Differential innate immune signalling via Ca^{2+} sensor protein kinases

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Abstract

Innate immunity represents the first line of inducible defense against microbial infection in plants and animals[1–3]. In both kingdoms, recognition of pathogen- or microbe-associated molecular patterns (PAMPs or MAMPs), such as flagellin, initiates convergent signalling pathways involving MAP kinase (MAPK) cascades and global transcriptional changes to boost immunity[1–4]. Although Ca^{2+} has long been recognized as an essential and conserved primary mediator in plant defense responses, how Ca^{2+} signals are sensed and relayed into early MAMP signalling is unknown[5,6]. Here, we use a functional genomic screen and genome-wide gene expression profiling to show that four calcium-dependent protein kinases (CDPKs) are Ca^{2+} sensor PKs critical to transcriptional reprogramming in plant innate immune signalling. Unexpectedly, CDPKs and MAPK cascades act differentially in four MAMP-mediated regulatory programs to control early genes involved in synthesis of defense peptides and metabolites, cell wall modifications and redox signalling. Transcriptome profile comparison suggests that CDPKs are the convergence point of signalling triggered by most MAMPs. Double, triple and quadruple *cpk* mutant plants display progressively diminished oxidative burst and gene activation induced by flg22, as well as compromised pathogen defense. In contrast to negative roles of calmodulin (CAM) and a CAM-activated transcription factor in plant defense[7,8], the present study reveals

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Ca^{2+} signalling complexity and demonstrates key positive roles of specific CDPKs in initial MAMP signalling.

Plants and animals sense invasion of potential microbial pathogens using pattern recognition receptors (PRRs) for diverse MAMPs and launch cascades of innate immune responses that are critical for fitness and survival[1–3]. Multiple MAMPs appear to trigger similar early responses via different PRRs, including Ca^{2+} influxes, MAPK cascade activation, oxidative burst and transcriptional reprogramming in various plants[1,3–6]. Bacterial flagellin epitope flg22 and other MAMPs can induce potent Ca^{2+} signatures in both cytoplasm and nucleus within minutes[9,10]. It has been shown that lipopolysaccharide (LPS)-mediated NO production is dependent on *Arabidopsis* CAM-like protein CML24[11]. However, no Ca^{2+} sensors have been identified in flg22 or other MAMP signalling, and their immediate downstream responses remain elusive.

There are three major types of known Ca^{2+} sensors in plants: CAMs/CMLs, calcineurin B-like proteins (CBLs) and CDPKs[11–14]. CDPKs represent a plant innovation encoded by a large gene family of 34 members in *Arabidopsis*[12,15] (Supplementary Fig. 1). To investigate the potential link between Ca^{2+} signatures and CDPKs in plant innate immune responses, we developed an in-gel kinase assay to detect flg22-induced endogenous Ca^{2+}-dependent PK activities using histone as a general substrate[12,15]. We observed transient activation of multiple putative CDPKs between 5 to 30 min after flg22 elicitation (Fig. 1a and data not shown). The putative 60 kD CDPKs are distinct, in molecular weight and kinetics, from the long-lasting (10 to 180 min) CDPK induced by Avr9-Cf9 interaction in cell death control in tobacco[16,17], but similar in size to the potato StCDPK4 and StCDPK5 that were recently shown to mediate oxidative burst by directly phosphorylating NADPH oxidase RBOHB (respiratory burst oxidase homolog)[18]. Significantly, flg22 activation of CDPKs was abolished in the *fls2* mutant and by Ca^{2+} blockers (La^{3+} and BAPTA) (Supplementary Fig. 2). To test whether transient Ca^{2+} signatures[9] and CDPK activation could be correlated with transcriptional activation of early flg22 responsive genes, we first selected a marker gene, *NHL10* (*NDR1/HIN1-like 10*)[19], that is highly induced by flg22 within 30 min (Fig. 1b). To simplify the *NHL10* transcription activation assay, the promoter of *NHL10* was fused to the firefly luciferase reporter gene (*LUC*) to generate a reporter for leaf protoplast transient assays, a well established cell system for MAMP signalling studies[4,20]. Importantly, flg22 activation of *NHL10-LUC* is absolutely dependent on the FLS2 receptor kinase (Fig. 1c), and is abolished by the general PK inhibitor K252a that could potentially inhibit multiple PKs in flg22 signalling (Fig. 1d). The Ca^{2+} channel blockers (La^{3+}, Gd^{3+}) that prevent influx of external calcium effectively diminished *NHL10-LUC* activation by flg22 (Fig. 1d), despite a possible Ca^{2+} release from internal stores. Thus, these data suggest that putative CDPKs could be activated by flg22 and involved in early transcriptional control downstream of the FLS2 receptor.

