LETTERS

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

Marie Boudsocq[1]†, Matthew R. Willmann[1]†, Matthew McCormack[1], Horim Lee[1], Libo Shan[1]†, Ping He[1]†, Jenifer Bush[1], Shu-Hua Cheng[1]‡ & Jen Sheen[1]

Innate immunity represents the first line of inducible defence against microbial infection in plants and animals[1–3]. In both kingdoms, recognition of pathogen- or microbe-associated molecular patterns (PAMPs or MAMPs, respectively), such as flagellin, initiates convergent signalling pathways involving mitogen-activated protein 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 defence responses, how Ca^{2+} signals are sensed and relayed into early MAMP signalling is unknown[5,6]. Using a functional genomic screen and genome-wide gene expression profiling, here we show that four calcium-dependent protein kinases (CDPKs) are Ca^{2+}-sensor protein kinases critical for 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 the synthesis of defence 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 the 22-amino-acid peptide flg22, as well as compromised pathogen defence. In contrast to negative roles of calmodulin and a calmodulin-activated transcription factor in plant defence[7,8], the present study reveals 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]. Several MAMPs seem to trigger similar early responses by different PRRs, including Ca^{2+} influxes, MAPK cascade activation, oxidative burst and transcriptional reprogramming in various plants[1,3–6]. The bacterial flagellin epitope flg22 and other MAMPs can induce potent Ca^{2+} signatures in both the cytoplasm and nucleus within minutes[9,10]. It has been shown that lipopolysaccharide (LPS)-mediated NO production is dependent on *Arabidopsis* calmodulin (CAM)-like protein CML24 (ref. 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 and CAM-like proteins, calcineurin B-like proteins, 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 protein kinase activities using histone as a general substrate[12,15]. We observed transient activation of several putative CDPKs between 5 and 30 min after flg22 elicitation (Fig. 1a and data not shown). The putative 60-kDa CDPKs are distinct, in molecular mass and kinetics, from the long-lasting (10–180 min) CDPK induced by Avr9–Cf9 interaction in cell death control in tobacco[16,17], but similar in size to the potato (*Solanum tuberosum*) StCDPK4 and StCDPK5 that were recently shown to mediate oxidative burst by directly phosphorylating NADPH oxidase RBOHB (respiratory burst oxidase homologue)[18]. Notably, 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

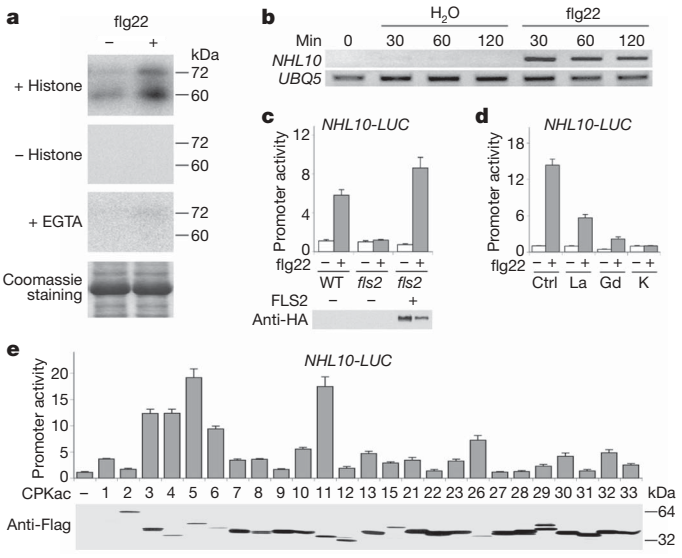

Figure 1 | Functional genomic screen for CDPKs in early flg22 signalling. **a**, Flg22 stimulates endogenous CDPK activities. Calcium-dependent histone phosphorylation, abolished by EGTA, is stimulated by flg22 at 15 min. **b**, *NHL10* is an early flg22-responsive gene. **c**, Flg22 activation of *NHL10-LUC* is dependent on the FLS2 receptor. The *fls2* mutant is complemented by FLS2–HA. Error bars, s.d. ($n = 8$). WT, wild type. **d**, Flg22 signalling requires calcium and protein kinase activity. Inhibitors: 1 mM $LaCl_3$ (La), 0.5 mM $GdCl_3$ (Gd), 2 μM K252a (K). Ctrl, control. 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.

