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# Calcium-dependent protein kinase 5 links calcium signaling with *N*-hydroxy-L-pipecolic acid- and *SARD1*-dependent immune memory in systemic acquired resistance

**Tiziana Guerra[1], Silke Schilling[1], Katharina Hake[1], Karin Gorzolka[2], Fabian-Philipp Sylvester[1], Benjamin Conrads[1], Bernhard Westermann[2] and Tina Romeis[1,2]**

[1]Department of Plant Biochemistry, Dahlem Centre of Plant Sciences, Institute for Biology, Freie Universität Berlin, Berlin 14195, Germany; [2]Leibniz Institute of Plant Biochemistry, Halle (Saale) 06120, Germany

Author for correspondence:
*Tina Romeis*
*Tel: +49 345 5582 1400*
*Email: tina.romeis@ipb-halle.de*

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## Summary

- Systemic acquired resistance (SAR) prepares infected plants for faster and stronger defense activation upon subsequent attacks. SAR requires an information relay from primary infection to distal tissue and the initiation and maintenance of a self-maintaining phytohormone salicylic acid (SA)-defense loop.
- In spatial and temporal resolution, we show that calcium-dependent protein kinase CPK5 contributes to immunity and SAR. In local basal resistance, CPK5 functions upstream of SA synthesis, perception, and signaling. In systemic tissue, CPK5 signaling leads to accumulation of SAR-inducing metabolite *N*-hydroxy-L-pipecolic acid (NHP) and SAR marker genes, including *Systemic Acquired Resistance Deficient 1* (*SARD1*)
- Plants of increased CPK5, but not CPK6, signaling display an 'enhanced SAR' phenotype towards a secondary bacterial infection. In the *sard1-1* background, CPK5-mediated basal resistance is still mounted, but NHP concentration is reduced and enhanced SAR is lost.
- The biochemical analysis estimated CPK5 half maximal kinase activity for calcium, K50 [$Ca^{2+}$], to be *c*. 100 nM, close to the cytoplasmic resting level. This low threshold uniquely qualifies CPK5 to decode subtle changes in calcium, a prerequisite to signal relay and onset and maintenance of priming at later time points in distal tissue. Our data explain why CPK5 functions as a hub in basal and systemic plant immunity.

## Introduction

Rapid and long-term activation of the plant immune system guarantees plant survival upon pathogen infection. Pathogen recognition initiates early intracellular responses at the local infection site, which involve changes in ion fluxes including an increase of the cytoplasmic calcium concentration, recruitment of signaling cascades, and the activation of transcriptional reprogramming. The local information of 'having been attacked' is subsequently relayed to distal plant parts. Based on phytohormone-mediated transcriptional and metabolic changes, resistance to the attacking pathogen is manifested in the entire plant. The plant may establish and maintain systemic acquired resistance (SAR) and thus prime an immune memory of 'being prepared to defend upon a subsequent attack' (Hake & Romeis, 2018).

During defense initiation, a microbial pathogen is recognized at the site of infection via nonspecies-specific pathogen-associated molecular patterns (PAMPs). PAMPs bind as ligands to specific receptors (pattern recognition receptors), intracellular defense responses become activated, and PAMP-triggered immunity (PTI) is established. In a species-specific context, a second layer of defense, effector-triggered immunity (ETI), has evolved to detect PTI-suppressing effectors from adapted pathogens. In ETI defense, response activation occurs either directly upon effector binding or indirectly by the recognition of a process of effector-target modification. ETI triggers a stronger, more long-term response and hypersensitive cell death reaction. PTI and ETI may result in 'priming' of SAR, rendering a plant prepared for repeated infection after a time gap. Primed plants show more rapid and stronger activation of defense responses upon a secondary infection even by unrelated pathogens (Conrath, 2006; Hilker *et al.*, 2015; Hake & Romeis, 2018).

Both local and systemic resistance depend on the synthesis, accumulation, and downstream signaling of the phenolic phytohormone salicylic acid (SA). SA biosynthesis is catalyzed by ISOCHORISMATE SYNTHASE 1 (ICS1), and SA transport from the chloroplast to the cytoplasm is mediated by ENHANCED DISEASE SUSCEPTIBILITY 5 (EDS5) (Wildermuth *et al.*, 2001; Serrano *et al.*, 2013). SA-dependent responses were shown to be under the control of PHYTOALEXIN DEFICIENT 4 (PAD4). PAD4, in concerted action with a lipase-like protein, ENHANCED DISEASE SUSCEPTIBILITY

1 (EDS1), is required for the expression of multiple defense responses in basal defense in PTI and ETI, as well as for ETI-associated hypersensitive response leading to systemic resistance (Glazebrook *et al.*, 1997; Wiermer *et al.*, 2005; Rietz *et al.*, 2011). SA is perceived by NON-EXPRESSOR OF PATHOGENESIS-RELATED GENES 1 (NPR1) as one of three known SA receptors (Fu *et al.*, 2012; Wu *et al.*, 2012; Yan & Dong, 2014; Manohar *et al.*, 2015; Y. Ding *et al.*, 2018). Continuous SA signaling will thus have an impact on changes in histone modification, gene expression, and metabolite production and lead to persistent plant resistance to pathogens.

This genetic framework subsequent to local pathogen attack in SA-mediated plant resistance is well characterized. Yet little is known about the switch into prolonged systemic continuous SA signaling and the establishment of SAR, a decision that brings with it a cost on development and severe growth retardation of the plant (Hake & Romeis, 2018).

The nature of plant signals that bear the information of 'having been attacked' from local infection sites to distal tissues is an ongoing matter of debate. Several metabolites have been described, whose requirement for distal SA accumulation and SAR have been proven, including nonproteinaceous amino acid pipecolic acid (Pip) (Návarová *et al.*, 2012). Pip had been recognized as a prominent signal molecule and marker for SAR. It is synthesized 12 h after pathogen infection from L-Lys through enzymes AGD2-LIKE DEFENSE RESPONSE PROTEIN 1 (ALD1) and SAR-DEFICIENT 4 (SARD4) (Bernsdorff *et al.*, 2016, P. Ding *et al.*, 2016; Hartmann *et al.*, 2017). Most recently, it was shown that Pip is the direct biosynthetic substrate for FLAVIN-DEPENDENT MONOOXYGENASE 1 (FMO1) to generate *N*-hydroxy-L-pipecolic acid (NHP) (Hartmann *et al.*, 2018). Thus, although Pip was shown to be sufficient to enforce prolonged SA biosynthesis in systemic plant tissue in an *ALD1*/*FMO1*/*ICS1* positive feed-forward loop during SAR, these recent data identified NHP as the causative SAR-inducing metabolite (Hartmann *et al.*, 2018; Hartmann & Zeier, 2019).

The manifestation of SAR requires the key transcriptional regulator SYSTEMIC ACQUIRED RESISTANCE DEFICIENT 1 (SARD1), which is induced late after a priming pathogen attack (Wang *et al.*, 2009; Truman & Glazebrook, 2012; Sun *et al.*, 2015). SARD1 is required for continuous SA synthesis in distal plant tissue, and *sard1* mutants show reduced SAR (Zhang *et al.*, 2010; Wang *et al.*, 2011), lacking a full memory of 'having been attacked'.

Calcium-dependent protein kinases (CDPKs, in *Arabidopsis thaliana*: CPKs) are calcium-sensor-protein kinase effector proteins in a single molecule. Upon calcium binding, a consensus enzyme with four EF-hand calcium-binding motifs undergoes a conformational change, adopts a kinase-active state and thus phosphorylates target proteins (Cheng *et al.*, 2002; Liese & Romeis, 2013; Schulz *et al.*, 2013). CDPKs have consequently been discussed as decoders of changes in the cytoplasmic calcium concentration [$Ca^{2+}$]. Accordingly, CDPKs were identified as positive regulators during the initiation of immune signaling, triggering local immune responses in PTI and ETI, and contributing to basal resistance (Kobayashi *et al.*, 2007, 2012; Boudsocq *et al.*, 2010; Dubiella *et al.*, 2013; Gao & He, 2013; Gao *et al.*, 2013). A negative regulatory role in immune signal initiation was demonstrated for CPK28 (Monaghan *et al.*, 2014, 2015; Wang *et al.*, 2018a).

Furthermore, CDPKs are implicated in the relay of an immune signal from local to distal sites. In particular, Arabidopsis CPK5 has been shown to drive a calcium- and reactive oxygen species (ROS)-based autopropagating signaling loop, which contributes to signal propagation from a local infection site to uninfected foliar tissue of a plant in PTI (Dubiella *et al.*, 2013; Seybold *et al.*, 2014; Hake & Romeis, 2018).

However, a specific requirement of calcium signal decoding during the manifestation of SAR in systemic plant tissue at later time points after a primary local pathogen attack is unknown. Plants overexpressing CPK5-yellow fluorescent protein (CPK5-YFP) show constitutive CPK5 enzyme activity. Enhanced CPK5 signaling leads to constitutive defense responses and increased SA-dependent disease resistance in these plants (Dubiella *et al.*, 2013). Independently, several alleles of *cpk5* have been identified in a forward genetic screen as suppressors of the autoimmune mutant *exo70B1* characterized by its resistance to multiple bacterial and fungal pathogens. *exo70B1* plants display constitutive SA-dependent defense responses reminiscent of those of enhanced CPK5 signaling. Resistance depends on the atypical immune receptor TN2, and TN2 protein interacts with CPK5, stabilizing the enzyme in a kinase-active state (Liu *et al.*, 2017). Taken together, these data suggest that CPK5 contributes to the control of long-term systemic resistance and SAR.

Here, we address in temporal and spatial resolution the role of CPK5 as a calcium-regulated key component for signaling in systemic resistance. CPK5 functions upstream of the SA signaling cascade comprising *ICS1*, *EDS5*, and *NPR1*, as well as *PAD4*, and upstream of the *ALD1*- and *FMO1*-dependent and SAR-inducing metabolite NHP-mediated immune responses in basal and systemic resistance. Enhanced CPK5, but not CPK6, signaling causes an increase in SAR at late time points in distal tissue, and this 'enhanced SAR' requires *SARD1*. In the *sard1* mutant background, CPK5-mediated enhanced basal resistance is still observed, but the accumulation of NHP is reduced, and priming is lost. CPK5 is capable of responding to even subtle calcium changes as a result of its low biochemical half maximal kinase activity for calcium (K50), at *c.* 100 nM. This feature predominantly qualifies CPK5 over CPK6 for signal propagation and for control of systemic defense manifestation through a switch in NHP- and *SARD1*-dependent SAR.

