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PECT1, a rate-limiting enzyme in phosphatidylethanolamine biosynthesis, is involved in the regulation of stomatal movement in *Arabidopsis*

Juntaro Negi[1,*], Tomoki Obata[1], Sakura Nishimura[1], Boseok Song[1], Sho Yamagaki[1], Yuhei Ono[1], Makoto Okabe[1], Natsumi Hoshino[2], Kohei Fukatsu[3], Ryo Tabata[4,5], Katsushi Yamaguchi[6], Shuji Shigenobu[6], Masashi Yamada[7], Mitsuyasu Hasebe[6,8], Shinichiro Sawa[4], Toshinori Kinoshita[3], Ikuo Nishida[2] and Koh Iba[1]

[1]*Department of Biology, Faculty of Science, Kyushu University, Fukuoka 812-8581, Japan,*
[2]*Graduate School of Science and Engineering, Saitama University, 338-8570, Saitama, Japan,*
[3]*Graduate School of Science and Institute of Transformative Bio-Molecules (WPI-ITbM), Nagoya University, Chikusa, Nagoya 464-8602, Japan,*
[4]*International Research Center for Agricultural and Environmental Biology, Kumamoto University, 2-39-1, Kumamoto, Japan,*
[5]*Graduate School of Bioagricultural Sciences, Nagoya University, Nagoya, Japan,*
[6]*National Institute for Basic Biology, Okazaki, Japan,*
[7]*Department of Biology and HHMI, Duke University, Durham, North Carolina 27710, USA, and*
[8]*Department of Basic Biology, School of Life Science, SOKENDAI (Graduate University for Advanced Studies), Okazaki 444-8585, Japan*

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*For correspondence (e-mail negi.juntaro.630@m.kyushu-u.ac.jp).

SUMMARY

An *Arabidopsis* mutant displaying impaired stomatal responses to CO_2, *cdi4*, was isolated by a leaf thermal imaging screening. The mutated gene *PECT1* encodes CTP:phosphorylethanolamine cytidylyltransferase. The *cdi4* exhibited a decrease in phosphatidylethanolamine levels and a defect in light-induced stomatal opening as well as low-CO_2-induced stomatal opening. We created RNAi lines in which *PECT1* was specifically repressed in guard cells. These lines are impaired in their stomatal responses to low-CO_2 concentrations or light. Fungal toxin fusicoccin (FC) promotes stomatal opening by activating plasma membrane H^+-ATPases in guard cells via phosphorylation. *Arabidopsis* H^+-ATPase1 (AHA1) has been reported to be highly expressed in guard cells, and its activation by FC induces stomatal opening. The *cdi4* and *PECT1* RNAi lines displayed a reduced stomatal opening response to FC. However, similar to in the wild-type, *cdi4* maintained normal levels of phosphorylation and activation of the stomatal H^+-ATPases after FC treatment. Furthermore, the *cdi4* displayed normal localization of GFP-AHA1 fusion protein and normal levels of *AHA1* transcripts. Based on these results, we discuss how PECT1 could regulate CO_2- and light-induced stomatal movements in guard cells in a manner that is independent and downstream of the activation of H^+-ATPases. [Correction added on 15 May 2023, after first online publication: The third sentence is revised in this version.]

Keywords: phosphatidylethanolamine, guard cell, CO_2, light, plasma membrane H^+-ATPase.

INTRODUCTION

Phosphatidylethanolamine (PE), a major class of non-bilayer-forming phospholipids, is widely distributed among various organelle membranes except plastids in plant cells (Douce et al., 1973). In the biological kingdom, PE can be synthesized via three metabolic pathways, the nucleotide pathway (Kennedy pathway), base-exchange pathway and phosphatidylserine decarboxylation pathway (Gibellini & Smith, 2010; Nakamura, 2021). In the plant kingdom, the nucleotide pathway is thought to be the main avenue for the *de novo* synthesis of PE and is regulated by a rate-limiting enzyme, CTP:phosphorylethanolamine cytidylyltransferase (PECT; EC 2.7.7.14; Wang & Moore Jr., 1991). In *Arabidopsis*, *PECT1* (At2g38670) encoding CTP:phosphorylethanolamine cytidylyltransferase is predominantly responsible for PE biosynthesis, and the null allele *pect1-6* has been shown to cause embryonic lethality (Mizoi et al., 2006).

The physiological aspects of PE have been critically studied in various species using mutants defective in PE biosynthesis. PE is required for the function of the membrane-associated lactose permease in *Escherichia coli* (Bogdanov & Dowhan, 1995). Yeast (*Saccharomyces cerevisiae*) cells require PE for targeting amino acid transporters to the plasma membrane (PM; Opekarova et al., 2002; Robl et al., 2001). In *Arabidopsis*, PE biosynthesis is required for normal embryogenesis (Mizoi et al., 2006) and regulation of flowering (Susila et al., 2021), but its role in other aspects of physiological functions remains uncovered.

Stomata are pores formed by pairs of guard cells, and they regulate gas exchange between plants and the atmosphere. The stomatal aperture is regulated by multiple environmental cues such as CO_2, light and humidity as well as endogenous hormone stimuli such as abscisic acid (ABA; Hetherington & Woodward, 2003). Molecular mechanisms of stomatal aperture regulation have been extensively studied, resulting in the identification of crucial singling factors and regulatory ion channels (Engineer et al., 2016; Inoue & Kinoshita, 2017; Kim et al., 2010). Phospholipids also play important roles in stomatal regulation: phosphatidic acid (PA) has been shown to regulate ABA-induced stomatal closure and to inhibit light-induced stomatal opening (Jacob et al., 1999; Takemiya & Shimazaki, 2010; Zhang et al., 2004); and phosphoinositide (PI) metabolism has been implicated in regulating stomatal closure through alteration of cytosolic calcium ion concentrations (Gilroy et al., 1990; Staxén et al., 1999) and pH values in vacuoles (Bak et al., 2013). However, the role of the major phospholipids such as PE on stomatal regulation has attracted little attention.

Leaf temperature monitoring has been used as a convenient tool for monitoring transpiration activity, and has been widely utilized for isolating mutants with altered stomatal responses to changes in ABA/humidity (Merlot et al., 2002, 2007; Xie et al., 2006) and CO_2 concentrations (Hashimoto et al., 2006; Hashimoto-Sugimoto et al., 2013; Negi et al., 2008, 2013, 2018; Sun et al., 2022) and blue light exposure (Takemiya et al., 2013; Yamauchi et al., 2016, 2019). In this study, we isolated a novel CO_2-insensitive mutant, *cdi4*, using a high-throughput leaf thermal imaging system (Negi et al., 2014), and found that *cdi4* eventually causes an amino acid substitution in a putative catalytic domain of PECT1. We also demonstrated that PE is more abundant in guard cells than in mesophyll cells, and is essential for low-CO_2- and light-induced stomatal opening. We showed that *cdi4* attenuates fusicoccin (FC)-induced activation of stomatal opening despite the levels of phosphorylation of PM H^+-ATPases and H^+-pumping activity in the guard cells being normal, suggesting that *cdi4* acts independently or downstream of the PM H^+-ATPases in the stomatal opening pathway. Our results showed a novel role of PE in the regulation of stomatal movements in response to environmental changes.

RESULTS

Isolation of the *Arabidopsis cdi4* mutant with impaired CO_2 responses in guard cells

We isolated a CO_2-insensitive mutant line, *cdi4*, from an M2 population of ~30 000 ethyl methanesulfonate (EMS)-mutagenized *Arabidopsis* plants using leaf infrared imaging thermography. This mutant exhibited higher leaf temperatures than the wild-type (WT) under low-CO_2 conditions (Figure 1a) due to its impaired response to CO_2-dependent stomatal movements (Figure 1b–d). The stomatal conductance responses to changes in CO_2 concentrations in the atmosphere were attenuated in *cdi4* plants compared with those in WT plants (Figure 1b–d). In intact leaves, decreasing CO_2 concentrations from 360 to 0 ppm induced a prominent increase in the stomatal conductance in WT plants, whereas its effect was relatively smaller in the *cdi4* mutant. These results suggested that *cdi4* mutants have a reduced sensitivity to decreasing CO_2 concentrations in the atmosphere. We also compared the stomatal responses of WT and *cdi4* plants to light: when WT and *cdi4* plants kept in the dark were shifted to light under ambient CO_2 concentrations (~350 ppm), WT plants exhibited a larger increase in stomatal conductance compared with the *cdi4* mutants (Figure 1e–g). In summary, these results suggested that *cdi4* stomata exhibit a reduced sensitivity to ambient stimuli including to decreasing CO_2 concentrations and changes in light conditions.

