MAP kinase signalling cascade in *Arabidopsis* innate immunity

Tsuneaki Asai*§, Guillaume Tena*§, Joulia Plotnikova*, Matthew R. Willmann*, Wan-Ling Chiu*, Lourdes Gomez-Gomez†, Thomas Boller‡, Frederick M. Ausubel* & Jen Sheen*

* *Department of Genetics, Harvard Medical School, and Department of Molecular Biology, Massachusetts General Hospital, Boston, Massachusetts 02114, USA*
† *Instituto de Desarrollo Regional, Sección de Biotecnología, Campus Universitario s/n, E-02071 Albacete, Spain*
‡ *Friedrich Miescher-Institute, PO Box 2543, CH-4002 Basel, Switzerland*
§ *These authors contributed equally to the work*

There is remarkable conservation in the recognition of pathogen-associated molecular patterns (PAMPs) by innate immune responses of plants, insects and mammals. We developed an *Arabidopsis thaliana* leaf cell system based on the induction of early-defence gene transcription by flagellin, a highly conserved component of bacterial flagella that functions as a PAMP in plants and mammals. Here we identify a complete plant MAP kinase cascade (MEKK1, MKK4/MKK5 and MPK3/MPK6) and WRKY22/WRKY29 transcription factors that function downstream of the flagellin receptor FLS2, a leucine-rich-repeat (LRR) receptor kinase. Activation of this MAPK cascade confers resistance to both bacterial and fungal pathogens, suggesting that signalling events initiated by diverse pathogens converge into a conserved MAPK cascade.

The innate immune system is the first line of inducible defence against infectious disease. A key function of innate immunity is the detection of PAMPs produced by infectious agents but not by host cells[1–3]. In insects and mammals, the recognition of PAMPs is often mediated by Toll and Toll-like receptors (TLRs), respectively, with extracellular LRRs[2,4]. In plants, LRR domains are also found in the products of disease resistance genes[5–8], which are receptors important for innate immunity.

Recent studies have identified bacterial flagellin as a PAMP that is recognized by the innate immune system in diverse organisms including insects, mammals and plants[9–11]. In mammals, TLR5 mediates the innate immune responses to flagellin[11]. In *Arabidopsis*, the LRR receptor kinase FLS2 is required for flagellin signalling[12]. A 22-amino-acid peptide flg22, corresponding to the most conserved domain of eubacterial flagellin, binds to FLS2 and induces defence responses in leaves[10,12,13].

In contrast to animals, the molecular mechanisms underlying the responses to PAMPs are largely unknown in plants[5–7]. In mammals, different TLRs specifically recognize distinct PAMPs but activate common and conserved signal transduction pathways[2,4]. In plants, many different types of pathogens and pathogen-derived elicitors also trigger similar defence responses[7,14–17]. The best candidates for components of convergent signalling pathways in plants are the mitogen-activated protein kinases (MAPKs)[7,16–19]. Although several MAPKs involved in plant defence response have been identified[14,20–25], the identity of the upstream receptors, MAPK kinases (MAPKKs) and MAPKK kinases (MAPKKKs), and the downstream transcription factors remain mostly unclear.

To elucidate MAPK signalling cascades in plant innate immune responses, we developed an *Arabidopsis* protoplast transient expression system in which transcription of early defence genes is activated by flg22 and in which the role of MAPK cascade components could be systematically tested and assigned. Using this system, we have identified a complete plant MAPK cascade and WRKY transcription factors acting downstream of the flagellin receptor FLS2. Our data also suggest that this signalling pathway functions in response to both fungal and bacterial pathogens and potentially could be engineered to enhance the disease resistance of crop plants to a wide range of pathogens.