## Materials and Methods

### Plant materials and growth conditions

*Arabidopsis thaliana* Col-0 wild-type, mutants, CPK5- and CPK6-overexpressing lines and derived crosses were grown under short-day conditions (8 h : 16 h light : dark) at 20°C, 60% relative humidity. The mutant plants represent *cpk5* (SAIL_657C06) and *cpk6* (SALK_025460C). *CPK5-YFP#7* was crossed with *ics1*

(SALK_088254), *npr1-1* (Cao *et al.*, 1997), *pad4* (SALK_089936), *eds5* (SALK_091541C), *sard1-1* (SALK_138476C), *ald1* (SALK_007673), and *fmo1* (SALK_026163) and subsequently genotyped in filial generations. Primers used for genotyping are listed in Supporting Information Table S1. Plant lines *ics1*, *pad4*, *eds5*, and *sard1* were obtained from the Nottingham Arabidopsis Stock Centre (NASC), *npr1-1* was kindly provided by C. Gatz (University of Göttingen, Germany), and *ald1* and *fmo1* were kindly provided by J. Zeier (University Düsseldorf, Germany). *CPK5-YFP#7* and CPK5mut-YFP#15 (expressing a kinase-deficient variant of CPK5) have been described previously (Dubiella *et al.*, 2013).

### Generation of CPK6-YFP-overexpressing line

The coding region of full-length CPK6 (AT2G17290) was amplified from Col-0 cDNA with primers CPK6-YFP-LP and CPK6-YFP-RP. The 1636 bp CPK6 fragment was cloned into the pENTR D/TOPO vector (Invitrogen, Thermo Fisher Scientific, Dreieich, Germany) and confirmed by sequencing. The C-terminal YFP-fusion construct was generated by LR-Gateway recombination into pXCSG-YFP. Transgenic *Arabidopsis* plants were generated by the floral dip method. The flowering *Arabidopsis thaliana* Col-0 plants were dipped into *Agrobacterium tumefaciens* GV3101 pMP90RK carrying pXCSG-CPK6-YFP. Seeds were harvested and selected for BASTA (phosphinothricin, glufosinate ammonium) resistance to gain independent transformants.

### Generation of CPK5-StrepII, CPK6-StrepII and CPK5-YFP-StrepII constructs

The C-terminal CPK6 StrepII-fusion construct was generated using the pENTR D/TOPO CPK6 vector via LR-Gateway recombination into pXCSG-StrepII. The coding regions of full-length CPK5 (AT4G35310) and CPK6 were amplified from pXCSG-CPK5-StrepII, pXCS-CPK5-YFP (Dubiella *et al.*, 2013) and pXCSG-CPK6-StrepII with primers CPK5-StrepII-LP and CPK5-StrepII-RP, primers CPK5-YFP-StrepII-LP and CPK5-YFP-StrepII-RP, and CPK6-StrepII-LP and CPK6-StrepII-RP, respectively. The 1749 bp CPK5-StrepII and 1707 bp CPK6-StrepII fragments were cloned into the pET30 expression vector derivate (Novagen, Merck, Darmstadt, Germany) denoted as pET30-CTH (Glinski *et al.*, 2003) via *Nde*I and *Xho*I restriction sites to replace the His-tag. To generate pET30-CPK5-YFP-StrepII, a fragment encompassing 264 bp of CPK5 (C-terminal part) plus 720 bp YFP was cloned into pET30-CPK5-StrepII using the internal restrictions sites *Mfe*I and *Bsa*I.

### Gene expression analysis by quantitative reverse transcription polymerase chain reaction (RT-qPCR)

To analyze transcript abundance, RNA was extracted from leaf tissue using the TRIzol method (ThermoFisher, Waltham, MA). Two micrograms of RNA was incubated with RNase-free DNase (Fermentas, ThermoFisher) and subjected to reverse transcription using SuperscriptIII SuperMix (ThermoFisher) according to the manufacturer's protocols. Real-time quantitative PCR analysis was performed in a final volume of 10 µl according to the manufacturer's instructions of Power SYBR Green PCR Master Mix (Applied Biosystems, ThermoFisher) using the CFX96 system (Bio-Rad). Amplification specificity was evaluated by post-amplification dissociation curves. *ACTIN2* (At3g18780) was used as the internal control for quantification of gene expression. Primer sequences are listed in Table S2.

### Protein expression and purification

The expression vector pET30 containing CPK5-YFP-StrepII, CPK5-StrepII or CPK6-StrepII was introduced into the *E. coli* BL21 (DE3) (Stratagene, Agilent, Santa Clara, CA) strain. Bacteria were grown at 37°C in LB media containing 50 µg ml$^{-1}$ kanamycin, and protein expression was induced at $OD_{600}$ 0.5–0.8 with 0.3 mM isopropylthiol-β-galactoside and incubated for an additional 2–2.5 h at 28°C. Cells were harvested by centrifugation. Cells were lysed in lysis buffer (100 mM Tris, pH 8.0, 150 mM NaCl, 20 mM dithiothreitol (DTT), 100 µg ml$^{-1}$ avidin and 50 µl protease inhibitor cocktail for histidine-tagged proteins per 1 g *E. coli*) by incubation for 20 min with 1 mg ml$^{-1}$ lysozyme and additional sonication. Cell debris was removed by centrifugation. The supernatant was loaded onto self-packed columns containing 1 ml Strep-Tactin-Macroprep (50% suspension; IBA, Göttingen, Germany) equilibrated with wash buffer (100 mM Tris, pH 8.0, 150 mM NaCl). Flow-through was loaded on columns three times. After washing columns with 3 ml wash buffer, proteins were eluted with 3 × 500 µl elution buffer (100 mM Tris, pH 8.0, 150 mM NaCl, 2 mM *d*-desthiobiotin). Protein purity and concentration were analyzed via Bradford (protein assay; Bio-Rad) and 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and Coomassie staining.

### *In vitro* kinase assay

*In vitro* kinase assay was performed with StrepII-tag affinity purified recombinant CPK5-variants and CPK6 with substrate peptides Syntide-2 or respiratory burst oxidase homolog protein D (RBOHD) (amino acids 141–150, encompassing S148: RELRRVFSRR). The final kinase reaction (30 µl) contained *c.* 25 nM CPK5-variants or CPK6 in a volume of 5 µl and either 20 µl buffer E (50 mM Tris-HCl, pH 8.0, 2 mM DTT, 0.1 mM EDTA) and 5 µl reaction buffer (60 mM $MgCl_2$, 60 µM RBOHD S148 or 60 µM Syntide2, 60 µM ATP, 3 µCi [γ-$^{32}$P] ATP, 0.2 × buffer E, 30 µM $CaCl_2$ or 12 mM egtazic acid (EGTA)) for time course analysis or 20 µl calcium buffer (30 mM Tris, pH 8.0, 10 mM EGTA, 150 mM NaCl and different concentrations of $CaCl_2$ as indicated) and 5 µl reaction buffer without additional $CaCl_2$ or EGTA for K50 determination. The reaction was either incubated for 20 min at 22°C for K50 determination or as indicated for time course analysis. The reaction was stopped by adding 3 µl 10% phosphoric acid. Radioactively labeled phosphorylation of peptides was determined by the P81 filter-binding method as previously described (Romeis *et al.*, 2000). Kinase activities are plotted against the $Ca^{2+}$ concentration using GraphPad Prism 4 software (GraphPad Software, San Diego, CA) in a four-parameter logistic equation. Relative kinase

activities as a percentage are fitted by a four-parameter logistic equation using GraphPad Prism 4.

### In-gel kinase assay

Phosphorylation events were monitored in unstressed *Arabidopsis* 2-wk-old seedlings as described previously (Dubiella *et al.*, 2013). Seedlings were grown on 0.5 MS + 1% sucrose for 2 wk and harvested in pools of 200 mg and directly frozen in liquid nitrogen.

### Bacterial growth *in planta*

To quantify bacterial growth in *Arabidopsis*, 6-wk-old plants grown on soil under short-day conditions were used. The different *Pseudomonas syringae* strains were grown in King's B media overnight at 28°C in a shaker at 200 rpm. The cells were harvested by centrifugation at 2000 ***g***, at 4°C for 15 min, and washed twice with 10 mM $MgCl_2$. Cells were resuspended in 10 mM $MgCl_2$ to a concentration of $10^4$ cfu $ml^{-1}$ used for infiltration into the leaf. For basal resistance, *Pseudomonas syringae* pv. *tomato* (*Pst*) DC3000 was used. In the case of SAR experiments, the primary infection and treatment with either bacterial suspension at $OD_{600}$ 0.005 (avirulent *Pseudomonas syringae* pv. *maculicola* ES4326 *avr Rpm1*) or the control of 10 mM $MgCl_2$ were conducted for 2 d in three fully developed 'primary' leaves, which were cut 24 h after infiltrations. For the secondary infection, the virulent strain *P. syringae* pv. *maculicola* ES4326 was used at a concentration of $10^4$ cfu $ml^{-1}$ for infiltration. Samples were harvested at days 0 and 3 after (the secondary) infection. At least eight plants were inoculated per *Arabidopsis* line. For the analysis of systemic signal propagation, 6-wk-old *Arabidopsis* plants were used. Experiments were performed as described previously (Dubiella *et al.*, 2013).

### Synthesis and characterization of NHP

*N*-Hydroxy-L-pipecolic acid was synthesized from L-pipecolic acid and analyzed as described in detail in Methods S1, S2 and Fig. S6.

### GC analysis of amino acid derivatives

The analysis of NHP and Pip was performed as described by Hartmann *et al.* (2018) and Návarová *et al.* (2012) by GC-MS, with minor modifications as described in detail in Methods S1, S2 and Fig. S6.

