expression to physiological stimuli, and the intracellular localization of the proteins were determined. Interestingly, the protein is associated with the plasma membrane, although it has no predicted transmembrane domain. PCaP1 has the capacity to bind Ca\(^{2+}\) and other metal ions. Thus the protein was named AtPCaP1 (*Arabidopsis* thaliana plasma-membrane associated cation-binding protein) (hereafter referred to as PCaP1). The present study provides information on this novel protein. The molecular properties and the physiological significance of PCaP1 are discussed.

**Materials and methods**

**Plant materials**

Seeds of *A. thaliana* (ecotype Col-0) were germinated on sterile gel plates and grown at 22 °C under continuous light. The gel plates contained Murashige and Skoog (MS) salt, 2.5 mM MES-KOH (pH 5.7), 2% (w/v) sucrose, and 0.25% Gellan gum (Wako Pure Chemical Ind., Osaka, Japan) (1× MS-sucrose). The effects of metal ions, sugars, and phytohormones were examined by growing the seedlings on sterile liquid medium (0.5× MS-sucrose) supplemented with the corresponding component. For examination of phytopathological responses, plantlets were treated with flagellin oligopeptide. The flagellin-derived peptide with 22 residues (flg22) was synthesized according to the consensus sequence for the flagellin-derived peptide with 22 residues (flg22) and used as a crude membrane fraction. Plasma membranes were isolated from crude membranes as described previously (Kobae et al., 2004).

For sucrose density gradient centrifugation, the crude membrane suspension (5 mg, 0.3 ml) was layered on a sucrose density gradient (10.4 ml, 15–45%), centrifuged at 77 000 × g for 19 h in a swing-bucket rotor, and collected in 0.45 ml fractions. The sucrose solution consisted of 10 mM Tricine-KOH (pH 7.5), 1 mM EGTA, and 2 mM EDTA.

**Preparation of antibodies and immunoblotting**

For antibody production, a peptide corresponding to the internal region of *Arabidopsis* PCaP1 (positions 152–166, TEPTGETSGEKE) was synthesized. The peptide was linked with the carrier protein (keyhole limpet haemocyanin) and injected into rabbits. The peptide and antibody were prepared by Operon Biotechnology (Tokyo, Japan). Anti-peptide antibodies to plasma membrane H\(^+\)-ATPase (AHA), subunit-a of vacuolar H\(^+\)-ATPase (VHA-a), and the ER luminal binding protein (BiP) of *Arabidopsis* have been described previously (Kobae et al., 2004). Protein samples were subjected to SDS-PAGE and immunoblotting. The blots were visualized with horseradish peroxidase-coupled protein A and western blotting detection reagents (Amersham Biosciences, Piscataway, NJ, USA). Protein concentration was determined using a BCA protein assay reagent kit (Pierce Biotechnology, Rockford, CA, USA).

**RNA preparation and mRNA quantification**

Organs or whole plants frozen in liquid nitrogen were homogenized in a mortar with a pestle. RNA extracted using an extraction kit (Clontech, Mountain View, CA, USA) was treated with DNase I and an RNase-free DNase set (Qiagen, Valencia, CA, USA). RNA (1 μg) was converted into cDNA using Superscript II RNase H\(^-\) reverse transcriptase (Invitrogen, Carlsbad, CA, USA) and oligo(dT)\(_20\).

Real-time polymerase chain reaction (PCR) analysis was performed with an iCycler iQ Real-Time PCR system (Bio-Rad, Hercules, CA, USA) using the iQ SYBR Green Supermix (Bio-Rad) as described previously (Kamiya et al., 2005). The primer sets used for real-time PCR were as follows: 5′-GTGGCTGAAACCACAAAGC-3′ (forward) and 5′-GGCTCTTTTGCATGGCTT-3′ (reverse) for PCaP1, and 5′-CGGCTACACATGCGAGA-3′ (forward) and 5′-GCTGGAATTACCGGGGTCT-3′ (reverse) for 18S rRNA. The specificity of these primers was confirmed by PCR. Standard plasmid that contained a sequence of PCaP1 was prepared. The standard curve for PCaP1 was generated by using these standard plasmids and gene-specific primers. Copy numbers of the products were calculated from the threshold cycles of triplicate real-time PCR assays using the standard curves. Relative mRNA contents were normalized with the 18S rRNA content. Values are expressed as means ± SD calculated for three assays.

