

A novel-type phosphatidylinositol phosphate-interactive, Ca-binding protein PCaP1 in *Arabidopsis thaliana*: stable association with plasma membrane and partial involvement in stomata closure

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Abstract The Ca²⁺-binding protein-1 (PCaP1) of *Arabidopsis thaliana* is a new type protein that binds to phosphatidylinositol phosphates and Ca²⁺-calmodulin complex as well as free Ca²⁺. Although biochemical properties, such as binding to ligands and *N*-myristoylation, have been revealed, the intracellular localization, tissue and cell specificity, integrity of membrane association and physiological roles of PCaP1 are unknown. We investigated the tissue and intracellular distribution of PCaP1 by using transgenic lines expressing PCaP1 linked with a green fluorescence protein (GFP) at the carboxyl terminus of PCaP1. GFP fluorescence was obviously detected in most tissues including root, stem, leaf and flower. In these tissues, PCaP1–GFP signal was observed predominantly in the plasma membrane even under physiological stress conditions but not in other organelles. The fluorescence was detected in the cytosol when the 25-residue N-terminal sequence was deleted from PCaP1 indicating essential contribution of *N*-myristoylation to the plasma membrane anchoring. Fluorescence intensity of PCaP1–GFP in roots was slightly decreased in seedlings grown in medium supplemented with high

concentrations of iron for 1 week and increased in those grown with copper. In stomatal guard cells, PCaP1–GFP was strictly, specifically localized to the plasma membrane at the epidermal-cell side but not at the pore side. A T-DNA insertion mutant line of PCaP1 did not show marked phenotype in a life cycle except for well growth under high CO₂ conditions. However, stomata of the mutant line did not close entirely even in high osmolarity, which usually induces stomata closure. These results suggest that PCaP1 is involved in the stomatal movement, especially closure process, in leaves and response to excessive copper in root and leaf as a mineral nutrient as a physiological role.

Keywords *Arabidopsis thaliana* · Calcium-binding protein · Phosphatidylinositol phosphate · Plasma membrane · Signal transducing protein · Stomata

Introduction

Many kinds of signal transducing proteins, ligand receptors, protein kinases, protein phosphatases, ion channels, phospholipases, and transcription factors are involved in homeostasis of cell and organs under various physiological conditions, defense processes to biotic stresses, and tolerance to abiotic stresses (Davière and Achard 2013; Hwang et al. 2012; Leivar and Monte 2014). Response of plants to changes in environmental conditions is achieved through a series of cellular processes including sensing of environmental stimuli, transduction of a primary signal into an intracellular protein, modification of proteins and enzymes such as phosphorylation, and activation of transcription factor in nucleus, although the signal pathway is varied with extra- and inter-cellular stimuli. Detailed information on receptors for the primary stimuli, protein kinases, and trans-acting elements has been reported

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for various organisms. Each cell in the plant receives many kinds of environmental signals simultaneously and adapts to biotic and abiotic stresses. There must be a cross talk of signals between the transducing systems.

We focused on a phosphatidylinositol phosphates (PtdInsPs)-interactive, Ca-binding protein of *Arabidopsis thaliana*, called PCaP1 (locus ID: At4g20260) (Ide et al. 2007) as a possible hub element of signal transduction and cross talk. PCaP1 is composed of 224 amino acid residues and has no known functional enzymatic motif. The protein has an *N*-myristoylation signal at its N-terminus and has been demonstrated to be myristoylated at Gly-2 (Nagasaki et al. 2008). A continuous region of the center and C-termini has notably high contents of proline, glutamate, valine, and lysine residues. The ability of PCaP1 to bind PtdInsPs, Ca^{2+} , and a Ca^{2+} -calmodulin complex is characteristic property. These ligands are key components of intracellular signaling (Meijer and Munnik 2003; Carlton and Cullen 2005; McCormack et al. 2005; De Falco et al. 2009; Kleerekoper and Putkey 2009; Luan 2009; Xue et al. 2009). Therefore, its biochemical properties suggest that PCaP1 mediates a signal from the Ca^{2+} -calmodulin complex to PtdInsPs. The intracellular localization, tissue and cell specificity, stability of membrane association and physiological roles of PCaP1 are unknown.

A PCaP1-related protein PCaP2 is composed of 168 amino acid residues with sequence similar to that of the *N*-myristoylation signal at the N-terminal region. PCaP2, which can bind PtdInsPs, Ca^{2+} , and a Ca^{2+} -calmodulin complex, is specifically expressed in root hairs (Kato et al. 2010a, b). The formation and polar cell growth of root hairs highly depend on PCaP2 (Kato et al. 2010a, b, 2013; Tanaka et al. 2014). It was estimated that changes in cytosolic Ca^{2+} concentrations at the root tip are transferred to signaling of PtdInsPs through binding of Ca^{2+} -calmodulin complex to PCaP2. Little information is available on the physiological role of PCaP1 except under specific pathological condition. In leaves infected with potyvirus, the potyviral protein P3N-PIPO interacts with PCaP1 in the host cells. In other words, PCaP1 helps cell-to-cell movement of P3N-PIPO (Vijayapalani et al. 2012).

Here, we prepared transgenic plants stably expressing PCaP1 tagged with green fluorescent protein (GFP) to visualize the protein in plants and found that PCaP1 was expressed in most tissues and cells both under stress and under normal conditions. It was localized in the plasma membrane. Analysis using proteins tagged with GFP in transgenic plants revealed the involvement of the N-terminal domain in the plasma membrane localization of PCaP1. PCaP1 was localized uniformly in the plasma membrane in all cells except the stomatal guard cells. We hypothesized that PCaP1 plays a fundamental role in all cells. To investigate this hypothesis we examined phenotypic properties

of a loss-of-function mutant *pcap1*. *A. thaliana* mutants that do not express PCaP1 were unable to close stomata as fast as the wild type. These results suggest a molecular mechanism by which plasma membrane associated protein PCaP1 is involved the stomatal closure and reveal the importance of signaling of Ca and PtdInsPs in this process. Thus we establish a novel aspect of the regulation of stomatal movement.

Materials and methods

Plant materials and growth conditions

Seeds of *A. thaliana* Columbia-0 (Col-0) were sown on sterile gel plates containing Murashige–Skoog (MS) salt, 2.3 mM MES–KOH (pH 5.7), 2 % (w/v) sucrose and 0.8 % Ina agar (Funakoshi, Tokyo, Japan) and kept at 22 °C under long-day conditions (a light/dark regime of 16 h/8 h, cool-white lamps, $90 \mu\text{mol m}^{-2} \text{s}^{-1}$) to germinate. In some experiments, seeds were sown on 1.5 % agar plate, which were placed vertically. The effects of ions, metals and phytohormones were examined on sterile agar gel plates containing 0.5 MS (one-half strength of MS salts) supplemented with 2 % sucrose.

T-DNA insertion lines of PCaP1 (*pcap1-1*, registration no. SALK_022955; *pcap1-4*, registration no. SAIL_241_A08) of *A. thaliana* (Col-0) were obtained from The Salk Institute Genomic Analysis Laboratory (<http://signal.salk.edu/index.html>) and the Syngenta Arabidopsis Insertion Library (Sessions et al. 2002). Information concerning the insertion mutants was obtained from the Salk Institute Genomic Analysis Laboratory website (<http://signal.salk.edu>). Homozygous transgenic lines were selected by PCR after the second back cross and the T-DNA insertion sites were confirmed. In some experiments, Col-0 and mutant lines were cultivated in growth chambers, which had CO_2 concentrations controlled at 380 or 700 ppm.

Preparation of PCaP1–GFP construct and its transformation to *A. thaliana* plants

Transgenic plants expressing a $PCaP1^{\text{pro}}::PCaP1\text{--}GFP$ construct were prepared as follows. A DNA segment of the *PCaP1* promoter followed by the open reading frame (ORF) of *PCaP1* was amplified from an *A. thaliana* genomic DNA preparation by PCR using a primer set: forward, 5'-CACCATGGGTTACTGGAATTC CAAG-3' (forward) and 5'-AGGCTTTGGTGGTTCAGCC-3' (reverse). The obtained fragments were inserted into pENTR/D-TOPO and then sub-cloned into the pGWB404 vector (Nakagawa et al. 2007), which contained a synthetic GFP whose Ser-65 was substituted with threonine. The resulting plasmid was termed $PCaP1^{\text{pro}}::PCaP1\text{--}GFP$.