We designed a functional genomic screen to identify CDPK candidates and elucidate their biological functions in innate immune signalling. In *Arabidopsis*, the 34 CDPK members can be classified into four subgroups (I–IV) based on sequence similarity[12,15] (Supplementary Fig. 1). We first determined the expression patterns of *Arabidopsis CPK*

genes using real-time quantitative RT-PCR (qRT-PCR) (Supplementary Fig. 3 and Table 1) and public microarray data[21]. The 25 members expressed in leaves were tested in a functional screen for their ability to activate the Ca^{2+}-dependent flg22 reporter *NHL10-LUC* in mesophyll protoplasts. A constitutively active form of each CDPK (CPKac) was generated by deleting both C-terminal Ca^{2+} regulatory and auto-inhibitory domains[22],[23], while retaining the N-terminal variable sequences potentially important for subcellular localization[12],[15]. When each CPKac was co-expressed with *NHL10-LUC* in a protoplast transient expression assay, we discovered that only specific CPKac could induce *NHL10-LUC* more than 5-fold, mimicking flg22 (Fig. 1e). Remarkably, five of them (CPKac 4, 5, 6, 11 and 26) belong to a closely related clade in subgroup I (Supplementary Fig. 1), suggesting potential redundancy along with the functional specificity. This finding was unexpected because the best studied CDPK known to play a critical role in plant defense is the tobacco NtCDPK2 involved in gene-for-gene (Avr9-Cf9) fungal resistance[17], but its *Arabidopsis* orthologues CPK1ac and CPK2ac did not significantly induce *NHL10-LUC* expression (Fig. 1e) despite their relatively high kinase activity (Supplementary Fig. 4). Thus, different CDPKs even within the same subgroup may play distinct roles in plant defense signalling. *NHL10-LUC* induction by CPK3ac, which belongs to subgroup II (Supplementary Fig. 1), might be associated with its higher protein expression and kinase activity (Fig. 1e, Supplementary Fig. 4). The five CDPK candidates in subgroup I displayed predicted molecular weights matching the putative 60 kD CDPKs activated by flg22 in leaf cells[15] (Fig. 1a), but *CPK26* showed relatively low endogenous expression in leaves, compared to the others (Supplementary Fig. 3, Table 1). Thus, we focused our studies on CPK4, 5, 6 and 11 and demonstrated that their kinase activity was required to induce *NHL10-LUC* expression (Supplementary Fig. 5a). Their full-length (FL) forms that absolutely required Ca^{2+} for activation could not activate *NHL10-LUC* in the absence of flg22 unless Ca^{2+} was artificially introduced into the cells using a Ca^{2+} ionophore A23187 to partially mimic flg22 signalling[22] (Supplementary Fig. 5b–d).

To further elucidate the function of *CPK4, 5, 6,* and *11* in flg22 signalling, we used ATH1 whole-genome GeneChips to identify potential CDPK early target genes[24]. CPK5 and CPK11 were selected as representative of the closely related gene pairs *CPK5/6* and *CPK4/11* (Supplementary Fig. 1), and constitutively active forms were transiently expressed in protoplasts to carry out RNA expression profiling. Potential target genes of CPK5ac and CPK11ac were identified by extensive microarray data analyses using multiple algorithms, stringent filtering, and marker gene validation by qRT-PCR (Fig. 2, 3, 4a, Supplementary Fig. 6, Table 2 and Methods). Significantly, the majority (70%, 171 out of 244) of CPK5ac and CPK11ac early target genes were also regulated by flg22 within 30 to 60 min in mesophyll protoplasts, seedlings and leaves[19] (Fig. 2a, Supplementary Tables 2–6). Many (81 out of 171) of these flg22-CDPK early target genes are shared by CPK5ac and CPK11ac, suggesting functional redundancy. However, unique target genes for CPK5ac (59 out of 171) or CPK11ac (31 out of 171) were also observed. CPK5ac-specific target genes (72) showed even higher positive correlation (82%, 59 of the 72) with early flg22 responsive genes. CPK11ac but not CPK5ac appeared to repress genes in early flg22 signalling (Fig. 2a, Supplementary Tables 4, 5). Notably, many flg22-CDPK target genes encode enzymes that modulate defense-related metabolites, cell wall and redox signalling (Supplementary Tables

3–5). For instance, recent biochemical and genetic evidence supports the involvement of *CYP81F2* (cytochrome P450 monooxygenase)*, PEN2* (myrosinase) and the transcription factor *MYB51* in glucosinolate and callose metabolism in innate immune responses[25],[26]. Three genes (*PROPEP1, 2, 3*) encoding endogenous defense peptide signals were also activated early by flg22, CPK5ac and CPK11ac[27] (Supplementary Tables 3–5). Strikingly, only a few transcription factors (TFs) and no significant marker genes for abscisic acid, methyljasmonate, ethylene or salicylate could be identified[28] (Supplementary Tables 3–5, data not shown). CDPKs may play a major role in rapid transcriptional reprogramming by directly regulating activity of TFs rather than their expression[29]. Identification of early flg22 responsive genes as CPK5ac and CPK11ac target genes further supports potential roles of specific CDPKs in primary flg22 signalling.