[1]Department of Genetics, Harvard Medical School and Department of Molecular Biology and Center for Computational and Integrative Biology, Massachusetts General Hospital, Massachusetts 02114, USA. †Present addresses: Institut des Sciences du Végétal, UPR2355 CNRS, 1 Avenue de la Terrasse, 91198 Gif s/ Yvette Cedex, France (M.B.); Department of Biology, University of Pennsylvania, 433 South University Avenue, Philadelphia, Pennsylvania 19104, USA (M.R.W.); Institute for Plant Genomics and Biotechnology, Texas A&M University, College Station, Texas 77843, USA (L.S., P.H.).
‡Deceased.

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* also known as *YLS9*)[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 *NHL 10-LUC* is absolutely dependent on the FLS2 receptor kinase (Fig. 1c), and is abolished by the general protein kinase inhibitor K252a that could potentially inhibit many protein kinases 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 determine their biological functions in innate immune signalling. In *Arabidopsis*, the 34 CDPK members can be classified into four subgroups (I–IV) on the basis of sequence similarity[12,15] (Supplementary Fig. 1). We first determined the expression patterns of *Arabidopsis CPK* genes using quantitative real-time reverse transcriptase PCR (qRT–PCR) (Supplementary Fig. 3 and Supplementary 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 the carboxy-terminal Ca^{2+} regulatory and auto-inhibitory domains[22,23], while retaining the amino-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 fivefold, mimicking flg22 (Fig. 1e). Remarkably, five of them (CPKac4, 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 have a critical role in plant defence 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 have distinct roles in plant defence 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 and Supplementary Fig. 4). The five CDPK candidates in subgroup I showed predicted molecular masses matching the putative 60-kDa 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 and Supplementary 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 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 the Ca^{2+} ionophore A23187 to partially mimic flg22 signalling[22] (Supplementary Fig. 5b–d).

To determine the function of *CPK4*, *CPK5*, *CPK6* and *CPK11* in flg22 signalling further, 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–CPK6* and *CPK4–CPK11* (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 (Figs 2, 3, 4a, Supplementary Fig. 6, Supplementary Table 2 and Supplementary Methods). Notably, most (70%, 171 out of 244) of the 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 and 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 seemed to repress genes in early flg22 signalling (Fig. 2a and Supplementary Tables 4 and 5). Notably, many flg22–CDPK target genes encode peptides, receptor kinases and enzymes that modulate defence-related metabolites (cytochrome P450, methyltransferase, tryptophan and phenylpropanoid pathway), cell wall (glycoside hydrolase, glucosyltransferase and pectinesterase) and redox signalling (peroxidase, oxidoreductase and glutathione transferase) (Supplementary Tables 3–5). For instance, recent biochemical and genetic evidence supports the involvement