CDI4 was identical to PECT1, a rate-limiting enzyme in PE biosynthesis

By map-based cloning, we identified the *CDI4* gene as *At2g38670*, which encodes PECT1, the rate-limiting enzyme in PE biosynthesis. The *cdi4* mutation was identified by whole-genome sequencing (Tabata et al., 2013) as a single C-to-T nucleotide substitution, which was expected to cause ^{245}Ser-to-Phe substitution in a putative catalytic domain of PECT1 (Figure 2a). Expression of a 1.2-kb *PECT1* cDNA fragment in *cdi4* mutants under the control of a CaMV 35S promoter (p35S) successfully restored the normal responses in the transformants to decreasing CO_2 concentrations and to light, verifying that *CDI4* is identical to *At2g38670* (Figure 1a–g).

We then examined if PECT1 shows expression in guard cells and, if so, its subcellular localization pattern within guard cells, using transgenic plants expressing β-glucuronidase under the control of a PECT1 promoter (pPECT1::GUS plants) and a translational fusion of full-length PECT1 and green fluorescent protein (GFP) under the control of p35S (p35S::PECT1–GFP plants). In pPECT1::GUS plants, GUS activity stained the whole plant bodies blue including the guard cells (Figure 2b). When p35S::PECT1–GFP plants were stained with the Mito Tracker dye, GFP fluorescence was detected in guard cells and it co-localized

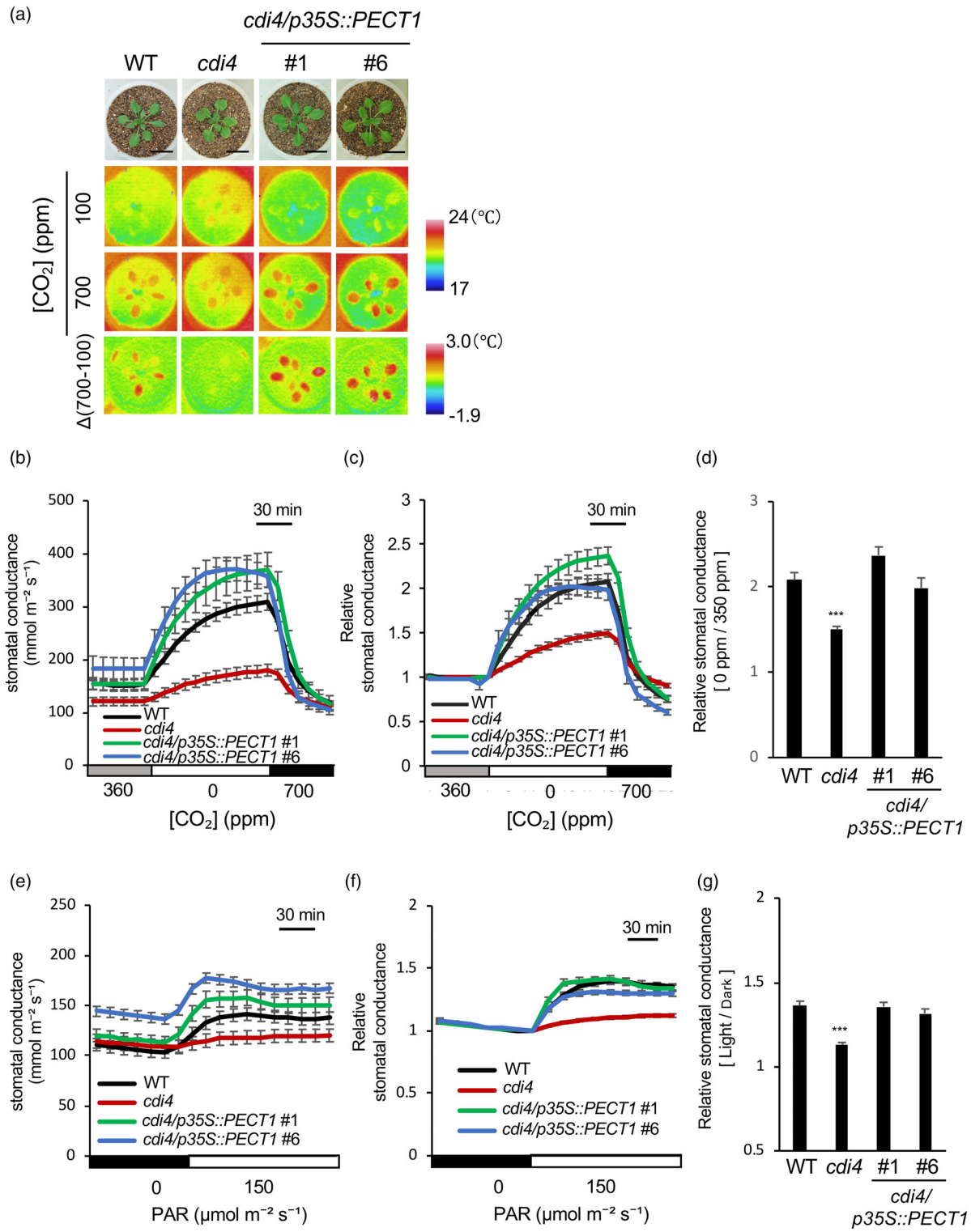

Figure 1. The *cdi4* mutant shows defects in the light- and low-CO_2-induced stomatal opening.
(a) The *cdi4* mutant showed impairments in CO_2-dependent leaf temperature changes. The *cdi4* transgenic lines, expressing p35S::PECT1, complemented the *cdi4* phenotype, as was evident from the recovery of normal CO_2-dependent leaf-temperature changes. Time-resolved stomatal conductance in response to [CO_2] changes (b) or light intensity changes (e) in the wild-type (WT), *cdi4* mutant and *cdi4/ p35S::PECT1*. Conductance was normalized to the average conductance at the last 360 ppm data point (c) and the last dark data point (f).
(d, g) Final steady-state relative stomatal conductance, whose date was obtained from (c) or (f), respectively. Values shown are means ± SE ($n > 4$). Asterisks denote comparison with WT, ***$P < 0.001$ by one-way ANOVA with Dunnett's *post hoc* test.