Because Ca^{2+} influxes are common early events downstream of multiple MAMP perception[9],[10], we compared the flg22-CDPK early target genes (Fig. 2a, Supplementary Tables 3–5) to published MAMP microarray datasets generated with ATH1 GeneChips (Fig. 2b, Supplementary Table 7 and Methods). In these experiments, *Arabidopsis* seedlings or leaves were treated with diverse MAMPs from bacteria (elf26, a 26 amino acid peptide of elongation factor EF-Tu; harpin; LPS and PGN, peptidoglycan), fungi (chitin) and oomycetes (NPP1, necrosis-inducing Phytophthora protein)[10],[20]. The results clearly showed that elf26, harpin, chitin and NPP1 activated similar early genes as flg22, CPK5ac and CPK11ac (Fig. 2b). Thus, in addition to the MAMP-activated convergent MAPK cascades[4],[20], specific CDPKs also play a crucial role in early MAMP signalling downstream of diverse PRRs[3],[9],[10]. Intriguingly, LPS activated very few flg22-CDPK target genes (19%) and PGN stimulated only 79 (46%), presumably due to eliciting distinct Ca^{2+} signatures[10],[11]. As a control, we showed that ABA activated hardly any flg22-CDPK target genes despite its role in defense gene regulation[28] (Fig. 2b). Thus, Ca^{2+} influx induced by elicitors, MAMPs, Avr effectors, and hormones may activate similar as well as different downstream defense responses. Future investigations will reveal more complex and diverse Ca^{2+} signalling mechanisms in plants.

To gain new insight into potential CDPK activation during plant-microbe interactions, we compared the flg22-CDPK target genes (Fig. 2a, Supplementary Tables 3–5) with published microarray datasets of genes activated during the infection processes by bacteria (*Pseudomonas syringae pv. phaseolicola*), fungi (*Botrytis cinerea*) and oomycetes (*Phytophthora infestans*) (Fig. 2c, Supplementary Table 8 and Methods) (http://arabidopsis.org/info/expression/ATGenExpress.jsp). Identification of co-regulated genes indicated that CDPK activation of early gene expression is likely a conserved natural response when plants encounter various microbes and launch MAMP-mediated innate immune signalling.

To uncover possible connections between CDPK signalling and MAPK cascades[3],[4],[10],[30], we analyzed by qRT-PCR the expression of selected flg22-inducible genes in protoplasts expressing constitutively active CPKac or MKK4a. Unexpectedly, our analysis revealed at least four regulatory programs for early flg22-responsive genes (Fig. 3a). Genes like *FRK1* (*FLG22-INDUCED RECEPTOR KINASE1*) was MAPK-specific[4] while *PHI1* (*PHOSPHATE-INDUCIBLE1*) appeared to be CDPK-specific. Other flg22 early genes were

either activated equally by both CPKac and MKK4a, such as *NHL10*, *PER62* (*PEROXIDASE62*) and *PER4*, or were induced to much higher levels by MKK4a, e.g., *CYP82C2*, *CYP81F2*, *WAK2* (*WALL-ASSOCIATED KINASE2*) and *FOX* (*FAD-LINKED OXIDOREDUCTASE*). When CPK5ac and MKK4a were co-expressed in protoplasts, CDPK-specific and MAPK-specific genes were partially antagonized by introduction of the other pathway (Fig. 3b). In contrast, for genes co-regulated by CDPK and MAPK signalling, the two pathways could act either synergistically or independently. Unlike in other defense signalling pathways[5],[6], Ca^{2+} blockers reduced but did not eliminate MAPK or *NHL10-LUC* activation by flg22 (Supplementary Fig. 7, Fig. 1d). Consistently, CPKac did not activate MPK3 or MPK6 when co-expressed in protoplasts (Supplementary Fig. 8). These data unravelled previously unrecognized complexity and dynamics in early transcriptional reprogramming mediated by differential activities of CDPKs and MAPK cascades in plant innate immunity.

In parallel to the comprehensive gain-of-function approach, we identified loss-of-function *cpk* single mutants from the T-DNA insertion collections (Supplementary Fig. 9a), and generated higher order *cpk* mutants to conduct molecular and physiological analyses of flg22 signalling. While T-DNA insertion into *CPK5, 6* and *11* created null mutants, all the putative *cpk4* mutant lines we examined showed only partially reduced transcript levels (Supplementary Fig. 9b and data not shown). We obtained the *cpk5,6* double and *cpk5,6,11* triple mutants by genetic crosses and generated the *cpk4,5,6,11* quadruple mutant by virus-induced gene silencing (VIGS)[24] (Supplementary Fig. 9). Single and higher order *cpk* mutants did not exhibit apparent phenotypes under normal growth conditions (data not shown). Consistent with the functional redundancy of subgroup I CDPKs (Fig. 1e, 2, 3a), we did not observe altered flg22 responses or pathogen susceptibility in single *cpk* mutants (Supplementary Fig. 10 and data not shown). Further mutant characterization was performed with double, triple and quadruple *cpk* mutants (Fig. 4a–c). Although *cpk5,6* and *cpk5,6,11* lost most flg22 activation of 60 kD CDPKs (Supplementary Fig. 11), MAPK activation by flg22 was not affected in *cpk5,6* and *cpk5,6,11* mutants (Supplementary Fig. 12), confirming the gain-of-function results (Supplementary Fig. 8). Thus, CDPKs and MAPK cascades are most likely activated independently downstream of the FLS2 receptor (Fig. 4d).