### Data availability

Sequence data from this article can be found in The Arabidopsis Information Resource or GenBank/EMBL databases under the following accession numbers: *CPK5* (AT4G35310), CPK6 (AT2G17290), *ICS1* (AT1G74710), *NPR1* (AT1G64280), *PAD4* (AT3G52430), *EDS5* (AT4G39030), *SARD1* (AT1G73805), *ALD1* (AT2G13810), and *FMO1* (AT1G19250).

## Results

### SA biosynthesis and signaling are required for CPK5-mediated defense marker gene expression and enhanced pathogen resistance

To address whether SA synthesis and SA signaling are required for CPK5-mediated enhanced resistance to bacterial pathogens, we performed crosses between the CPK5-overexpressing line *CPK5-YFP#7* and defense mutants *ics1*, *eds5, npr1-1*, *pad4*, and *sard1*. The phenotypic analysis revealed that reduced rosette diameter, necrosis, and crinkled leaves, which are attributed to enhanced CPK5-activity in line #7, are no longer evident in resulting lines in the absence of SA biosynthesis (*ics1*), transport (*eds5*) or signaling (*npr1*) (Fig. 1a). This phenotypic reversion is corroborated by the expression of *PR1*, indicative of SA signaling, and *FRK1*, an flg22-responsive marker gene. *CPK5-YFP#7* is characterized by constitutive high levels of *PR1*, *FRK1*, and *ICS1* (Dubiella *et al.*, 2013) (Figs 1b, S1, S2 (left panel)). In mutant backgrounds, these basal defense gene expressions revert, as for *PR1*, nearly to Col-0 wild-type levels, and in *ics1* even fall below those (Fig. S2). Accordingly, in pathogen growth assays with the virulent pathogen *Pst* DC3000, enhanced bacterial resistance indicative of *CPK5-YFP#7* is compromised in the mutant backgrounds, showing either bacterial counts similar to the Col-0 wild-type (× *npr1*) or becoming even more susceptible than Col-0 (× *ics1*, × *eds5*) (Fig. 1f,g). The expression and presence of active CPK5-YFP protein kinase were confirmed by Western blot and in-gel kinase assay, as, for example, shown for the *ics1* and *npr1* backgrounds (Fig. S3). These data indicate that SA biosynthesis and SA downstream signaling are predominantly responsible for CPK5-mediated defense reactions.

Likewise, *PAD4*, a key component and positive regulator of defense responses in the context of ETI upon activation of TIR-NB-LRR proteins, is required for CPK5-dependent and SA-mediated bacterial resistance. In line *pad4* × CPK5-YFP#7, the plant rosette diameter, defense marker gene expression, and pathogen growth are reverted to Col-0 phenotypes (Fig. 1). *edr1* × *CPK5-YFP#7* shows reduced *PR1* and *FRK1* defense gene expression compared with *CPK5-YFP#7*, but still elevated compared with Col-0 (Fig. S2) and retains its increased higher basal resistance, accompanied by the phenotype of a small plant rosette and the development of necrosis symptoms (Fig. 1h). Compared with an increased susceptibility of *ics1* and *npr1* single mutants, the expression of CPK5-YFP in these backgrounds triggers a certain benefit of enhanced CPK5 signaling, resulting in less bacterial growth (Fig. 1f). These data hint at an additional contribution of CPK5-triggered defense activation.

### The expression of SAR marker genes depends on CPK5

We next assessed SAR marker gene expression *ALD1* and *FMO1* in a temporally and spatially defined manner using a modified systemic signaling assay. Three local (proximal) leaves were stimulated by either mock infiltration (10 mM $MgCl_2$) or infiltration

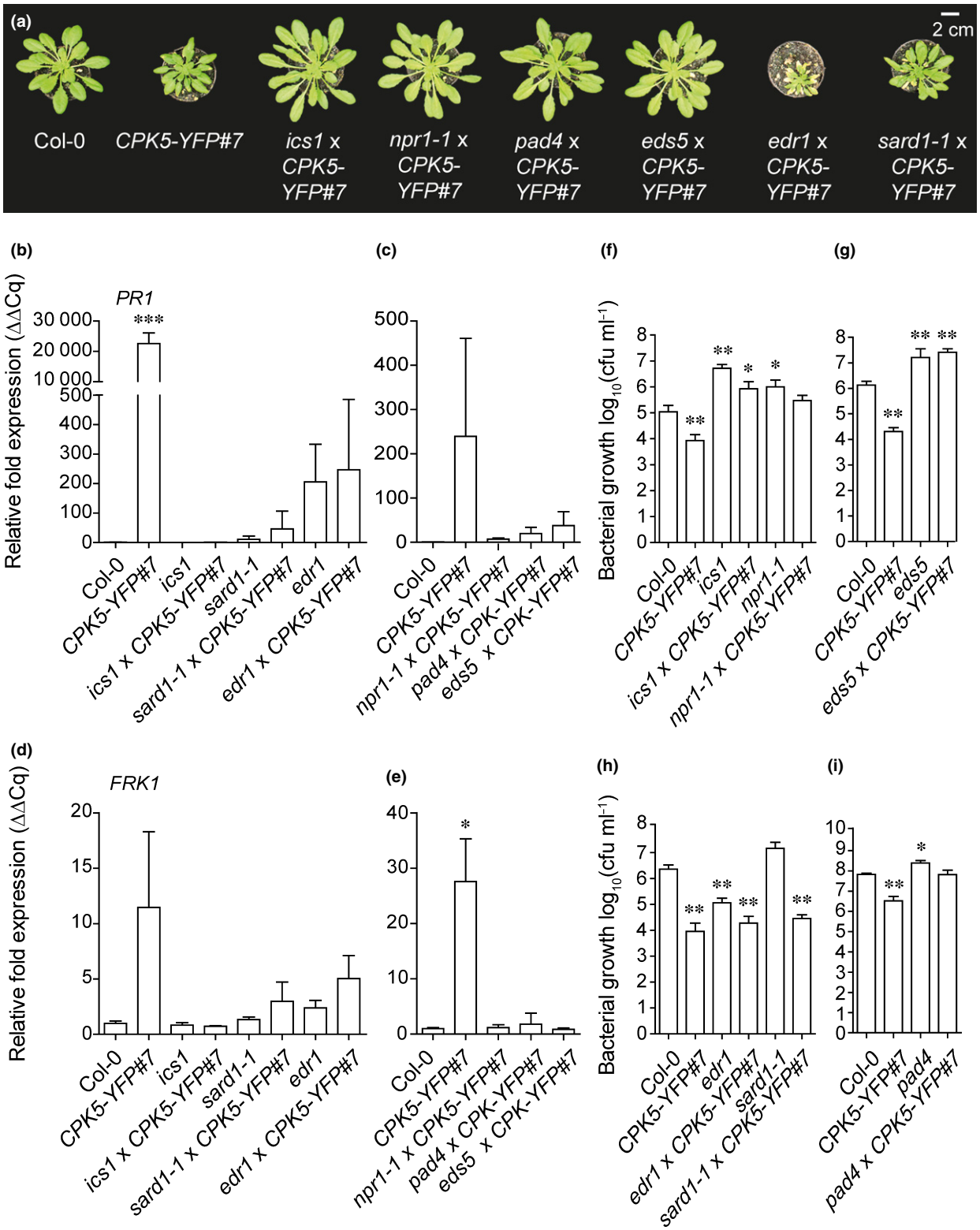


**Fig. 1** *Arabidopsis thaliana* CPK5 signaling-dependent basal pathogen resistance requires salicylic acid biosynthesis and signaling but is independent of *Systemic Acquired Resistance Deficient 1* (*SARD1*). (a) Six-week-old plants of Col-0, *CPK5-YFP#7*, and of derived crosses with *ics1*, *npr1*, *pad4*, *eds5*, *edr1*, *and sard1*. Bar, 2 cm. (b–e) Basal *PR1* and *FRK1* gene expression of plants, as shown in (a) was analyzed by quantitative reverse transcription polymerase chain reaction. Bars are means ± SEM of three biological replicates. The asterisks indicate statistically significant differences in comparison to Col-0 (one-way ANOVA, Dunnett post-test; *, $P < 0.05$; ***, $P < 0.001$;). (f–i) Six-week-old plants, as shown in (a) were inoculated with a concentration of $10^4$ cfu ml$^{-1}$ *Pseudomonas syringae* pv. *tomato* (*Pst*) DC3000. Bacterial numbers were monitored at 0 dpi (data not shown) and 3 dpi. Bars are means ± SEM of 12 biological replicates. The asterisks indicate statistically significant differences in comparison to Col-0 (one-way ANOVA, Dunnett post-test; *, $P < 0.05$; **, $P < 0.01$). The experiment was repeated three times with similar results.

with 200 nM flg22. Samples of systemic tissue were harvested 2 d after the local infiltration (2 dpi) and gene expression was monitored via RT-qPCR. Enhanced CPK5 signaling in *CPK5-YFP#7* resulted in high constitutive *ALD1* and *FMO1* expression, irrespective of whether it was mock or flg22 treatment (Fig. 2a). Likewise, *NHL10*, a specific marker gene for rapid CPK5-dependent defense signal propagation, is constitutively expressed in systemic tissue. Col-0 and *CPK6-YFP#23* do not display elevated SAR marker gene expression. Also, *cpk5* and, less prominently, *cpk6* single mutants show some reduction in basal and systemic *ALD1, FMO1*, and *NHL10* expression at 2 dpi after local flg22 stimulus (Fig. 2b). These data were further corroborated by comparing systemic gene expression in *cpk5*, *cpk6*, and *cpk5 cpk6* double mutant plants after exposure to mock, flg22, or bacterial (*Psm avrRpm1*) treatment (Fig. S4). At 2 dpi, low systemic *NHL10* expression is observed in the absence of *CPK5* in accordance with published data (Dubiella *et al.*, 2013). Likewise, flg22-induced and CPK5-dependent systemic *FMO1* and *ALD1* expression is observed, which is absent in *cpk5* and *cpk5 cpk6* plants. Interestingly, upon *Psm avrRpm1* bacterial treatment, subsequent systemic *FMO1* and *ALD1* gene expression at 2 dpi depends on both *CPK5* and *CPK6*.