In some experiments, PCaP1–GFP and its modified variant were expressed under the control of promoter of guard cell-specific gene GC1 (At1g22690) (Yang et al. 2008). For preparation of $\Delta N25PCaP1$, which lacks the N-terminal region (2nd to 25th residues), the ORF of *PCaP1* was amplified from the genomic DNA preparation by PCR using a primer set: 5'-CACCTCTCTAGAAGCTGCTGAAGCTACCAAG-3' (forward) and 5'-AGGCTTTGGTGGTTCAG-3' (reverse). The obtained fragment was sub-cloned into the pGWB404 vector to prepare the DNA fragment $\Delta N25PCaP1$ –GFP. The DNA fragment was then inserted into the down-stream of *GCI*^{pro} of *Agrobacterium* binary vector pPZP211. The plasmid obtained was named *GCI*^{pro}:: $\Delta N25PCaP1$ –GFP.

Arabidopsis thaliana Col-0 plants were transformed by the floral dip method using *Agrobacterium tumefaciens* strain C58C1 carrying the *PCaP1*^{pro}::*PCaP1*–GFP binary vector (Clough and Bent 1998). PCaP1–GFP transformants were grown on half strength MS-agarose plates containing 50 $\mu\text{g ml}^{-1}$ kanamycin. T1 transformants were selected by GFP fluorescence. The obtained T2 plants were used for fluorescence observation by confocal laser scanning microscopy (CLSM).

Histochemical assay of *PCaP1* promoter-*GUS* gene expression Plants expressing *PCaP1* promoter-*GUS* gene were prepared as described previously (Ide et al. 2007). An upstream region that contained a 5'-noncoding region of 2,025 bp and a 18-bp section of the coding region of *PCaP1* was amplified from genomic DNA by PCR and was ligated into the binary vector pGWB203 (Nakagawa et al. 2007), which contains the DNA sequence for β -glucuronidase (*GUS*). The chimeric constructs were introduced into the *Agrobacterium tumefaciens* strain pMP90 (C58C1) by electroporation and used to transform *A. thaliana* plants. Transformants were selected on plates containing 40 $\mu\text{g ml}^{-1}$ Benlate (Sumitomo Chemical, Osaka, Japan), 0.20 mg ml⁻¹ Cefotax (Chugai Pharmaceutical, Tokyo, Japan), 50 $\mu\text{g ml}^{-1}$ hygromycin, and 30 $\mu\text{g ml}^{-1}$ kanamycin. T2 transformants were grown on the medium containing MS salt, 2.3 mM MES–KOH (pH 5.7), 2 % (w/v) sucrose and 0.25 % Gellan gum (Wako Pure Chemicals, Osaka, Japan) and then assayed for *GUS* activity. *GUS* activity was visualized by treatment of whole seedlings with 90 % (v/v) acetone for 30 min at $-4\text{ }^{\circ}\text{C}$. After washing with 100 mM sodium phosphate buffer (pH 7.0), the samples were infiltrated with a solution containing 100 mM sodium phosphate buffer (pH 7.0), 0.5 mM $\text{K}_3\text{Fe}(\text{CN})_6$, 0.5 mM $\text{K}_4\text{Fe}(\text{CN})_6$, 10 mM EDTA, 2 mM 5-bromo-4-chloro-3-indolyl- β -D-glucuronide, 20 % (v/v) methanol and 0.1 % (w/v) Triton X-100 under vacuum for 30 min, and then incubated at 37 $^{\circ}\text{C}$ until blue histochemical stain was confirmed. The regular incubation period was 12 h.

Confocal laser scanning microscopy (CLSM)

Observations were conducted with an upright FV1000/BX61 confocal laser scanning microscope (Olympus, Tokyo, Japan) using a set of BA465–495 (excitation) and BA505–550 (emission) filters. For imaging the plasma membrane, tissues were treated with 10 $\mu\text{g ml}^{-1}$ propidium iodide (Sigma Aldrich, Steinheim, Germany) for staining of cell walls. Propidium iodide red fluorescence was visualized with a FV1000/BX61 confocal laser scanning microscope set at 561 nm for excitation and at 550–650 nm for emission. The intensities of green fluorescence were extracted from CLSM images using FX10-ASW (<http://www.aswsoftware.com/>).

Measurement of stomatal aperture

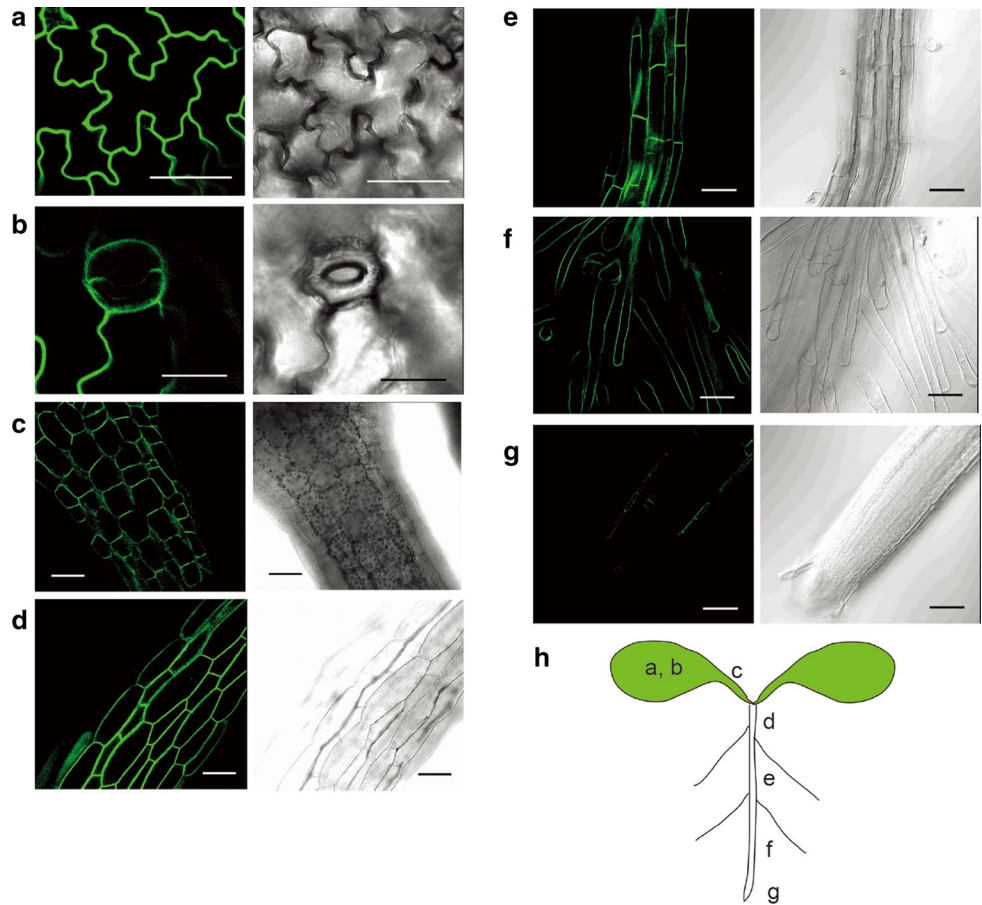
Stomatal aperture of wild-type (Col-0), *pcap1-1* and *pcap1-4* plants was assayed using leaves of 3-week-old plants grown on agar plates as described previously (De Angeli et al. 2013). The whole leaves were detached just before the light period started and glued the abaxial side onto a coverslip using a non-toxic medical adhesive (Medical Adhesive B Liquid, VM 355-1, Ulrich, Swiss). The adaxial epidermis and mesophyll layers were gently removed. The coverslips with the glued abaxial epidermis were placed in a Petri dish containing a 200 mOsm buffer (stomata-opening buffer). The 200 mOsm buffer contained 10 mM MES–KOH, pH 6.1, 30 mM KCl and 100 mM sorbitol. A 600 mOsm buffer contained 10 mM MES–KOH, pH 6.1, 30 mM KCl and 500 mM sorbitol. The epidermis was equilibrated for 2 h in light in the 200 mOsm buffer and measured for stomatal aperture (the first assay). Then the assay buffer was changed to the 600 mOsm buffer. Finally, the buffer for the same epidermis was changed to the 200 mOsm buffer (the second assay) to confirm the reversibility of stomata movement. Five leaves from Col-0, *pcap1-1* and *pcap1-4* were analyzed in parallel. About 3 pictures per leaf and per time point were taken using an inverted microscope (BX51/DP72, Olympus) at a $\times 40$ magnification. Each picture contains approximately 20 stomata. Stomatal apertures (ratio of the width to the length of stomata) were measured manually using the ImageJ software (Schneider et al. 2012). For each time point, more than 300 stomatal apertures were measured.