Using qRT-PCR, we confirmed that flg22 induction of the MAPK-specific target gene *FRK1* was not altered, whereas all eight predicted flg22-CDPK target genes displayed differentially impaired flg22 induction in double, triple and quadruple *cpk* mutants (Fig. 4a). CPK4, 5, 6, and 11 were all important for regulation of *PHI1, NHL10, PER62* and *PER4* as their flg22 induction decreased progressively in the double, triple and quadruple *cpk* mutants (Fig. 4a). For *CYP81F2*, *WAK2* and *FOX* activation by flg22, CPK5,6 and the MAPK cascades played equal but independent roles (Fig. 3, 4a). Interestingly, CPK5 and CPK6 were essential for flg22 activation of *CYP82C2*, which uniquely required the synergistic CDPK and MAPK functions (Fig. 3b, 4a). The oxidative burst induced by flg22 was gradually reduced in *cpk5,6, cpk5,6,11* and *cpk5,6,11,4^{VIGS}* mutants to a similar level as in the flg22 signalling mutant *bak1* (Fig. 4b), suggesting a role for these CDPKs in regulating ROS production, potentially by directly phosphorylating the NADPH oxidase RBOHB[18]. We evaluated the role of CDPKs in *Arabidopsis* plant susceptibility to a bacterial pathogen,

and showed increased growth of *Pseudomonas syringae pv. tomato* (*Pst)* DC3000 in *cpk5,6* and *cpk5,6,11* mutants (Supplementary Fig. 13), likely due to impairment in multiple MAMP signalling pathways[20] (Fig 2a, b). Moreover, flg22-induced immunity against *Pst* DC3000[19],[20] was also compromised in a seedling pathogen assay in *cpk5,6* and *cpk5,6,11* mutants compared to those observed in *bak1* and *fls2* mutants (Fig. 4c). Interestingly, all four CPK-GFP fusions were localized in both cytoplasm and nucleus[29] (Supplementary Fig. 14), supporting their potentially versatile functions in response to flg22, including the activation of NADPH oxidase and the modulation of gene expression (Fig. 4d).

By integrating a functional genomic screen, transcriptome profiling and reverse genetics, we have presented compelling evidence for the identification of a specific subgroup of CDPKs that regulate MAMP-triggered immunity in *Arabidopsis*. Since CDPKs are evolutionarily conserved, these findings might offer new tools to enhance disease resistance in crop plants. The apparent functional redundancy of closely related CDPKs and their complex interactions with MAPK cascades illustrate the challenges in dissecting the MAMP signalling network. The different Ca^{2+} signatures associated with diverse MAMPs[9],[10] may potentially be decoded by distinct CDPKs and other Ca^{2+} sensors, such as CML24[11], in various subcellular locales and partially account for differential MAMP responses. We provide genomic evidence that CDPK activities are the convergence point of signalling triggered by multiple but not all MAMPs.

METHODS SUMMARY

DNA constructs

The full-length and truncated *Arabidopsis* CPK constructs were fused to the FLAG tag or GFP at the C-terminus in a plant expression vector[4],[20],[22] (Supplementary Tables 9–11). A 1632-bp promoter region of *NHL10* was fused to the *LUC* reporter gene to generate *NHL10-LUC*. The effector constructs *FLS2-HA, MKK4a-MYC, MPK3-HA* and *MPK6-HA* have been described[4],[20].

Mesophyll protoplast transient expression assay

Protoplast isolation and transient expression assays were carried out as described[4],[20]. For promoter activities, protoplasts were co-transfected with *UBQ10-GUS* as an internal control. Protein expression was monitored by immunoblot using commercially available monoclonal antibodies raised against HA (Roche), MYC (Roche) and FLAG (Sigma) epitope tags. *In vitro* and in-gel kinase assays were carried out as described[4], using MBP and histone as substrates for MAPK and CDPK activities, respectively.

Gene expression analysis

Real-time and semi-quantitative RT-PCR were carried out using the primers listed in Supplementary Tables 12 and 13. *UBQ5* (At3g62250), *TUB4* (At5g44340) and *EIF4a* (At3g13920) were used as control genes. Global gene expression analyses were performed with *Arabidopsis* ATH1 GeneChip arrays (Affymetrix) and were compared to publicly available microarray data. For details on data processing and analyses, see Supplementary Information.

Analysis of *cpk* mutants

The loss-of-function *cpk* mutants were isolated from ABRC resources (Supplementary Table 14). Higher order *cpk* mutants were generated either by crossing or VIGS. Bacterial growth assays were performed in leaves and seedlings using *Pst DC3000*. The oxidative burst was measured using a luminol-based assay[3].