## CPK5 contributes to priming and causes NHP accumulation

To address whether the observed constitutive *ALD1* gene expression correlates with SAR metabolites, we determined Pip concentrations by GC-MS analysis in Col-0 and *CPK5-YFP#7* plants. A statistically significant accumulation of Pip was measured in CPK5-overexpressing lines in the absence of pathogen exposure (Fig. S5). Pip has recently been identified as a direct biosynthetic precursor for the genuine SAR-inducing metabolite NHP, an enzymatic step catalyzed by FMO1 (Hartmann *et al.*, 2018). Therefore, we analyzed the accumulation of NHP by GC-MS analysis using chemically synthesized NHP for standardization (Fig. S6). In the absence of further pathogen-related stimulation or treatment with bacteria, NHP concentrations are constitutively elevated to *c.* 0.5 μg $mg^{-1}$ FW in *CPK5-YFP#7* plants compared with Col-0 or plants expressing *CPK6-YFP#23*. Very low NHP concentrations are observed in *cpk5 cpk6* double mutant plants (Fig. 3). Next, we conducted SAR bacterial growth assays where plants were exposed to a priming infection with avirulent *P. syringae* pv. *maculicola* ES 4326 avrRpm1 *(Psm avrRpm1)* in local (proximal) leaves, followed 2 d later by a triggering infection with virulent *Psm* ES 4326 in distal leaves (see Figs 4, S7). The reported benefit of priming as a reduced bacterial growth upon secondary infection is observed in Col-0 plants (Figs 4, 5d, 6b) (Durrant & Dong, 2004; Mishina & Zeier, 2006; Fu & Dong, 2013; Gruner *et al.*, 2013; Shah & Zeier, 2013). CPK5-YFP-expressing plants are overall more resistant to bacterial pathogens (Dubiella *et al.*, 2013) (Figs 1f–i, 5c,d, 6b). The degree of bacterial growth reduction may differ in independent experiments because external growth conditions could affect CPK5 protein abundance in individual plants (Fig. 4b).

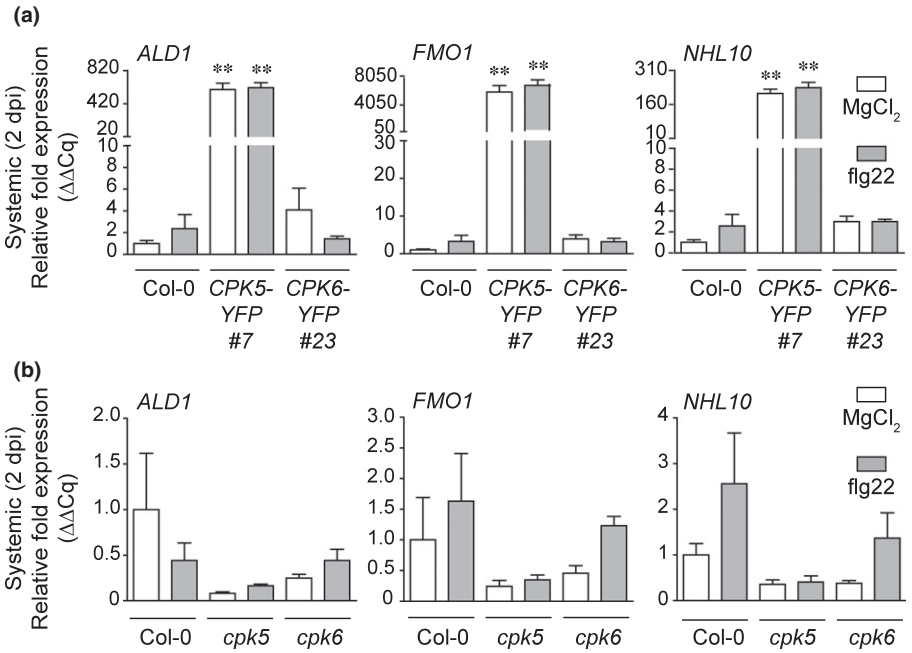


**Fig. 2** *Arabidopsis thaliana* CPK5 but not CPK6 is involved in late systemic defense signaling. (a) Enhanced CPK5 signaling results in constitutive systemic expression of *ALD1*, *FMO1*, and *NHL10* at 2 dpi. Three local leaves of 6-wk-old plants of Col-0, *CPK5-YFP#7*, and *CPK6-YFP#23* were infiltrated with mock (10 mM $MgCl_2$, open bars) or 200 nM flg22 (closed bars). After 2 d (2 dpi) three systemic leaves were harvested, and gene expression was analyzed by quantitative reverse transcription polymerase chain reaction. Bars are means ± SEM of three biological replicates. The asterisks indicate statistically significant differences in comparison to Col-0 mock (one-way ANOVA, Dunnett post-test; **, $P < 0.01$). The experiment was repeated with similar results. (b) Systemic defense gene expression at 2 dpi is reduced in *cpk5*. Col-0, *cpk5*, and *cpk6* leaves were analyzed as described in (a). Bars are means ± SEM of three biological replicates. The experiment was repeated with similar results.

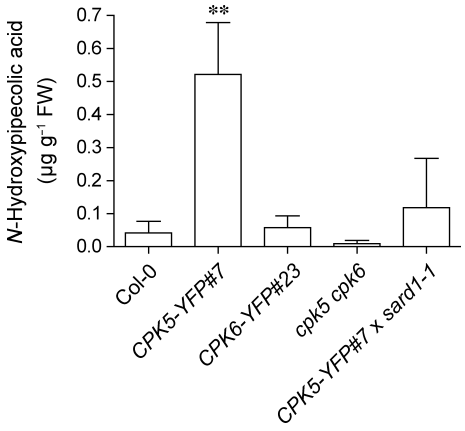


**Fig. 3** Enhanced CPK5 signaling triggers accumulation of *N*-hydroxypipecolic acid in *Arabidopsis thaliana*. Five six-week-old plants of Col-0, *CPK5-YFP#7*, *CPK6-YFP#23*, *cpk5 cpk6*, and *CPK5-YFP#7* × *sard1-1* were analyzed for their *N*-hydroxy-L-pipecolic acid contents using GC-MS. Bars are means ± SEM of five biological replicates (except for *CPK5-YFP#7* × *sard1-1* with four replicates). The asterisk indicates statistically significant differences in comparison to Col-0 (one-way ANOVA; Dunnet post-test; **, $P < 0.01$). The experiment was repeated with similar results.

Interestingly, this basal amount of CPK5-mediated resistance, already seen upon infection with the virulent strain, mimics the SAR in primed Col-0 plants. Remarkably, when *CPK5-YFP#7* is subjected to a combination of priming and triggering infections, a status of hyper-resistance (enhanced SAR) is observed, resulting in biologically even lower bacterial growth (Figs 4b, 5d, 6b). No alteration in priming is observed in plants overexpressing CPK6 (*CPK6-YFP#23*) (Fig. 4d). Also no statistically significant reduction of priming occurs in respective *cpk5* and *cpk6* single mutants (Fig. 4a,c,e). By contrast, *cpk5 cpk6* double mutant lines can no longer be primed (Fig. 4e). This is in agreement with the absence of systemic *FMO1* and *ALD1* gene expression in *cpk5* and *cpk6* single and *cpk5 cpk6* double mutant plants after priming incubation with *Psm avrRpm1* (Fig. S4). These data are consistent with previous reports in which single *cpk* mutants did not show compromised resistance to infections with bacterial pathogens (Boudsocq *et al.*, 2010; Gao *et al.*, 2013).

## CPK5-mediated priming depends on *ALD1*- and *FMO1*-signaling

We next generated crosses between *CPK5-YFP#7* and *ald1* and *fmo1* mutant lines, and double homozygous plants were selected. In both backgrounds, *CPK5-YFP#7* no longer triggered the reduced growth phenotype manifested in a smaller rosette, disordered leaf shape, and lesion development caused by enhanced CPK5 signaling (Fig. 5a). Also, basal expression of marker genes *ICS1* and *NHL10*, both elevated in *CPK5-YFP#7*, revert to the Col-0 wild-type level in the *ald1* and *fmo1* mutant backgrounds (Fig. 5b).

In standard bacterial growth assays using *Pst* DC3000 both single mutant lines, *ald1* and *fmo1,* are more susceptible to bacterial pathogens than is Col-0. In both backgrounds, the enhanced resistance of *CPK5-YFP#7* is lost. Bacterial growth in *ald1* × *CPK5-YFP#7* reverts to Col-0 wild-type values, and in *fmo1* × *CPK5-YFP#7*, bacterial growth resembles that of the *fmo1* single mutant (Fig. 5c). These data demonstrate that full CPK5-mediated resistance depends on *ALD1* and *FMO1*.

To address the dependency of CPK5-mediated SAR on *ALD1* and *FMO1*, priming experiments were conducted in temporal and spatial resolution as described earlier. Priming, as observed in Col-0 and the enhanced SAR phenotype of *CPK5-YFP#7* was absent in *ald1* × *CPK5-YFP#7* and *fmo1* × *CPK5-YFP#7* (Fig. 5d). Instead, inoculated plants showed increased susceptibility, like unprimed Col-0, or became even more susceptible (*fmo1* × *CPK5-YFP#7*). These data are consistent with the FMO1 enzyme catalyzing the synthesis of the SAR-inducing metabolite NHP from the precursor Pip (Hartmann *et al.*, 2018).

## CPK5-mediated priming depends on *SARD1*

We next assessed whether CPK5-mediated enhanced SAR requires the key transcription factor *SARD1*. Our expression analysis revealed a high constitutive level of *SARD1* transcript in line *CPK5-YFP#7* compared with Col-0. *SARD1* transcript accumulation is absent in the priming-deficient *ald1* and *fmo1* single mutants but also when CPK5-YFP is expressed in these backgrounds. Basal *SARD1* expression is slightly reduced in *cpk5* compared with Col-0 (Fig. 6a).