Results

Stable localization of PCaP1 on the plasma membrane of tissues

PCaP1 is a soluble protein and was detected in plasma membrane fraction after subcellular fractionation (Ide et al.

Fig. 1 Intracellular localization of PCaP1–GFP in seedlings. CLSM images of various tissues of 7-day-old seedlings expressing *PCaP1^{pro}::PCaP1–GFP* (left panel in each set) and DIC images (right) are shown. **a** Leaf epidermal cells, **b** guard cells, **c** epidermal cells of petiole, **d** epidermal cells of stem, **e** epidermal cells of root at middle region, **f** root hairs, and **g** a root tip. Bars 50 μm (a, c–g) or 10 μm (b)



2007). To investigate the intracellular localization, we prepared the *PCaP1–GFP* construct and expressed it in Col-0 under the control of the own promoter (*PCaP1^{pro}::PCaP1–GFP*). GFP was linked to the C-terminus of PCaP1, because the N-terminal glycine residue (Gly-2) is myristoylated after removal of the first methionine as described previously (Nagasaki et al. 2008). Transformation of the construct was confirmed by PCR using primer sets for PCaP1 and GFP DNAs (Supplemental Fig. S1). Transgenic lines 1 and 2 shown in Fig. S1 were used for further experiments. Plants expressing *PCaP1^{pro}::PCaP1–GFP* grew normally and showed clear green fluorescence in various tissues of 5-day-old seedlings. GFP green fluorescence was observed in cotyledon epidermal cells, petioles, hypocotyls, roots, and root hairs (Fig. 1). PCaP1–GFP was detected specifically in the plasma membrane but neither in the membranes of other organelles nor the cytoplasm. PCaP1–GFP was detected uniformly in the plasma membranes in most cells but unevenly detected exceptionally in guard cells (Fig. 1b).

Plants at the reproducible stage, 30-day-old, also showed clear fluorescence in rosette and cauline leaves, stems, petals, stomata, sepals, styles, anthers, filaments, stigma, and seedpods (Fig. 2a). Root hairs showed clear fluorescence

of PCaP1–GFP in 7-day-old (Fig. 1f) and 21-day-old seedlings (Fig. 2c). Leaf trichomes also showed fluorescence (Fig. 2b). Clear fluorescence was detected predominantly in the plasma membrane. At the vegetative growth stage examined, the same images were obtained in inner cells of each tissue (data not shown). These observations indicate constitutive expression throughout the life cycle of the plant and stable association of PCaP1 with the plasma membranes and suggest that PCaP1 is a common element in various tissues.

Side-specific localization of PCaP1 on the plasma membrane of guard cells

In the guard cells, PCaP1–GFP was clearly observed in the plasma membrane only on the outer side facing the epidermal cells, but not that of the inner side facing the pore, in the pair of guard cells (Figs. 1b, 2a). There is a possibility that guard cells do not express PCaP1 but surrounding epidermal cells do. Thus, this side-specific localization of PCaP1 was examined by quantitative analysis of the fluorescence intensity (Fig. 3). Fluorescence image of the surface area of a guard cell and its cross section image of the same cell (marked by a line from i to ii) are shown in

Fig. 2 Intracellular localization of PCaP1 in cells in plant tissues. Images of indicated tissues of 30-day-old plants (a) and 14-day-old plantlets (b, c) expressing *PCaP1^{Pro}::PCaP1-GFP* were acquired by CLSM (left panel in each set) and by DIC (right)

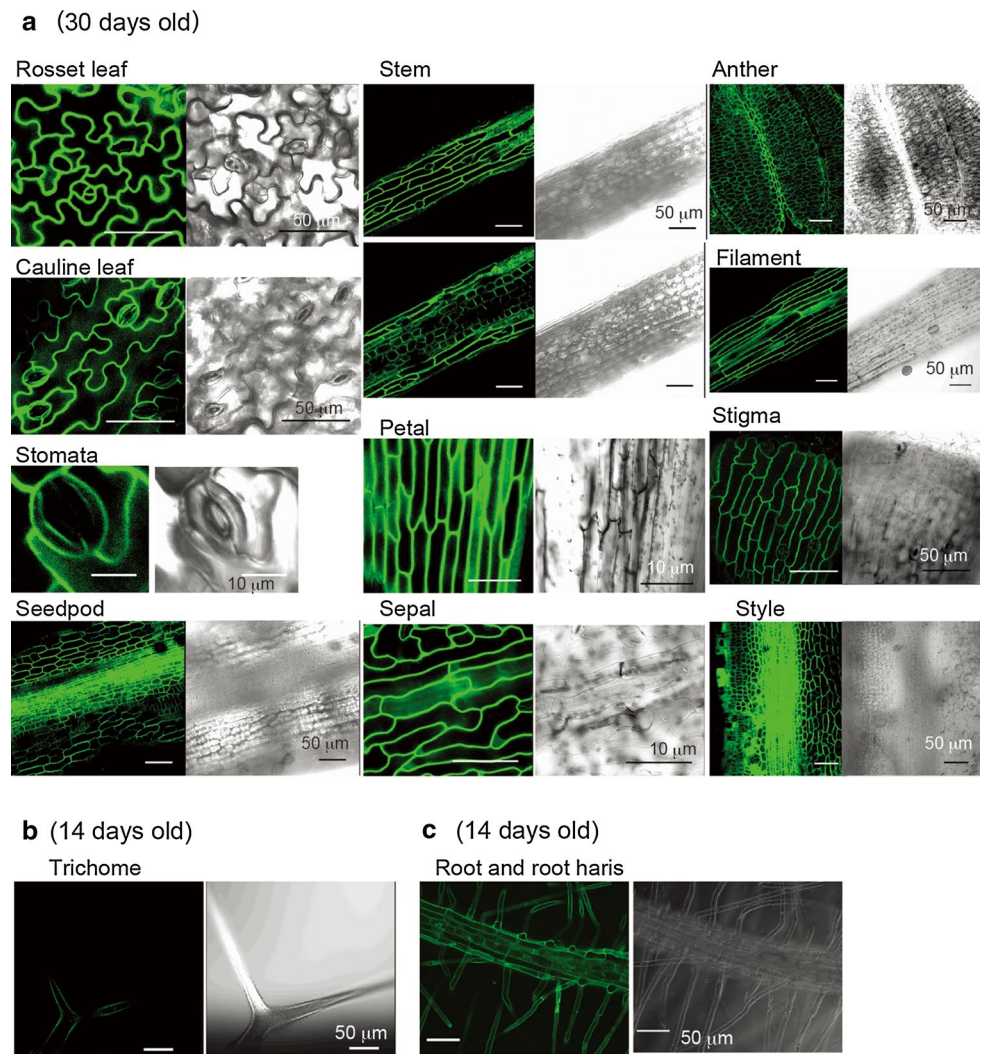


Fig. 3a and b, respectively. Clear green fluorescence was detected in the plasma membrane of guard cell in the cross section of the stomata, which could be distinguished from that of neighbor epidermal cells (Fig. 3b). In this image the fluorescent intensity at the adjacent region was relatively high compared with other parts. Furthermore, fluorescent peaks corresponding to guard cells and epidermal cells were detected individually when analyzed for some specific region with appropriate distance between the two cells (Fig. 3c, marked by a line from iii to iv in Fig. 3a). The fluorescent intensity in the outer side of the plasma membrane, which face to the epidermal cell, was markedly higher than that of inner pore side (Fig. 3d). These results indicate that PCaP1 is localized predominantly on the outer side of the guard cell plasma membrane.

To examine whether or not the side-specific plasma membrane localization of PCaP1 in the guard cells depends on modification with a myristoyl moiety, we introduced the modified construct without the N-terminal myristoylation site of PCaP1 ($\Delta N25PCaP1-GFP$) into Col-0 (Fig. 4a).