METHODS

Plant material and growth conditions

We isolated homozygous T-DNA insertions mutants[31], *cpk5* (sail_657C06), *cpk6* (salk_025460) and *cpk11* (salk_054495), using seeds obtained from ABRC. The T-DNA insertions were confirmed by sequencing (Supplementary Fig. 9a). While T-DNA insertions abolished gene expression of *CPK5*, *CPK6* and *CPK11*, VIGS of *CPK4* strongly reduced gene expression of *CPK4* (Supplementary Fig. 9b). Wild-type (WT) Col-0, *fls2* (salk_093905), *bak1* (salk_116202) and *cpk* mutants were grown on soil in a growth chamber at 23°C with a 13 h photoperiod for 4 weeks before bacterial inoculation or protoplast isolation. The *cpk5,6* double and *cpk5,6,11* triple mutants were generated by genetic crosses. For in-gel kinase assay with seedlings, WT and *cpk* mutants were grown in liquid medium $0.5 \times$ MS, 0.5% sucrose, for 10 days at 23°C with a 13 h photoperiod.

Bacterial growth assays

The bacterial growth assays were performed as previously described[32],[33]. Briefly, *Arabidopsis* leaves of 4-week-old plants were infiltrated with *Pst* DC3000 at 5×10^4 cfu/ml and bacterial growth was monitored 4 days after inoculation using serial dilution plating of ground leaf disks. For seedling assay, 5-day-old seedlings grown in liquid $0.5 \times$ MS were treated with 100 nM flg22 for 1 day prior to co-cultivation with *Pst* DC3000 at 1×10^7 cfu/ml for 3 days. Surface sterilized seedlings were ground for bacterial counting as above. The experiments were repeated three times with similar results.

Effector and reporter constructs

All primers used for cloning and mutagenesis are listed in Supplementary Tables 9–11 and PCR products were checked by sequencing. For CDPK effector constructs, the coding region was amplified from *Arabidopsis* Col-0 complementary DNA, fused to FLAG epitope tag or GFP at the C-terminus, and cloned between a 35S-derived promoter and NOS terminator[4],[20],[22],[32],[34]. For the *NHL10-LUC* reporter construct, the 1632-bp sequence immediately upstream of the translation start codon of *NHL10* was amplified by PCR from *Arabidopsis* (Col-0) genomic DNA and fused to the luciferase coding region.

Mesophyll protoplast transient expression assay

The detailed protocol was described in Yoo et al (2007)[34]. Typically, 0.1 ml protoplasts at a density of 2×10^5/ml were transfected with 20 µg total DNA including different effector and reporter constructs. The ratio of effector and reporter DNA was 1:1. For promoter activities, *UBQ10-GUS* was co-transfected as an internal control. The results were presented as LUC/GUS ratio and normalized to the values obtained without the treatment or effector

expression. To induce flg22 responses, transfected protoplasts were incubated for 30 min before elicitation with 100 nM flg22 for various time periods as indicated. To examine the effect of various effectors (e.g., CDPKs) on reporter expression, transfected protoplasts were incubated for 6 h. For experiments with FL CPK5 and Ca^{2+}/A23187, protoplasts were incubated for 4 h to allow CDPK protein accumulation, and then cultured with the ionophore for 3 h. For protein expression and kinase assays, 0.2 ml protoplasts were transfected with 40 µg total DNA and collected 6 h later. All experiments showed similar results when repeated 3–5 times with duplicated or triplicated samples.

Subcellular localization

Protoplasts from cell suspension were isolated and transfected as reported[35]. GFP fluorescence was observed by confocal microscopy (LEICA SP2 inverted confocal microscope).

Protein kinase assays

Kinase assays were carried out as previously described[4],[32]. The in-gel CDPK assay was modified from the MAPK assay using histone as the substrate without cold ATP and EDTA.

RT-PCR analysis

Primers used for qRT-PCR and semi-quantitative RT-PCR are listed in Supplementary Tables 12 and 13. Total RNA was isolated from protoplasts using RNeasy Plant Mini kit (Qiagen). The cDNA was synthesized from 1 µg of total RNA using oligo(dT) primer and reverse transcriptase (Promega). RT-PCR was run for 30 cycles and *UBQ5* (At3g62250) was used as a control gene. Quantitative RT-PCR analysis was carried out with an iCycler iQ real-time PCR system using iQ SYBR Green supermix (Bio-Rad), with *TUB4* (At5g44340) and *EIF4a* (At3g13920) as control genes.

Microarray analysis

Protoplasts transfected with control DNA, CPK5ac or CPK11ac, were collected after 6 h expression. For flg22 treatment, untransfected protoplasts were incubated for 4 h before induction with 100 nM flg22 at 28°C for 30 and 60 min, and 8-day-old seedlings grown in liquid $0.5 \times$ MS were induced with 2 nM flg22 for 30 and 60 min. Total RNA was extracted using RNeasy Plant Mini kit (Qiagen) according to the manufacturer's protocol. Eight µg of total RNA were amplified, biotinylated and fragmented using the BioArray™ HighYield™ RNA transcript labelling kit (Enzo) according to the manufacturer's instructions. Hybridization to Arabidopsis ATH1 GeneChip arrays (Affymetrix) and scanning were conducted by the microarray facility of Harvard-Partners Center for Genetics and Genomics (Boston, USA). Data were pre-processed in GCOS 1.2 (Affymetrix GeneChip Operating Software) as well as being imported into FlexArray and processed with MAS5.0 (Affymetrix), dChip (www.dchip.org), and RMA (Bioconductor-R, http://www.bioconductor.org/) algorithms. Gene level variability across duplicate and triplicate arrays was then determined separately with Cyber-T, as well as SAM (Significance analysis of Microarrays) and t-test. Results of the different methods were compared based on the validated marker genes (Fig. 3, 4a). Details for microarray dataset sources, data analyses and

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identification of CDPK target genes (Supplementary Tables 2–5) are described in Supplementary Methods. The original GeneChip files will be available at Gene Expression Omnibus.

