Two Redundant Receptor-Like Cytoplasmic Kinases Function Downstream of Pattern Recognition Receptors to Regulate Activation of SA Biosynthesis[1][OPEN]

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Salicylic acid (SA) serves as a critical signaling molecule in plant defense. Two transcription factors, SARD1 and CBP60g, control SA biosynthesis through regulating pathogen-induced expression of *Isochorismate Synthase1*, which encodes a key enzyme for SA biosynthesis. Here, we report that Pattern-Triggered Immunity Compromised Receptor-like Cytoplasmic Kinase1 (PCRK1) and PCRK2 function as key regulators of SA biosynthesis. In the *pcrk1 pcrk2* double mutant, pathogen-induced expression of *SARD1*, *CBP60g*, and *ICS1* is greatly reduced. The *pcrk1 pcrk2* double mutant, but neither of the single mutants, exhibits reduced accumulation of SA and enhanced disease susceptibility to bacterial pathogens. Both PCRK1 and PCRK2 interact with the pattern recognition receptor FLS2, and treatment with pathogen-associated molecular patterns leads to rapid phosphorylation of PCRK2. Our data suggest that PCRK1 and PCRK2 function downstream of pattern recognition receptor in a signal relay leading to the activation of SA biosynthesis.

In their natural environment, plants are continuously exposed to various microbial organisms. During the course of evolution, plants have developed sophisticated immune systems to combat pathogen invasion. Recognition of pathogen-associated molecular patterns (PAMPs) by membrane-localized pattern recognition receptors initiates PAMP-triggered immunity (PTI), which serves as the first line of plant defense and plays a pivotal role in defending plants from microbial invasion (Boller and Felix, 2009). Some of the well-studied PAMP receptors belong to the receptor-like kinase (RLK) family (Monaghan and Zipfel, 2012). For example, FLAGELLIN-SENSITIVE2 (FLS2) and Elongation Factor-TU RECEPTOR (EFR) recognize flagellin and EF-Tu from bacteria, respectively (Gómez-Gómez and Boller, 2000; Zipfel et al., 2006), CHITIN ELICITOR RECEPTOR KINASE1 (CERK1) recognizes both the fungal cell wall component chitin as well as the bacterial cell wall component peptidoglycan (Miya et al., 2007; Wan et al., 2008; Gimenez-Ibanez et al., 2009; Willmann et al., 2011), and the S-domain-1 receptor-like kinase LORE recognizes lipopolysaccharide from Gram-negative bacteria (Ranf et al., 2015). Another RLK, BRASSINOSTEROID INSENSITIVE1-ASSOCIATED RECEPTOR KINASE1 (BAK1), functions as a coreceptor for FLS2 and EFR (Chinchilla et al., 2007; Heese et al., 2007).

Plants also have a large number of receptor-like cytoplasmic kinases (RLCKs) that are evolutionarily related to RLKs but without a transmembrane domain (Shiu and Bleecker, 2001; Lin et al., 2013). Early studies on the RLCKs Pto and PBS1 implicated these proteins play important roles in plant defense (Martin et al., 1993; Swiderski and Innes, 2001). In tomato, targeting of Pto by the bacterial effector protein AvrPto triggers activation of defense responses (Martin et al., 2003). Cleavage of Arabidopsis (*Arabidopsis thaliana*) PBS1 by AvrPphB activates RPS5-mediated immune responses (Shao et al., 2003). In addition to Pto and PBS1, RLCKs such as BIK1, PBL1, RIPK, and OsRLCK185 are also targeted by pathogen effector proteins (J. Zhang et al., 2010; Liu et al., 2011; Feng et al., 2012; Yamaguchi et al., 2013), further supporting the importance of RLCKs in plant immunity.

Several RLCKs have been shown to associate with PAMP receptors to transduce defense signals. BIK1

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interacts with multiple PAMP receptors, including FLS2, EFR, and CERK1 (Lu et al., 2010; J. Zhang et al., 2010). It promotes reactive oxygen species (ROS) production by phosphorylating the NADPH oxidase D (Kadota et al., 2014; Li et al., 2014). Another RLCK, BSK1, also interacts with FLS2 and is required for flagellin-induced ROS production (Shi et al., 2013). In rice (*Oryza sativa*), the RLCK OsRLCK185 associates with OsCERK1 (Yamaguchi et al., 2013). It is phosphorylated by OsCERK1 after chitin treatment and is required for chitin-induced immune responses. A recent study showed that PBL27, an Arabidopsis ortholog of OsRLCK185, regulates chitin-induced defense responses in Arabidopsis (Shinya et al., 2014).

Salicylic acid (SA) is a signaling molecule that plays pivotal roles in plant defense response (Vlot et al., 2009; Dempsey et al., 2011). Following pathogen infection, SA accumulates in both local and systemic tissues. Both local resistance and systemic acquired resistance (SAR) are compromised in transgenic plants expressing the SA-degrading enzyme salicylate hydroxylase (Gaffney et al., 1993; Delaney et al., 1994). Similarly, mutants deficient in pathogen-induced SA biosynthesis exhibit defects in SAR as well as basal resistance against pathogens (Nawrath and Métraux, 1999; Nawrath et al., 2002). In Arabidopsis, pathogen-induced SA is mainly produced from chorismate via ISOCHORISMATE SYNTHASE1 (ICS1; Wildermuth et al., 2001). Infection by pathogens leads to rapid induction of *ICS1* expression and activation of biosynthesis of SA. The induction of *ICS1* expression is mainly controlled by the transcription factors SARD1 and CBP60g (Wang et al., 2009, 2011; Y. Zhang et al., 2010). Some other transcriptional regulators, including WRKY28, NTM1-like9, CCA1 hiking expedition, Nonexpressor of Pathogenesis-Related genes1, Ethylene Insensitive3, and Ethylene Insensitive3-Like1, also influence the expression of *ICS1* (Wildermuth et al., 2001; Chen et al., 2009; van Verk et al., 2011; Zheng et al., 2015). Recently, SARD1 and CBP60g were also reported to function as master regulators of plant immunity that regulate the expression of a large number of plant defense regulators in addition to *ICS1* (Sun et al., 2015).

The transcription of both *SARD1* and *CBP60g* is strongly induced by pathogens (Wang et al., 2009; Y. Zhang et al., 2010), although the mechanism by which their expression is activated is unknown. In this study, we have shown that the two redundant receptor-like cytoplasmic kinases PCRK1 and PCRK2 function downstream of PAMP receptors and contribute to the induction of *SARD1* and *CBP60g* expression and SA biosynthesis.

RESULTS

Identification of Knockout Mutants for *PCRK1* and *PCRK2*

Genetic redundancy is a major problem in classic forward genetic screen. To identify genes that function redundantly in plant immunity, we carried out a systematic reverse genetic analysis of genes that encodes proteins with >80% amino acid sequence identity (Qu et al., 2010). At3g09830 and At5g03320 belong to receptor-like cytoplasmic kinase subfamily VII and share 81% sequence identity. Since At3g09830 was recently designated as PATTERN-TRIGGERED IMMUNITY COMPROMISED RECEPTOR-LIKE CYTOPLASMIC KINASE1 (PCRK1; Sreekanta et al., 2015), we named At5g03320 as PCRK2. To determine the functions of these two kinases, T-DNA insertion mutants for *PCRK1* (*pcrk1-2*, SALK_145629) and *PCRK2* (*pcrk2-1*, SAIL_129_D02) were obtained from the Arabidopsis Biological Resource Center, and the *pcrk1-2 pcrk2-1* double mutant (designated as *pcrk1 pcrk2*) was generated by crossing the two single mutants. The T-DNA insertion in *pcrk1-2* is in the third exon and the insertion in *pcrk2-1* is in the first exon (Fig. 1A). Analysis of the expression levels of *PCRK1* and *PCRK2* in the single and double mutants showed that their transcript levels were significantly reduced by the T-DNA insertions (Fig. 1, B and C).

pcrk1 pcrk2 Mutant Plants Exhibit Compromised Basal Resistance to Pathogens

To determine whether PCRK1 and PCRK2 function in plant immunity, we challenged wild-type and mutant plants with the virulent bacterial pathogen *Pseudomonas syringae* pv *maculicola* (*Pma*) ES4326. As shown in Figure 1D, growth of *Pma* ES4326 in *pcrk1 pcrk2* is significantly higher than in the wild type and single mutants, whereas the bacterial growth in the two single mutants is comparable to that in the wild-type plants. Similar results were observed when the wild-type and mutant plants were challenged with a different bacterial pathogen, *Pseudomonas syringae* pv *tomato* (*Pto*) DC3000 (Fig. 1E).