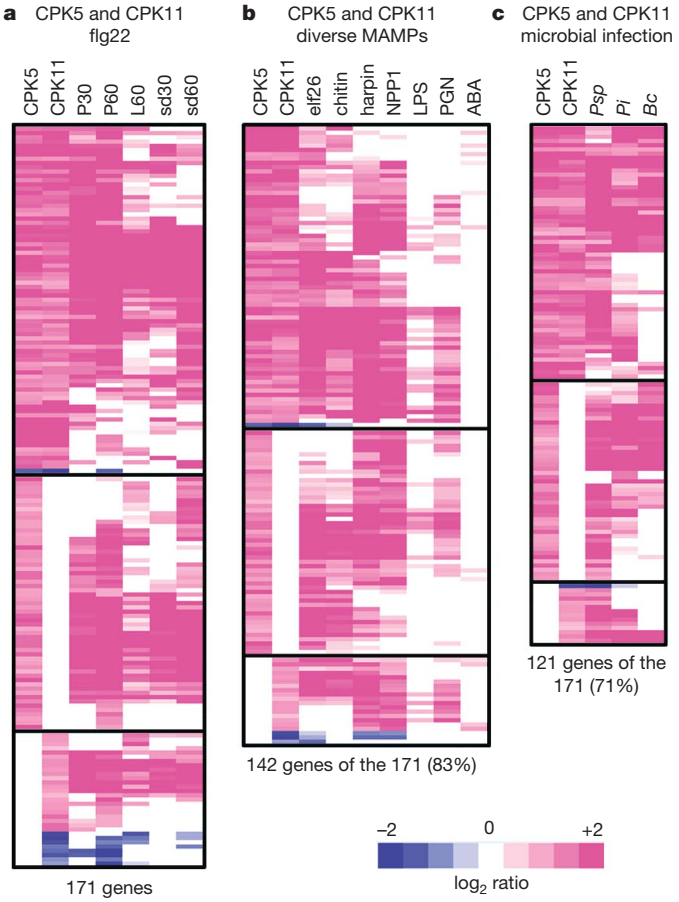

Figure 2 | Transcriptome profiling of CPK5ac and CPK11ac target genes in flg22, multiple MAMP and microbial signalling. **a**, Hierarchical clustering analysis of CPK5ac and CPK11ac target genes and early flg22-responsive genes. The genes were identified from protoplasts transiently expressing constitutively 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. *Bc*, *Botrytis cinerea*; *Pi*, *Phytophthora infestans*; *Psp*, *Pseudomonas syringae* pv. *phaseolicola*. Genes are clustered into three groups: shared by CPK5 and CPK11 (top panel), CPK5-specific (middle panel) and CPK11-specific (bottom panel). The number of genes co-regulated by CPK5ac and/or CPK11ac is indicated at the bottom of each panel.

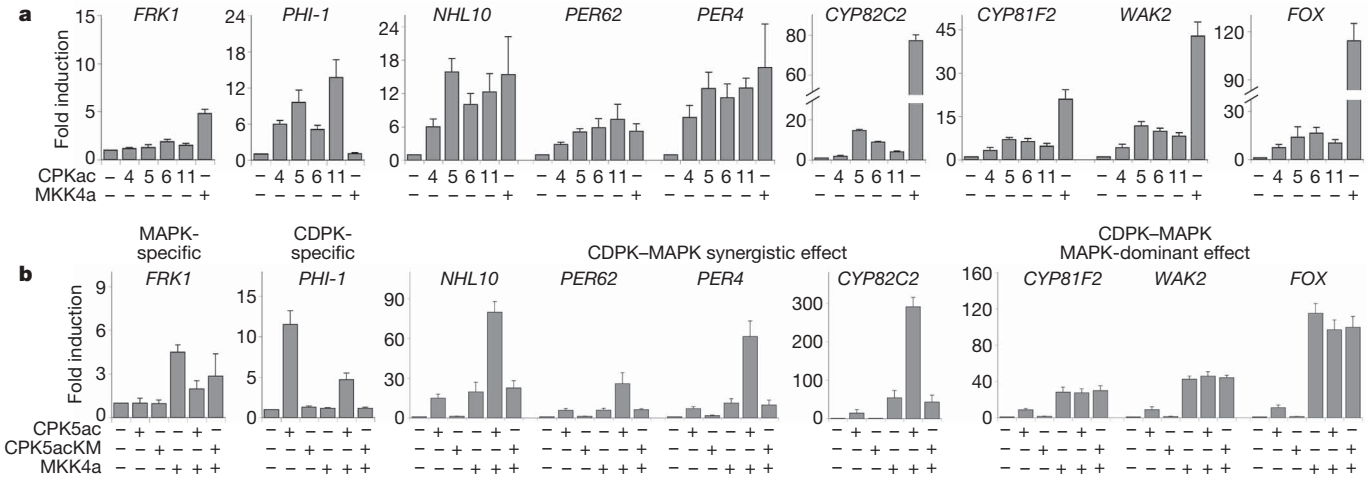

Figure 3 | Interaction between CDPK and MAPK cascades in flg22 signalling. **a**, **b**, CDPKs and MAPKs differentially regulate four flg22-responsive gene programs. The induction of endogenous genes was monitored by qRT–PCR in protoplasts expressing individual kinases (**a**) or co-expressing CPKac and MKKa (**b**). CPKac and MKK4a are constitutively active kinases, CPK5acKM is an inactive kinase mutant. Error bars, s.d. ($n = 6$). Marker gene details are shown in the Supplementary Information.