Three constructs, namely free *GFP*, *PCaP1-GFP*, and $\Delta N25PCaP1-GFP$, were expressed under the control of the guard cell specific promoter *GCI* (Yang et al. 2008). Only the *PCaP1-GFP* construct showed the plasma membrane localization, especially in their outer side (Fig. 4b). Free GFP was detected predominantly in nuclei and the cytoplasm and $\Delta N25PCaP1-GFP$ in the cytoplasm. These observations indicate that the plasma membrane association and its side specific localization depend on the N-terminal myristoylation.

Quantitative and spatial response of PCaP1 to excess metals

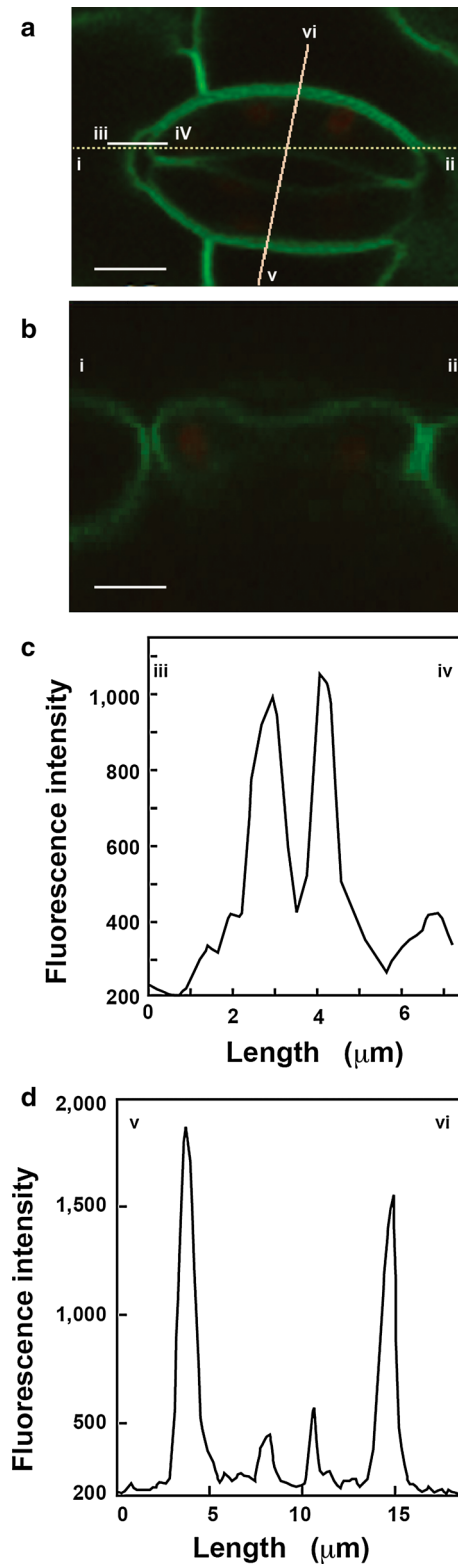
Iron is an essential micronutrient of plants and can be toxic at excessive levels (Connolly and Guerinet 2002). The plants grown under normal conditions for 21 days were transplanted to the medium containing 0.5 mM $FeSO_4$ to examine the response of PCaP1 to excessive iron, the fluorescence was enhanced after 2 h, especially in root hairs

Fig. 3 Localization of PCaP1 in the outer rim side of plasma membrane of guard cells. Images of leaf stomata of 21-day-old plant expressing *PCaP1^{pro}::PCaP1-GFP* were acquired by CLSM. **a** a surface image of a pair of guard cells. **b** a cross-section image of a portion indicated by a horizontal line (i–ii) in the same guard cells shown in (a). Globular structures with slight red color are chloroplasts in the guard cell. Plasma membranes of cells adhering to each other can be distinguished. Chloroplasts are shown in slightly red due to their autofluorescence. Bars in (a) and (b), 5 μm . **c** Fluorescence intensity on a short line (iii–iv) to show the plasma membranes of guard cell and adjacent cell. **d** Fluorescence intensity on a diagonal line (v–vi) to show the difference between the outer and inner side of the guard cell plasma membrane

(Figs. S2a, S2b). The plasma membrane localization was not altered even after the treatment. When plants were germinated and grown under excessive iron conditions, the growth of shoots and roots was remarkably suppressed (Fig. S2c). In contrast to the short-period treatment (Figs. S2a, S2b), the fluorescence intensity was decreased to 60 % of that of control plants after 7 days under the excessive iron (Figs. S2d, S2e).

Copper is also an essential micronutrient. Increase in PCaP1 transcription by excessive copper was reported previously (Ide et al. 2007). Furthermore, PCaP1 has been reported to bind Cu^{2+} and change its higher order structure (Nagasaki-Takeuchi et al. 2008). To examine effect of copper on the expression of *PCaP1*, seeds of plant expressing *PCaP1^{pro}::PCaP1-GFP* were germinated and grown under excessive copper conditions at 0.1 mM CuSO_4 . In both treatments with Cu^{2+} , green fluorescence intensity was increased by 90 % in roots compared with roots grown under normal conditions (Fig. 5a, b). Enhancement of *PCaP1* expression by excessive Cu^{2+} was observed in the first rosette leaf (Fig. 5c, d), which did not contact with the gel solution. This was consistent with the effect on transgenic lines expressing *PCaP1^{pro}::GUS*, which were cultivated in the presence of 0.1 mM CuSO_4 for 2 weeks (Fig. S3). Plant growth was markedly suppressed, indicating that the concentration of 0.1 mM CuSO_4 was toxic for plants. Enhancement of gene expression *PCaP1*, especially in cotyledons, leaves and stomata, by the treatment with CuSO_4 was also detected (Fig. S3g, S3h, S3i).

Flagellin oligopeptide 22 (flg22) has been used as a trigger of pathological reaction in plants (Zipfel et al. 2004). Generally, tissues respond to a pathological elicitor within a few minutes and the transcriptional levels of the related genes for defense are raised within 1 h (Navarro et al. 2004). We treated rosette leaves of 21-day-old plants with 10 μM flg22 and monitored fluorescent image of the leaf epidermal tissue at 5, 10, 20, and 30 min after the start of treatment. No marked difference in fluorescent intensity and intracellular localization of PCaP1-GFP was detected (Fig. S4). These results show that flg22 does not induce *PCaP1* within 30 min.



Marked enhancement of expression (Fig. S3) and accumulation of PCaP1 protein (Fig. 5) by excessive copper led us to examine the physiological effect of the loss of PCaP1 on copper tolerance. We prepared homozygous

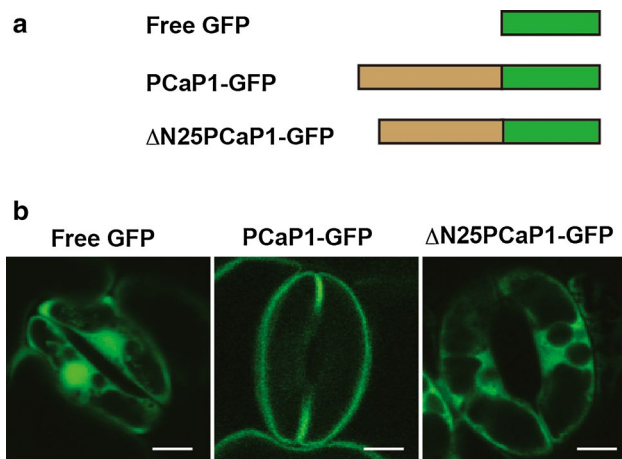


Fig. 4 Intracellular localization of a modified PCaP1. **a** Schematic constructions of free GFP, PCaP1-GFP, and Δ N25PCaP1-GFP. Δ N25PCaP1 is a truncated PCaP1 that lacks N-terminal 25 residues. These constructs were expressed under the control of guard cell specific promoter *GCI*. **b** CLSM images of stomata in rosette leaves in 21-day-old plants expressing *GCI*^{pro}::GFP, *GCI*^{pro}::PCaP1-GFP, or *GCI*^{pro}:: Δ N25PCaP1-GFP under the control of *GCI* promoter. Bars in (b), 10 μ m

T-DNA-inserted loss-of-function mutants of PCaP1, *pcap1-1* (SALK_022955) and *pcap1-4* (SAIL_241_A08), in which T-DNA was inserted into the third intron and the fourth exon, respectively (Fig. S5a). Neither mRNA (data not shown) or protein (Fig. S5b) of PCaP1 was detected in these mutants. Under excessive copper conditions, growth of Col-0 was suppressed 50 % (Fig. 6). The *pcap1-1* mutant was highly sensitive to excessive copper compared with Col-0 (Fig. 6e, f). This result suggests the involvement of PCaP1 in tolerance to excessive copper.