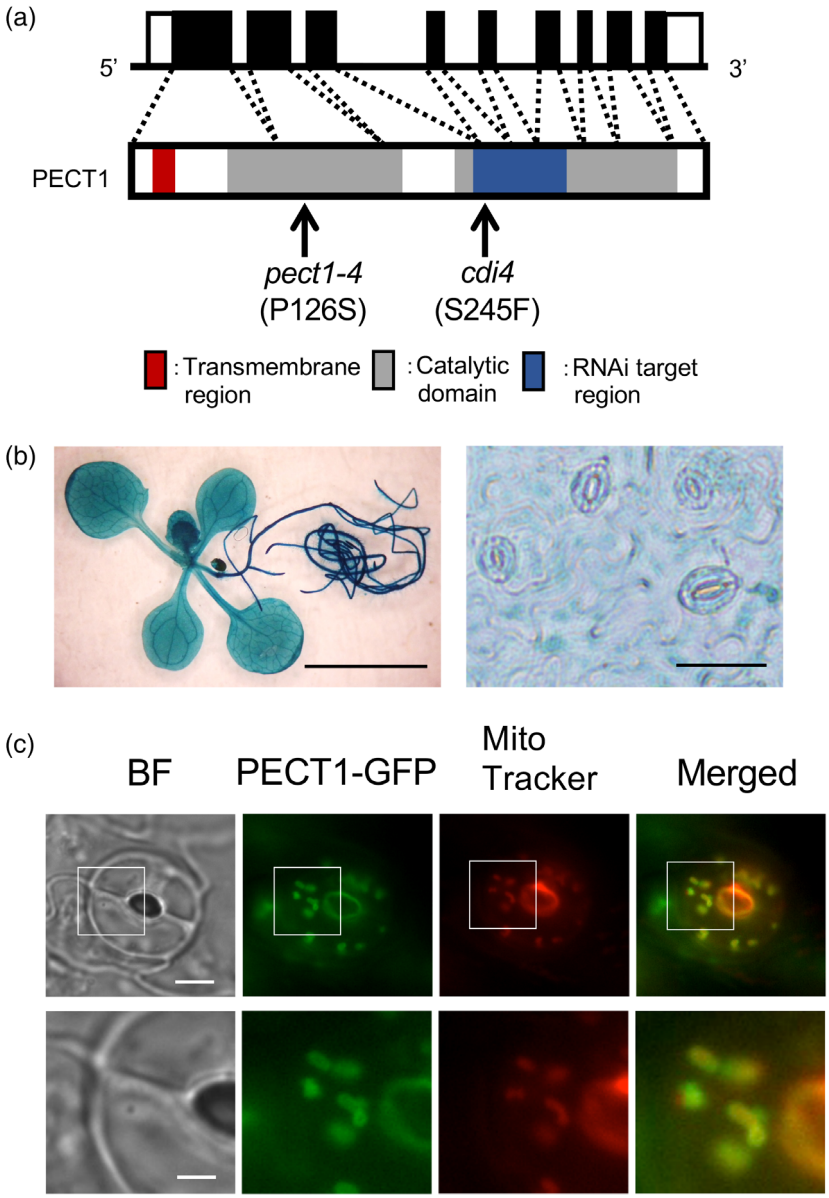

Figure 2. The *CDI4* is identical to *PECT1*, which encodes CTP:phosphorylethanolamine cytidylyltransferase.
(a) Schematic representation of the structure of the *PECT1* gene (upper) and the PECT1 protein (lower). Arrows indicate *cdi4* and *pect1-4* mutation sites. The protein structure includes a putative transmembrane region (dark red box), the conserved catalytic domains (light gray boxes) and the target region for RNAi (dark blue box).
(b) GUS staining of pPECT1::GUS transformants shows that PECT1 is expressed in various tissues, including the guard cells. Scale bars: 5 mm (left panel); 40 μm (right panel).
(c) Subcellular localization of PECT1-green fluorescent protein (GFP) protein in guard cells. Mitochondria were stained by MitoTracker (red). The images in the bottom panel are magnified images of the area surrounded by white squares in the images above. Scale bars: 10 μm (upper panel); 4 μm (lower panel).

with MitoTracker fluorescence within the guard cells (Figure 2c). A PECT1-enhanced yellow fluorescence protein fusion (PECT1-EYFP) has been reported to localize on the outer surface of mitochondria in root epidermal cells of transgenic pPECT1::PECT1-EYFP plants (Mizoi et al., 2006). Taken together, these results suggested that PECT1 could function in guard cells in association with mitochondria.

PE levels were decreased in the *cdi4* and allelic mutant *pect1-4*

Because PECT1 is a rate-limiting enzyme in PE biosynthesis, we examined whether *cdi4* mutation affects the PE content in plants. Total lipids were extracted from rosette leaves of WT, *cdi4* and *cdi4 p35S::PECT1* plants, and each

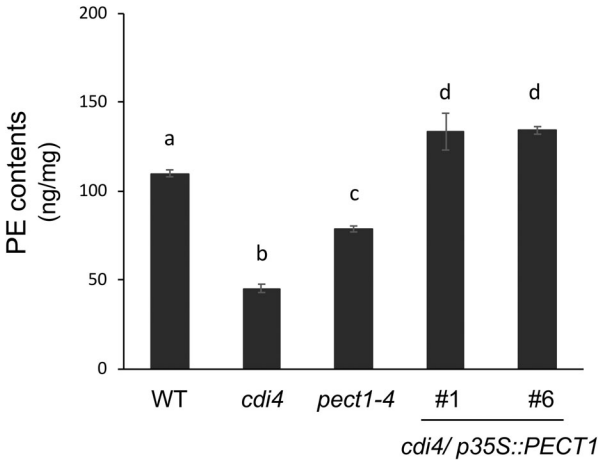

Figure 3. Phosphatidylethanolamine (PE) levels were decreased in the *cdi4* and *pect1-4* mutants compared with wild-type (WT).
Phosphorylethanolamine contents in rosette leaves measured by liquid chromatography–mass spectrometry (LC–MS). The statistical significance was determined by a one-way ANOVA with Tukey–Kramer multiple comparison tests. The same letters indicate no significant difference ($P > 0.05$).

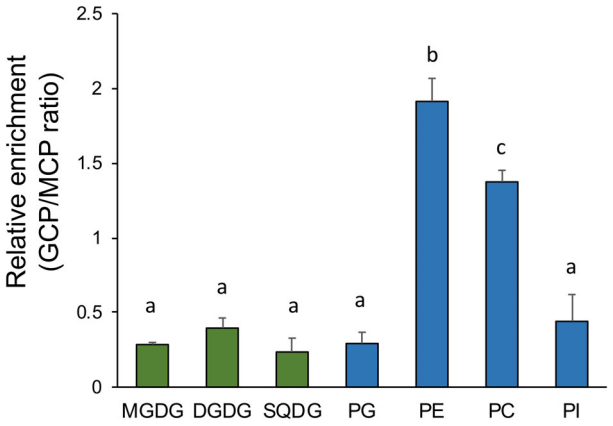

Figure 4. Differences in glycerolipids composition between guard cell protoplasts (GCPs) and mesophyll cells protoplasts (MCPs).
The ratio of the glycerolipid contents in GCPs to that in MCPs was determined. Phosphatidylethanolamine (PE) content was higher in guard cells than in mesophyll cells. MGDG, monogalactosyldiacylglycerol; DGDG, digalactosyldiacylglycerol; SQDG, sulfoquinovosyldiacylglycerol; PG, phosphatidylglycerol; PE, phosphatidylethanolamine; PC, phosphatidylcholine; PI, phosphatidylinositol. Glycolipids are represented in green and phospholipids are shown in blue. The statistical significance was determined by a one-way ANOVA with Tukey–Kramer multiple comparison tests. The same letters indicate no significant difference ($P > 0.05$).

lipid class was determined using liquid chromatography–mass spectrometry (LC–MS; Figure 3). PE levels were significantly lower in *cdi4* plants than in WT ($P < 0.05$, by 58.6%), whereas PE levels were restored in *cdi4 p35S::PECT1* plants. The content of phosphatidylcholine (PC) in *cdi4* mutant was higher than that in WT, but the other polar glycerolipid classes did not change compared with the WT levels in *cdi4* mutant (Figure S1). These results suggested that *cdi4* mutation could cause a significant decrease in PE contents in guard cells.

An allelic mutant, *pect1-4*, was selected by the TILLING (targeting-induced local lesions in genomes) method as a line carrying a point mutation within PECT1 (Mizoi et al., 2006; Figure 2a). To investigate whether the reduced responsiveness of *cdi4* stomata to light and CO_2 was due to a functional defect in PE synthesis, we measured the stomatal response and lipid composition in *pect1-4*. Similar to that in *cdi4* mutants, stomatal response to light and CO_2 were impaired in *pect1-4* mutants (Figure 5), and their PE content was also reduced (Figure 3). This result suggests that PE is involved in the stomatal response.