To investigate whether *SARD1* is required for CPK5-mediated priming and enhanced SAR, a cross between *sard1-1* and *CPK5-YFP#7* was generated and double homozygous plants were analyzed. In the *sard1-1* background, neither local nor systemic *ALD1* and *FMO1* gene expression at 2 dpi, constitutively elevated in *CPK5-YFP#7*, occurs (Fig. 6c). A similar reversion of gene expression is observed for *PR1*, *FRK1* (Fig. 1b,d), and *ICS1* (Fig. S8). Remarkably, in the *sard1-1* background, systemic *ALD1* and *FMO1* expression is low in unstimulated conditions (resembling Col-0), but upon flg22-stimulation both gene transcripts accumulate to high levels, comparable to those of the CPK5-overexpressing line (Fig. S1, right panel). These data correlate with the growth phenotype of these plants, which display a reduced rosette diameter and lesion development of *CPK5-YFP#7* in the *sard1-1* background compared with *CPK5-YFP#7* alone (Fig. 1a), correlating with a still functional constitutive basal resistance to the virulent bacterial pathogen *Pst* DC3000 (Fig. 1h).

Accordingly, in priming experiments, these *sard1-1* and *CPK5-YFP#7* plants are still able to repress bacterial growth to the level of *CPK5-YFP#7*. However, in the absence of *SARD1*, these plants are unable to induce enhanced SAR (Fig. 6b). Thus, although a basal defense is already activated by CPK5-YFP throughout the plant and in distal leaves, an additional SARD1-dependent signal, probably NHP itself, is required for enhanced SAR.

## CPK5 is a highly responsive calcium-activated enzyme

The calcium concentration at which a CDPK displays half-maximal phosphorylating kinase activity, K50 [$Ca^{2+}$], reflects an

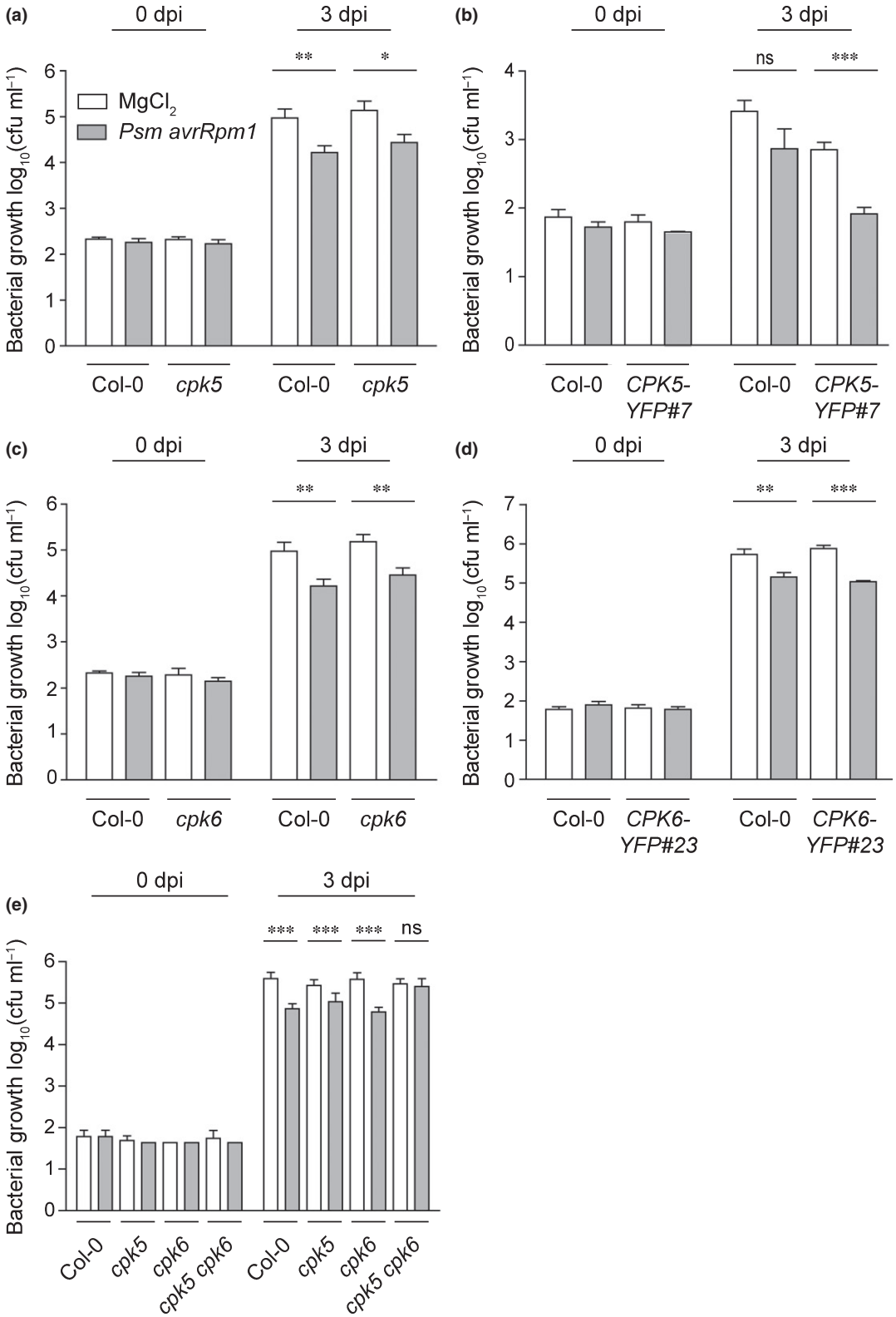


**Fig. 4** Enhanced CPK5 signaling triggers immune priming in *Arabidopsis thaliana*. (a–e) Six-week-old plants of Col-0, *cpk5* (a), *CPK5-YFP#7* (b), *cpk6* (c), *CPK6-YFP#23* (d), and *cpk5 cpk6* (e) were infiltrated with mock (10 mM $MgCl_2$; open bars) or priming infection with the avirulent bacterial strain *Pseudomonas syringae* pv. *maculicola* ES 4326 avrRpm1 *(Psm avrRpm1*) ($OD_{600}$ = 0.005; closed bars) in local leaves. After 2 d, distal leaves were subjected to triggering infection with virulent strain *Psm* ES 4326 ($10^4$ cfu $ml^{-1}$). Bacterial numbers of the triggering strain were monitored at 0 and 3 dpi. Bars are means ± SEM of 12 biological replicates. The asterisks indicate statistically significant differences in comparison to mock at 3 d postinfection (dpi; Student's *t*-test; ***, $P < 0.001$; **, $P < 0.01$; *, $P < 0.05$; ns, not significant). The experiments were repeated three times with similar results.

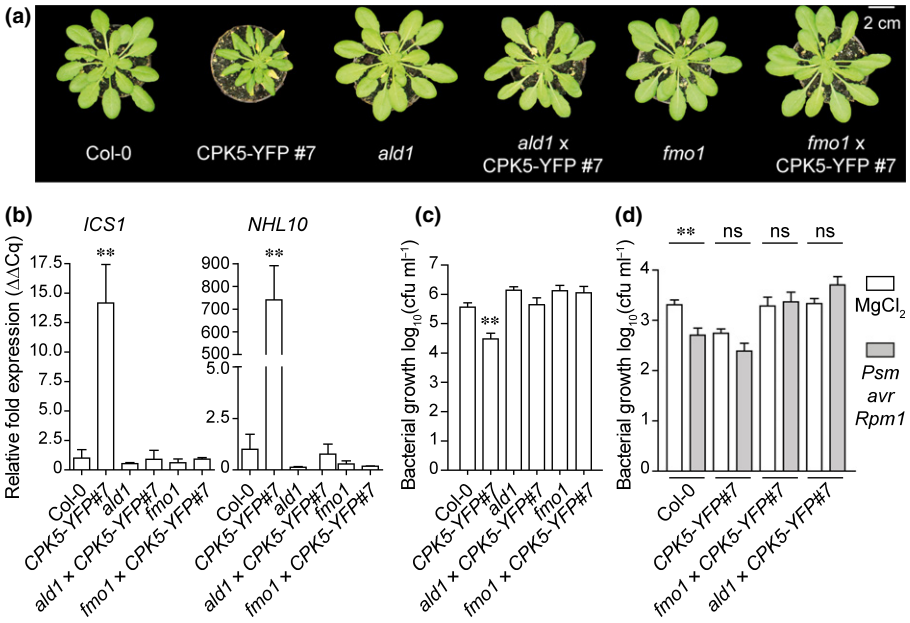


**Fig. 5** *Arabidopsis thaliana* CPK5-mediated resistance is dependent on *ALD1* and *FMO1*. (a) Six-week-old plants of Col-0, *CPK5-YFP#7*, and derived crosses with *ald1* and *fmo1*. Bar, 2 cm. (b) Basal *ICS1* and *NHL10* gene expression of plants as shown in (a) was analyzed by quantitative reverse transcription polymerase chain reaction. Bars are means ± SEM of three biological replicates. The asterisks indicate statistically significant differences in comparison to Col-0 (one-way ANOVA, Dunnett post-test; **, $P < 0.01$). (c) Six-week-old plants, as shown in (a) were inoculated with a concentration of $10^4$ cfu ml$^{-1}$ *Pseudomonas syringae* pv. *tomato* (*Pst*) DC3000. Bacterial numbers were monitored at 0 dpi (data not shown) and 3 dpi. Bars are means ± SEM of 12 biological replicates. The asterisks indicate statistically significant differences in comparison to Col-0 (one-way ANOVA, Dunnett post-test; **, $P < 0.01$). The experiment was repeated three times with similar results. (d) Six-week-old plants, as shown in (a) were subjected to mock (10 mM $MgCl_2$; open bars) or priming infection with avirulent bacterial strain *Pseudomonas syringae* pv. *maculicola* (*Psm*) ES 4326 avrRpm1 ($OD_{600}$ = 0.005; closed bars) in local leaves. After 2 d, distal leaves were subjected to triggering infection with virulent strain *Psm* ES 4326 ($10^4$ cfu ml$^{-1}$). Bacterial numbers of the triggering strain were monitored at 0 d postinfection (dpi; data not shown) and 3 dpi. Bars are means ± SEM of 12 biological replicates. The asterisks indicate statistically significant differences in comparison to mock at 3 dpi (Student's *t*-test, **, $P < 0.01$; ns, not significant). The experiment was repeated with similar results.

isoform-specific biochemical parameter and differs even between close homologs within the CDPK gene family. A low K50 for calcium is indicative of an enzyme that has adopted its catalytically active conformation at low concentrations of calcium. In standard *in vitro* kinase assays with recombinant enzymes towards synthetic peptide substrate Syntide-2, the K50 [$Ca^{2+}$] for CPK5 is as low as *c.* 102 nM, and that for CPK6 is *c.* 186 nM (Fig. 7a, c). The intracellular calcium concentration in unstimulated plants is reported to be *c.* 100 nM (Stael *et al.*, 2012; Costa *et al.*, 2018). Thus, at the resting cytoplasmic calcium concentration, CPK5, but not yet CPK6, activity is already highly responsive to subtle concentration changes. A similarly low K50 [$Ca^{2+}$] is observed with peptide substrate encompassing S148 from the CPK5 *in vivo* phosphorylation target RBOHD (Dubiella *et al.*, 2013; Kadota *et al.*, 2014) (Fig. 7b,d). Likewise, a similarly low K50 [$Ca^{2+}$] is determined for fusion protein CPK5-YFP for both substrates (Fig. S9).