To determine whether the compromised bacterial resistance in *pcrk1 pcrk2* is caused by loss of function of PCRK1 and PCRK2, we generated transgenic plants expressing PCRK1-GFP or PCRK2-GFP fusion proteins in the *pcrk1 pcrk2* background under their native promoters. When challenged with *Pma* ES4326 or *Pto* DC3000, bacterial growth in the transgenic lines was comparable to that observed in wild type (Fig. 1, D and E), suggesting that the enhanced disease susceptibility phenotype of *pcrk1 pcrk2* is complemented by the transgenes. These data suggest that PCRK1 and PCRK2 are required for basal resistance against bacterial pathogens and play redundant roles in the regulation of plant immunity.

A Mutation in a Conserved Residue in the ATP-Binding Site of PCRK2 Abolishes Its Function in Plant Defense

To determine whether the kinase activity of PCRK2 is required for its function in plant immunity, we mutated a conserved Lys residue (K115) in the ATP-binding site of PCRK2 that is predicted to be essential for kinase activity. Transgenic lines were generated to express $PCRK2^{K115E}$-GFP in the *pcrk1 pcrk2* background under

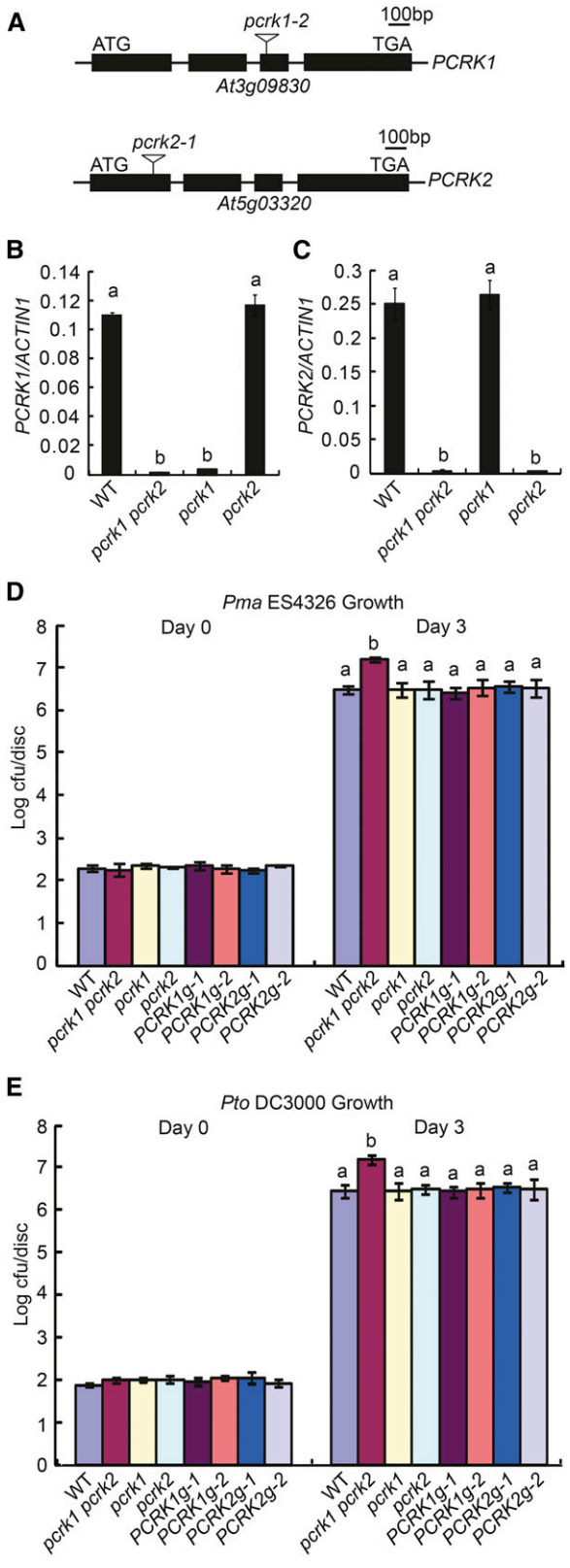

Figure 1. Loss of basal resistance in *pcrk1 pcrk2*. A, T-DNA insertion sites in *PCRK1* and *PCRK2*. *pcrk1-2*, SALK_145629; *pcrk2-1*, SAIL_129_D02. Black boxes indicate exons, and lines represent introns. B and C, Gene expression levels of *PCRK1* (B) and *PCRK2* (C) in wild type (WT), *pcrk1 pcrk2*, *pcrk1-2*, and *pcrk2-1* determined by quantitative PCR. Samples were collected from 12-d-old seedlings grown on half-strength MS plates. The values were normalized to the expression of *ACTIN1*. Error bars represent standard deviations ($n = 3$). Statistical differences among different samples are labeled with different letters ($P < 0.001$, ANOVA). D and E, Growth of *Pma* ES4326 (D) and *Pto* DC3000 (E) on the indicated genotypes. *PCRK1g-1* and *PCRK1g-2* are two independent lines expressing *PCRK1-GFP* under its native promoter in *pcrk1 pcrk2*. *PCRK2g-1* and *PCRK2g-2* are two independent lines expressing *PCRK2-GFP* under its own promoter in *pcrk1 pcrk2*. Four-week-old plants grown under short-day conditions were infiltrated with *Pma* ES4326 or *Pto* DC3000 at a dose of $OD_{600} = 0.0001$. Samples were taken at 0 h (day 0) and 72 h (day 3), respectively. Error bars represent standard deviations of six samples. Statistical differences among different genotypes are labeled with different letters ($P < 0.01$, ANOVA). Cfu, Colony forming unit. Similar results were obtained in two independent experiments.

its own promoter. Analysis of three independent transgenic lines showed that they support a similar amount of *Pma* ES4326 growth as *pcrk1 pcrk2* (Fig. 2A), suggesting that PCRK2^{K115E}-GFP is unable to complement the phenotype of *pcrk1 pcrk2*. The transcript levels of *PCRK2^{K115E}-GFP* in these lines are similar to those in wild-type *PCRK2-GFP* transgenic lines shown in Figure 1 (Fig. 2B). Surprisingly, the PCRK2^{K115E}-GFP protein accumulates to considerably lower levels than the wild-type PCRK2-GFP protein (Fig. 2C). When PCRK2^{K115E}-HA and PCRK2-HA were transiently expressed in *Nicotiana benthamiana* under *Cauliflower mosaic virus* 35S promoter, PCRK2^{K115E}-HA also accumulated to lower levels than the wild-type protein (Fig. 2D), suggesting that the K115E mutation affects the accumulation of PCRK2.