**Transient transformation of Arabidopsis root and cultured cells with GFP-tagged PCaP1**

To construct green fluorescent protein (GFP) fusion proteins, cDNA for PCaP1 was amplified by PCR using the primer set as Ishikawa et al., 2005). The tissues were homogenized in a 5-fold volume of a medium containing 50 mM TRIS-acetate (pH 7.5), 250 mM sorbitol, 1 mM EGTA, 2 mM dithiothreitol (DTT), 1% (w/v) polyvinylpyrrolidone, and 20 μM p-(amidinophenyl) methanesulphonyl fluoride hydrochloride. The homogenate was filtered through two layers of Miracloth (EMD Biosciences, Darmstadt, Germany) and centrifuged at 10 000 g for 10 min. After centrifugation of the supernatant at 100 000 g for 30 min, the pellet obtained was suspended in 20 mM TRIS-acetate (pH 7.5), 250 mM sorbitol, 1 mM EGTA, 2 mM MgCl\(_2\), and 2 mM DTT, and used as a crude membrane fraction. Plasma membranes were isolated from crude membranes as described previously (Kobae et al., 2004).

For sucrose density gradient centrifugation, the crude membrane suspension (5 mg, 0.3 ml) was layered on a sucrose density gradient (10.4 ml, 15–45%), centrifuged at 77 000 × g for 19 h in a swing-bucket rotor, and collected in 0.45 ml fractions. The sucrose solution consisted of 10 mM Tricine-KOH (pH 7.5), 1 mM EGTA, and 2 mM EDTA.
follows: 5'-CACCTAGGTTACTGGAATTCACAAG-3' (forward) and 5'-AGGTTTGTGGTTGTCGCCC-3' (reverse). The resulting fragments were inserted into pENTR/D-TOPO (Invitrogen) followed by insertion in the destination vector pGWB5 or pGWB6 (developed by Dr. Tsuyoshi Nakagawa, Shimane University, Japan) in order to generate a fusion construct with GFP at the C-terminus of PCaP1.

Expression of the GFP–PCaP1 fusion construct in Arabidopsis suspension-cultured cells (Deep cells) was done as described previously (Kobae et al., 2004; Ishikawa et al., 2005). The GFP fluorescence was visualized with a Fluoview FV500 confocal laser-scanning microscope (Olympus) using a set of BA465–495 (excitation) and BA505–550 (excitation) filters.

Promoter–GUS constructs and histochemical analysis
The putative promoter of PCaP1 (~2025 to +18 from the predicted start codon) was amplified from genomic DNA by PCR using the primers: 5'-CACCTAGGTTACTGGAATTCACAAG-3' (forward) and 5'-GGATTCCAGTAAACCCATCTT-3' (reverse). The resulting fragments with the additional sequence CACC at the 5' termini were ligated into the binary vector pGWB203 (developed from Invitrogen Directional TOPO pENTR vector by Dr. Tsuyoshi Nakagawa, Shimane University, Japan), which contains the DNA sequence for ß-glucuronidase (GUS), in order to produce a translational fusion product. The chimeric constructs were introduced into Agrobacterium tumefaciens strain pMP90 (CS51C) by electroporation and used to transform Arabidopsis plants (Ishikawa et al., 2005). Transformants were selected on plates containing 40 mg/l kanamycin. T₂ plants were used for the GUS analysis. Plant materials were incubated with 90% (v/v) acetic acid for 30 min at ~20°C and then washed with 100 mM phosphate buffer (pH 7.0). The samples were infiltrated with a solution containing 100 mM sodium phosphate buffer (pH 7.0), 0.5 mM K₃Fe(CN)₆, 0.5 mM K₄Fe(CN)₆, 10 mM EDTA, 2 mM 5-bromo-4-chloro-3-indolyl-ß-D-galacturonic acid, 20% (v/v) methanol, and 0.3% (w/v) Triton X-100 under vacuum for 30 min, and then incubated at 37°C until blue histochemical stain was confirmed (~6–12 h). The stained tissues were incubated sequentially in 30, 50, and 70% ethanol for 1 h. In order to produce thin sections, the stained tissues were dehydrated, embedded in HistoResin Plus (Leica, Heidelberg, Germany), and sectioned (40 µm in thickness) using a microtome. Root sections were counter-stained with 0.01% Safranine O (Waldeck GmbH & Co. KG, Muenster, Germany) before observation.