Virus-induced gene silencing

VIGS was performed as described[24],[36] using *Arabidopsis* WT, *cpk5,6* and *cpk5,6,11* mutants. The control VIGS construct using a GFP fragment has already been reported[24]. The *CPK4* VIGS construct was generated using a gene specific 500-bp fragment. The mutant plants were identified by RT-PCR (Supplementary Table 13) and the VIGS experiments were repeated 4 times with similar results.

Oxidative burst measurement

The production of ROS was measured with a luminol-based assay[37],[38]. Leaf disks from 4-week-old plants were floated on water overnight and transferred to assay tubes with 100 μl reaction buffer (20 μM luminol, 1 μg horseradish peroxidase, 100 nM flg22). Luminescence was measured every 3 min for 30 min using an LB 9507 luminometer (Berthold technologies). The experiments were repeated 4 times with similar results.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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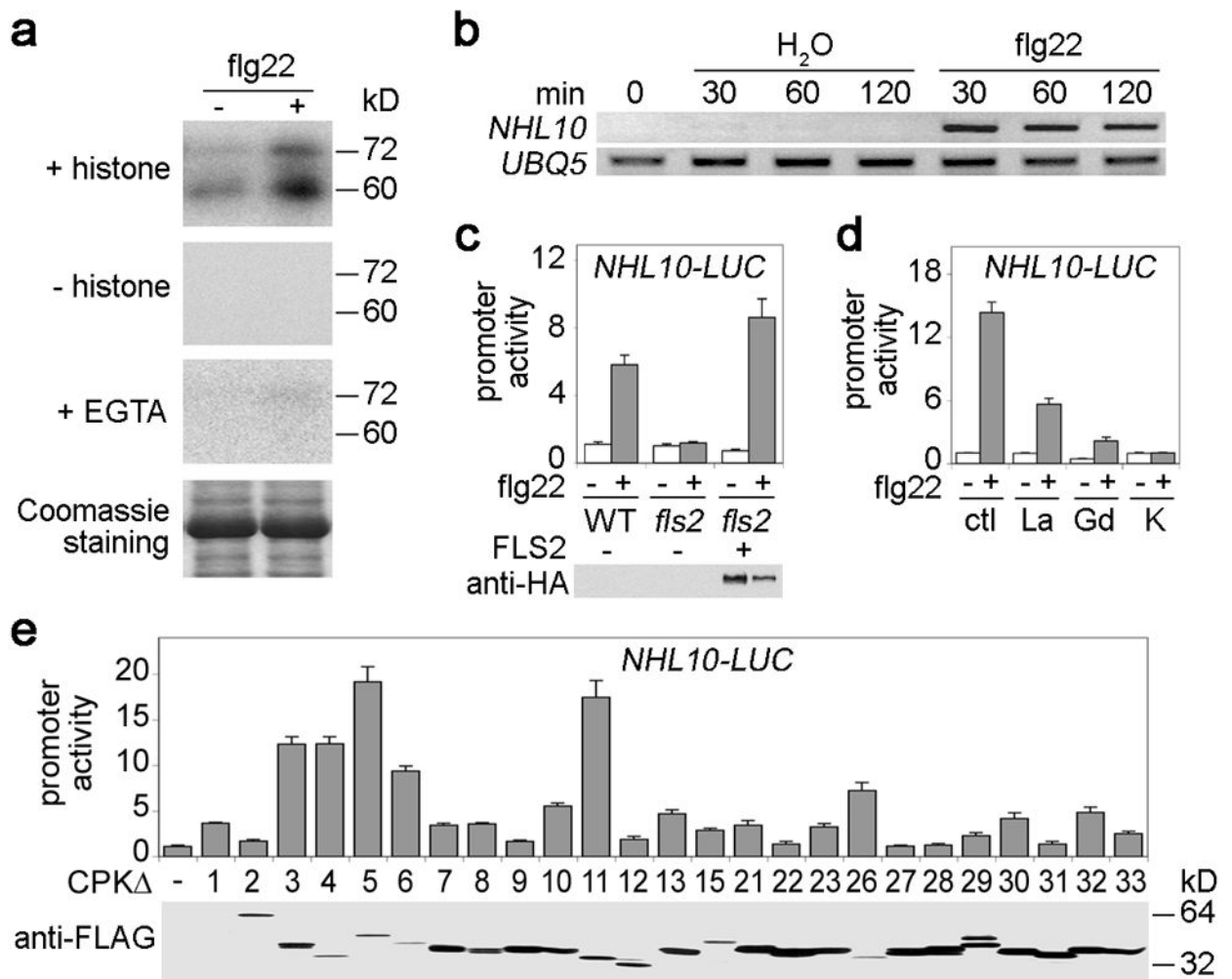