Guard cell protoplasts (GCPs) contain larger amount of PE than mesophyll cell protoplasts (MCPs)

In plants, plastidial glycolipids are synthesized via two distinct pathways: the plastidic pathway and the endoplasmic reticulum (ER) pathway (Roughan et al., 1980; Somerville & Browse, 1991), which can be discriminated from each other by the unique fatty acid compositions of the respective diacylglycerol backbones. The relative contributions of the plastidic and the ER pathways to the plastidial glycolipid synthesis differ among various plant tissues, and our previous studies have shown that the ER pathway is more extensively utilized in guard cells than in mesophyll cells for the synthesis of chloroplast glycolipids (Negi et al., 2018; Obata et al., 2021). Because PE as well as PC in the extraplastidial membranes are also synthesized via the ER pathway, we suspected that PE could be synthesized more abundantly in guard cells than in mesophyll cells. Thus, we determined the glycerolipid compositions in guard cells and mesophyll cells using isolated protoplasts. Figure 4 summarizes the relative enrichment of respective glycerolipids in GCPs over those in MCPs. PE content in GCPs was almost twice compared with MCPs, whereas the plastidial glycolipids monogalactosyldiacylglycerol (MGDG), digalactosyldiacylglycerol (DGDG) and sulfoquinovosyldiacylglycerol (SQDG), and the plastidial phospholipid phosphatidylglycerol (PG) in GCPs were less than half compared with MCPs. These results supported our view that PE could have more substantial contributions than other glycerolipids to stomatal function in guard cells (Figure 4).

PECT1 expressed in guard cells is responsible for stomatal CO_2 and light responses

PECT1 is expressed in various tissues, including the guard cells (Figure 2b). Therefore, it is possible that the *pect1* mutation in tissues other than stomata inhibits stomatal CO_2 and light responses. To eliminate this possibility and to further probe the function of PECT1 in guard cells, we created transgenic plants in which *PECT1* was repressed constitutively

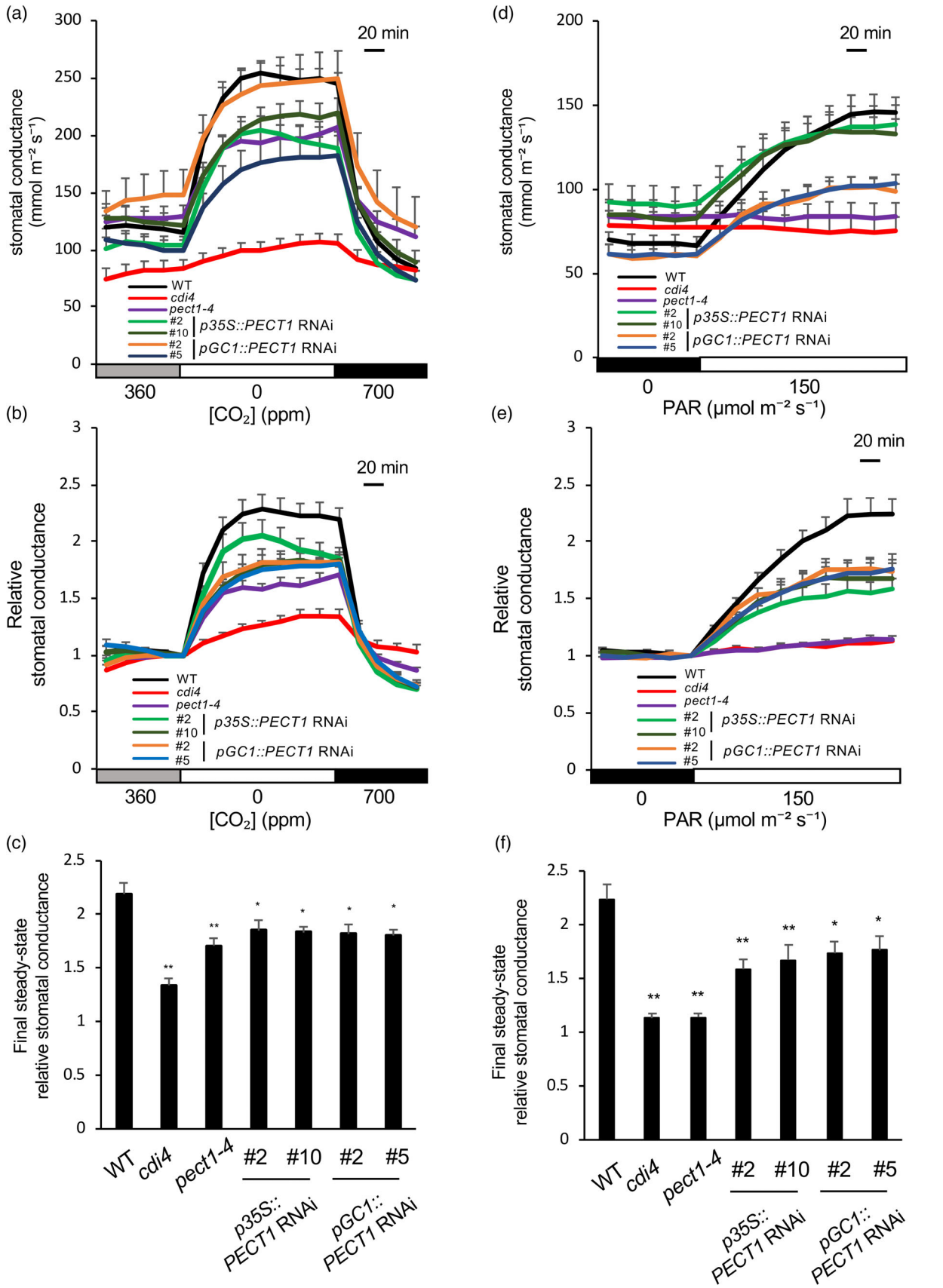

Figure 5. The RNAi lines where *PECT1* expression is suppressed in a guard cell- specific manner showed decreased stomatal responsiveness to light and low-CO_2.
Time course of stomatal conductance in p35S::PECT1 RNAi lines showing suppressed *PECT1* expression in whole plants and pGC1::PECT1 RNAi lines, suppressed *PECT1* expression in guard cells specifically in response to changes in CO_2 concentrations (a) or in light intensity (d). Stomatal conductance was normalized to the average conductance at the last 360 ppm CO_2 data point (b) and the last 0 μmol m^{-2} sec^{-1} PAR data point (e).
(c, f) Final steady-state relative stomatal conductance, whose data were obtained from (b) or (e), respectively. Values shown are means ± SE ($n > 5$). Asterisks denote comparison with wild-type (WT), $^{**}P < 0.01$ and $^{*}P < 0.05$ by one-way ANOVA with Dunnett's *post hoc* test.

throughout whole plants (p35S::PECT1 RNAi plants) or specifically in guard cells (pGC1::PECT1 RNAi plants) by antisense RNA technology. Quantitative polymerase chain reaction (qPCR) analysis using *PECT1*-specific primers demonstrated that the levels of *PECT1* transcripts were decreased to ~20 and ~30% of the control levels in whole rosettes (Figure S2a) and GCPs (Figure S2b), respectively, in the respective transgenic plants. We also confirmed that PE levels were significantly lower in these transgenic plants compared with those in WT (Figure S3). In the stomatal responses to CO_2, p35S::PECT1 RNAi plant lines #2 and #10 showed significantly reduced (~20%) on the relative stomatal conductance compared with WT. Similarly, the relative stomatal conductance in pGC1::PECT1 RNAi plant lines #2 and #5 was significantly reduced (~20%) compared with WT (Figure 5a–c). In the stomatal responses to the light, p35S::PECT1 plant lines #2 and #10 and pGC1::PECT1 RNAi plant lines #2 and #5 exhibited significantly reduced (~30%) on the relative stomatal conductance compared with WT (Figure 5d–f). Thus, stomatal CO_2 and light responses of these plants were inhibited to a similar extent in all transgenic plants. These results suggested that PECT1 in guard cells participates, at least in parts, in the regulation of CO_2- and light-induced stomatal movements.

cdi4 mutation inhibited FC-induced stomatal opening, but did not affect the expression, localization or activity of PM H^+-ATPase

The activation of the PM H^+-ATPases is primarily important for stomatal opening. Because stomatal opening by low-CO_2 and light was inhibited in the *cdi4* mutant (Figure 1), we examined the effects of *cdi4* on the expression and activity of PM H^+-ATPase. We first examined the stomatal responses to FC, a fungal toxin that constitutively activates PM H^+-ATPase in guard cells (Figure 6). We defined the parameter 'relative stomatal aperture (RSA)' for assessing the positive effect of FC on stomata opening. As shown in Figure 6(b), the RSA value for WT was nearly 1.8, whereas the RSA values were significantly reduced to 1.2–1.3 ($P < 0.05$) in *cdi4* mutants, the p35S::PECT1 RNAi lines #2 and #10, and the pGC1::PECT1 RNAi lines #2 and #5. These results suggested that *cdi4* as well as downregulation of *PECT1* could attenuate the FC-induced stomatal opening.