Preparation of recombinant PCaP1
cDNA for PCaP1 was amplified by PCR with the primers: 5'-GGAATTCTATGTTGATCCGAATTCACGG-3' (forward) and 5'-ATGCGGCGCCCTAGTGGTGTGAGGCTGTTGTTGTCGCCC-3' (reverse); NdeI and NotI sites are underlined) using KOD-Plus DNA polymerase (Toyobo, Osaka, Japan). The amplified DNA fragment was inserted into a plasmid vector pZErO-2 (Invitrogen) and then ligated into the NdeI/NotI site of the pET23b expression vector (Novagen, Madison, WI, USA). After confirmation of the nucleotide sequence, the expression vector was introduced into Escherichia coli BL21/DE3 (Novagen). Transformants were grown in LB broth for 3 h at 30°C after induction with 0.4 mM isopropylthio-ß-D-galactopyranoside.

Cells were harvested by centrifugation and suspended in 20 mM TRIS-acetate (pH 7.5) containing 20% (v/v) glycerol, 0.2 mg/ml lysozyme, 10 mM 2-mercaptoethanol, and protein inhibitor cocktail (0.5× Complete™, EDTA-free) (Roche Applied Science, Mannheim, Germany). The cells were disrupted by sonication for 12.5 min on ice. After removal of cell debris by centrifugation at 100,000 g for 30 min, the supernatant was applied to an Ni-NTA Superflow column (Qiagen, Valencia, CA, USA) equilibrated with 20 mM imidazole, 20 mM TRIS-acetate (pH 7.5), 20% (v/v) glycerol, and 2 M NaCl. Protein was eluted with 300 mM imidazole, 20 mM TRIS-acetate (pH 7.5), and 2 M NaCl. PCaP1-enriched fractions were tested by SDS-PAGE and then applied to a HiTrap Phenyl HP column (Amersham Biosciences) equilibrated with 20 mM TRIS-acetate (pH 7.5) and 2 M NaCl. PCaP1 was recovered in the flow-through fraction. After desalting with a gel filtration column of Sephadex G-25 (Amersham Biosciences), the protein was applied to a column of Sephacryl S-300 HR (Amersham Biosciences).

45Ca²⁺ overlay assay
Purified preparations of IgG, calmodulin, and recombinant PCaP1 were incubated at 25°C for 10 min. In some experiments, PCaP1 was heated at 95°C for 10 min. The proteins were blotted onto a poly(vinylidene difluoride) membrane using a slot blot apparatus (Bio-Rad). The membrane sheet was washed twice with 10 mM MES-KOH, pH 6.5, 5 mM MgCl₂, and 60 mM KCl; incubated in the same buffer (1 ml) supplemented with 1 mM CaCl₂ and 3.7 MBq of ⁴⁵Ca²⁺ (as CaCl₂) at 25°C for 30 min; washed twice in 10 ml of 50% (v/v) ethanol and dried at room temperature. An autoradiogram of the ⁴⁵Ca²⁺-labelled proteins on the membrane was obtained by exposure to an X-ray film for 3 d at ~80°C.

Results

Identification and characteristics of PCaP1 protein
New CaBP family members have been examined to elucidate their role in Ca²⁺ signalling and calcium homeostasis in plant cells. PCaP1 has been found as a protein with some sequence similarity to RVCaB in a search of the Arabidopsis database. PCaP1 (protein ID, AT128768; locus ID, At4g20260) is predicted to consist of 225 amino acid residues (Fig. 1). Lack of cysteine and histidine residues and high contents of glutamate, lysine, and valine residues are common properties between PCaP1 (glutamate, 44; lysine, 35; valine, 25) and RVCaB. Charged residues make up 20% of RVCaB and 36% of PCaP1. The estimated pI value of PCaP1 is 4.79. PCaP1 and RVCaB contain four or nine repeats, respectively, of a motif VEEKK including variations (IEEKK, VEETK, VEEESK, VEEETK, and VEEKK). PCaP1 was suggested to have two possible myristoylation sites at the N-terminus and the internal part (position 77–82) by sequence analysis by the The Mendel Site (http://mendel.imp.univie.ac.at/myristate/) and ScanProsite (http://kr.expasy.org/tools/scanprosite/) (Fig. 1). The internal site is not likely to be used biologically even though its residues were predicted because of the nature of the myristoylation reaction mechanism. Myristate might be linked to a glycolipid residue at the N-terminus.