PCaP1 controls stomata closure

Side-specific localization in the plasma membrane of guard cells (Fig. 3) led us to examine physiological role of PCaP1 in stomatal movement. Stomatal aperture was examined under normal conditions in light using epidermal strips from leaves of Col-0, *pcap1-1*, and *pcap1-4*. Stomata in all leaves from wild type and mutant plants fully opened in 200 mOsm sorbitol buffer after the exposure to 2 h light (Fig. S6). No difference in the width-length ratio of stomata was observed between Col-0 and the mutants (Fig. 7). The width-length ratio obtained in this study was normal and consistent with values reported for *A. thaliana* previously (De Angeli et al. 2013). In general, stomata close when treated with high concentration solutions even under light conditions (De Angeli et al. 2013). Therefore, we performed a stomata closing assay on epidermal strips from two independent *pcap1* lines. In the assay medium was changed the medium from 200 mOsm buffer to 600 mOsm

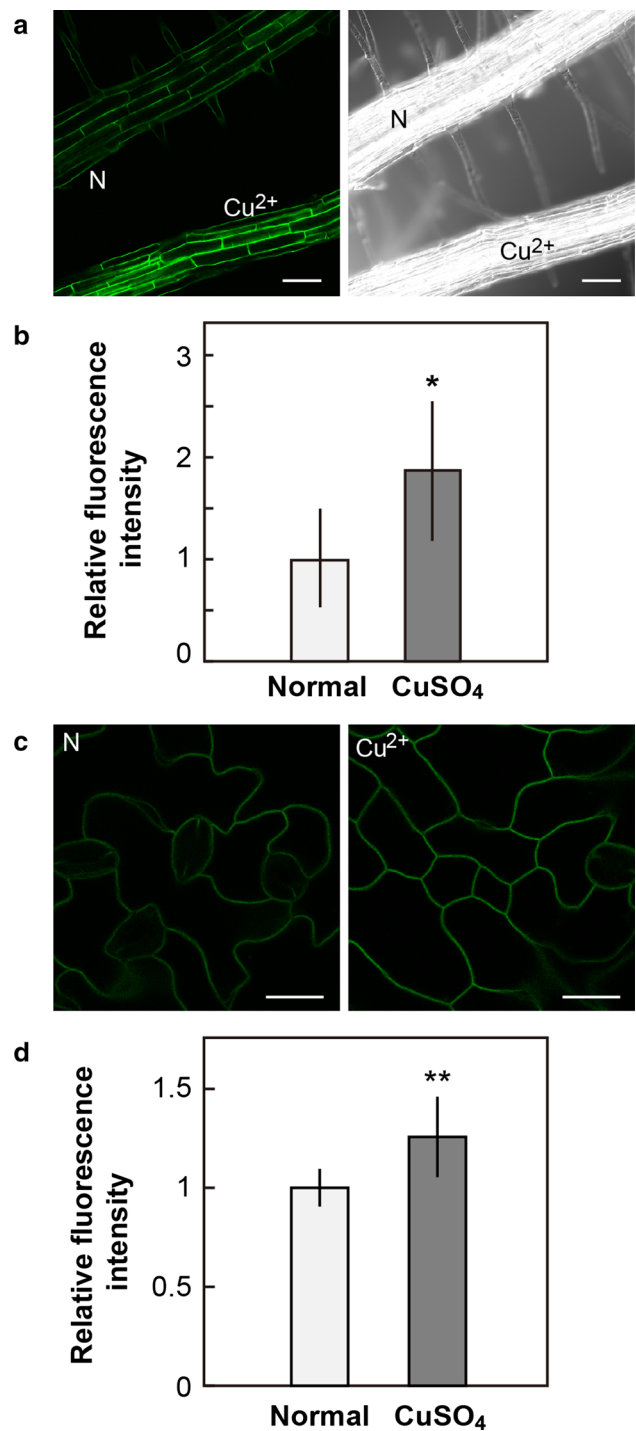
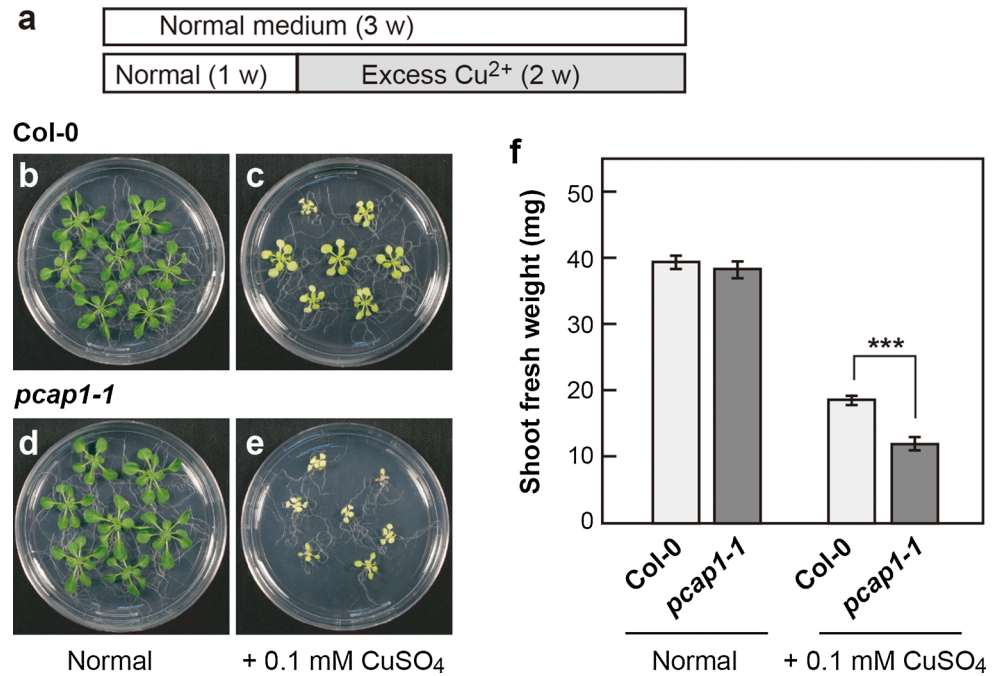


Fig. 5 Increase in PCaP1 under excessive copper. **a** Plants were grown in normal agar plates (N) or agar plates supplemented with 0.1 mM CuSO₄ for 7 days. Bars 100 μ m. **b** Fluorescence intensity of seedling roots grown in 0.1 mM CuSO₄ was quantified by CLSM and is shown as relative intensity to that of roots grown in normal medium for 7 days. Values are expressed as mean \pm SD; n = 30. *P < 0.05 (t test). **c** Plants were grown in normal agar plates (N) or agar plates supplemented with 0.1 mM CuSO₄ (Cu²⁺) for 14 days. Bars 20 μ m. **d** Fluorescence intensity of the first leaf grown in 0.1 mM CuSO₄ was quantified by CLSM and is shown as relative intensity to that of the leaf grown in normal medium for 14 days. Values are expressed as mean \pm SD; n = 10. **P < 0.01 (t test)

Fig. 6 Effect of excessive copper on the growth of Col-0 and its loss-of-function mutant *pcap1-1*. Col-0 (**b** and **c**) and loss-of-function mutant *pcap1-1* plants (**d**, **e**) grown on the normal medium for 1 week were transplanted to the plates containing 0.1 mM CuSO₄ (**c**, **e**) and grown for 2 weeks (**a**). The other plants were grown in the absence of CuSO₄ (**b**, **d**) Plants on the plates were photographed (**b–e**). **f** Fresh weight of 3-week-old shoots of Col-0 and *pcap1-1* grown for total 3 weeks was determined and shown in a graph. Values are expressed as mean ± SD; *n* = 20. Asterisks indicate significant difference at ****P* < 0.001 between Col-0 and *pcap1-1* (*t* test)



buffer, stomata in Col-0 leaves clearly closed (Fig. 7). However, stomata in the *pcap1-1* and *pcap1-4* mutant did not close completely. The width-length ratios of *pcap1-1* and *pcap1-4* were 34 and 30 % higher than that of Col-0, respectively, indicating dysfunction of these mutants in stomata closure. The guard cells of the mutants were not damaged because the ratio was completely recovered to the open state when the assay buffer was changed to the normal 200 mOsm buffer. The present data indicate that PCaP1 is involved in the stomata closing process in response to high osmolarity.