Remarkably, the CPK5-YFP fusion protein displays higher kinase activity at low [$Ca^{2+}$] concentrations compared with CPK5 in a rapid kinetic assay (Fig. 7e). This is consistent with an interpretation that CPK5-YFP undergoes a more rapid (and at lower cytoplasmic calcium concentrations) calcium-induced conformational change to adapt and stabilize the active conformation compared witih the native enzyme. In accordance with this analysis, CPK5-YFP transgenic plants display constitutive biochemical (in-gel kinase) activity, and a further increase in kinase activity was induced upon flg22 treatment of these plants (Dubiella *et al.*, 2013).

## Discussion

Systemic acquired resistance represents a status of preparedness of the entire plant foliage to a broad spectrum of microbial pathogens induced by a preceding infection at a local site. This preparedness is corroborated by more rapid and vigorous activation of defense responses. Because long-term defense activation may come at the cost of growth retardation and a delay in plant development, the decision to switch in the SAR mode has to be tightly controlled. SA plays a pivotal role in plant immunity, and SA accumulation is undoubtedly a key executor to establish and maintain SAR. However, SA is not considered to be the only signal molecule to be transported through the plant. Thus, the switch to SAR requires an information relay of the 'having been attacked' perception from local to distal plant sites as well as the initiation and maintenance of an SA-accumulation loop that manifests 'being prepared when it happens again' information (Hake & Romeis, 2018).

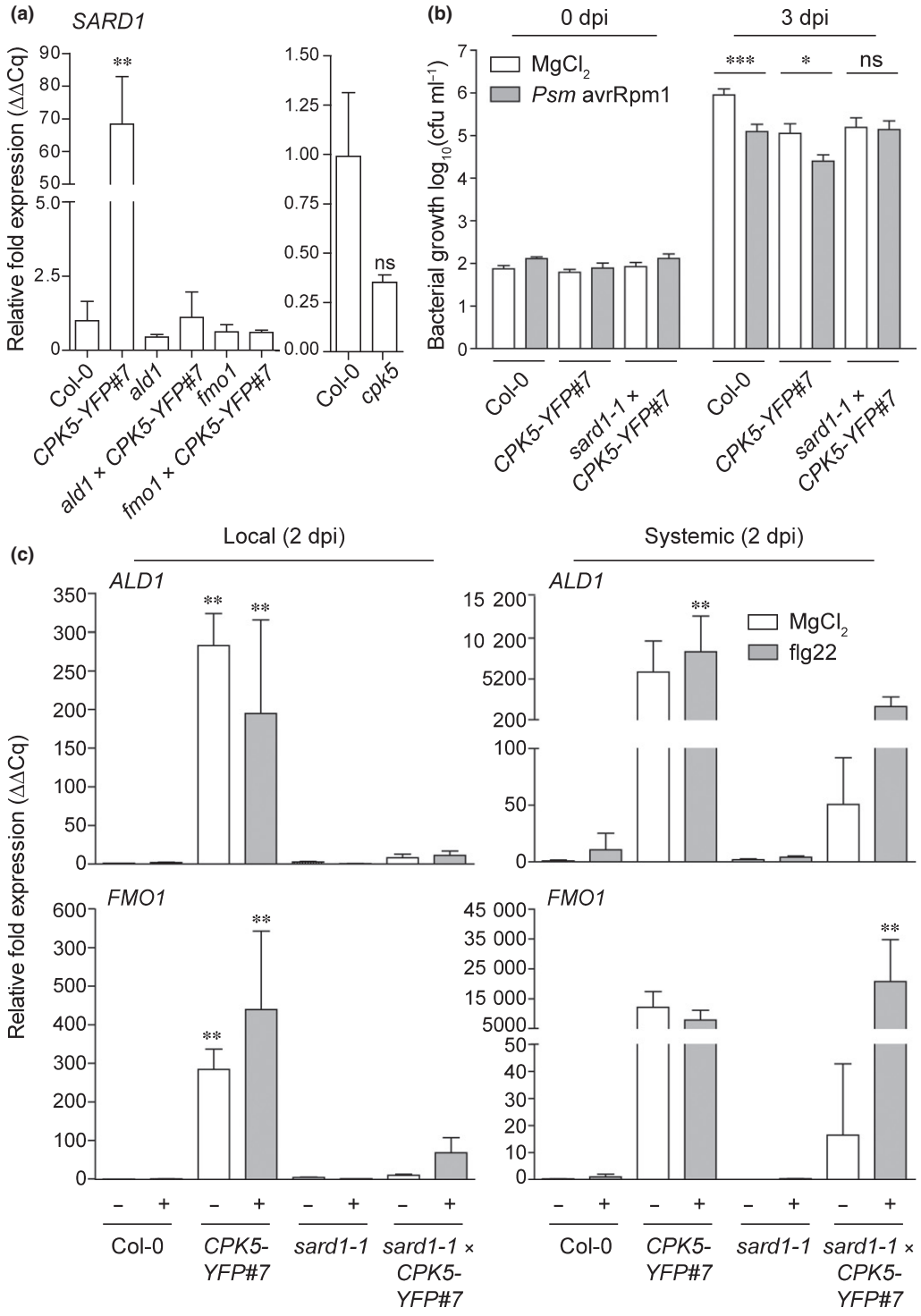


**Fig. 6** *Arabidopsis thaliana* CPK5-dependent 'enhanced systemic acquired resistance (SAR)' requires *Systemic Acquired Resistance Deficient 1* (*SARD1*). (a) Basal *SARD1* gene expression in 6-wk-old plants of Col-0, *CPK5-YFP#7*, and derived crosses with *ald1*, *fmo1* (left panel), and *cpk5* (right panel) were analyzed by quantitative reverse transcription polymerase chain reaction (qRT-PCR). Bars are means ± SEM of three biological replicates. The asterisks indicate statistically significant differences in comparison to Col-0 (one-way ANOVA, Dunnett post-test; **, $P < 0.01$; ns, not significant). (b) Six-week-old plants of Col-0, *CPK5-YFP#7*, and *sard1-1* × *CPK5-YFP#7* were subjected to mock (10 mM $MgCl_2$, open bars) or priming infection with avirulent bacterial strain *Pseudomonas syringae* pv. *maculicola* (*Psm*) ES 4326 avrRpm1 ($OD_{600}$ = 0.005; closed bars) in local leaves. After 2 d, distal leaves were subjected to triggering infection with virulent strain *Psm* ES 4326 ($10^4$ cfu $ml^{-1}$). Bacterial numbers of the triggering strain were monitored at 0 and 3 d postinfection (dpi). Bars are means ± SEM of 12 biological replicates. The asterisks indicate statistically significant differences in comparison to mock at 3 dpi (Student's *t*-test; ***, $P < 0.001$; **, $P < 0.01$; *, $P < 0.05$; ns, not significant). The experiment was repeated with similar results. (c) Local and systemic *ALD1* and *FMO1* expression of Col-0, *CPK5-YFP#7*, *sard1-1*, and *sard1-1* × *CPK5-YFP#7* were analyzed by qRT-PCR at 2 dpi after mock (10 mM $MgCl_2$, open bars) or 200 nM flg22 (closed bars) stimulus as described in Fig. 2(a). Bars are means ± SEM of three biological replicates. The asterisks indicate statistically significant differences in comparison to Col-0 mock (one-way ANOVA, Dunnett post-test; **, $P < 0.01$). The experiment was repeated with similar results.