The ability of PCaP1 to bind Ca²⁺ was tested by ⁴⁵Ca²⁺ overlay analysis. In the present experiment, recombinant PCaP1 was examined because of the difficulty in obtaining purified native PCaP1 due to the low abundance of the...
protein in planta. The recombinant PCaP1 expressed in E. coli cells was completely purified, as shown in Fig. 2A. Membrane sheets blotted with purified PCaP1 were incubated in $^{45}$Ca$^{2+}$ in the presence of 5 mM MgCl$_2$ and 60 mM KCl, and then rinsed with 50% ethanol. The recombinant PCaP1 reproducibly gave a clear positive signal of $^{45}$Ca$^{2+}$ (Fig. 2B). Purified PCaP1 heated to 95 $^\circ$C also gave the same level of signal (data not shown). Thus, PCaP1 retained Ca$^{2+}$-binding activity even after heat treatment. Calmodulin showed a positive signal in this assay, but immunoglobulin, a negative control, gave no signal. The amount of PCaP1 could not be determined because PCaP1 was partially trapped on the membrane filter like RVCaB (Yuasa and Maeshima, 2000).

Organ- and cell-specific expression

The tissue-specific expression was examined by promoter–GUS analysis of PCaP1. The putative promoter region (~2 kb in length) was fused in-frame with the GUS gene and transformed into Arabidopsis. Three-week-old plants were stained for GUS. There was strong GUS activity for PCaP1 throughout the plant including roots and rosette leaves (Fig. 3A). The promoter–GUS analysis is consistent with the results of mRNA quantification, and taken together these assays show that the PCaP1 gene is expressed in all organs. In the cross-section of root, all of the tissues including epidermis and cortex, endodermis, and root hairs were strongly stained (Fig. 3B). The epidermis and leaf veins also showed strong GUS activity in the section of leaf. In flower organ, pistils and anthers showed GUS activity.

The mRNA level of PCaP1 was quantified by real-time PCR and compared in several organs (Fig. 3C). PCaP1 was expressed in both shoots and roots, with relatively low mRNA levels in flowers and siliques. This result was in agreement with the transcript levels obtained by DNA microchip analysis (www.genevestigator.ethz.ch) (Zimmermann et al., 2004), indicating the reliability of the present analysis.

Changes in mRNA level in response to salts and sugars

Recent proteomic and genomic analyses revealed that a set of genes is regulated by temperature, drought, and salt stress under the control of intracellular signalling (Xiong...
et al., 2002). As demonstrated above, since PCaP1 has 
Ca\(^{2+}\)-binding capacity, the transcription level of PCaP1 is 
changed in response to Ca\(^{2+}\). The effects of Ca\(^{2+}\) and 
other metals were first examined to investigate the physio-
logical function of PCaP1. Figure 4 shows the mRNA 
level in whole plantlets after treatment with CaCl\(_2\), NaCl, 
KCl, MgCl\(_2\), or CuCl\(_2\). The plants grew normally under 
these conditions. The level of PCaP1 mRNA was relatively 
constant after treatment with all salts except for CuCl\(_2\); 
after treatment with 0.1 mM CuCl\(_2\), expression of PCaP1 
decreased transiently by 50% at 2 h but then increased 
3.5-fold at 18 h. The effect of long-term supply of metal 
ions was also examined. The PCaP1 mRNA level in-
creased >2-fold after treatment with 100 mM NaCl, 
100 mM KCl, 100 mM MgCl\(_2\), or 0.5 mM FeCl\(_3\) for 2 d 
(data not shown).

The effect of deficiency of metal ions on the mRNA 
levels was examined by changing the growth medium from 
the salt-containing medium to 0.5 \(\times\) MS medium, which 
contains low concentrations of salts, such as 0.75 mM 
MgCl\(_2\), 50 \(\mu\)M MnSO\(_4\), and 0.05 \(\mu\)M CuSO\(_4\). Suppression 
of Mg\(^{2+}\) concentration caused a moderate increase in the 
mRNA level after 6 h (data not shown). In the presence of 
other ions, the mRNA levels were relatively constant. 