We then examined if PM H^+-ATPase is a direct target of *cdi4* mutation effects. In *Arabidopsis*, PM H^+-ATPases in guard cells are encoded by the isogenes *AHA1*–*AHA11* (Ueno et al., 2005), among which *AHA1* encodes the major isoform and is responsible for blue-light-induced stomatal opening (Yamauchi et al., 2016). Therefore, we examined the levels of *AHA1* transcripts in WT, *cdi4*, p35S::PECT1 RNAi and pGC1::PECT1 RNAi plants by quantitative reverse transcriptase (qRT)-PCR (Figure 7a,b), and the intracellular localization of AHA1-GFP in transgenic p35S::AHA1-GFP and *cdi4* p35S::AHA1-GFP plants (Figure 7c). *cdi4* did not significantly decrease the levels of *AHA1* transcripts in rosette leaves compared with WT, and similar trends were seen in p35S::PECT1 RNAi and pGC1::PECT1 RNAi plants (Figure 7a), although pGC1::PECT1 RNAi plant line #5 showed a significant increase. The levels of *AHA1* transcripts were not significantly different between WT and pGC1::PECT1 RNAi lines in GCPs (Figure 7b). *cdi4* mutation did not appear to affect the subcellular localization of AHA1-GFP in guard cells (Figure 7c). Thus, we concluded that neither *cdi4* nor RNAi suppression of *PECT1* significantly affect the levels of *AHA1* transcripts and localization.

It has been reported that PM H^+-ATPase is activated by phosphorylation of threonine at the penultimate residue, threonine, from the C-terminus (Kinoshita & Shimazaki, 1999). Therefore, we conducted immunohistochemical analyses of phosphorylation levels of PM H^+-ATPases using intact leaves. In *cdi4* mutants, the phosphorylation level of PM H^+-ATPases by blue light was lower than that in WT (Figure 8a,b), indicating that the prosses that leads to the activation of PM H^+-ATPases via phosphorylation is also affected in the mutant. The phosphorylation of PM H^+-ATPases after FC treatment occurred at normal levels in *cdi4* stomata (Figure 8c,d). We also examined whether or not FC-induced H^+-pumping occurs in the mutant as in WT. The FC-induced H^+-pumping was found in the *cdi4* mutant to a similar extent as in WT plants (Figure 8e,f). These results indicate that FC-induced phosphorylation and H^+-pumping of the PM H^+-ATPases were not affected, but the phosphorylation of PM H^+-ATPases by blue light was attenuated in the *cdi4* mutant.

DISCUSSION

In this study, we revealed that downregulation of PE synthesis negatively affects the stomatal opening responses to the decreasing concentrations of ambient CO_2 and the initiation of light. Our previous studies have shown that guard cells

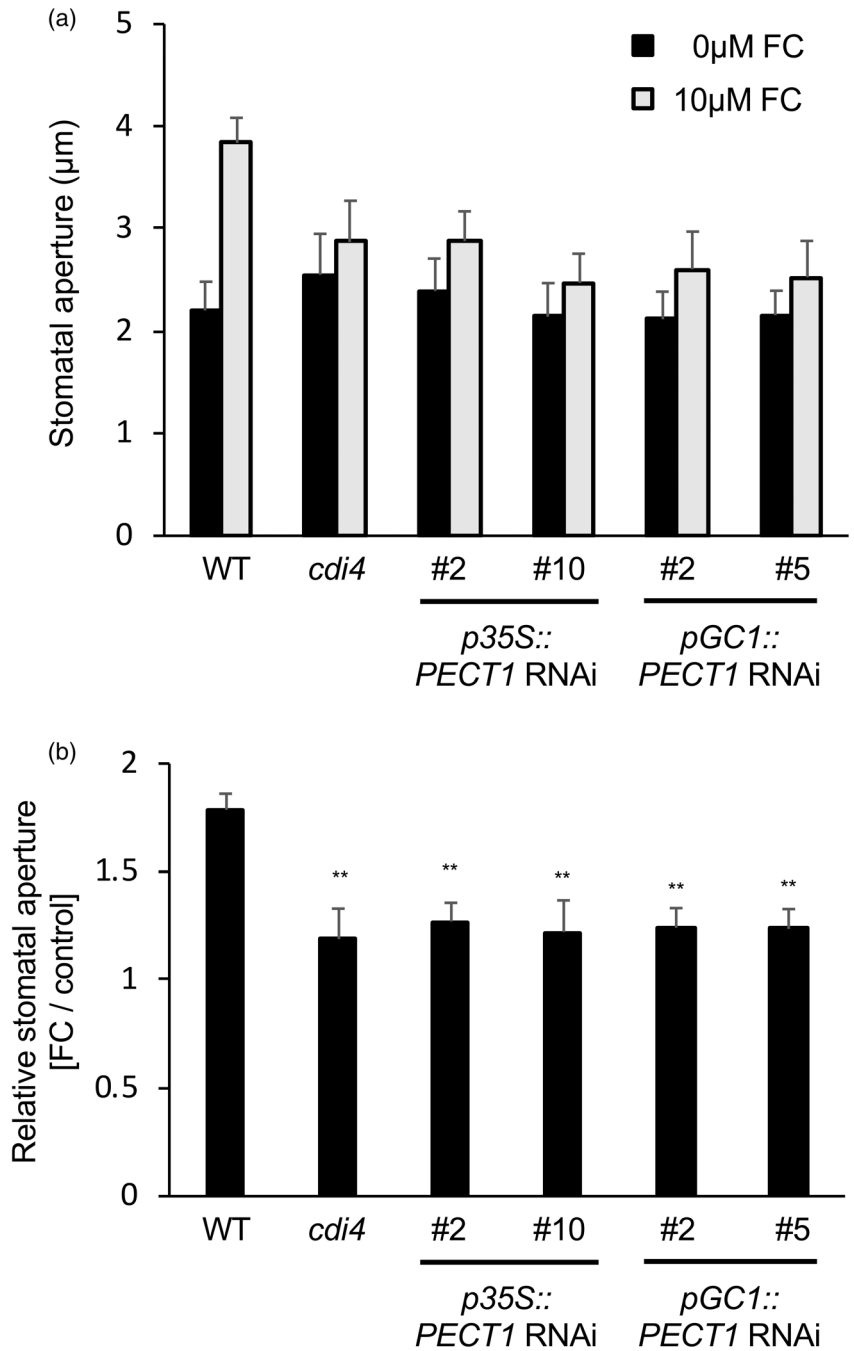

Figure 6. Stomatal opening by fusicoccin (FC) was inhibited in *cdi4* and the *PECT1* repressor lines.
(a) Stomatal aperture changes in response to FC. The black and gray bars indicate stomatal aperture measurements after mock [0.1% dimethylsulfoxide (DMSO)] or 10 μM FC treatment. Relative stomatal aperture (RSA) measurements (b) were calculated as (stomatal aperture after FC treatment)/(stomatal aperture after mock treatment). Values shown are means ± SE. Asterisks denote comparison with wild type (WT) $^{**}P < 0.01$ by one-way ANOVA with Dunnett's *post hoc* test.