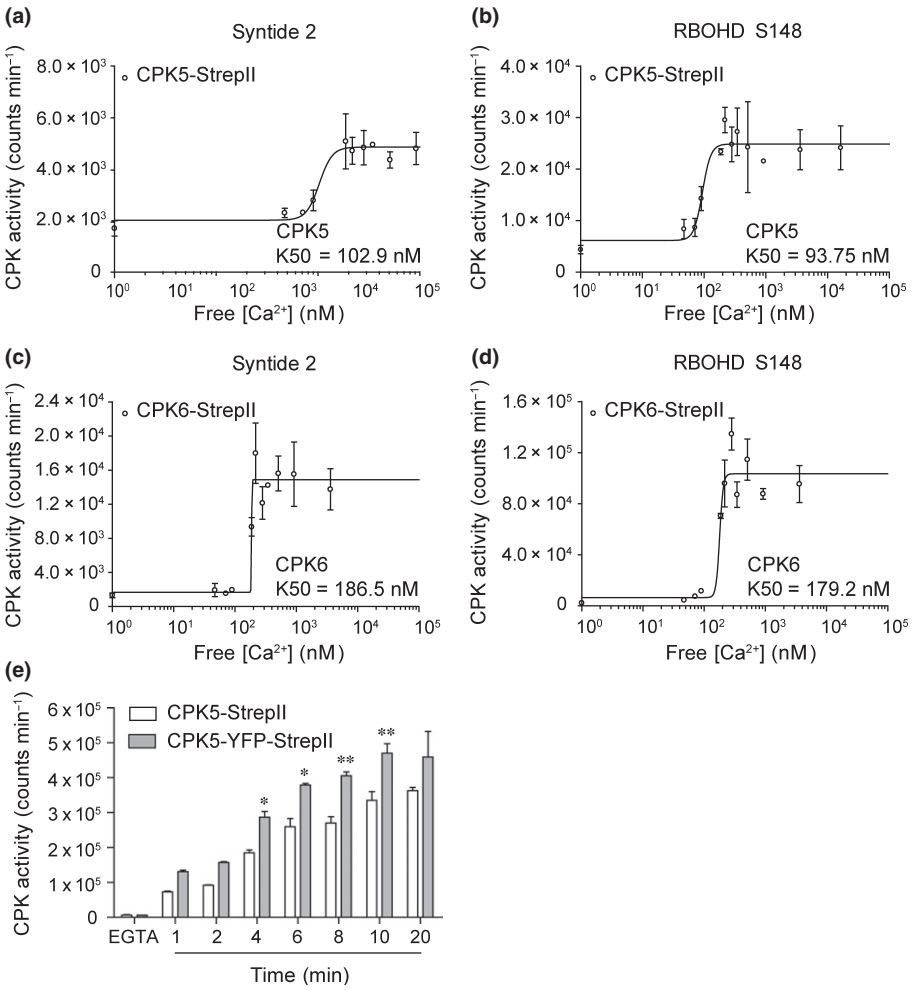


**Fig. 7** *Arabidopsis thaliana* CPK5 displays high kinase activity at low $Ca^{2+}$ concentrations. (a, b) $Ca^{2+}$-dependent protein kinase activity of CPK5-StrepII. Kinase activity of affinity-purified recombinant protein CPK5-StrepII (25 nM) was analyzed in an *in vitro* kinase assay towards 10 μM substrate peptide Syntide 2 (a) and respiratory burst oxidase homolog protein D (RBOHD) S148 (10-amino-acid peptide encompassing S148) (b), for 20 min in a series of increasing $Ca^{2+}$ concentrations as indicated and half maximal kinase activity for calcium (K50) was determined. Data are means ± SEM of two technical replicates. The experiment was repeated with similar results. (c, d) $Ca^{2+}$-dependent protein kinase activity of CPK6-StrepII. Kinase activity of affinity-purified recombinant protein CPK6-StrepII (25 nM) was analyzed in an *in vitro* kinase assay towards Syntide 2 (c) and RBOHD S148 (d), as described for (a, b). (e) $Ca^{2+}$-dependent protein kinase activity of affinity-purified recombinant CPK5-StrepII (open bars) or CPK5-YFP-StrepII (closed bars) (25 nM protein) was assessed over a fast time kinetic from 0 to 20 min in an *in vitro* kinase assay with 10 μM substrate peptide w S148 without calcium or at a fixed saturating $Ca^{2+}$ concentration of 0.5 μM. Bars are means ± SEM of two technical replicates. The asterisks indicate statistically significant differences of CPK5-YFP-StrepII in comparison to CPK5-StrepII (two-way ANOVA, Bonferroni post-test; *, $P < 0.05$; **, $P < 0.01$). The experiment was repeated with similar results.

## CPK5 signaling triggers *SARD1*-dependent resistance and mediates enhanced SAR

Plants overexpressing CPK5-YFP show increased basal resistance to the virulent bacterial pathogen *Pst*. CPK5 signaling-mediated immunity is thereby dependent on SA. In crosses of the *CPK5-YFP#7* line to mutants *ics1, eds5*, *pad4*, and *npr1-1* implicated in SA biosynthesis and signaling in PTI and ETI, CPK5-dependent defense marker gene expression and resistance to virulent *Pst* are compromised, and plant growth reverts to that of the wild-type (Fig. 1). CPK5 can therefore be placed upstream of PAD4 and the cascade of SA-dependent defense reactions.

Here we show that enhanced CPK5 signaling results in constitutive expression of SAR marker genes *ALD1* and *FMO1* as well as SAR key transcription factor *SARD1* at late time points in distal leaves, and these plants also accumulate NHP, the causative SAR-inducing metabolite (Figs 3, S6). Plants with enhanced CPK5 signaling therefore contain all essential components for an NHP- and SA-autoactivating loop required for the onset and maintenance of priming (Hartmann & Zeier, 2019). Consistently, *CPK5-YFP#7* plants show an enhanced SAR phenotype (Figs 4–6). Priming and enhanced SAR of CPK5 signaling in *CPK5-YFP#7* is entirely lost in the mutant backgrounds of *ald1* and *fmo1* (Fig. 5d) for basal and systemic resistance (Fig. 5c,d). Additionally, enhanced CPK5 signaling results in a constitutive accumulation of *SARD1* transcript, validating the constitutive high *ALD1* and *FMO1* gene expression levels but also NHP metabolite accumulation in *CPK5-YFP#7*, even in the absence of

any pathogen-related stimulation. Low systemic *SARD1, ALD1*, and *FMO1* transcript abundances are detected in *cpk5* (Figs 2b, 6a, S4). Plants that overexpress *SARD1* are reported to be more resistant to bacterial infection in basal immunity and to accumulate SA (Zhang *et al.*, 2010), similar to what is observed in CPK5-overexpressing lines (Dubiella *et al.*, 2013). Interestingly, in the *sard1-1* background, high constitutive transcript abundances of *ALD1* and *FMO1* from *CPK5-YFP#7* are significantly reduced (Fig. 6c), mimicking to a degree the *sard1* single mutant (Sun *et al.*, 2015). Likewise, accumulation of NHP observed in *CPK5-YFP#7* is reduced in the *sard1-1* background (Fig. 3). In response to the flg22 immune stimulus systemic, *ALD1* and *FMO1* expression at 2 dpi is induced to a high level, as seen for enhanced CPK5 signaling in *CPK5-YFP#7* (Fig. 6c). These data are in agreement with the interpretation that a local bacterial pathogen attack triggers basal resistance with contributions of CPK5 and CPK6. In addition, CPK5 participates in immune signal propagation and the onset of a distal switch into NHP-mediated SAR. In the absence of *SARD1*, high NHP concentrations are not maintained and SAR cannot be established.

This interpretation is mirrored by SAR bacterial growth phenotypes. In *ald1* and *fmo1* backgrounds, *CPK5-YFP#7* lost both the CPK5-mediated enhanced basal resistance and systemic enhanced SAR (Fig. 5c,d). By contrast, in a *sard1* background, CPK5-dependent basal resistance in *CPK5-YFP#7* is retained, but the memory of 'having been attacked' is lost (Fig. 6b). These data link CPK5 signaling with NHP metabolite and SARD1 transcription factor functions, and all are required to mount and maintain the primed plant state manifesting an immune memory.

SARD1 is a key regulatory transcription factor that binds to the promoters of a large number of genes, for which a positive or negative role in systemic plant resistance can be attributed, including *ICS1*. Its expression is tightly controlled by positive and negative regulation in a temporally and spatially manner and occurs late in systemic tissue (Wang *et al.*, 2011; Zheng *et al.*, 2015; Sun *et al.*, 2018; Zhou *et al.*, 2018). It is unclear yet whether *SARD1* expression is also controlled by post-transcriptional mechanisms that may directly impact *SARD1*-dependent SAR, for example by phosphorylation through CPK5.

## CPK5 links the calcium regulatory network with immune signaling

The role of calcium signaling, well characterized during local PAMP-induced immune reactions, is less clear in late systemic signaling and the switch to SAR. Regulatory calcium-binding proteins such as CMLs and AGP5, in particular, those that are under transcriptional control of *SARD1/CBP60 g* (Truman & Glazebrook, 2012; Aldon *et al.*, 2018), may depend on temporal and spatial distinct intracellular calcium conditions different from those of initiating local calcium burst. Interestingly, calmodulin-dependent transcriptional regulators CAMTA3 and CBP60a have been described as negative regulators in the control of long-term transcriptional reprogramming of defense genes (Galon *et al.*, 2008; Truman & Glazebrook, 2012; Sun *et al.*, 2015). These data imply that the intracellular calcium status is essential also in the control of SAR.

CPK5 and CPK6 belong to the CPDK subfamily I and comply with a consensus CDPK enzyme with four canonical EF-hand motifs (Cheng *et al.*, 2002). In biochemical assays, the catalytic activities of CPK5 and CPK6 are calcium-dependent. A remarkably low K50 [$Ca^{2+}$] of 102 nM was determined for CPK5 and a K50 [$Ca^{2+}$] of 186 nM was determined for CPK6 (Fig. 7a,c). These data indicate that CPK5, in particular, is most sensitive to subtle [$Ca^{2+}$] changes around the intracellular resting calcium concentration. Thus, this low K50 [$Ca^{2+}$] may explain why CPK5 (but not CPK6) is part of a signal propagation mechanism from local to distal sites via a CPK5/RBOHD-driven autoactivation circuit (Dubiella *et al.*, 2013), why overexpression of CPK5 (but not of CPK6) induces SAR, and why CPK5 (but not CPK4, CPK6, and CPK11) is required for defense responses in autoimmune mutants such as *exo70B1* (Liu *et al.*, 2017). Thus, CPK6 (here: K50 [$Ca^{2+}$] of 186 nM) and other CPKs such as CPK4 and CPK11 (reported K50 [$Ca^{2+}$] of *c.* 3 and *c.* 4 μM, respectively (Boudsocq *et al.*, 2012)), may become fully activated and contribute to defense activation upon an intracellular calcium burst, for example, as a consequence of a direct local priming pathogen attack, exposure to flg22, or a secondary (triggering) pathogen stimulation. But these enzymes may not be suited to decoding subtle calcium changes when propagating a signal or activating SAR in distal tissue. CPK6, the phylogenetically closest homolog to CPK5, has been predominantly implicated in guard cell function during the control of the stomatal aperture (Mori *et al.*, 2006; Brandt *et al.*, 2012; Ye *et al.*, 2013). *CPK6-YFP#23* plants show neither constitutive SAR marker gene expression nor elevated NHP concentrations, and these plants do not display an enhanced SAR phenotype towards bacterial pathogens (Figs 2a, 3, 4d). Consistently, ROS generation driving immune signal propagation is compromised in *cpk5* but not in *cpk6* (Dubiella *et al.*, 2013).

## CPK5 protein amount and catalytic activity contribute to the manifestation of priming

The underlying mechanism that manifests SAR is a matter of ongoing research, and the synthesis and accumulation of SAR-inducing signaling molecules, modifications at the chromatin level and control of the transcriptome may all contribute to immune memory (Conrath *et al.*, 2015; Hilker *et al.*, 2015; Martinez-Medina *et al.*, 2016; Reimer-Michalski & Conrath, 2016; Hake & Romeis, 2018; Hartmann *et al.*, 2018; Hartmann & Zeier, 2019).