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*, *PROPEP2* and *PROPEP3*) encoding endogenous defence peptide signals were also activated early by flg22, CPK5ac and CPK11ac[27] (Supplementary Tables 3–5). Notably, only a few transcription factors and no significant marker genes for abscisic acid (ABA), methyljasmonate, ethylene or salicylate could be identified[28] (Supplementary Tables 3–5 and data not shown). CDPKs may have a crucial involvement in rapid transcriptional reprogramming by directly regulating the activity of transcription factors rather than their expression[29]. Identification of early flg22-responsive genes as CPK5ac and CPK11ac

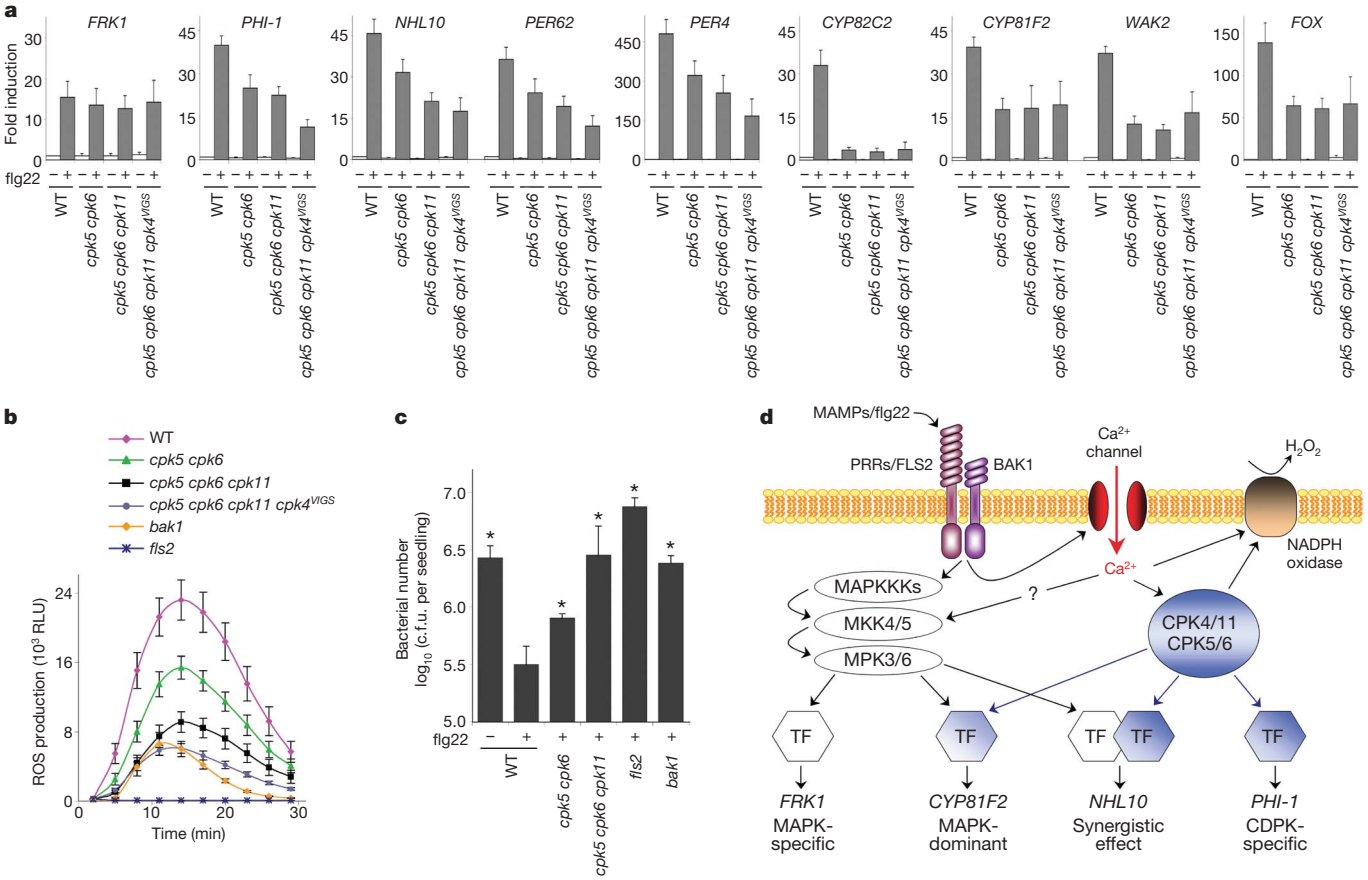

Figure 4 | CDPKs are critical positive regulators in flg22 signalling. **a**, Flg22 induction of gene expression is diminished in *cpk* mutants. Gene expression was analysed by qRT–PCR in protoplasts treated with 2 nM flg22 for 30 min. Error bars, s.d. ($n = 8$). **b**, The *cpk* mutants exhibit impaired 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. c.f.u., colony-forming units. Error bars, s.d. ($n = 6$). *$P < 0.001$, significant difference from wild type plus flg22. **d**, Model for convergent activation of specific CDPKs and MAPK cascades in initial MAMP signalling. TF, transcription factors.

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 and Supplementary Tables 3–5) to published MAMP microarray data sets generated with ATH1 GeneChips (Fig. 2b, Supplementary Table 7 and Supplementary 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 peptidoglycan (PGN)), 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 have 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 owing 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 defence 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 defence 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 and Supplementary Tables 3–5) with published microarray data sets 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 Supplementary Methods) (http://arabidopsis.org/info/expression/ATGenExpress.jsp). Identification of co-regulated genes indicated that CDPK activation of early gene expression is probably 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 analysed by qRT–PCR the expression of selected flg22-inducible genes in protoplasts expressing constitutively active CPKac or MKK4a. Unexpectedly, our analysis showed at least four regulatory programs for early flg22-responsive genes (Fig. 3a). Genes such as *FRK1* (*FLG22-INDUCED RECEPTOR KINASE1*) were MAPK-specific[4], whereas *PHI-1* (*PHOSPHATE-INDUCED 1*) seemed to be CDPK-specific. Other flg22 early genes were either activated equally by both CPKac and MKK4a, such as *NHL10*, *PER62* (*PEROXIDASE62*, also known as At5g39580) and *PER4* (At1g14540), or were induced to much higher levels by MKK4a, for example, *CYP82C2*, *CYP81F2*, *WAK2* (*WALL-ASSOCIATED KINASE 2*) and *FOX* (*FAD-LINKED OXIDOREDUCTASE*, At1g26380). When CPK5ac and MKK4a were co-expressed in protoplasts, CDPK- and MAPK-specific genes were partially antagonized by the 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 defence signalling pathways[5,6], Ca^{2+} blockers reduced but did not eliminate MAPK or *NHL10-LUC* activation by flg22 (Supplementary