Figure 1. Functional genomic screen for CDPKs in early flg22 signalling
a, Flg22 stimulates endogenous CDPK activities. Calcium-dependent histone phosphorylation, abolished by EGTA, is stimulated by flg22 at 15 min. **b,** *NHL10* is an early flg22 responsive gene. **c,** Flg22 activation of *NHL10-LUC* is dependent on the FLS2 receptor. The *fls2* mutant is complemented by FLS2-HA. Error bars, s.d. (n=8). **d,** Flg22 signalling requires calcium and PK activity. Inhibitors: 1 mM LaCl$_3$ (La), 0.5 mM GdCl$_3$ (Gd), 2 μM K252a (K). Error bars, s.d. (n=6). **e,** Specific CPKac can activate *NHL10-LUC*. Error bars, s.d. (n=6). Mesophyll protoplasts (**a–d**) were treated with 100 nM flg22. The expression level of FLS2-HA and CPKac-FLAG was monitored by immunoblot.
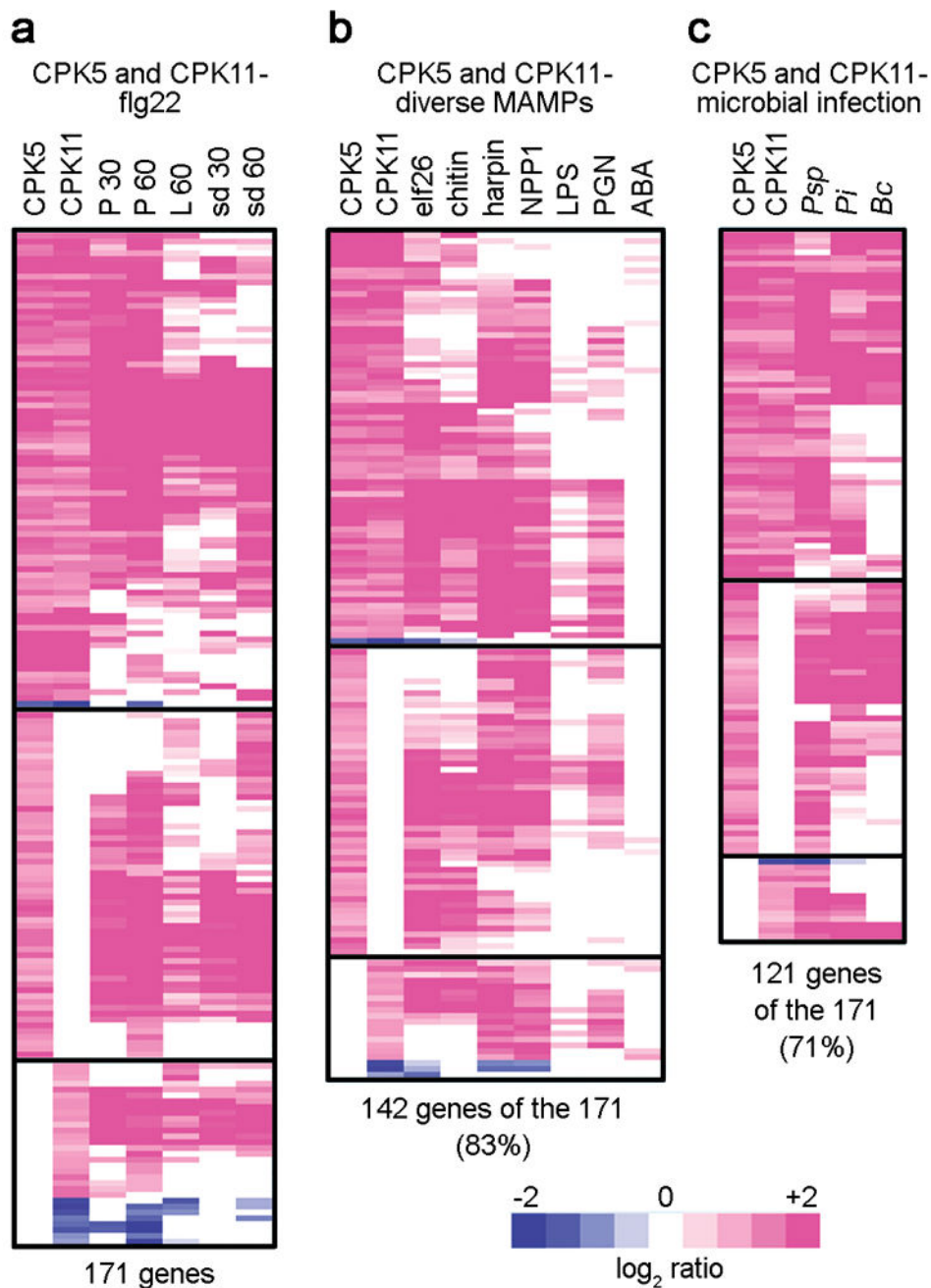
Figure 2. Transcriptome profiling of CPK5ac and CPK11ac target genes in flg22, multiple MAMP and microbial signalling