Stomatal aperture is regulated by various environmental factors, such as humidity, temperature, light, water state of leaf, and abscisic acid through calcium signaling (Allen et al. 2001; Kim et al. 2010). Under osmotic stresses, malate metabolism and malate transporters, such as SLAC1 and AtABC14, have been shown to play an important role in stomatal response to physiological stimuli (Asai et al. 2000; Saji et al. 2008; Lee et al. 2008; Negi et al. 2008; Vahisalu et al. 2008; Medeiros et al. 2016). Furthermore, a high atmospheric CO₂ condition is known to close the leaf stomata (Brearley et al. 1997; Hanstein and Felle 2002; Kim et al. 2010). We compared the growth of Col-0 with that of *pcap1* mutant under normal and high CO₂ conditions. Growth of *pcap1* mutant was approximately 19 % better than that of Col-0 on day 10 under normal CO₂ conditions (380 ppm) (Fig. 8). High CO₂ conditions (700 ppm) stimulated growth of both Col-0 and *pcap1*. Shoot fresh weight of *pcap1* was 21 % greater than that of Col-0 on day 14.

Discussion

Distribution in tissues and intracellular localization of PCaP1

The aim of the present study was to clarify the distribution of PCaP1 proteins in the cells and tissues and to investigate its physiological roles. We prepared a construct of PCaP1-GFP with its own promoter and introduced it into Col-0 to visualize PCaP1 in plants under normal and stress conditions. Recently it has been noted that a tendency of dimerization of normal GFP causes a formation of artifactual intracellular structure in plants expressing high levels of GFP-linked membrane proteins (Segami et al. 2014). Therefore, we selected transgenic lines with relatively low expression of the transgenic gene to escape the side effects of excessive accumulation of PCaP1-GFP.

The expression level of PCaP1-GFP in the transgenic lines was enough to recognize GFP fluorescence (Figs. 1, 2). The PCaP1-GFP was detected in most tissues and cells in seedlings and mature plants and could be observed by CLSM. Our observation is considerably consistent with in silico data of transcriptional level (Arabidopsis eFP Browser, http://bar.utoronto.ca/efp_arabidopsis/cgi-bin/efpWeb.cgi, Winter et al. 2007). PCaP1 was also detected in highly specialized cells including trichomes, root hairs, stigma, and anther. In all of these cells PCaP1 is clearly localized in the plasma membrane. The stable association of PCaP1 to the plasma membrane depends on the N-terminal region,

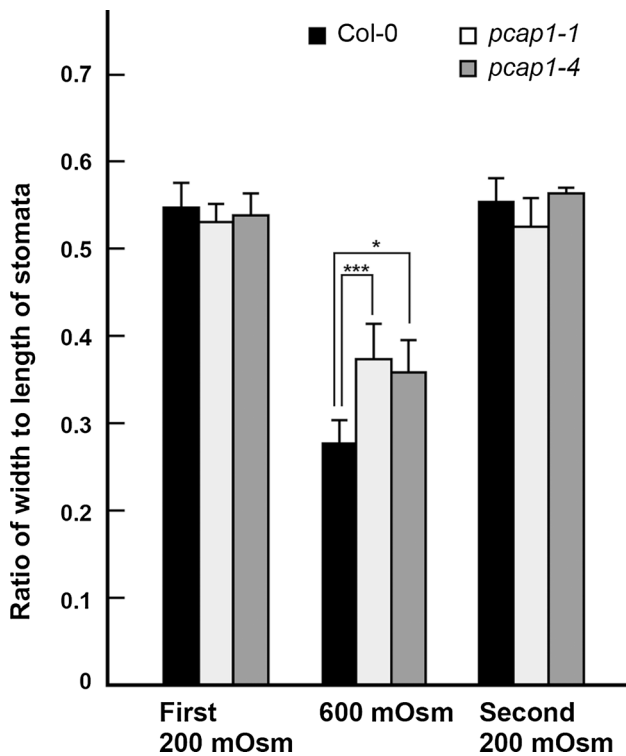


Fig. 7 Defect of *pcap1* in stomatal closure in *A. thaliana*. Stomatal aperture was measured on stripped epidermis in the presence of 200 or 600 mOsm sorbitol buffer in *pcap1-1* and *pcap1-4* knockout mutants and wild-type plants. The stripped epidermis was exposed to 2 h light in 200 mOsm buffer (1st treatment) and then in 600 mOsm buffer. It was again placed in 200 mOsm buffer (2nd treatment). In presence of 600 mOsm buffer, the knockout mutants showed increased aperture of 34 % (*pcap1-1*, ****P* < 0.005 by *t* test; *n* = 5) and 30 % (*pcap1-4*, **P* < 0.05 by *t* test; *n* = 5) compared with the corresponding wild types. Measurements performed in the presence of 200 mOsm buffer in the 1st and 2nd treatments showed no significant difference in stomatal apertures between Col-0 and *pcap1* lines. Each point is the mean of values from five experiment in each experiment. At least 60 stomata were analyzed for each treatment

because the protein without the N-terminal 25 residues was not associated with the membrane but not in the cytoplasm (Fig. 4). The *N*-myristoylation and the N-terminal lysine-rich region might be essential for stable association of the protein to the membrane as suggested by in vitro analyses (Nagasaki-Takeuchi et al. 2008). In addition to the *N*-myristoylation, the N-terminal polybasic cluster may be key properties for interaction of the region with the negatively charged PtdInsPs (McLaughlin and Murray 2005). Finally, it should be noted that PCaP1 is a good candidate of a new marker protein of the plasma membrane in various experiments, because the transgenic plants expressing PCaP1-GFP (this study) and PCaP1 specific antibodies (Ide et al. 2007; Nagasaki et al. 2008) are available.

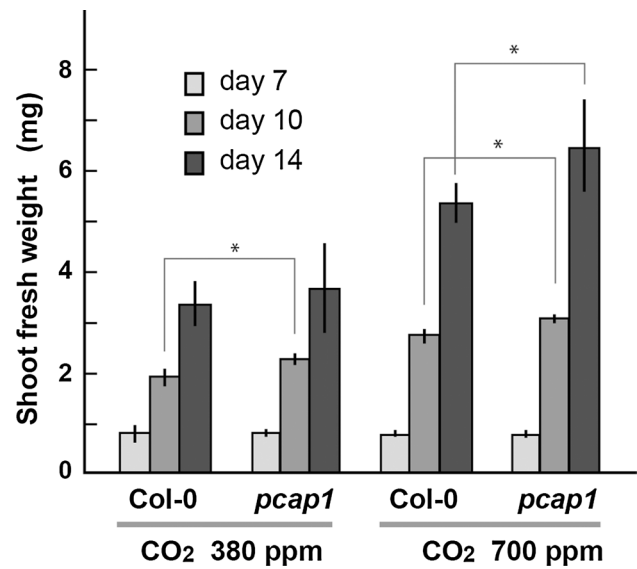


Fig. 8 Growth of *pcap1* mutant under normal and high CO₂ conditions. Wild-type plant (Col-0) and *pcap1-1* and *pcap1-4* knockout mutants were grown on normal agar plates under the normal (380 ppm) and high CO₂ (700 ppm) conditions. Shoot fresh weight was measured on day 7, 10, and 14. Values are expressed as mean ± SD; *n* = 15. Asterisks indicate significant difference at **P* < 0.05 by *t* test between Col-0 and *pcap1-1*