PAMP-Triggered Immunity Is Compromised in *pcrk1 pcrk2*

To test whether PTI is affected in *pcrk1* and *pcrk2* mutant plants, we challenged wild-type and mutant plants with *Pto* DC3000 *hrcC*$^-$, a bacterial strain that is deficient in delivery of type III effectors and often used as an indicator of PTI. As shown in Figure 3A, growth of *Pto* DC3000 *hrcC*$^-$ in *pcrk1*, *pcrk2*, and wild type is comparable, but the *pcrk1 pcrk2* double mutant supports considerably higher growth of the bacteria. The enhanced susceptibility to *Pto* DC3000 *hrcC*$^-$ observed in the double mutant can be complemented by both *PCRK1* and *PCRK2* (Fig. 3A). These data suggest that PCRK1 and PCRK2 function redundantly in the positive regulation of PTI.

To determine whether PCRK1 and PCRK2 are required for resistance responses mediated by FLS2, we analyzed bacterial growth in wild-type and *pcrk1 pcrk2* plants pretreated with flg22, a peptide derived from bacterial flagellin (Gómez-Gómez and Boller, 2000). As shown in Figure 3B, flg22-induced resistance to *Pto* DC3000 was compromised in *pcrk1 pcrk2* compared to wild-type plants.

Recognition of PAMPs by their receptors triggers rapid induction of ROS production and activation of

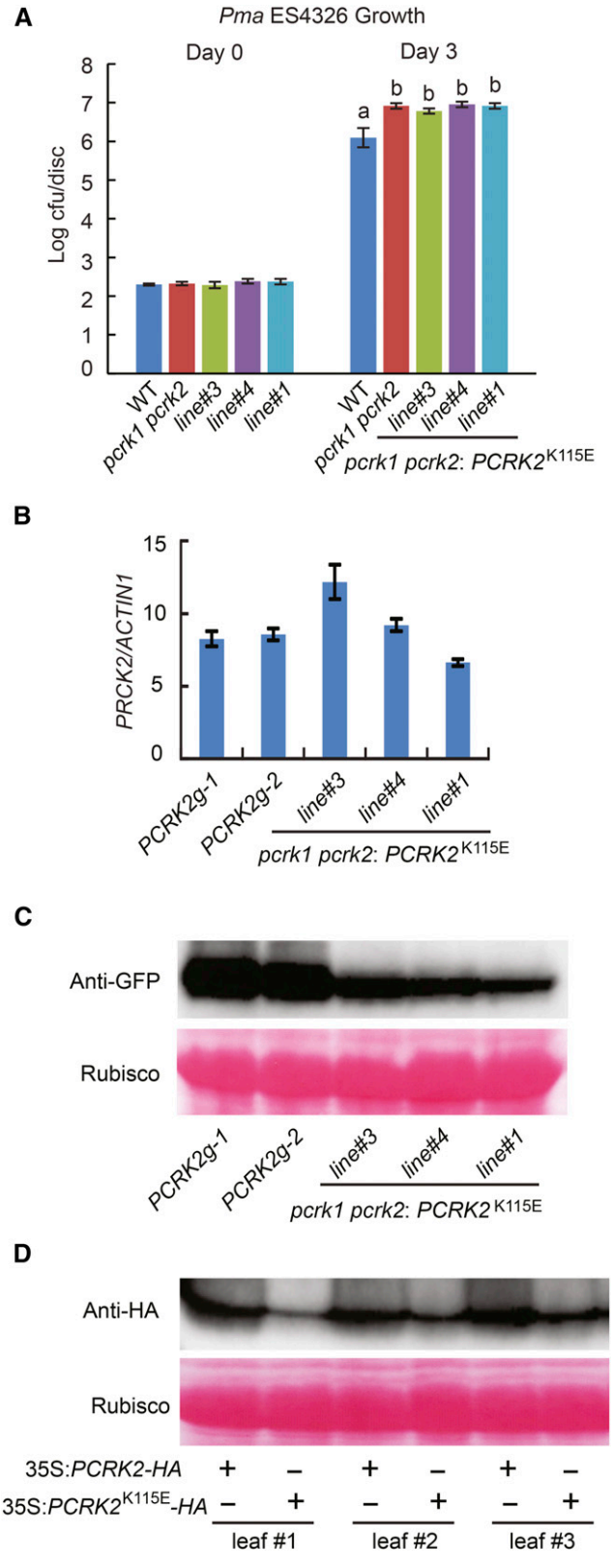

Figure 2. A conserved Lys (K115) of PCRK2 is required for its accumulation and function in plant defense. A, Growth of *Pma* ES4326 on wild type (WT), *pcrk1 pcrk2*, and three independent *PCRK2*^{K115E}*-GFP* transgenic lines in *pcrk1 pcrk2* background. Four-week-old plants grown under short-day conditions were infiltrated with *Pma* ES4326 (OD_{600} = 0.0001). Samples were taken at 0 h (day 0) and 72 h (day 3). Error bars represent standard deviations of six samples. Statistical differences among different samples are labeled with different letters ($P < 0.01$, ANOVA). B, Expression levels of *PCRK2* in transgenic lines. *PCRK2g-1* and *PCRK2g-2* are two independent lines expressing *PCRK2-GFP* under its own promoter in *pcrk1 pcrk2* background as mentioned in Figure 1D. RNA was extracted from 12-d-old seedlings grown on half-strength MS. The expression levels were normalized to *ACTIN1*. Error bars represent SD (n = 3). C, Western-blot analysis of PCRK2-GFP or PCRK2^{K115E}-GFP protein levels in total protein extracts of indicated transgenic lines in *pcrk1 pcrk2* background using an anti-GFP antibody. D, Protein levels of PCRK2-HA and PCRK2^{K115E}-HA in *N. benthamiana*. Suspension solutions (OD_{600} = 0.2) of *Agrobacterium* GV3101 carrying constructs expressing PCRK2-HA or PCRK2^{K115E}-HA were infiltrated into the same leaf (half leaf for each strain). After 2 d, tissue from the inoculated area was collected individually and analyzed by western blot using an anti-HA antibody. PCRK2-HA and PCRK2^{K115E}-HA protein levels in three individual leaves are shown. Experiments in this figure have been repeated at least twice with similar results.

MAP kinases. To test whether PCRK1 and PCRK2 are required for PAMP-induced oxidative burst and activation of MAP kinases, we compared flg22-induced ROS production and MAP kinase activation in wild type and *pcrk1 pcrk2*. As shown in Supplemental Figure S1, ROS production in the *pcrk1 pcrk2* double mutant is similar to that observed in wild type. However, flg22-induced MAPK activation appears to be modestly reduced in the *pcrk1 pcrk2* double mutant compared to wild type (Fig. 3C). Analysis of the expression of *FRK1* and *WRKY29*, two defense marker genes downstream of MPK3 and MPK6, showed that their induction by flg22 is also reduced in *pcrk1 pcrk2* (Fig. 3, D and E). These results further support that PCRK1 and PCRK2 are critical regulators of PAMP-induced immune responses.