A role of mitogen-activated protein kinases MPK3 and MPK6 has been demonstrated in chemically induced resistance. Priming was correlated with enhanced *MPK* transcript abundance and the accumulation of (as yet inactive) MPK3 and MPK6 proteins. Exposure to a triggering stimulus not only led to enhanced MPK biochemical activation but also increased MPK-dependent downstream signaling, resulting in more prominent defense reactions. Consistently, priming responses were compromised or lost in mutant lines of *mpk3*

and *mpk6* (Beckers *et al.*, 2009). MPK3 and MPK6 were shown to contribute to SAR via an *ALD1*-Pip regulatory loop (Wang *et al.*, 2018b). Furthermore, an interplay between MPK and CPK signaling has been demonstrated for innate immune signaling in PTI (Boudsocq *et al.*, 2010), where exemplary defense gene activation was shown to be either MPK-specific or CPK-specific, or to be controlled through joint MPK and CPK signaling. CPK5 signaling contributed to the activation of transcriptional regulators WRKY8, 28, and 48 upstream of WRKY46 and CPK5 phosphorylate recombinant WRKY28 and WRKY48 proteins (Gao *et al.*, 2013). Thus, it is conceivable that transcriptional regulators downstream of protein kinase signaling become increasingly essential in the transition from PTI and ETI to SAR. Interestingly, chromatin immunoprecipitation sequencing analysis (Chip-Seq) revealed promoter sequences recognized by SARD1 in primed plants. Among SARD1-binding sequences were promoters of signaling genes *MPK3* and *CPK4*, in addition to promoters of genes of the SA amplification loop (*ALD1*/*FMO1*/*ICS1*), and promoters of genes mediating general SA-dependent PTI and ETI defense signaling (*EDS1*, *PAD4*, *NPR1)* (Sun *et al.*, 2015). These data support the fact that the accumulation of (potentially not yet fully active) signaling proteins such as MPKs and CPKs as part of the repertoire to manifest a primed plant status.

In *CPK5-YFP#7* plants of enhanced CPK5 signaling, the enzyme accumulates to a high protein amount that is even stronger in line *CPK5-YFP#2* (Dubiella *et al.*, 2013). Additionally, the CPK5-YFP fusion protein is biochemically more active at a given low cytoplasmic [$Ca^{2+}$] level than native CPK5, which correlates with observed constitutive biochemical phosphorylation activity of CPK5-YFP in these lines (Dubiella *et al.*, 2013). Taken together, our data are consistent with the interpretation that CPK5-YFP mimics a preformed ('primed'), phosphorylation competent state (synonymous with a so-called 'protein mark'), which, upstream of NHP and *SARD1*, contributes to the enhanced SAR phenotype.

Our data are in accordance with a model where CPK5 guards the cytoplasmic calcium status in resting cells facilitated by its low K50 [$Ca^{2+}$] (Fig. 8). Following a primary pathogen attack and subsequent rise in cytoplasmic [$Ca^{2+}$], CPK5 largely acquires its open conformation, and local defenses leading to basal immunity are activated, whereby other CPKs, such as CPK6, contribute. The message of 'having been attacked' is spread via a CPK5-mediated calcium/ROS propagation mechanism and generates the command of 'having to defend' mediated through the synthesis and accumulation of SA and NHP, which are both required to switch into and maintain SAR in systemic plant tissue in dependency of *SARD1*. It is conceivable that during the immune memory, CPK5 adopts a primed enzyme state. This is probably manifested through a distinct intramolecular pattern of protein phosphorylation combined with a change in protein conformation. Upon a triggering stimulus such as a secondary bacterial attack, a more rapid CPK5 enzyme transition to the fully

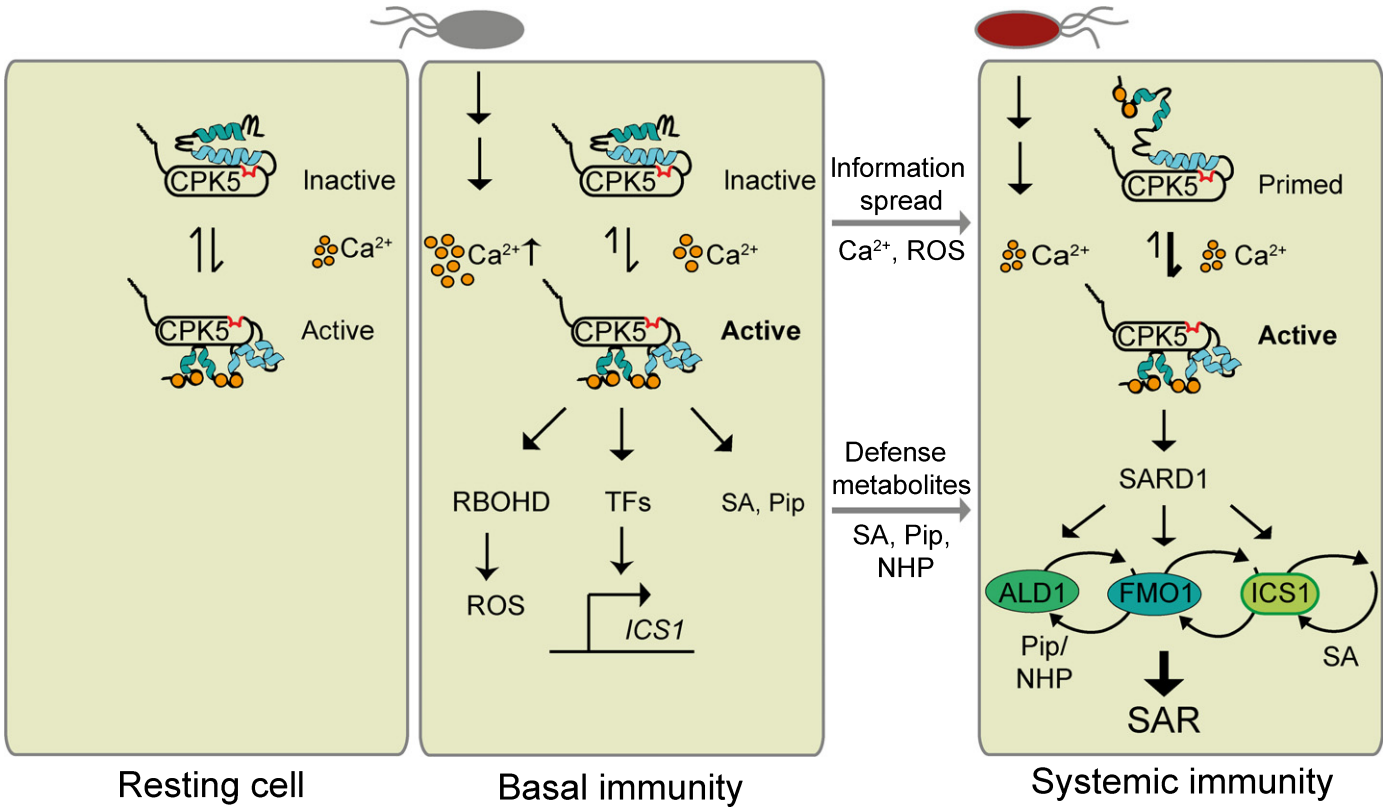


**Fig. 8** Scheme of $Ca^{2+}$-dependent CPK5 activation and function in immune responses, priming, and systemic acquired resistance (SAR). Pip, pipecolic acid; NHP, *N*-hydroxy-L-pipecolic acid; SA, salicylic acid; ROS, reactive oxygen species; TFs, transcription factors.

active state may thus lead to a faster and stronger defense activation. Also, in distal tissue, full SAR may require a subsequent contribution of CPK6 signaling activated by raising [$Ca^{2+}$] following a secondary bacterial attack (Fig. 8).

In summary, CPK5 signaling, already required for the spatial immune signal spread into the entire foliage of a plant, also controls the temporal switch via the linkage of calcium signaling with SA and NHP metabolite synthesis and with *SARD1* transcript accumulation, to induce (reversible) SAR. Whether *SARD1* is solely activated at the transcriptional level, or whether it is also activated at the post-translational level (e.g. upon direct interaction and phosphorylation of the SARD1 protein through CPK5), remains to be shown.

## Acknowledgements

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## Author contributions

TG and TR conceived and designed the research. TG, SS, KH, KG, F-PS, BC and BW, performed experiments. TG, BC, KH, KG, F-PS and TR analyzed the data. TG and TR wrote the manuscript.

## ORCID

Tiziana Guerra https://orcid.org/0000-0002-0199-7354
Katharina Hake https://orcid.org/0000-0003-0179-2516
Tina Romeis https://orcid.org/0000-0002-0838-0031
Bernhard Westermann https://orcid.org/0000-0002-6228-5991

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## Supporting Information

Additional Supporting Information may be found online in the Supporting Information section at the end of the article.

**Fig. S1** Enhanced CPK5 signaling results in *ICS1* transcript accumulation.

**Fig. S2** CPK5 signaling-dependent defense gene expression in basal pathogen resistance.

**Fig. S3** CPK5-YFP is expressed and retains constitutive protein kinase activity in mutant backgrounds.

**Fig. S4** Comparative systemic defense and SAR marker gene expression in *cpk5*, c*pk6*, and *cpk5 cpk6* mutant lines.

**Fig. S5** Enhanced CPK5 signaling results in the accumulation of pipecolic acid.

**Fig. S6** NMR and GC-MS analyses of *N*-hydroxy-L-pipecolic acid.

**Fig. S7** Experimental outline to assess temporal and spatial distinct responses required for priming of SAR.

**Fig. S8** Systemic defense gene expression at 2 dpi is reduced in *sard1-1*.

**Fig. S9** $Ca^{2+}$-dependent protein kinase activity of CPK5-YFP-StrepII.

**Methods S1** Synthesis of *N*-hydroxy-L-pipecolic acid.

**Methods S2** GC-MS analysis of *N*-hydroxy-L-pipecolic acid.

**Table S1** Primers used for genotyping of T-DNA insertion lines and cloning.

**Table S2** Primers used for RT-qPCR analysis.

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