Carbon source nutrients are known to affect the expres-
sion of a large number of genes (Rolland et al., 2002). 
The effect of four different sugars at a concentration of 
150 mM was examined (Fig. 5). Mannitol and sorbitol 
increased the mRNA level 2-fold and 3-fold, respectively. 
On the other hand, sucrose and glucose showed no effect 
on PCaP1.

Response to physiological and physical stimuli

The effect of salicylic acid, abscisic acid, and gibberellic 
acid on the mRNA level of PCaP1 was examined (Fig. 6). 
These hormone treatments did not affect the mRNA level 
of PCaP1.

The flagellin oligopeptide flg22, which is the most 
highly conserved region in the N-terminus (QRLSTGSRI-
NSAKDDAAGLQIA) of eubacterial flagellin (Felix et al., 
1999), induces pathological responses in Arabidopsis and 
other plants (Nuhse et al., 2003; Zipfel et al., 2004). In 
this experiment, 3-week-old plantlets were incubated in a 
0.5 \(\times\) MS-sucrose medium containing 10 \(\mu\)M flagellin 
peptide. Treatment with the peptide increased the 

![Fig. 3. (A, B) Expression of the PCaP1 promoter::GUS fusion in Arabidopsis. (A) Plants grown for 7 d were incubated in GUS staining solution for 3.5 h (a) or 1 h (b–e). (a, b) Whole plant. (c) Surface of the cotyledon. (d) Root tip and elongation zone. (e) Mature zone of the root. Bars: 2 mm (a), 1 mm (b), 50 \(\mu\)m (c), 200 \(\mu\)m (d), 100 \(\mu\)m (e).](https://academic.oup.com/jxb/article-abstract/58/5/1173/491327)
Fig. 4. Changes in the levels of PCaP1 mRNA in response to inorganic ions. Plants grown on Gellan gum plates containing 0.5x MS-sucrose medium for 3 weeks under constant light were transferred to liquid medium containing 5 mM CaCl2 (A), 50 mM CaCl2 (B), 50 mM NaCl (C), 50 mM KCl (D), 50 mM MgCl2 (E), or 0.1 mM CuCl2 (F) for the indicated period. The mRNA levels were determined by real-time PCR and expressed as percentages of the value at time 0. The half-strength MS-sucrose medium contained 1.5 mM CaCl2, 0.75 mM MgSO4, 50 mM CuCl2, 0.1 mM Na+, 10.7 mM K+, and other inorganic ions.

Fig. 5. Effect of sugars on the PCaP1 mRNA level. Plants grown for 3 weeks in 0.5x MS-sucrose (1% sucrose) under constant light were treated with water (A), sucrose (B), glucose (C), mannitol (D), or sorbitol (E) at a concentration of 150 mM for up to 18 h. The mRNA levels are expressed as percentages of the value at time 0. Each value is expressed as the mean ± SD calculated for three assays.
transcript amount 2.4-fold (Fig. 6). This observation suggests the involvement of PCaP1 in the response to microbial pathogens.

To examine the effect of temperature, 3-week-old plantlets grown at 22 °C were kept for 6 h at 36 °C or 4 °C. The transcript level of PCaP1 was not changed under either temperature. Dehydration partially suppressed the expression of PCaP1.

**Immunoochemical detection of PCaP1 in tissues**

A polyclonal antibody was prepared against the conjugate of keyhole limpet haemocyanin and the internal sequence of PCaP1 (Fig. 1). The anti-PCaP1 antibody reacted specifically with the antigen in crude membranes of Arabidopsis (Fig. 7A). The corresponding authentic peptide completely inhibited the immunochemical reaction, indicating the specificity of the antibody. The antibody recognized a recombinant PCaP1 expressed in E. coli (data not shown). Therefore, this antibody was used to probe the PCaP1 protein in the following experiments.

Crude membrane fractions prepared from cauline leaves, rosette leaves, and stems showed clear immunostained bands at 36 kDa (Fig. 7B). On the other hand, the fraction of flowers and siliques showed only a slight immunostained band. Judging from the intensity of immunostaining, the relative content of PCaP1 on the basis of the weight of membrane protein in leaves was similar to that in stems. Plasma membranes purified from crude membranes gave an intense immunostained band at 36 kDa (Fig. 7C), which suggested that PCaP1 was localized in the plasma membrane.