PCRK1 and PCRK2 Associate with FLS2 in Vivo

Both PCRK1 and PCRK2 contain putative palmitoylation sites in their N terminus. Analysis of transgenic plants or protoplasts expressing PCRK1-GFP and PCRK2-GFP fusion proteins by confocal microscopy suggests that they are localized on the plasma membrane (Fig. 4A; Supplemental Fig. S2). FLS2 is a membrane-localized PAMP receptor that plays critical roles in immunity against bacterial pathogens (Gómez-Gómez and Boller, 2000; Zipfel et al., 2004). To test whether FLS2 interacts with PCRK1 and PCRK2, we coexpressed FLAG-tagged FLS2 with FLAG-ZZ-tagged PCRK1 or HA-tagged PCRK2 in *N. benthamiana*. The ZZ tag was derived from the B domain of Protein A, which binds IgG with high affinity. Immunoprecipitation was carried out using IgG agarose beads that recognizes the ZZ tag or anti-HA conjugated agarose beads. The precipitated proteins were detected by anti-HA and anti-FLAG antibodies. As shown in Figure 4, B and C, FLS2-FLAG coimmunoprecipitated with PCRK1-FLAG-ZZ as well as with PCRK2-HA,

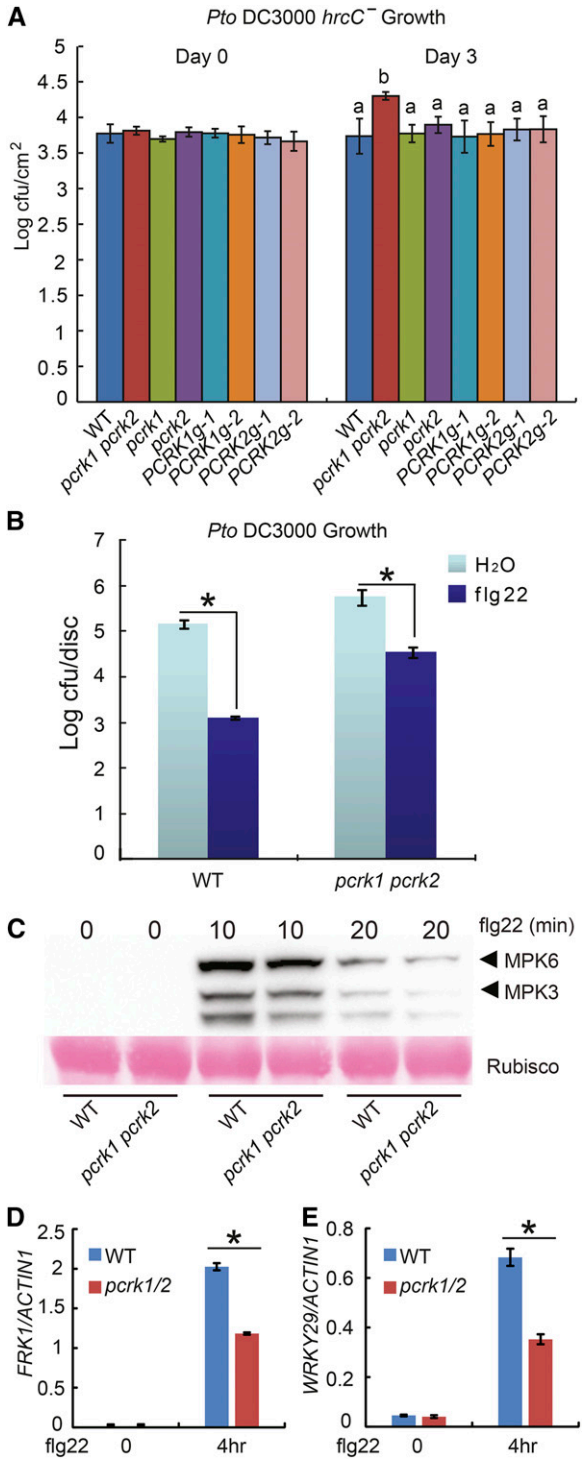

Figure 3. PCRK1 and PCRK2 contribute to PAMP-triggered immunity. A, Growth of *Pto* DC3000 *hrcC*⁻ on wild-type (WT), *pcrk1 pcrk2*, *pcrk1*, *pcrk2* and indicated transgenic lines. *PCRK1g-1* and *PCRK1g-2* are lines expressing *PCRK1-GFP* in *pcrk1 pcrk2* under the native promoter. *PCRK2g-1* and *PCRK2g-2* are lines expressing *PCRK2-GFP* in *pcrk1 pcrk2* under its own promoter. Four-week-old plants grown under short-day conditions were infiltrated with *Pto* DC3000 *hrcC*⁻ at a dose of OD_{600} = 0.001. Growth of the bacteria was analyzed on samples taken at 0 h and 72 h. Error bars represent standard deviations of six samples. Statistical differences among different genotypes are labeled with different letters ($P < 0.01$, ANOVA). B, flg22-Induced resistance against *Pto* DC3000 in wild type and *pcrk1 pcrk2*. Leaves of 4-week-old plants were infiltrated with H_2O or 1 μM flg22. The treated leaves were infiltrated with *Pto* DC3000 at a dose of OD_{600} = 0.001 24 h later. Growth of the bacteria was determined on samples taken 72 h postinoculation. Error bars represent standard deviations of six samples. Significant differences compared with flg22-triggered immunity in wild type: $*P < 0.01$. C, flg22-Induced MAPKs activation. Twelve-day-old seedlings grown on half-strength MS medium plates were sprayed with 10 nM flg22. Samples were harvested at 0, 10, and 20 min after flg22 treatment. Protein bands were analyzed by immunoblots using anti-Erk antibody (Cell signaling; #4370S). D and E, Induction of *FRK1* (D) and *WRKY29* (E) in wild type and *pcrk1 pcrk2*. Leaves of two-week-old seedlings grown on half-strength MS plates were sprayed with 1 μM flg22. Values were normalized to the expression of *ACTIN1*. Error bars represent standard deviations ($n = 3$). Significant differences compared with wild type: $*P < 0.01$. Experiments in this figure have been repeated at least twice with similar results.

suggesting that FLS2 and PCRK1/PCRK2 associate with each other.

To test whether FLS2 and PCRK1 interact with each other in Arabidopsis, we generated transgenic plants expressing PCRK1 with a FLAG-ZZ double tag under its own promoter. Immunoprecipitation was carried out on protein extracts from the PCRK1-FLAG-ZZ transgenic plants using IgG agarose beads. The precipitated proteins were detected by anti-FLAG and anti-FLS2 antibodies. As shown in Figure 4D, FLS2 coimmunoprecipitated with the PCRK1-FLAG-ZZ protein, confirming that FLS2 and PCRK1 associate with each other in planta.

PAMP Treatment Induces Phosphorylation of PCRK2

Protein phosphorylation plays critical roles in the activation of protein kinases during signal transduction. To test whether PCRK2 is phosphorylated upon flg22-treatment, we transiently expressed PCRK2-HA in protoplasts prepared from *fls2* and wild-type plants. After treatment with flg22, a mobility shift of PCRK2-HA was detected by western blot in wild type but not in the *fls2* mutant samples (Fig. 5A). The shifted PCRK2-HA was eliminated by treatment with λ-PMPase phosphatase, suggesting that the mobility shift was caused by phosphorylation of PCRK2. Similarly, treatment with elf18 also induces a mobility shift of PCRK2-HA in wild type but not in the *efr* mutant samples (Fig. 5B). The mobility shift is not affected by the K115E mutation predicted to eliminate the kinase activity of PCRK2 (Fig. 5, C and D).

Next we generated transgenic plants expressing PCRK2-HA under its own promoter for phosphorylation analysis. As shown in Supplemental Figure S3A, a mobility shift of PCRK2-HA was detected when the transgenic plants were treated with flg22 and the shifted PCRK2-HA was eliminated by treatment with λ-PMPase phosphatase. Similarly, treatment with elf18 also induces a mobility shift of PCRK2-HA