preferentially utilize the ER pathway rather than the plastidic pathway for glycolipid synthesis (Negi et al., 2018), and when the contribution of the ER pathway is blocked by *gles1* mutation, chloroplast development in guard cells is severely inhibited. However, our understanding of the physiological significance of the ER pathway in guard cells remains to be deepened. In this study, we showed that PE, being exclusively produced in the ER pathway, is enriched in guard cells (Figure 4), and is essential for stomatal opening (Figures 1, 5 and 6). Our results suggested that the ER pathway is enforced more in guard cells than in mesophyll cells in order to maintain sufficient amounts of not only glycolipids for

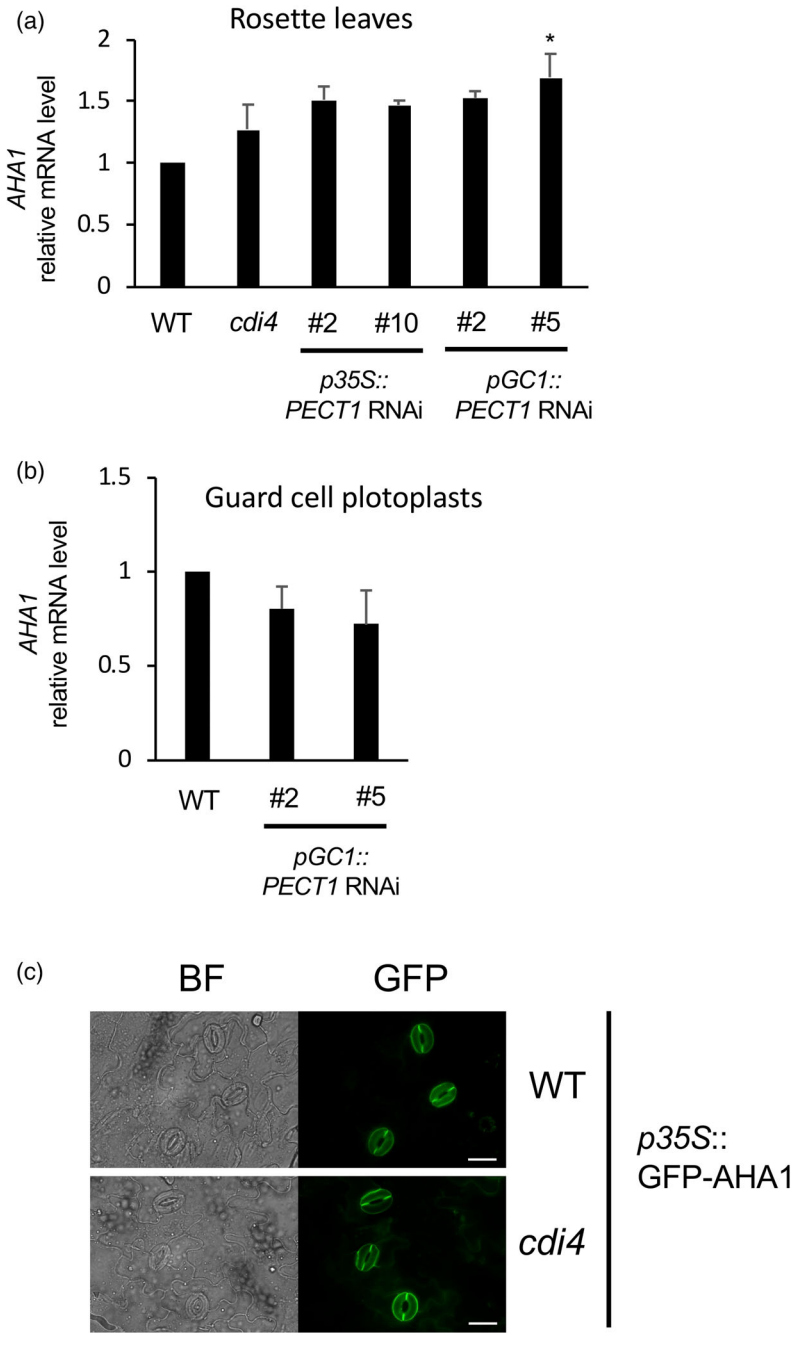

Figure 7. The *cdi4* mutation does not affect localization and expression of AHA1, one of the major plasma membrane (PM) H^+-ATPase in guard cells.
AHA1 relative transcriptional levels in rosette leaves (a) and guard cell protoplasts (b) were determined in *cdi4* and PECT1 repression lines. Values shown are means ± SE ($n > 4$). Asterisk denotes comparison with wild type (WT) $*P < 0.05$ by one-way ANOVA with Dunnett's *post hoc* test. (c) GFP fluorescence and the bright field images (BF) of epidermis of WT/p35S::GFP-AHA1 and *cdi4*/p35S::GFP-AHA1 are shown as representative examples. Scale bars represent 20 μm.

chloroplast maintenance but also PE, which we have demonstrated here to be essential for stomatal movements.

At present, the molecular mechanisms behind the involvement of PE in stomatal opening remain unclear, but we here raise the following four hypotheses.

1 Transporter conformation hypothesis: it has been reported that the activity of the PM H^+-ATPase varies depending on the phospholipid composition of the membrane (Kasamo & Yamanishi, 1991). Because PE is a non-bilayer-forming lipid having a cone-shape structure,