Most of the PCaP1 protein was recovered in the crude membrane fraction but not in the cytosol fraction (Fig. 7D). The specific content of PCaP1 in the cytosol was estimated to be <3% of that in the crude membrane fraction. Thus, it was estimated that PCaP1 is predominantly located in the membranes.
Plasma membrane localization of PCaP1 protein

To determine the subcellular localization of PCaP1, two DNA constructs encoding a fusion protein of PCaP1 with GFP, which was at the N- (GFP–PCaP1) and C-termini of PCaP1 (PCaP1–GFP), were prepared and transiently expressed in *Arabidopsis* suspension-cultured cells. More than 50 cells were observed for each construct, and typical images are shown in Fig. 8. The green fluorescence from GFP of PCaP1–GFP was clearly detected at the plasma membrane of suspension-cultured cells. A similar expression pattern was observed in *Arabidopsis* roots. Some fluorescence could be seen in the cytosol, but none was seen in vacuoles or nuclei. However, GFP fluorescence was detected in the cytosol for the GFP–PCaP1 construct. The difference in the fluorescence images between the two constructs may be related to the function of the N- and C-terminal parts of PCaP1 in intracellular localization, as discussed later. For the control experiment, the fluorescence of the cells expressing free GFP was dispersed throughout the cytosol and nucleus of suspension-cultured cells (Fig. 8I). In contrast to the control experiment, the green fluorescence from GFP–PCaP1 was not observed in nuclei, suggesting that the fluorescence of GFP–PCaP1 was not from the free GFP cleaved from the fusion protein.

To test the plasma membrane localization of endogenous PCaP1 protein under natural conditions, the crude membrane fraction prepared from the homogenate of 3-week-old plantlets was subjected to equilibrium sucrose density gradient centrifugation (Fig. 9). PCaP1 protein was recovered in fractions 10–17 together with the AHA, VHA-a and the ER luminal protein BiP were recovered in lighter fractions 2–9. Thus, these results suggest that PCaP1 is localized in the plasma membrane, but not in the vacuolar or ER membranes.

Tight association of PCaP1 with plasma membranes

PCaP1 is a highly charged protein and has no predicted transmembrane domain or hydrophobic segment in its sequence; this is inconsistent with its plasma membrane localization. The conditions for stimulating the dissociation of the protein from the membrane were examined. PCaP1 was released from membranes by treatment with 100 mM Na₂CO₃, but not by sonication in 100 mM KCl (Fig. 10A). These results suggest that PCaP1 is not bound to the membrane by an electrostatic interaction.

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**Fig. 8.** Expression of PCaP1–GFP fusion proteins in protoplasts. Constructs of PCaP1–GFP (A–D), GFP–PCaP1 (E–H), and free GFP (I, J) were transiently expressed in *Arabidopsis* suspension-cultured cells. The green fluorescence of GFP-tagged proteins was viewed by confocal laser scanning microscopy (A, C, E, G, I). Nomarski images (B, D, F, H, J) were also recorded. Bars, 10 μm.
The effects of various ions on the association of PCaP1 with the membrane were examined. Treatment with EDTA, NaCl, or KCl did not release PCaP1 from membranes even at 50 mM or 100 mM (Fig. 10B, C, D). However, addition of CaCl2 and/or MgCl2 stimulated the release of PCaP1 at concentrations >10 mM (Fig. 10E, F). Mg2+ and Ca2+ co-exist in the cytosol of living cells under physiological concentrations of ~5 mM and 0.1 μM, respectively (Sanders et al., 2002). Under the conditions of 5 mM MgCl2 and 0.05–1 mM CaCl2, most PCaP1 was retained in the membrane (data not shown). These observations suggest that PCaP1 tightly associates with the membrane under physiological concentrations of Ca2+ and Mg2+. The result is consistent with the observation of GFP-linked PCaP1 (Fig. 8) and the cell fractionation experiment (Fig. 9).

To examine the reversibility of the interaction of PCaP1 with membranes, free PCaP1 was incubated with the PCaP1-depleted membranes (Fig. 11). PCaP1 was completely removed from the membrane by incubation with 100 mM CaCl2. The PCaP1-depleted membrane (Ppt1Ca) was incubated with the PCaP1-containing supernatant (Sup1Ca). When the mixture was treated with EDTA to remove free Ca2+ and other metal ions, PCaP1 was recovered in the precipitate (Fig. 11B, lane 3). When the Sup1Ca was re-centrifuged without mixing with Ppt1Ca, PCaP1 was recovered in Sup2 (lane 8). These results clearly indicate re-association of the released PCaP1 with the membrane.