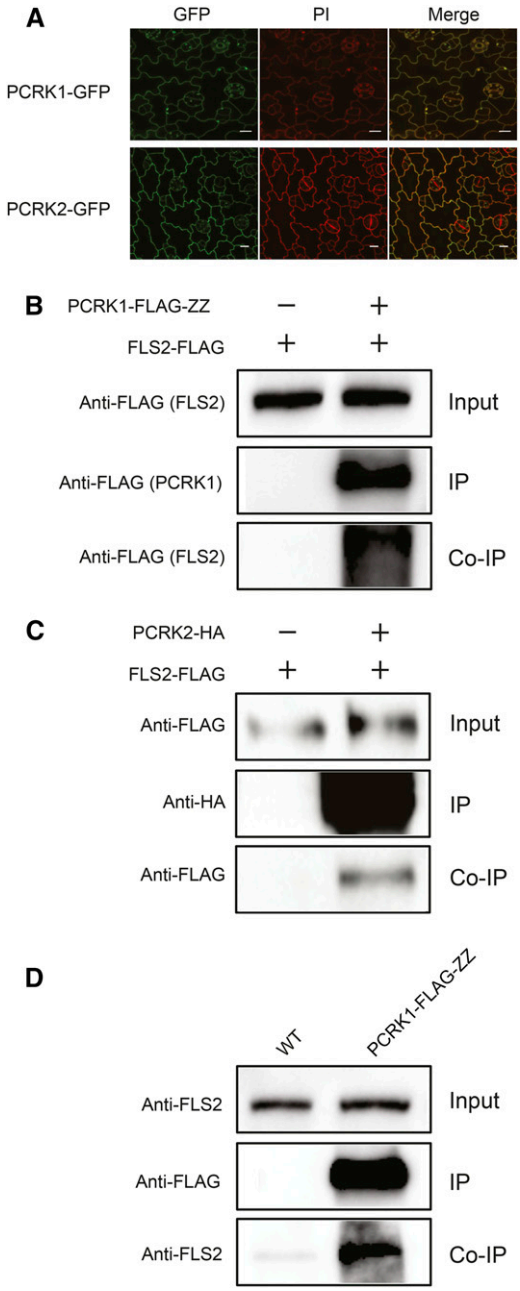

Figure 4. PCRK1 and PCRK2 associate with FLS2. A, GFP fluorescence in epidermal cells of transgenic plants expressing PCRK1-GFP or PCRK2-GFP under their own promoters. Cell wall was stained red by PI. Bar = 10 μm. B, PCRK1 associates with FLS2 in planta. PCRK1- FLAG-ZZ and FLS2-FLAG were expressed in *N. benthamiana* by infiltrating leaves of 4-week-old plants with *Agrobacterium* (OD_{600} = 0.6) carrying plasmids expressing the PCRK1 and FLS2 fusion proteins. Samples were harvested 48 h postinoculation. Total protein was immunoprecipitated with IgG agarose beads that bind the ZZ tag. PCRK1-FLAG-ZZ and FLS2-FLAG were detected by immunoblot with anti-FLAG antibody. C, PCRK2 associates with FLS2 in planta. PCRK2-HA and FLS2-FLAG were expressed in *N. benthamiana* by infiltrating leaves of 4-week-old plants with *Agrobacterium* (OD_{600} = 0.6) carrying plasmids expressing the PCRK2 and FLS2 fusion proteins. Samples were harvested 48 h postinoculation. Total protein was immunoprecipitated with anti-HA conjugated agarose beads. PCRK2-HA and FLS2-FLAG were detected by immunoblot using anti-HA and anti-FLAG antibodies, respectively. D, PCRK1 associates with FLS2 in Arabidopsis. Total protein extracted from wild type plants or PCRK1-FLAG-ZZ transgenic plants was immunoprecipitated with IgG agarose beads. PCRK1-FLAG-ZZ and FLS2 were detected by immunoblot with anti-FLAG and anti-FLS2 antibodies, respectively. Experiments in this figure have been repeated at least twice with similar results.

in the PCRK2-HA transgenic plants (Supplemental Fig. S3B).

PCRK1 and PCRK2 Regulate Pathogen-Induced SA Biosynthesis

SA plays an essential role in defense against bacterial pathogens. To test whether the enhanced disease susceptibility phenotype observed in the *pcrk1 pcrk2* double mutant is caused by reduced SA accumulation, we measured pathogen-induced SA levels in *pcrk1 pcrk2* plants. As shown in Figure 6A, *Pma* ES4326-induced SA accumulation in *pcrk1 pcrk2* is approximately one-half of the amount observed in wild-type plants. SA level after induction by *Pto* DC3000 *hrcC*$^-$ is also significantly lower in *pcrk1 pcrk2* than in wild-type plants (Fig. 6B).

In Arabidopsis, pathogen-induced SA is synthesized by ICS1 (Wildermuth et al., 2001). To determine whether PCRK1 and PCRK2 are required for pathogen-induced expression of *ICS1*, we compared the expression levels of *ICS1* in wild-type and *pcrk1 pcrk2* mutant plants. As shown in Figure 7A, the expression level of *ICS1* is considerably lower in *pcrk1 pcrk2* than in wild type after induction by *Pma* ES4326. Moreover, induction of *ICS1* expression by *Pto* DC3000 *hrcC*$^-$ is almost completely blocked in *pcrk1 pcrk2* (Fig. 7B).

PCRK1 and PCRK2 Are Required for Full Induction of *SARD1* and *CBP60g* during Pathogen Infection

Previous studies showed that transcription factors SARD1 and CBP60g regulate pathogen-induced *ICS1* expression (Wang et al., 2009; Y. Zhang et al., 2010). The expression of *SARD1* and *CBP60g* is dramatically increased after bacterial infection. As shown in Figure 7, C and D, induction of *SARD1* by *Pma* ES4326 and *Pto* DC3000 *hrcC*$^-$ is compromised in *pcrk1 pcrk2*. Induction of *CBP60g* by *Pma* ES4326 and *Pto* DC3000 *hrcC*$^-$ is also greatly reduced in *pcrk1 pcrk2* compared to wild-type plants (Fig. 7, E and F), suggesting that PCRK1 and PCRK2 regulate pathogen-induced induction of *SARD1* and *CBP60g*.

PCRK1 and PCRK2 Contribute to the Induction of *ALD1* and *FMO1* during Pathogen Infection

ALD1 and *FMO1* are two target genes of SARD1 and CBP60g (Sun et al., 2015). SARD1 and CBP60g are required for induction of *ALD1* and *FMO1* following pathogen infection. To determine whether the expression

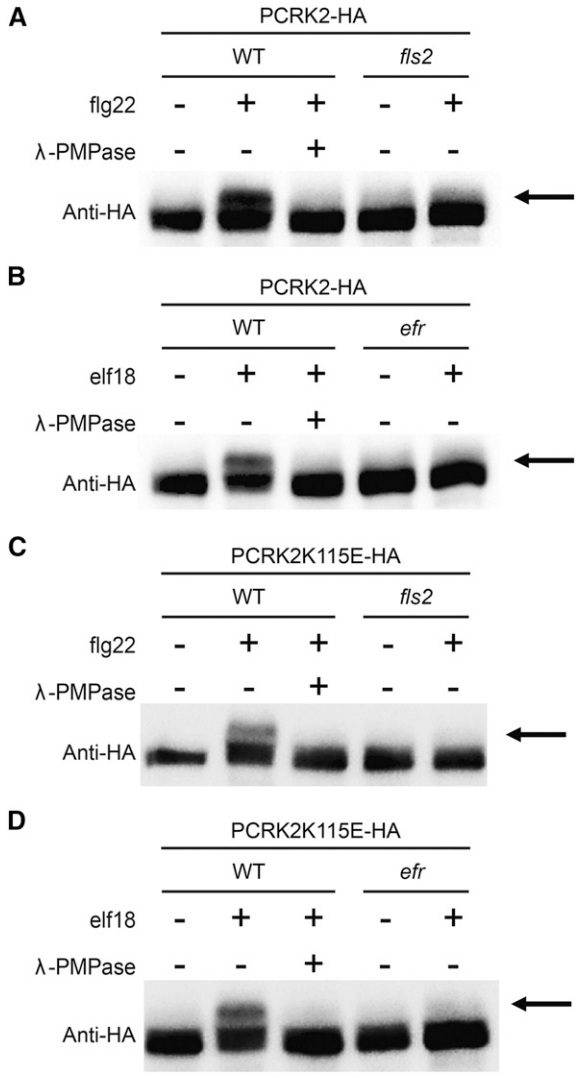

Figure 5. flg22 and elf18 induce rapid phosphorylation of PCRK2 and PCRK2^{K115E}. A and B, Rapid phosphorylation of PCRK2 induced by flg22 (A) and elf18 (B). C and D, Rapid phosphorylation of PCRK2^{K115E} induced by flg22 (C) and elf18 (D). Arabidopsis mesophyll protoplasts isolated from wild type, *fls2*, or *efr* plants were transformed with 35S-PCRK2-HA or 35S-PCRK2^{K115E}-HA constructs. After incubation for 16 to 20 h, transformed protoplasts were treated with 1 μM flg22 or 1 μM elf18 for 15 min. Total protein extracts were treated with or without λ-PMPase phosphatase and detected by immunoblot using the anti-HA antibody to analyze the phosphorylation of PCRK2^{K115E}. Similar results were obtained in two independent experiments.