Fig. 7 and 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. Although T-DNA insertion into *CPK5*, *CPK6* and *CPK11* 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 cpk6* double and *cpk5 cpk6 cpk11* triple mutants by genetic crosses and generated the *cpk4 cpk5 cpk6 cpk11* 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 (Figs 1e, 2 and 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 cpk6* and *cpk5 cpk6 cpk11* lost most flg22 activation of 60-kDa CDPKs (Supplementary Fig. 11), MAPK activation by flg22 was not affected in *cpk5 cpk6* and *cpk5 cpk6 cpk11* mutants (Supplementary Fig. 12), confirming the gain-of-function results (Supplementary Fig. 8). Thus, CDPKs and MAPK cascades are probably 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 showed differentially impaired flg22 induction in double, triple and quadruple *cpk* mutants (Fig. 4a). CPK4, CPK5, CPK6 and CPK11 were all important for regulation of *PHI-1*, *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 had equal but independent roles (Figs 3 and 4a). Notably, CPK5 and CPK6 were essential for flg22 activation of *CYP82C2*, which uniquely required the synergistic CDPK and MAPK functions (Figs 3b and 4a). The oxidative burst induced by flg22 was gradually reduced in *cpk5 cpk6*, *cpk5 cpk6 cpk11* and *cpk5 cpk6 cpk11 cpk4*^{VIGS} mutants to a similar level as in the flg22 signalling mutant *bak1* (Fig. 4b), suggesting a role for these CDPKs in regulating reactive oxygen species (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 cpk6* and *cpk5 cpk6 cpk11* mutants (Supplementary Fig. 13), probably due to impairment in several MAMP signalling pathways[20] (Fig. 2a, b). Moreover, flg22-induced immunity against *Pst* DC3000 (refs 19, 20) was also compromised in a seedling pathogen assay in *cpk5 cpk6* and *cpk5 cpk6 cpk11* mutants compared to those observed in *bak1* and *fls2* mutants (Fig. 4c). Interestingly, all four CPK–GFP (green fluorescent protein) fusions were localized in both the 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*. Further characterization of the functions of CDPK and MAPK target genes, such as peptides, receptor kinases and enzymes involved in antimicrobial chemical production, cell wall modifications and redox signalling, will help elucidate the diverse primary responses in innate immune signalling. Because 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 (ref. 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 several 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 1,632-base-pair (bp) promoter region of *NHL10* was fused to the *LUC* reporter gene to generate *NHL10-LUC*. The effector constructs *FLS2-HA* (haemagglutinin), *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* (β-glucuronidase) as an internal control. Protein expression was monitored by immunoblot using commercially available monoclonal antibodies raised against haemagglutinin (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 Arabidopsis Biological Resource Center (ABRC) resources (Supplementary Table 14). Higher order *cpk* mutants were generated either by crossing or by VIGS. Bacterial growth assays were performed in leaves and seedlings using *Pst* DC3000. The oxidative burst was measured using a luminol-based assay[3].