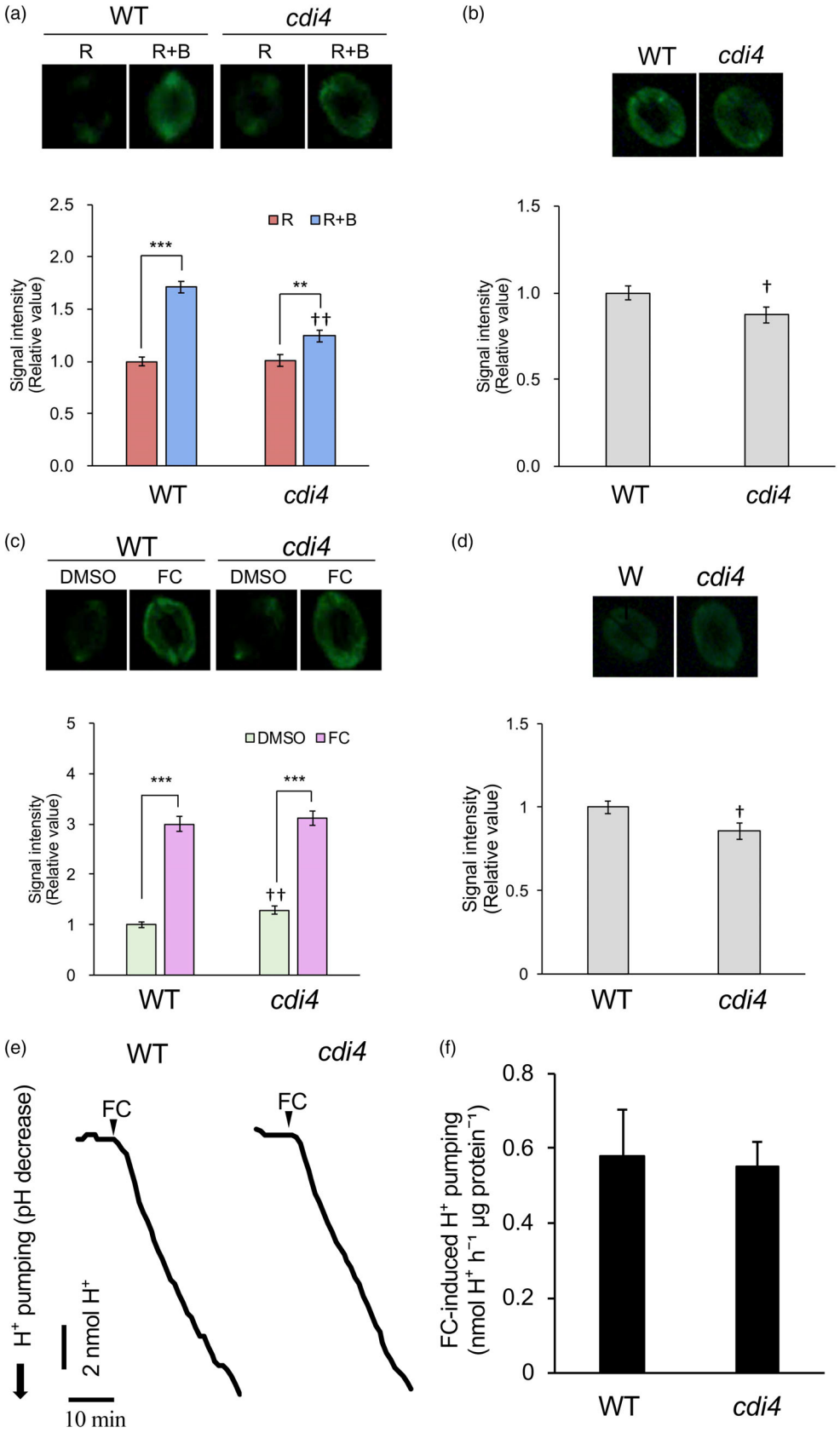

Figure 8. The *cdi4* mutation does not affect fusicoccin (FC)-induced plasma membrane (PM) H^+-ATPase phosphorylation and activation.
(a–d) Immunohistochemical detection of blue-light- or FC-induced phosphorylation (a, c) and amount of PM H^+-ATPase (b, d) in guard cells using whole leaves from wild-type (WT) and *cdi4*. Phosphorylation or amount of the protein was detected using anti-pThr or anti-H^+-ATPase antiserum, respectively. Typical fluorescence images of stomata in the epidermis using anti-pThr or anti-H^+-ATPase antiserum (upper), and quantification of fluorescence images of the stomata (lower) are shown. (a, b) Leaves were incubated under red light for 30 min (R), then blue light was superimposed for 1 min on the background red light (R + B). Values shown are means ± SE (150 stomata from three independent experiments). Asterisks denote comparison of R and R + B in each plant; daggers indicate comparison with the same treatment of WT with Student's *t*-test (***$P < 0.001$, **$P < 0.01$, $^{\dagger\dagger}P < 0.01$, $^{\dagger}P < 0.05$). (c, d) Leaves were floated in buffer and treated with dimethylsulfoxide (DMSO) or 10 μM FC, and incubated in the dark for 30 min. Values shown are means ± SE (150 stomata from three independent experiments). Asterisks denote comparison of DMSO and FC treatment; daggers indicate comparison with the same treatment of WT using Student's *t*-test (***$P < 0.001$, $^{\dagger\dagger}P < 0.01$, $^{\dagger}P < 0.05$). (c) FC-dependent H^+ pumping of guard cell protoplasts (GCPs) from WT and *cdi4*. GCPs were incubated with light illumination and 10 μM FC was applied. (d) Quantification of maximum rate of FC-induced H^+ pumping from GCPs. Values shown are means ± SE of four experiments.

PE has a potential to properly fix or glue a membrane protein within the membrane bilayer (van den Brink-van der Laan et al., 2004). Here, we show that a decrease in PE did not affect the total transcripts, polypeptides, and FC-induced phosphorylation and H^+-pumping of PM H^+-ATPases (Figures 7 and 8). However, the decrease in PE levels appears to attenuate the FC response (Figure 6), suggesting that PE may affect the stomatal opening mechanisms downstream of PM H^+-ATPases. It is possible that PE may contribute to the stabilization of potassium ion channels, which are known to be activated by membrane hyperpolarization caused by the activation of PM H^+-ATPases. Further studies involving measurement of potassium ion channel activity in *cdi4* mutants are needed to verify this hypothesis.

2 Signal hypothesis: in our study, the phosphorylation of PM H^+-ATPases by blue light was suppressed in the *cdi4* mutant (Figure 8a,b). Phospholipids such as PE are hydrolyzed by phospholipases to lysophospholipids and free fatty acids. Several studies have reported that the lysophospholipids are involved in stomatal opening responses. In light-induced stomatal opening responses, phospholipase A2β (PLA2β) has been suggested to generate lysophospholipids (Seo et al., 2008), which are considered to be signaling molecules that activate one of the PM H^+-ATPases, AHA2 (Wielandt et al., 2015). Therefore, it is possible that PE synthesized by PECT1 in guard cells is hydrolyzed by a phospholipase to produce lysoPE for activation of a PM H^+-ATPase.

3 Mitochondrial hypothesis: PECT1 is localized to mitochondria (Mizoi et al., 2006; Figure 2c) and PE is the major glycerolipid in the mitochondrial membrane (Michaud et al., 2017). PECT1 regulates mitochondrial PE levels and modulates cytochrome *c* oxidase activity to maintain respiration capacity (Otsuru et al., 2013). Thus, the reduced stomatal opening response in *cdi4* could be explained by reduced respiratory activity or a decrease in energy supply caused by an alteration in the mitochondrial membrane lipid compositions.

4 Cytoskeletal hypothesis: a previous study reported that the stomatal opening response by FC is associated with the depolymerization of actin filaments in guard cells (Eun & Lee, 2000). Because FC-induced stomatal opening responses were inhibited in *cdi4* and *PECT1* RNAi lines (Figure 6), PE in guard cells could be involved in depolymerization of actin filaments. In animals, the movement of PE from the intracellular to the extracellular layer during telophase of cell division is thought to promote the dissociation and inactivation of RhoA from the membrane and the depolymerization of actin filaments (Emoto et al., 2005). Flippase is known as an enzyme that produces an asymmetric distribution of lipid molecules between inner and outer membrane layers. The flippase ALA10 is expressed in guard cells and has been reported to take up labeled phospholipids into guard cells, and the *ala10* mutants have a significantly reduced transpiration rate in both light and dark conditions (Poulsen et al., 2015). Thus, we suspect that in *cdi4* mutants the flippase of guard cells is unable to maintain proper PE distributions across the PM bilayer, so that depolymerization of actin filaments and hence the opening of the stomatal pores become inhibited.

In this study, we have shown that PECT1 is involved in stomatal regulation in *Arabidopsis* using *cdi4* and *pect1-4* mutants, which exhibited an impaired stomatal response to CO_2. Both mutants exhibit a decrease in PE level and a defect in stomatal movement, including decreased responses to alteration in CO_2-concentration- and light-induced stomatal opening. Our results also indicate that PECT1 in guard cells regulates stomatal movement. Although the exact molecular mechanism for this regulation remains unclear, we have raised several hypotheses, which are to be tested in future studies.

EXPERIMENTAL PROCEDURES

Plant materials and growth conditions

All *Arabidopsis* lines used in this study, including a transgenic line expressing GFP-AHA1 (Hashimoto-Sugimoto et al., 2013), were derived from the Columbia (Col-0) ecotype. EMS-mutagenized M2 seeds were purchased from Lehle Seeds (Round Rock, TX, USA). Transgenic plants were generated by *Agrobacterium*-mediated transformation as previously described (Clough & Bent, 1998). Plants were grown on solid Murashige–Skoog medium for 19 days in a growth chamber (constant white light of 35 μmol m^{-2} sec^{-1} at

22°C, 70% RH), and then transplanted into vermiculite pots supplemented with mineral nutrients.

Isolation of *cdi4* mutant and map-based cloning

To identify genes that function in CO_2 responses in guard cells, we isolated CO_2-insensitive mutants (*ht1-ht2* and *cdi1-cdi10*) through a high-throughput leaf thermal imaging (Hashimoto et al., 2006; Negi et al., 2008). The *cdi4* mutant was isolated as one of these mutant lines. Genetic mapping of 162 chromosomes localized CDI4 to the 1100-kb region between two single-nucleotide polymorphisms (SNPs), CER427813 and CER424914. Sequencing of this region combined with genetic complementation of the *cdi4* mutant identified a single open reading frame, At2g38670, as the CDI4 gene.