Stimulated PCaP1 expression in response to excessive copper

This study showed that the PCaP1 protein exists at a constant level in the plasma membrane in most cells. The transcript level of *PCaP1* has been reported to be relatively constant in *A. thaliana* (Ide et al. 2007) and is consistent with an open database on an *Arabidopsis* eFP browser (Winter et al. 2007). The transcription is enhanced several folds by a few physiological stimuli. Treatment with 0.1 mM CuCl₂ for 18 h increased the *PCaP1* mRNA level 3-folds (Ide et al. 2007). The present study demonstrated the twice increment of PCaP1-GFP in roots by cultivation in 0.1 mM CuSO₄ for a week (Fig. 5), suggesting stimulation of *PCaP1* gene and/or stabilization of PCaP1 in cells by Cu²⁺. Copper ion is known to have an elicitor effect in plants (Schützendübel and Polle 2002). However, application of flg22, a pathological trigger, to the leaf surface did not enhance fluorescent intensity of PCaP1-GFP in leaf epidermal cells (Fig. S4). Thus, the enhancement of PCaP1 accumulation by Cu²⁺ might be independent of the elicitor effect and related to biochemical characteristics of PCaP1. PCaP1 protein has a capacity to bind Cu²⁺ ions in vitro experiments (Nagasaki-Takeuchi et al. 2008). Thus, the enhancement of PCaP1 might be a reaction for adaptation and tolerance to excessive Cu²⁺. Indeed, Cu²⁺ at 0.1 mM

suppressed growth of seedlings and enhanced PCaP1 expression especially in leaves (Fig. S3). Leaves might be sensitive to excessive Cu^{2+} , because, copper content is high in roots and low in shoots under normal cultivation conditions (Greger 2004).

On the other hand, iron slightly enhanced the protein accumulation of PCaP1 at 0.5 mM within 2 h of the treatment (Fig. S2). Exposure to the excessive Fe^{2+} for 7 days resulted in 40 % reduction of PCaP1 accumulation in roots and reduced growth of plants. Although the response of PCaP1 was varied with metal ions, it should be noted that PCaP1 was stably associated the plasma membrane under these stress conditions. There are possibilities that PCaP1 suppresses the activity of Cu uptake transporter and stimulates the activity of Cu export transporter at the plasma membrane.

Physiological significance of PCaP1 in guard cells

In guard cells, PCaP1 has a localization polarity in the plasma membrane. PCaP1–GFP was strictly, specifically localized to the plasma membrane at the epidermal-cell side, outer rim of the stomata, but not at the pore side (Figs. 1b and 3). The loss-of-function mutant lines of PCaP1 have a defect in stomata closure, although the stomata of the mutant lines opened normally (Figs. 7 and S6). Also, growth of plants under high CO_2 conditions increased at the middle growth stage (Fig. 8). Usually leaves close their stomata at high CO_2 , for example 700 ppm (Young et al. 2006). It has been discussed that abnormally elevated concentrations of CO_2 activated anion channels and K_{out}^+ efflux channels in *Vicia faba* guard cells, and induced depolarization in intact leaves (Allen et al. 2001; Brearley et al. 1997; Hanstein and Felle 2002; Kim et al. 2010). Young et al. (2006) reported that Ca^{2+} signaling is involved in CO_2 -induced stomatal closure (Young et al. 2006). Therefore, there is a possibility that elevated-[CO_2]- induced Ca^{2+} signaling is transferred to ion channels, such as Cl^- and K_{out}^+ efflux channels, through signal transduction by PCaP1. In addition to Cl^- and K^+ , it should be noted that malate metabolism and malate transport system also play a critical role in guard cell movement under high CO_2 and other abiotic stresses (Asai et al. 1999, 2000; Saji et al. 2008; Lee et al. 2008; Negi et al. 2008; Vahisalu et al. 2008; Medeiros et al. 2016). Further studies are required to examine direct or indirect interaction of PCaP1 with these ion channels and malate transporters and whether or not the stomata of *pcap1* mutants show defect in stomata closure at high CO_2 .

Furthermore, several investigators have emphasized the importance of cortical microtubules for stomata movement (Marcus et al. 2001; Lahav et al. 2004). Furthermore there is a strong correlation between number of cortical microtubules in guard cells and stomatal aperture in *A. thaliana*

(Eisinger et al. 2012). Microtubule structure crosses the guard cells from the pore side to the epidermal side. This special arrangement might be important for opening the pore of the stomata. If the microtubules are arranged vertically, cell enlargement of guard cells induced by environmental factors such as blue light does not result in the pore opening.

PCaP1 is a microtubule-destabilizing protein 25 (MDP25) and a negative regulator of hypocotyl cell elongation (Li et al. 2011). Therefore, there are two possible mechanisms in the contribution of PCaP1 to the stomata closure. First, PCaP1 may be involved in regulation of microtubule structure in guard cells with consideration of microtubule-destabilization function according to a previous report (Li et al. 2011). The second possibility depends on binding ability of PCaP1 to Ca^{2+} -calmodulin complex and PtdInsPs. Stomatal closure by abscisic acid depends on the Ca^{2+} signaling in the guard cell (Kim et al. 2010). PCaP1 is involved in the stomata closure through calcium and PtdInsP signaling, which may affect solute transporters, ion channels, and/or aquaporins in the plasma membrane. A recent study clearly demonstrated that PtdIns(3,5) P_2 affects the vacuolar structural change in guard cells during stomata closing (Bak et al. 2013). The microtubule structure needs to be examined under various physiological conditions including the dark and presence of abscisic acid. Studies are required to determine how PCaP1 regulates the association and dissociation of tubulin subunits and anchoring of the cortical microtubules to the plasma membrane of guard cells. Further studies are needed to reveal the pathway from increment of cytosolic Ca^{2+} concentration to PtdInsP signaling and the functional regulation of the membrane transporters and channels.

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References

- Allen GJ, Chu SP, Harrington CL, Schumacher K, Hoffmann T, Tang YY, Grill E, Schroeder JI (2001) A defined range of guard cell calcium oscillation parameters encodes stomatal movements. *Nature* 411:1053–1057
- Asai N, Nakajima N, Kondo N, Kamada H (1999) The effect of osmotic stress on the solutes in guard cells of *Vicia faba* L. *Plant Cell Physiol* 40:843–849
- Asai N, Nakajima N, Tamaoki M, Kamada H, Kondo N (2000) Role of malate synthesis mediated by phosphoenolpyruvate carboxylase in guard cells in the regulation of stomatal movement. *Plant Cell Physiol* 41:10–15