of *ALD1* and *FMO1* is affected in *pcrk1 pcrk2*, we compared the expression levels of *FMO1* and *ALD1* in *pcrk1 pcrk2* and wild-type plants. As shown in Figure 8, A and B, the expression levels of *ALD1* after *Pma* ES4326 and *Pto* DC3000 *hrcC*$^-$ infection are considerably lower in *pcrk1 pcrk2* than in wild-type plants. Similarly, induction of *FMO1* by *Pma* ES4326 and *Pto* DC3000 *hrcC*$^-$ is also significantly reduced in *pcrk1 pcrk2* compared to wild-type plants (Fig. 8, C and D).

DISCUSSION

Arabidopsis has 147 RLCKs that are divided into 11 subfamilies (Shiu and Bleecker, 2001). Several members in subfamily VII including BIK1, PBL1, and PBL27 have been shown to function as important signaling components in plant immunity (Lu et al., 2010; J. Zhang et al., 2010; Kadota et al., 2014; Li et al., 2014; Ranf et al., 2014; Shinya et al., 2014). In this study, we found two additional members in this subfamily, PCRK1 and PCRK2, which are also required for plant defense

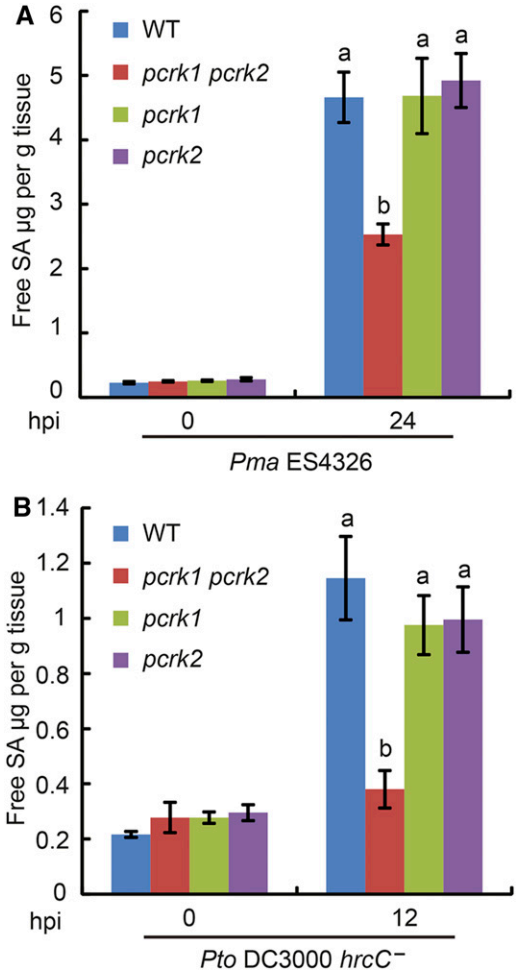

Figure 6. Pathogen-induced salicylic acid biosynthesis is reduced in *pcrk1 pcrk2*. A, SA levels in wild type, *pcrk1-2*, *pcrk2-1*, and *pcrk1 pcrk2* induced by *Pma* ES4326. Five-week-old plants grown under short-day conditions were infiltrated with *Pma* ES4326 at a dose of OD_{600} = 0.01. Samples were harvested at 0 and 24 h after infiltration. B, SA levels in wild type, *pcrk1-2*, *pcrk2-1*, and *pcrk1 pcrk2* induced by *Pto* DC3000 *hrcC*$^-$. Five-week-old plants grown under short-day conditions were infiltrated with *Pto* DC3000 *hrcC*$^-$ at a dose of OD_{600} = 0.05. Samples were harvested at 0 and 12 h after infiltration. SA was extracted and measured with HPLC as previously described (Li et al., 1999). hpi, Hours post inoculation. Error bars indicate standard deviations (n = 4). Statistical differences among different genotypes are labeled with different letters ($P < 0.01$, ANOVA). Similar results were obtained in two independent experiments.

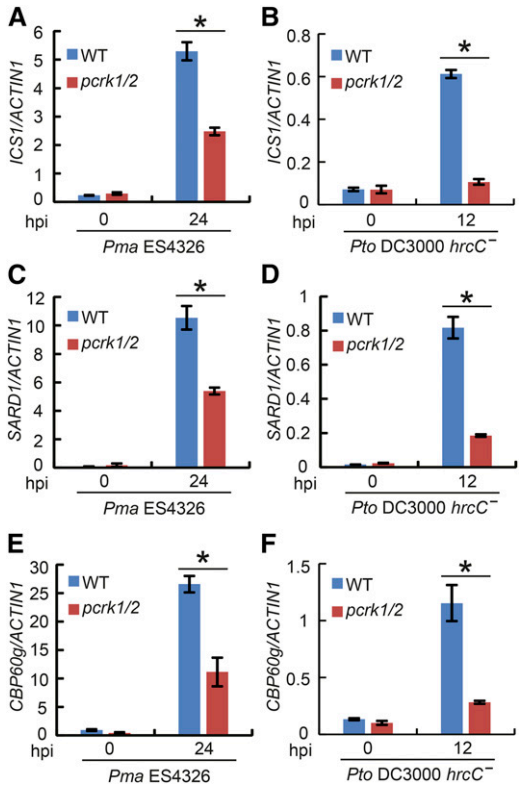

Figure 7. PCRK1 and PCRK2 contribute to induction of *ICS1*, *SARD1*, and *CBP60g* by pathogens. A and B, Induction of *ICS1* expression by *Pma* ES4326 (A) or *Pto* DC3000 *hrcC*$^-$ (B) in wild type and *pcrk1 pcrk2* (*pcrk1/2*). C and D, Induction of *SARD1* expression by *Pma* ES4326 (C) or *Pto* DC3000 *hrcC*$^-$ (D) in wild type and *pcrk1/2*. E and F, Induction of *CBP60G* expression by *Pma* ES4326 (E) or *Pto* DC3000 *hrcC*$^-$ (F) in wild type and *pcrk1/2*. Leaves of 3-week-old plants grown in short-day conditions were infiltrated with *Pma* ES4326 at a dose of OD_{600} = 0.001 or *Pto* DC3000 *hrcC*$^-$ at a dose of OD_{600} = 0.05. hpi, hours post inoculation. Values were normalized to the expression of *ACTIN1*. Error bars represent standard deviations (n = 3). Significant differences compared with wild type: *$P < 0.01$. Similar results were obtained in three independent experiments.

against pathogens. Plants lacking both PCRK1 and PCRK2 exhibit enhanced susceptibility to bacterial pathogens.