Thermal imaging

Plants grown in a growth chamber were transferred to a growth cabinet (constant white light of 80 μmol m^{-2} sec^{-1} at 22°C, 43% RH) equipped with an automatic CO_2 control unit (FR-SP; Koito Co. Ltd., Tokyo, Japan). After 90 min of adaptation to low [CO_2] (0 ppm), thermal images were captured under high [CO_2] (700 ppm) using a thermography apparatus (TVS-8500, NEC/Avio, Tokyo, Japan).

Stomatal conductance

The whole plant stomatal conductance to water vapor (g_s) was measured with a portable gas exchange fluorescence system (GFS-3000; Heinz Walz, Effeltrich, Germany) equipped with a 3010-A *Arabidopsis* chamber. The response of g_s to CO_2 was measured at constant light intensity (150 μmol m^{-2} sec^{-1}) and 40% RH. The g_s response to light was measured under constant [CO_2] (350 ppm) and 60% RH; after dark incubation for 3 h, the plant was kept under illumination (150 μmol m^{-2} sec^{-1}) for 2 h. Temperature in the chamber and the flow rate in the system were kept constant (22°C, 750 μmol sec^{-1}, respectively) throughout the gas exchange experiments. All measurements were taken once per minute.

Microscopic analysis of stomatal responses

Abaxial epidermal peels of 3-week-old plants were used to measure stomatal apertures. In the FC treatment, epidermal peels were floated on a test medium containing 30 mM KCl, 10 mM MES-KOH (pH 6.15) and 0.1 mM $CaCl_2$, and were incubated in the growth chamber. FC (10 μM) or 0.1% (v/v) dimethylsulfoxide (DMSO) was added to the solution after 1 h of illumination, and stomatal apertures were measured 2 h later. The stomatal apertures in epidermal peels were observed with a fluorescence microscope (IX71; Olympus, Tokyo, Japan).

Construction of binary vectors for plant transformation

All primers used for plasmid construction are listed in Table S1. To construct a binary vector for functional complementation of the *cdi4* mutant, the PCR-amplified *PECT1* ORF was inserted between *Bam*HI and *Sac*I sites of pBI121. For the construction of p35S::PECT1-GFP, the PCR-amplified *PECT1* ORF was inserted into the binary vector pMDC83 (Curtis & Grossniklaus, 2003). To construct a binary vector for pPECT1::GUS, a 0.5-kb *Sal*I/*Bam*HI fragment including the 5′ upstream region and 5′ UTR of *PECT1* was inserted between *Sal*I and *Bam*HI of pBI101. To construct a plasmid for 35S::*PECT1* RNAi, a region of the *PECT1* cDNA (nucleotides 720–1030) was amplified by PCR and inserted into the pANDA vector (Miki & Shimamoto, 2004). To generate pGC1::*PECT1* RNAi, the CaMV35S promoter was replaced by 1140 bp of the GC1 promoter regions (5) from the 35S::*PECT1* RNAi vector.

Transgene expression analysis

Histochemical staining of GUS activity in the pPECT1::GUS transformants was performed as described previously (Negi et al., 2008). Leaf epidermal peels prepared from 3-week-old plants expressing the PECT1-GFP fusion protein were analyzed using a fluorescence microscope (BZ-9000; Keyence, Osaka, Japan). To stain mitochondria, epidermal peels were stained with 500 nm MitoTracker (Invitrogen, Waltham, MA, USA) for 30 min. The GFP-3035B-KEY-ZERO (exciter, 472/30–25; emitter, 520/35–25) and TRITC (exciter, 540/25; emitter, 605/55) filter sets were used for observing GFP and MitoTracker fluorescence signals, respectively.

Isolation of GCPs and MCPs

The GCPs and MCPs were prepared enzymatically from 4- to 5-week-old *Arabidopsis* leaves as described previously (Negi et al., 2013; Yamamoto et al., 2016).

Measurement of lipid contents

For measurement of lipid contents by LC–MS, lipids were extracted as described previously (Obata et al., 2021) and the lipid samples were diluted with the extraction solvent (methanol [MeOH]:methyl tert-butyl ether [MTBE] = 1:3, v/v). LC–MS was performed with Prominence (Shimadzu, Kyoto, Japan) coupled to LCMS-8050 (Shimadzu). An aliquot of the extracted sample (2 μl) was injected into a Discovery HS F5-3 column (150 × 2.1 mm, 3 μm; Supelco) for MGDG, DGDG analysis, or into a Kinetex C8 column (150 × 2.1 mm, 2.6 μm; Phenomenex, Torrance, CA, USA) for SQDG, PG, PE, PC, PI analysis, with mobile phases containing solvent A (water) and solvent B (acetonitrile:2-propanol = 7:3). Both solvents were supplemented with 0.1% acetic acid and 10 mM ammonium acetate. The gradient program was as follows: 65% solvent B for the first 1 min; 65–95% solvent B in the next 9 min; and 95% solvent B for 6 min, with a flow rate of 0.2 ml min^{-1}. The column oven temperature was set at 40°C. LC–MS data were analyzed in LabSolutions LCMS (Shimadzu). The lipids in each class were quantified in comparison to standards, such as MGDG (Avanti, Alabaster, AL, USA; 840523P), DGDG (Avanti; 840524P), SQDG (Avanti; 840525P), 18:0–18:1-PG (Avanti; 840503P), 16:0–18:1-PE (Avanti; 850757P), 16:0–18:1-PC (Avanti; 850457P), Soy PI (Avanti; 840044P).

qRT-PCR

Total RNA prepared from leaves and GCPs was used for cDNA synthesis using the oligo (dT) primer. qPCR was performed using SYBR Green QPCR Master Mixes (Agilent Technologies, Santa Clara, CA, USA) and the Mx3000P Real-Time QPCR System (Stratagene, La Jolla, CA, USA). Gene-specific signals were normalized relative to *UBQ10* expression. Oligonucleotides used are listed in Table S1.

Measurement of H^+ pumping

The FC-dependent H^+-pumping activity in GCPs was determined with a glass pH electrode as described previously (Ueno et al., 2005; Yamauchi et al., 2016). The reaction mixture (1 ml) contained 0.125 mM MES-KOH (pH 6.0), 1 mM $CaCl_2$, 0.4 M mannitol, 10 mM KCl and GCPs (50 μg proteins).

Immunohistochemical detection of the PM H^+-ATPase in guard cells

The phosphorylation levels at the penultimate C-terminal residue of AHA2 polypeptide, ^{974}Thr, and the amount of PM H^+-ATPase in

guard cells were determined using *Arabidopsis* whole rosette leaves as described previously (Ando & Kinoshita, 2018).

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CONFLICT OF INTEREST

No conflicts of interest declared.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author (J.N.), upon reasonable request.

SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article.

Figure S1. Contents of glycerolipids other than PE in rosette leaves. Glycerolipid contents in rosette leaves were measured by LC–MS. The statistical significance was determined by a one-way ANOVA with Tukey–Kramer multiple comparison tests. Same letters indicate no significant difference ($P > 0.05$).

Figure S2. Gene expression of the *PECT1* in PECT1 RNAi lines. The *PECT1* relative transcriptional levels in rosette leaves (a), GCPs (b) and MCPs (c) were determined in *cdi4* and *PECT1* repression lines. Values shown are means ± SE ($n > 4$). Asterisks denote comparison with WT, $*P < 0.05$ and $**P < 0.01$ by one-way ANOVA with Dunnett's *post hoc* test.

Figure S3. PE levels were significantly lower in PECT1 RNAi plants than in WT. PE contents in rosette leaves (a) and GCPs (b) were determined in *PECT1* repression lines. Asterisks denote comparison with WT, $*P < 0.05$ by one-way ANOVA with Dunnett's *post hoc* test.

Table S1 Oligonucleotide primers used in this study

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