**Discussion**

A novel Ca2+-binding protein PCaP1 from *Arabidopsis* has been identified and characterized. The Ca2+-binding capacity of PCaP1 was clearly demonstrated, but since PCaP1 was released from the membrane in response not only to Ca2+ but also to Mg2+, whether it bound specifically to Ca2+ or could bind some other metal ions was not clear. Thus, the protein was named a plasma membrane-associated cation-binding protein (PCaP). PCaP1 is rich in glutamate and lysine residues, and also contains a relatively large number of proline and valine residues (Fig. 1). PCaP1 contains a unique VEExK motif (variations: IEEKK and VEETKK). The main acidic residues are glutamate (44 residues). There is only a single aspartate residue. The VEExK motif is a likely candidate for binding of Ca2+ or other metal ions. This hypothesis should be

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**Fig. 9.** Subcellular localization of PCaP1. Microsomal fractions were prepared from 3-week-old plants and subjected to sucrose density gradient centrifugation. (A) Sucrose (filled circles) and protein (open circles) concentrations in the fractions. (B) Immunoblots of the fractions with antibodies to PCaP1, plasma membrane H+-ATPase (AHA), BiP, and subunit-a of V-ATPase (VHA-a).

**Fig. 10.** Cation-dependent release of PCaP1 from membranes. Crude membrane fractions were prepared from 3-week-old plants (A) or suspension-cultured cells (B–F) in the presence of EDTA and then used for analysis of PCaP1 distribution. (A) The crude membrane fraction was incubated in 100 mM KCl or 100 mM Na2CO3, sonicated, and then centrifuged for 20 min at 100 000 g. The supernatants (Sup) and precipitates (Ppt) were analysed by immunoblotting with the anti-PCaP1 antibody. The pellets were suspended in the same volume as the original membrane suspension. (B) Effect of 50 mM EDTA on the distribution of PCaP1. Water was added to the mixture for the control experiment. (C–F) Effects of NaCl (C), KCl (D), CaCl2 (E), and MgCl2 (F) at the indicated concentrations.
Fig. 11. Reversible association of PCaP1 with membranes. (A) Crude membranes prepared from suspension-cultured cells were treated with 50 mM EDTA or 100 mM CaCl2 for 20 min at room temperature and then centrifuged at 100,000 g for 20 min. The supernatants (Sup1Ca and Sup1EDTA) were stored and the precipitates (Ppt1Ca and Ppt1EDTA) were suspended in the same volume as the original membrane suspensions. The Sup1Ca and Ppt1Ca obtained after treatment with CaCl2 were mixed, supplemented with 200 mM EDTA, and then centrifuged at 100,000 g for 20 min. The supernatant (Sup2) was stored and the precipitate (Ppt2) was suspended in the same volume as the original membrane suspension. (B) The fractions were analysed by immunoblotting with anti-PCaP1 antibody. Lane 1, crude membranes; lanes 2 and 3, Sup1EDTA and Ppt1EDTA; lanes 4 and 5, Sup1Ca and Ppt1Ca; lanes 6 and 7, Sup2 and Ppt2 as described above; lanes 8 and 9, Sup2 and Ppt2, which were obtained by centrifugation of Sup1Ca.

tested using a synthetic polypeptide containing multiple VEEK motifs. Further protein chemical studies are necessary to elucidate which cations it binds and its kinetics.

The tissue specificity of PCaP1 was examined by real-time PCR, promoter–GUS histochemical analysis, and immunohistochemical analysis of the tissue homogenate. PCaP1 was expressed in all organs examined (Fig. 3) and PCaP1 was constant under different conditions of metal ions (except for Cu2+), sucrose, glucose, phytohormones, and low and high temperatures (Figs 4, 5, 6). The relatively small enhancement of gene expression and its expression in most organs suggests that PCaP1 is a constitutive element of the Arabidopsis plant.