a, Hierarchical clustering analysis of CPK5ac and CPK11ac target genes and early flg22 responsive genes. The genes were identified from protoplasts transiently expressing constitutively active CPK5 or CPK11, protoplasts (P30 and P60), leaves (L60) and seedlings (sd30 and sd60) treated with flg22 for 30 and 60 min. **b,** Diverse MAMPs activate CPK5ac and CPK11ac target genes. ABA treatment was used as a control. **c,** Different microbes activate CPK5ac and CPK11ac target genes. *Psp: Pseudomonas syringae pv. phaseolicola, Pi: Phytophthora infestans, Bc: Botrytis cinerea*. Genes are clustered into 3 groups: shared
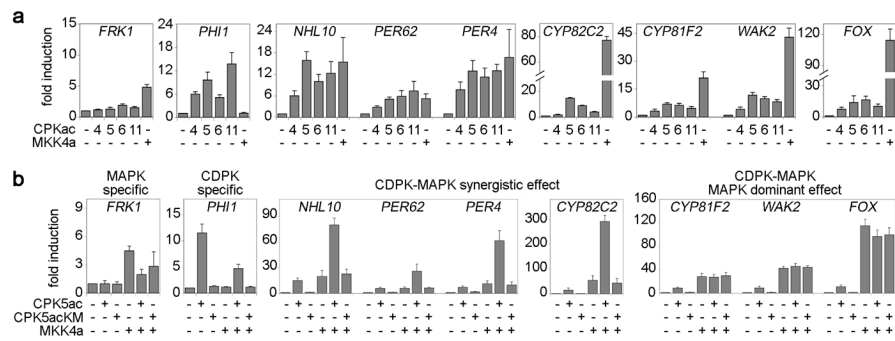
by CPK5 and CPK11 (upper panel), CPK5-specific (middle panel) and CPK11-specific (lower panel). The number of genes co-regulated by CPK5ac and/or CPK11ac is indicated at the bottom of each panel.

Figure 3. Interplay between CDPK and MAPK cascades in flg22 signalling
CDPKs and MAPKs differentially regulate four flg22-responsive gene programs. The induction of endogenous genes was monitored by qRT-PCR in protoplasts expressing individual kinases (a) or co-expressing CPKac and MKKa (b). CPKac and MKK4a: constitutively active kinases, CPK5acKM: inactive kinase mutant. Error bars, s.d. (n=6). Marker gene details are shown in the Supplementary Information.
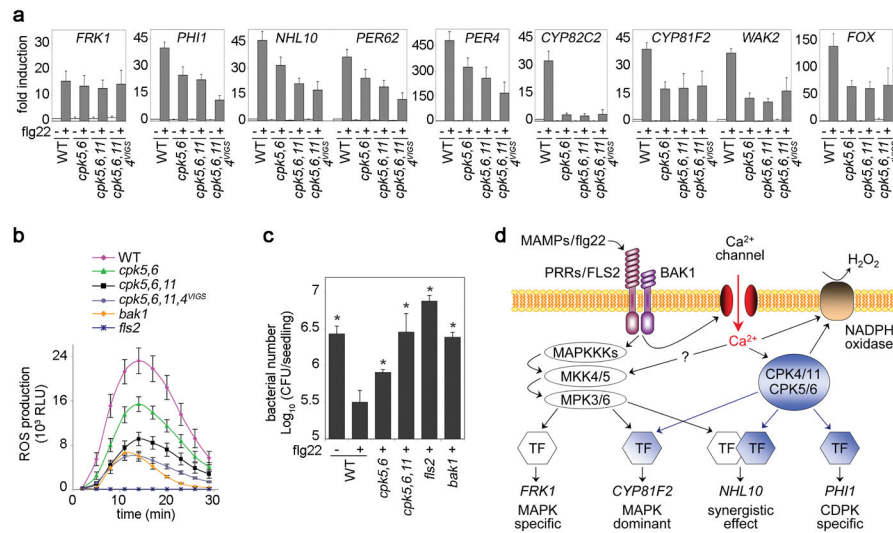
Figure 4. CDPKs are critical positive regulators in flg22 signalling
a, Flg22 induction of gene expression is diminished in *cpk* mutants. Gene expression was analyzed by qRT-PCR in protoplasts treated with 2 nM flg22 for 30 min. Error bars, s.d. (n=8). **b,** The *cpk* mutants exhibit impaired flg22-induced oxidative burst. RLU: relative light units. Error bars, s.d. (n=8). **c,** The *cpk* mutants are impaired in flg22-induced resistance to the bacterial pathogen *Pst* DC3000. Seedlings pre-treated with flg22 were inoculated with *Pst* DC3000 3 days before bacterial count. Error bars, s.d. (n=6). * Significantly different from WT +flg22 (p<0.001). **d,** Model for convergent activation of specific CDPKs and MAPK cascades in initial MAMP signalling.