Full Methods and any associated references are available in the online version of the paper at www.nature.com/nature.

Received 24 August 2009; accepted 5 January 2010.
Published online 17 February 2010.

1. Nürnberger, T., Brunner, F., Kemmerling, B. & Piater, L. Innate immunity in plants and animals: striking similarities and obvious differences. *Immunol. Rev.* **198,** 249–266 (2004).
2. Akira, S., Uematsu, S. & Takeuchi, O. Pathogen recognition and innate immunity. *Cell* **124,** 783–801 (2006).
3. Boller, T. & Felix, G. A renaissance of elicitors: perception of microbe-associated molecular patterns and danger signals by pattern-recognition receptors. *Annu. Rev. Plant Biol.* **60,** 379–406 (2009).
4. Asai, T. *et al.* MAP kinase signalling cascade in *Arabidopsis* innate immunity. *Nature* **415,** 977–983 (2002).
5. Lecourieux, D., Ranjeva, R. & Pugin, A. Calcium in plant defence-signalling pathways. *New Phytol.* **171,** 249–269 (2006).
6. Ma, W. & Berkowitz, G. A. The grateful dead: calcium and cell death in plant innate immunity. *Cell. Microbiol.* **9,** 2571–2585 (2007).
7. Kim, M. C. *et al.* Calmodulin interacts with MLO protein to regulate defence against mildew in barley. *Nature* **416,** 447–451 (2002).
8. Du, L. *et al.* Ca^{2+}/calmodulin regulates salicylic-acid-mediated plant immunity. *Nature* **457,** 1154–1158 (2009).
9. Lecourieux, D. *et al.* Proteinaceous and oligosaccharidic elicitors induce different calcium signatures in the nucleus of tobacco cells. *Cell Calcium* **38,** 527–538 (2005).
10. Gust, A. A. *et al.* Bacteria-derived peptidoglycans constitute pathogen-associated molecular patterns triggering innate immunity in *Arabidopsis*. *J. Biol. Chem.* **282,** 32338–32348 (2007).
11. Ma, W., Smigel, A., Tsai, Y. C., Braam, J. & Berkowitz, G. A. Innate immunity signaling: cytosolic Ca^{2+} elevation is linked to downstream nitric oxide generation through the action of calmodulin or a calmodulin-like protein. *Plant Physiol.* **148,** 818–828 (2008).
12. Harper, J. F. & Harmon, A. Plants, symbiosis and parasites: a calcium signalling connection. *Nature Rev. Mol. Cell Biol.* **6,** 555–566 (2005).
13. Boudsocq, M. & Sheen, J. in *Abiotic Stress Adaptation in Plants: Physiological, Molecular and Genomic Foundation* (eds Pareek, A., Sopory, S. K., Bohnert, H. J. & Govindjee) Ch. 4 (Springer, 2009).
14. Luan, S. The CBL-CIPK network in plant calcium signaling. *Trends Plant Sci.* **14,** 37–42 (2009).
15. Cheng, S. H., Willmann, M. R., Chen, H. C. & Sheen, J. Calcium signaling through protein kinases. The *Arabidopsis* calcium-dependent protein kinase gene family. *Plant Physiol.* **129,** 469–485 (2002).
16. Romeis, T., Piedras, P. & Jones, J. D. G. Resistance gene-dependent activation of a calcium-dependent protein kinase in the plant defense response. *Plant Cell* **12,** 803–815 (2000).
17. Romeis, T., Ludwig, A. A., Martin, R. & Jones, J. D. G. Calcium-dependent protein kinases play an essential role in a plant defence response. *EMBO J.* **20,** 5556–5567 (2001).
18. Kobayashi, M. *et al.* Calcium-dependent protein kinases regulate the production of reactive oxygen species by potato NADPH oxidase. *Plant Cell* **19,** 1065–1080 (2007).