The level of PCaP1 mRNA increased following treatment with Cu2+, sorbitol, mannitol, and flagellin oligopeptide, and was constant under different conditions of metal ions (except for Cu2+), sucrose, glucose, phytohormones, and low and high temperatures (Figs 4, 5, 6). The relatively small enhancement of gene expression and its expression in most organs suggests that PCaP1 is a housekeeping protein. However, stimulation of PCaP1 translation by treatment with the flagellin peptide and Cu2+ also suggests the involvement of PCaP1 in the pathologic response, because Cu2+ is known to have an elicitor effect (Schützendübel and Polle, 2002). This hypothesis remains to be fully examined by using a knockout mutant of PCaP1 and several kinds of pathogens.

Interestingly, PCaP1 is associated with the plasma membrane under physiological conditions judging from the fluorescence of the fusion proteins with GFP, and immunohistochemical detection after subcellular fractionation (Figs 8, 9). In fluorescence microscopic detection of PCaP1–GFP, green fluorescence was clear at the plasma membrane and weak in the cytosol. Furthermore, all of the PCaP1 protein in the crude membrane fraction was recovered in the plasma membrane, suggesting that PCaP1 was not released from the membrane during sucrose density gradient centrifugation (Fig. 9). These results suggest that PCaP1 stably associates with the plasma membrane under physiological conditions.

Interaction of PCaP1 with the membrane was retained even in the presence of NaCl or KCl at 100 mM (Fig. 10), suggesting that the association is not due to electrostatic attraction. Alkaline treatment with Na2CO3 released PCaP1 from the membrane. PCaP1 was also dissociated from the membrane by treatment with extremely high concentrations of Mg2+ and Ca2+, and reversibly associated with the membrane. Thus, it is proposed that PCaP1 stably associates with membrane integral component(s), such as membrane proteins or membrane lipids, under natural conditions.

In general, the sequence Met-Gly-X1-X2-X3-(Ser/Cys) at the N-terminus is a probable candidate for a protein myristoylation site. Myristic acid, a short saturated fatty acid, is linked to glycine in the motif after removal of the first methionine with catalysis using methionine amino peptidase and N-myristoyl transferase (Farazi et al., 2001). For PCaP1, the N-terminal part (GYWNSKVVKFKK) may be the myristoylation site (Fig. 1). The MGYWNS motif of PCaP1 is followed by a KVVKFKK sequence, which is rich in lysine residues. A cluster of positively charged residues such as lysine around the myristoylation site is thought to interact with negatively charged phospholipids in biomembranes. Thus, myristoylation may be a probable mechanism for the association of PCaP1 with plasma membranes. It should be noted that GFP–PCaP1 was localized to the cytosol and PCaP1–GFP to the plasma membrane (Fig. 8). From the motif search, there is another candidate for the myristoylation site in the internal part (Fig. 1). If this site is acylated, the size of the protein must be reduced by proteolysis at Gly77. However, such a small band was not found on the immunoblot. Thus, it is unlikely that the second myristoylation site is used.

Kawamura and Uemura (2003) reported that At4g20260, which encodes PCaP1, a plasma membrane protein from the leaf, transiently increased in abundance during cold acclimation. They reported that the At4g20260 protein has 55% identity with tobacco DREPP2 (developmentally regulated plasma membrane protein 2) (215 amino acid residues). The biochemical properties of DREPP2 have not been described previously. In the present study, cold treatment did not enhance the mRNA level of PCaP1 (Fig. 7). Thus there is a possibility that the protein
identified by Kawamura and Uemura (2003) should be annotated as another protein encoded by an isogene. If their annotation is correct, there is a possibility that the protein level does not reflect the transcript level.

In conclusion, a novel cation-binding protein PCaP1 was found and its gene expression profile was determined. PCaP1 has a unique primary sequence different from that of known Ca2+-binding proteins. The expression level of the PCaP1 gene is relatively constant in most organs. PCaP1 protein is stably associated with the plasma membrane under physiological conditions even though it has no transmembrane domain. The amino acid sequence of PCaP1 indicated that the plasma membrane association is probably mediated by the N-terminal myristoylation. Further understanding of the biochemical function of PCaP1 requires examination of myristoylation of PCaP1, identification of a partner or interactive component in the membrane, and determination of the structural properties and metal-binding kinetics. The response of PCaP1 to flagellin peptide and copper ion suggests its involvement in the pathological response. Further investigation of this unique membrane-associated protein should provide insight into the physiological role of PCaP1 in the plasma membrane.

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