- Bak G, Lee EJ, Lee Y, Kato M, Segami S, Heven S, Maeshima M, Hwang JU, Lee Y (2013) A role of phosphatidylinositol 3,5-bisphosphate in vacuolar structure change in guard cells of closing stomata. *Plant Cell* 25:2202–2216
- Brearley J, Venis MA, Blatt MR (1997) The effect of elevated CO₂ concentrations on K⁺ and anion channels of *Vicia faba* L. guard cells. *Planta* 203:145–154
- Carlton JG, Cullen PJ (2005) Coincidence detection in phosphoinositide signaling. *Trends Cell Biol* 15:540–547
- Clough SJ, Bent AF (1998) Floral dip: a simplified method for *Agrobacterium*-mediated transformation of *Arabidopsis thaliana*. *Plant J* 16:735–743
- Connolly EL, Guerinot ML (2002) Iron stress in plants. *Genome Biol* 3:1024.1–1024.4
- Davière J-M, Achard P (2013) Gibberellin signaling in plants. *Development* 140:1147–1151
- De Angeli A, Zhang J, Meyer S, Martinoia E (2013) AtALMT9 is a malate-activated vacuolar chloride channel required for stomatal opening in *Arabidopsis*. *Nat Commun* 4:1804
- De Falco TA, Bender KW, Snedden WA (2009) Breaking the code: Ca²⁺ sensors in plant signaling. *Biochem J* 425:27–40
- Eisinger W, Ehrhard D, Briggs W (2012) Microtubules are essential for guard-cell function in *Vicia* and *Arabidopsis*. *Mol Plant* 5:301–610
- Greger M (2004) Metal availability, uptake, transport and accumulation in plants. In: Prasad MNV (ed) Heavy metal stress in plants: from biomolecules to ecosystems, 2nd edn. Springer, Berlin, pp 1–27
- Hanstein SM, Felle HH (2002) CO₂-triggered chloride release from guard cells in intact fava bean leaves: kinetics of the onset of stomatal closure. *Plant Physiol* 130:940–950
- Hwang I, Sheen J, Müller B (2012) Cytokinin signaling networks. *Annu Rev Plant Biol* 63:353–380
- Ide Y, Nagasaki N, Tomioka R, Suito M, Kamiya T, Maeshima M (2007) Molecular properties of a novel, hydrophilic cation-binding protein associated with the plasma membrane. *J Exp Bot* 58:1173–1183
- Kato M, Nagasaki-Takeuchi N, Ide Y, Maeshima M (2010a) An *Arabidopsis* hydrophilic Ca²⁺-binding protein with a PEVK-rich domain, PCaP2, is associated with the plasma membrane and interacts with calmodulin and phosphatidylinositol phosphates. *Plant Cell Physiol* 51:366–379
- Kato M, Nagasaki-Takeuchi N, Ide Y, Tomioka R, Maeshima M (2010b) PCaPs, possible regulators of PtdInsP signals on plasma membrane. *Plant Signal Behav* 5:848–850
- Kato M, Aoyama T, Maeshima M (2013) A Ca²⁺-binding protein PCaP2 located on the plasma membrane is involved in root hair development as a possible signal transducer. *Plant J* 74:690–700
- Kim TH, Bohmer M, Hu H, Nishimura N, Schroeder JI (2010) Guard cell signal transduction network: advances in understanding abscisic acid, CO₂, and Ca²⁺ signaling. *Annu Rev Plant Biol* 61:561–916
- Kleerekoper QK, Putkey JA (2009) PEP-19, an intrinsically disordered regulator of calmodulin signaling. *J Biol Chem* 284:7455–7464
- Lahav M, Abu-Abied M, Belausov E, Schwartz A, Sadot E (2004) Microtubules of guard cells are light sensitive. *Plant Cell Physiol* 45:573–582
- Lee M, Choi Y, Burla B, Kim Y-Y, Jeon B, Maeshima M, Yoo J-Y, Martinoia M, Lee Y (2008) The ABC transporter AtABC14 is a malate importer and modulates stomatal response to CO₂. *Nature Cell Biol* 10:1217–1223
- Leivar P, Monte E (2014) PIFs: systemis integrators in plant development. *Plant Cell* 26:56–78
- Li J, Wang X, Qin T, Zhang Y, Liu X, Sun J, Zhou Y, Zhu L, Zhang Z, Yuan M, Mao T (2011) MDP25, a novel calcium regulatory protein, mediates hypocotyl cell elongation by destabilizing cortical microtubules in *Arabidopsis*. *Plant Cell* 23:4411–4427
- Luan S (2009) The CBL-CIPK network in plant calcium signaling. *Trends Plant Sci* 14:37–42
- Marcus AI, Moore RC, Cyr RJ (2001) The role of microtubules in guard cell function. *Plant Physiol* 125:387–395
- McCormack E, Tsai YC, Braam J (2005) Handling calcium signaling: *Arabidopsis* CaMs and CMLs. *Trends Plant Sci* 10:383–389
- McLaughlin S, Murray D (2005) Plasma membrane phosphoinositide organization by protein electrostatics. *Nature* 438:605–611
- Medeiros DB, Martins SCV, Cavalcanti JHF, Daloso D, Martinoia E, Nunes-Nesi A, DaMatta F, Fernie AR, Araujo W (2016) Enhanced photosynthesis and growth in atguac1 knockout mutants are due to altered organic acid accumulation and an increase in both stomatal and mesophyll conductance. *Plant Physiol* 170:86–101. doi:10.1104/pp.15.01053
- Meijer HJ, Munnik T (2003) Phospholipid-based signaling in plants. *Annu Rev Plant Biol* 54:265–306
- Nagasaki N, Tomioka R, Maeshima M (2008) A hydrophilic cation-binding protein of *Arabidopsis thaliana* AtPCaP1 is localized to plasma membrane via *N*-myristoylation and interacts with calmodulin and the phosphatidylinositol phosphates, PtdIns(3,4,5)P₃ and PtdIns(3,5)P₂. *FEBS J* 275:2267–2282
- Nagasaki-Takeuchi N, Miyano M, Maeshima M (2008) A plasma membrane-associated protein of *Arabidopsis thaliana* AtPCaP1 binds copper ions and changes its higher order structure. *J Biochem* 144:487–497
- Nakagawa T, Suzuki T, Murata S et al (2007) Improved gateway binary vectors: high performance vectors for creation of fusion constructs in transgenic analysis of plants. *Biosci Biotechnol Biochem* 71:2095–2100
- Navarro L, Zipfel C, Rowland O, Keller I, Robatzek S, Boller T, Jones JG (2004) The transcriptional innate immune response to flg22: interplay and overlap with *Avr* gene-dependent defense responses and bacterial pathogenesis. *Plant Physiol* 135:1113–1128
- Negi J, Matsuda O, Nagasawa T, Oba Y, Takahashi H, Kawai-Yamada M, Uchimiya H, Hashimoto M, Iba K (2008) CO₂ regulator SLAC1 and its homologues are essential for anion homeostasis in plant cells. *Nature* 452:483–486
- Saji S, Bathula S, Kubo A, Tamaoki M, Kanna M, Aono M, Nakajima N, Nakaji T, Takeda T, Asayama M, Saji H (2008) Disruption of a gene encoding C₄-dicarboxylate transporter-like protein increases ozone density through deregulation of the stomatal response in *Arabidopsis thaliana*. *Plant Cell Physiol* 49:2–10
- Schneider CA, Rasband WS, Eliceiri KW (2012) NIH Image to ImageJ: 25 years of image analysis. *Nat Methods* 9:671–675
- Schützendübel A, Polle A (2002) Plant responses to abiotic stresses: heavy metal-induced oxidative stress and protection by mycorrhization. *J Exp Bot* 53:1351–1365
- Segami S, Makino S, Miyake A, Asaoka M, Maeshima M (2014) Dynamics of vacuoles and H⁺-pyrophosphatase visualized by monomeric green fluorescent protein in *Arabidopsis*: artifactual bulbs and native intravacuolar spherical structures. *Plant Cell* 26:3416–3434
- Sessions A, Burke E, Presting G, Aux G, McElver J, Patton D, Dietrich B, Ho P, Bacwaden J, Ko C, Clarke JD, Cotton D, Bullis D, Snell J, Miguel T, Hutchiso D, Kimmerly B, Mitzel T, Katarigi F, Glazebrook J, Law M, Goff SA (2002) A high-throughput *Arabidopsis* reverse genetics system. *Plant Cell* 14:1985–2994
- Tanaka N, Kato M, Tomioka R, Kurata R, Fukao Y, Aoyama T, Maeshima M (2014) Characteristics of a root hair-less line of *Arabidopsis thaliana* under physiological stresses. *J Exp Bot* 65:1497–1512
- Vahisalu T, Kollist H, Wang Y-F, Nishimura N, Chan W-Y, Valerio G, Lamminmäki A, Brosché M, Moldau H, Desikan R, Schroeder

- JI, Kangasjärvi J (2008) SLAC1 is required for plant guard cell S-type anion channel function in stomatal signaling. *Nature* 452:487–491
- Vijayapalani P, Maeshima M, Nagasaki-Takekuchi N, Miller WA (2012) Interaction of the trans-frame potyvirus protein P3 N-PIPO with host protein PCaP1 facilitates potyvirus movement. *PLoS Pathogen* 8:e1002639
- Winter D, Vinegar B, Nahal H, Ammar R, Wilson GV, Provart NJ (2007) An “Electronic Fluorescent Pictograph” browser for exploring and analyzing large-scale biological data sets. *PLoS One* 2:e718
- Xue HW, Chen X, Mei Y (2009) Function and regulation of phospholipid signalling in plants. *Biochem J* 421:145–156
- Yang Y, Costa A, Leonhardt N, Siegel RS, Schroeder JI (2008) Isolation of a strong Arabidopsis guard cell promoter and its potential as a research tool. *Plant Meth* 4:6
- Young JJ, Mehta S, Israelsson M, Godoski J, Grill E, Schroeder JI (2006) CO₂ signaling in guard cells: calcium sensitivity response modulation, a Ca²⁺-independent phase, and CO₂ insensitivity of the *gca2* mutant. *Proc Natl Acad Sci USA* 103:7506–7511
- Zipfel C, Robatzek S, Navarro L, Oakely EJ, Jones JD, Felix G, Boller T (2004) Bacterial disease resistance in Arabidopsis through flagellin perception. *Nature* 428:764–767