19. Zipfel, C. *et al.* Bacterial disease resistance in *Arabidopsis* through flagellin perception. *Nature* **428,** 764–767 (2004).
20. Shan, L. *et al.* Bacterial effectors target the common signaling partner BAK1 to disrupt multiple MAMP receptor-signaling complexes and impede plant immunity. *Cell Host Microbe* **4,** 17–27 (2008).
21. Zimmermann, P., Hennig, L. & Gruissem, W. Gene-expression analysis and network discovery using Genevestigator. *Trends Plant Sci.* **10,** 407–409 (2005).
22. Sheen, J. Ca^{2+}-dependent protein kinases and stress signal transduction in plants. *Science* **274,** 1900–1902 (1996).
23. Uno, Y., Rodriguez Milla, M. A., Maher, E. & Cushman, J. C. Identification of proteins that interact with catalytically active calcium-dependent protein kinases from *Arabidopsis*. *Mol. Genet. Genomics* **281,** 375–390 (2009).
24. Baena-González, E., Rolland, F., Thevelein, J. M. & Sheen, J. A central integrator of transcription networks in plant stress and energy signalling. *Nature* **448,** 938–942 (2007).
25. Bednarek, P. *et al.* A glucosinolate metabolism pathway in living plant cells mediates broad-spectrum antifungal defense. *Science* **323,** 101–106 (2009).
26. Clay, N. K., Adio, A. M., Denoux, C., Jander, G. & Ausubel, F. M. Glucosinolate metabolites required for an *Arabidopsis* innate immune response. *Science* **323,** 95–101 (2009).
27. Huffaker, A. & Ryan, C. A. Endogenous peptide defense signals in *Arabidopsis* differentially amplify signaling for the innate immune response. *Proc. Natl Acad. Sci. USA* **104,** 10732–10736 (2007).
28. Nemhauser, J. L., Hong, F. & Chory, J. Different plant hormones regulate similar processes through largely nonoverlapping transcriptional responses. *Cell* **126,** 467–475 (2006).
29. Zhu, S. Y. *et al.* Two calcium-dependent protein kinases, CPK4 and CPK11, regulate abscisic acid signal transduction in *Arabidopsis*. *Plant Cell* **19,** 3019–3036 (2007).
30. Sheen, J. *et al.* in *Biology of Plant Microbe Interactions* Vol. 6, Ch. 86 (IS-MPMI Symposium Proceedings, 2008).

Supplementary Information is linked to the online version of the paper at www.nature.com/nature.

Acknowledgements We thank K. H. Liu and E. Baena-González for contributing to the CPK clone collection, O. R. Patharkar for initial microarray data analysis, B. Mueller and G. Tena for offering qPCR primers, M. N. Soler and S. Bolte for assistance on confocal microscopy, C. Laurière for discussions, S. P. Dinesh-Kumar for VIGS vectors, K. Schreiber and D. Desveaux for the seedling pathogen assay, and A. Gust and T. Nürnberger for the original PGN microarray data. We thank the Salk Institute, Syngenta Biotechnology and A. Sessions for sharing the *Arabidopsis* T-DNA collections, and the *Arabidopsis* Biological Resource Center for providing *Arabidopsis* mutant seeds. This research has been supported by a Marie Curie International fellowship within the 6th European Community Framework Program to M.B., an NSF predoctoral fellowship to M.R.W., and grants from the National Science Foundation and the National Institute of Health, and the MGH CCIB fund to J.S.