Recently, it was reported that knockout mutants of *PCRK1* support higher growth of bacterial pathogens *Pma* ES4326 and *Pto* DC3000 as well as the nonpathogenic bacteria *Pto* DC3000 *hrcC*$^-$ (Sreekanta et al., 2015). A double mutant of *PCRK1* and *PCRK2* was also generated in the study. However, the T-DNA insertion in the *pcrk2* mutant (GABI_323E12) used was downstream of the 3′-UTR of PCRK2, which may not affect the function of gene. In our study, we used a knockout mutant of *PCRK2* (*pcrk2-1*) with a T-DNA insertion in the first exon of the gene, and we consistently observed increased growth of *Pma* ES4326 and *Pto* DC3000 in the *pcrk1-1 pcrk2-1* double mutant, but not in the *pcrk1-2* and *pcrk2-1* single mutants. Some of this inconsistency is probably caused by different plant growth conditions. The difference in bacterial growth between wild type and the *pcrk1* single mutant under our growth conditions may be too small to quantify. Since the enhanced susceptibility to *Pma* ES4326, *Pto* DC3000, and *Pto* DC3000 *hrcC*$^-$ in the *pcrk1-2 pcrk2-1* double mutant can all be complemented by either *PCRK1* or *PCRK2*, *PCRK1* and *PCRK2* clearly function redundantly in the activation of plant defense against pathogens.

SA is one of the most important signaling molecules in plant defense. In the *pcrk1 pcrk2* double mutant, SA biosynthesis following infections by *Pma* ES4326 and *Pto* DC3000 *hrcC*$^-$ was greatly reduced. Since blocking SA biosynthesis leads to enhanced susceptibility to bacterial pathogens (Nawrath and Métraux, 1999), reduced pathogen-induced SA biosynthesis is most likely a major factor contributing to the increased bacterial growth in the *pcrk1 pcrk2* double mutant. However, the *pcrk1* single mutants were found to exhibit increased disease susceptibility without affecting SA accumulation (Sreekanta et al., 2015), suggesting that PCRK1 also regulates defense responses that are independent of SA.

Following pathogen infection, SA levels increase rapidly. The expression of *SARD1* and *CBP60g*, which encode transcriptions factors for activation of *ICS1* expression, is also dramatically induced after pathogen infection (Wang et al., 2009; Y. Zhang et al., 2010). How perception of pathogens leads to induction of *SARD1* and *CBP60g* and activation of SA biosynthesis

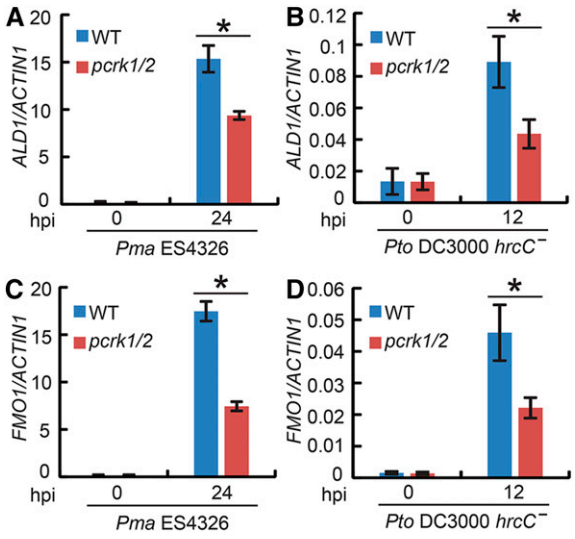

Figure 8. Pathogen-induced expression of *ALD1* and *FMO1* is compromised in *pcrk1 pcrk2*. A and B, Induction of *ALD1* expression by *Pma* ES4326 (A) or *Pto* DC3000 *hrcC*$^-$ (B) in wild type and *pcrk1 pcrk2* (*pcrk1/2*). C and D, Induction of *FMO1*expression by *Pma* ES4326 (C) or *Pto* DC3000 *hrcC*$^-$ (D) in wild type and *pcrk1/2*. Leaves of 3-week-old plants grown in short-day conditions were infiltrated with *Pma* ES4326 at a dose of OD_{600} = 0.001 or *Pto* DC3000 *hrcC*$^-$ at a dose of OD_{600} = 0.05. hpi, Hours postinoculation. Values were normalized to the expression of *ACTIN1*. Error bars represent standard deviations (n = 3). Significant differences compared with wild type: *$P < 0.01$. Similar results were obtained in three independent experiments.

is largely unknown. In the *pcrk1 pcrk2* double mutant, the expression of both *SARD1* and *CBP60g* is reduced following bacterial infection, suggesting that PCRK1 and PCRK2 regulate SA biosynthesis by influencing the expression of *SARD1* and *CBP60g*.

In addition to regulating SA biosynthesis, SARD1 and CBP60g are recently reported to regulate SA-independent defense responses as well (Sun et al., 2015). Chromatin immunoprecipitation-sequencing analysis identified many regulators of plant immunity such as *FMO1* and *ALD1* as target genes of SARD1 and CBP60g. FMO1 has been shown to function in SA-independent pathogen resistance (Bartsch et al., 2006), whereas ALD1 plays a critical role in biosynthesis of pipecolic acid and plant defense responses (Song et al., 2004; Návarová et al., 2012). Consistent with reduced expression of SARD1 and CBP60g in *pcrk1 pcrk2*, induction of *FMO1* and *ALD1* by bacterial infection is also compromised in *pcrk1 pcrk2* plants. It is likely that attenuation of SA-independent defense responses also contributes to the enhanced disease susceptibility phenotype in *pcrk1 pcrk2*.

Multiple lines of evidence suggest that PCRK1 and PCRK2 function downstream of PAMP receptors. Both PCRK1 and PCRK2 are required for resistance against the nonpathogenic bacteria *Pto* DC3000 *hrcC*$^{-}$. Pathogen resistance induced by flg22 was found to be compromised in *pcrk1 pcrk2* mutant plants. Similar to BIK1, PCRK1 and PCRK2 interact with the PAMP receptor FLS2. Consistent with its function in PTI, PCRK2 is rapidly phosphorylated after treatment with flg22 and efl18, suggesting that it is activated through phosphorylation by upstream RLKs. Unlike BIK1, which is required for PAMP-induced oxidative burst but not MAP kinase activation (Lu et al., 2010; J. Zhang et al., 2010), PCRK1 and PCRK2 are required for full activation of MAP kinases but do not appear to play a major role in the induction of ROS production, suggesting that different RLCKs may transduce signals from upstream PAMP receptors to different downstream signaling components to activate distinct defense pathways.

Since a *pcrk2* mutant predicted to lack kinase activity failed to complement the enhanced disease susceptibility phenotype in the *pcrk1 pcrk2* double mutant, the kinase activity of PCRK2 is probably critical for its function in pathogen resistance. Similarly, a *pcrk1* mutation predicted to abolish its kinase activity also compromises the function of PCRK1 in plant immunity (Sreekanta et al., 2015). These results suggest that the kinase activity of PCRK1 and PCRK2 is critical for their functions, and they probably transduce defense signals through phosphorylation of their protein substrates. Interestingly, the K115E mutation in PCRK2 also affects its accumulation in plants through an unknown mechanism. It remains to be determined whether accumulation of the similar mutant in PCRK1 (Sreekanta et al., 2015) is also reduced and whether reduced accumulation of the mutant proteins affects their functions in plant defense. BIK1 protein level was shown to be regulated by proteasome-mediated degradation (Monaghan et al., 2014). It will be interesting to determine whether PCRK2 regulates its own accumulation by inhibiting its degradation through auto-phosphorylation or phosphorylation of a component required for its degradation. A key question remaining to be answered in the future is what the target proteins of PCRK1 and PCRK2 are. Identification of the substrates of PCRK1 and PCRK2 will help us better understand how they activate the expression of SARD1 and CBP60g.