Author Contributions J.S. and M.R.W. initiated the project; M.B., M.R.W. and J.S. designed the experiments; M.R.W., M.B. and S.-H.C. built the CPK clone collection; M.B., M.R.W., H.L., L.S., P.H. and J.S. conducted the experiments and analysed the data; M.M., J.S. and M.B. analysed microarray data; J.B. managed plants; M.B., J.S. and M.M. prepared the manuscript with inputs from all co-authors.

Author Information All microarray data are available at the Gene Expression Omnibus (GEO; http://www.ncbi.nlm.nih.gov/geo/) under accession number GSE16557. Reprints and permissions information is available at www.nature.com/reprints. The authors declare no competing financial interests. Correspondence and requests for materials should be addressed to M.B. (boudsocq@molbio.mgh.harvard.edu) or M.R.W. (willmann@sas.upenn.edu).

METHODS

Plant material and growth conditions. We isolated homozygous T-DNA insertion mutants[31], *cpk5* (sail_657C06 from Syngenta), *cpk6* (salk_025460) and *cpk11* (salk_054495) using seeds obtained from the ABRC. The T-DNA insertions were confirmed by sequencing (Supplementary Fig. 9a). Although 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 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 cpk6* double and *cpk5 cpk6 cpk11* triple mutants were generated by genetic crosses. For in-gel kinase assay with seedlings, wild-type and *cpk* mutants were grown in liquid medium 0.5× 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]. In brief, *Arabidopsis* leaves of 4-week-old plants were infiltrated with *Pst* DC3000 at 5×10^4 c.f.u. ml^{-1} 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× MS were treated with 100 nM flg22 for 1 day before co-cultivation with *Pst* DC3000 at 1×10^7 c.f.u. ml^{-1} 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 a Flag epitope tag or GFP at the C terminus, and cloned between a 35S-derived promoter and a NOS terminator[4,20,22,32,34]. For the *NHL10-LUC* reporter construct, the 1,632-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 previously[34]. Typically, 0.1 ml protoplasts at a density of 2×10^5 ml^{-1} 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 the 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 (for example, CDPKs) on reporter expression, transfected protoplasts were incubated for 6 h. For experiments with full-length 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 duplicate or triplicate 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 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. qRT–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× 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 micrograms of total RNA was 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. 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 (http://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 on the basis of the validated marker genes (Figs 3 and 4a). Details for microarray data set sources, data analyses and 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* wild-type, *cpk5 cpk6* and *cpk5 cpk6 cpk11* 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 four 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 four times with similar results.

31. Alonso, J. M. & Stepanova, A. N. T-DNA mutagenesis in *Arabidopsis*. *Methods Mol. Biol.* **236,** 177–188 (2003).
32. He, P. *et al.* Specific bacterial suppressors of MAMP signaling upstream of MAPKKK in *Arabidopsis* innate immunity. *Cell* **125,** 563–575 (2006).
33. Schreiber, K., Ckurshumova, W., Peek, J. & Desveaux, D. A high-throughput chemical screen for resistance to *Pseudomonas syringae* in *Arabidopsis*. *Plant J.* **54,** 522–531 (2008).
34. Yoo, S. D., Cho, Y. H. & Sheen, J. *Arabidopsis* mesophyll protoplasts: a versatile cell system for transient gene expression analysis. *Nature Protocols* **2,** 1565–1572 (2007).
35. Boudsocq, M., Barbier-Brygoo, H. & Laurière, C. Identification of nine sucrose nonfermenting 1-related protein kinases 2 activated by hyperosmotic and saline stresses in *Arabidopsis thaliana*. *J. Biol. Chem.* **279,** 41758–41766 (2004).
36. Burch-Smith, T. M., Schiff, M., Liu, Y. & Dinesh-Kumar, S. P. Efficient virus-induced gene silencing in *Arabidopsis*. *Plant Physiol.* **142,** 21–27 (2006).
37. Felix, G., Duran, J. D., Volko, S. & Boller, T. Plants have a sensitive perception system for the most conserved domain of bacterial flagellin. *Plant J.* **18,** 265–276 (1999).
38. Chinchilla, D. *et al.* A flagellin-induced complex of the receptor FLS2 and BAK1 initiates plant defence. *Nature* **448,** 497–500 (2007).