MATERIALS AND METHODS

Plant Materials and Mutant Characterization

Plants were grown at 23°C under 16 h light/8 h dark (long day) or 12 h light/12 h dark (short day). *pcrk1-2* (SALK_145629) and *pcrk2-1* (SAIL_129_D02) were obtained from the Arabidopsis Biological Resource Center. *pcrk1-2* was crossed with *pcrk2-1*, and the *pcrk1 pcrk2* double mutant was isolated from the F2 population by genotyping. All mutants used are in the Columbia-0 (Col-0) background. Four-week-old Col-0, *fls2* (SALK_141277; Xiang et al., 2008), or *efr* (SALK_044334; Zipfel et al., 2006) plants grown in short-day conditions were used for protoplast isolation.

For gene expression analysis, total RNA was extracted from 12-d-old seedlings grown on half-strength Murashige and Skoog (MS) plates using RNAiso reagent (Takara). Reverse transcription was performed using M-MLV reverse transcriptase (Takara). Real-time PCR was conducted on the cDNA with SYBR Premix Ex *Taq*II kit (Takara). Primers used for real-time PCR analysis are listed in Supplemental Table S1.

Construction of the Plasmids

For transgene complementation, a *PCRK1* genomic fragment containing its promoter and coding region was amplified by primers PCRK1-F and PCRK1-R, and a *PCRK2* genomic fragment containing its promoter and coding region was amplified with primers PCRK2-F and PCRK2-R. The fragments were cloned into pCAMBIA1305-GFP. The resulting constructs were transformed into *pcrk1 pcrk2* by *Agrobacterium*-mediated transformation. The *PCRK1* genomic fragment mentioned above was also subcloned into pCAMBIA1305-3FLAG-ZZ, and the *PCRK2* genomic fragment was also subcloned into pCAMBIA1305-3HA. The resulting constructs were transformed into wild-type Col-0 plants through *Agrobacterium*-mediated transformation.

To construct the PCRK2^{K115E} mutant plasmid, two overlapping *PCRK2* genomic fragments were amplified using primers PCRK2-F and PCRK2-K115E-R or primers PCRK2-K115E-F and PCRK2-R. The *PCRK2*^{K115E} mutant DNA containing the native promoter, and the coding region of *PCRK2* was obtained by overlapping PCR using the two DNA fragments as template and cloned into pCAMBIA1305-GFP. The *pcrk1 pcrk2:PCRK2*^{K115E} transgenic plants were generated by transforming the construct into *pcrk1 pcrk2* plants.

For coimmunoprecipitation in *Nicotiana benthamiana*, the *PCRK2* cDNA fragment was amplified by primers PCRK2-F1 and PCRK2-R1 and cloned into pCambia1300-35S-3HA. *PCRK2*^{K115E} cDNA fragment was amplified from the total cDNA of a *pcrk1 pcrk2:PCRK2*^{K115E} transgenic line and cloned into pCambia1300-35S-3HA. The *FLS2* cDNA fragment was amplified with primers FLS2-F and FLS2-R and cloned into pCambia1300-35S-3FLAG.

Confocal Microscopy

Nine-day-old seedlings grown on half-strength MS medium plates were used for GFP fluorescence analysis. Leaves were stained in propidium iodide (PI) for 3 min to visualize the cell wall. A Nikon ECLIPSE 80i confocal microscope was used to take the GFP and PI fluorescence images.

Detection of PCRK2 and PCRK2^{K115E} Phosphorylation

Arabidopsis (*Arabidopsis thaliana*) mesophyll protoplasts were isolated and transfected with pCambia1300-35S-PCRK2-3HA or pCambia1300-35S-PCRK2^{K115E}-3HA construct using a protocol described by Yoo et al. (2007). Then 15 μg of each construct was used for transforming 5×10^4 cells. After

incubation in WI buffer (0.5 M mannitol, 20 mM KCl, 4 mM MES, pH 5.7) for 16 to 20 h, transformed protoplasts were treated with 1 μM flg22 or 1 μM elf18 for 15 min. cell pellets were collected by spinning at 150*g* for 2 min. For λ-PMPase assay, cell pellet was added with 30 μL of 1× λ-PMPase buffer plus 1 mM $MnCl_2$, 1% (v/v) Triton X-100, and 1× Protease Inhibitor Cocktail (Roche) and incubated on ice for 10 min. Then 1 μL of λ-PMPase (New England Biolabs) was added to the lysate and then incubated at 30°C for 2.5 h. The reaction was stopped by adding 10 μL of 4× SDS loading buffer and followed by immunoblot analysis.

For PAMP-induced PCRK2 phosphorylation in plants, 12-d-old *PCRK2-HA* transgenic plants grown on half-strength MS medium plates were sprayed with 1 μM flg22 or 1 μM elf18. Samples were taken 0 and 10 min after treatment. For λ-PMPase assay, 0.3 g of tissue was ground thoroughly in liquid nitrogen. Then 30 μL of grinding buffer (50 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 150 mM NaCl, 0.1% (v/v) Nonidet P-40, 1 mM PMSF, and 1× Protease Inhibitor Cocktail from Roche) was subsequently added to suspend the tissue. The samples were spun at 13,200 rpm for 10 min, and 23.1 μL of the supernatant was transferred into a new tube. Then 3 μL of 10× λ-PMPase buffer, 3 μL of 10 mM $MnCl_2$, and 0.9 μL of λ-PMPase were added to the supernatant and the sample was incubated at 30°C for 2 h. The reaction was stopped by adding 30 μL of 2× SDS loading buffer and followed by immunoblot analysis.

Coimmunoprecipitation

For coimmunoprecipitation in Arabidopsis, 2-week-old Col-0 and PCRK1-FLAG-ZZ transgenic seedlings in the *pcrk1 pcrk2* background grown on half-strength MS media were collected, ground in liquid nitrogen, and suspended in 1 volume of grinding buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% (v/v) Nonidet P-40, 1 mM NaF, 1 mM Na_3VO_4, 1 mM EDTA, 1 mM dithiothreitol, 1× Protease Inhibitor Cocktail from Roche). The samples were spun at 13,200 rpm for 10 min at 4°C, and the supernatant was incubated with 50 μL of IgG beads (GE Healthcare, catalog no. 17-0969-01) overnight with constant rotation. The beads were collected by centrifugation, washed three times with grinding buffer, and eluted by adding 50 μL of 1× SDS loading buffer (preheated to 95°C) followed with 5-min incubation at room temperature. The eluted proteins were subsequently analyzed by western blot using anti-FLAG antibody (Sigma) or anti-FLS2 antibody (J. Zhang et al., 2010).

Pathogen Infection Assays

For bacterial growth assays, two fully extended leaves of each 4-week-old plant grown in short-day conditions were infiltrated with *Pma* ES4326 at a dose of OD_{600} = 0.0001 or with *Pto* DC3000 *hrcC*$^-$ at a dose of OD_{600} = 0.001. Samples were collected at 0 and 3 d after infiltration. One leaf disc was excised from each infected leaf, and two leaf discs from each plant were collected as one sample. The samples were ground, diluted serially in 10 mM $MgCl_2$, and plated on Lysogeny broth agar plates. After incubation at 28°C for 36 h, bacterial colonies were counted from selected dilutions, and the colony numbers were used to calculate colony forming units. For protection assays, leaves of 4-week-old plants were infiltrated with H_2O or 1 μM flg22 24 h before the same treated leaves were infiltrated with *Pto* DC3000 at a dose of OD_{600} = 0.001. Samples were collected 3 d post *Pto* DC3000 inoculation and analyzed as above.

Supplemental Data

The following supplemental materials are available.

Supplemental Figure S1. Flg22-induced oxidative burst in WT and *pcrk1 pcrk2*.

Supplemental Figure S2. Localization of PCRK1-GFP and PCRK2-GFP in protoplasts.

Supplemental Figure S3. flg22 and elf18 induce rapid phosphorylation of PCRK2 in transgenic plants.

Supplemental Table S1. Primers used in this work.

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