Early defence gene transcription

To dissect the early signal transduction pathways in plant innate immune responses, we first established a leaf cell assay based on flg22 inducible transcription of early response genes in *Arabidopsis* mesophyll protoplasts[26]. Few reporter genes have been developed for the dissection of the early stages of defence signalling pathways in plants[27–29]. To identify genes that are induced by flg22-activated defence signalling, we created a subtracted complementary DNA library that represented messenger RNA species induced at various times by the elicitor flg22 in *Arabidopsis* mesophyll protoplasts[30]. Using flg22 rather than a pathogen or natural elicitors avoided the possibility that multiple elicitors could be functioning in parallel[12]. Furthermore, synchronous elicitation by flg22 is achieved more reproducibly in homogeneous mesophyll protoplasts than in intact leaves[26]. In the library we found well described defence genes, such as *PAL1* (At2g37040 in the *Arabidopsis* Genome Initiative nomenclature), *GST1* (Atlg02930), *PR1* (At2g19990) and *PR5* (Atlg75040), which are induced by a variety of pathogens and elicitors at different stages of the defence response in many plant species[28,29,31]. These genes were also induced in *Arabidopsis* leaves infiltrated with flg22 (not shown), suggesting that similar defence responses are induced by flg22 in isolated leaf protoplasts and in leaves of intact plants.

To develop new reporter genes that are expressed early in the primary defence response, we focused on flg22-activated genes identified in the subtraction library that are expressed at early time points (see below) and code for putative regulatory factors, including *WRKY29* (At4g23550)[32] and *FRK1* (*FLG22-INDUCED RECEPTOR-LIKE KINASE 1*; At2g19190), which encode a WRKY transcription factor (with a conserved WRKY DNA-binding domain) and a LRR receptor kinase, respectively. Induction of WRKY transcription factors and LRR receptor kinase genes by pathogens, pathogen-derived elicitors or salicylic acid has been demonstrated in the leaves of several plant species including *Arabidopsis*[28,29,32–34]. These results indicate that *Arabidopsis* protoplasts treated with flg22 increase transcription of defence-related genes that are also expressed in intact plants after pathogen infection.

Polymerase chain reaction after reverse transcription of RNA (RT-PCR) analysis showed that *WRKY29*, *FRK1* and *GST1* mRNA levels were increased in *Arabidopsis* protoplasts within 30 min after flg22 treatment (Fig. 1a), whereas induction of the extensively studied *PR1* and *PR5* genes occurred much later (Fig. 1a). Two stress-regulated genes, H_2O_2-inducible *GST6* (At2g47730) and ABA-, cold-, or drought-responsive *RD29A* (AT5g52310)[35], were

not induced by flg22 over basal levels of expression (Fig. 1a). In contrast to the common assumption that protoplasts isolated from fresh leaves are badly damaged, stressed and dying, or are already activated in defence responses, these data show that *Arabidopsis* mesophyll protoplasts respond specifically to flg22, similarly to the response observed in intact plants[13].

To examine whether *de novo* protein synthesis is required for flg22-induced defence gene expression, protoplasts were preincubated with the protein synthesis inhibitor cycloheximide (CHX) and then treated with flg22. RT-PCR analysis showed that induction of the early response genes *WRKY29*, *FRK1* and *GST1* by flg22 was not significantly affected by CHX, whereas the inhibitor blocked induction of the late response genes *PR1* and *PR5*, but not the expression of other control genes (Fig. 1a). To determine whether the early-defence genes are activated transcriptionally, the promoters of the *WRKY29*, *FRK1*, *GST1*, *GST6* and *RD29A* genes were fused to the firefly luciferase reporter gene (*LUC*)[35] and tested for their response to flg22 in transiently transfected protoplasts[35]. Consistent with the RT-PCR data obtained with the endogenous genes, the *WRKY29*, *FRK1* and *GST1* promoters, but not the *GST6* and *RD29A* promoters, were induced by flg22 (Fig. 1b). As a parallel control, the 23-amino-acid peptide flg23RM, corresponding to the flg22-homologous sequence of *Rhizobium meliloti* flagellin, activated none of these promoters (not shown). *Rhizobium meliloti* is a plant symbiont that fails to activate host innate immunity[10,13]. These results suggest that in response to pathogen-derived flagellin, *WRKY29*, *FRK1* and *GST1* are rapidly induced by pre-existing signalling molecules. Because the H_2O_2-responsive gene *GST6* (ref. 35) was not induced by flg22 (Fig. 1a, b), it is likely that although flg22 induces an oxidative burst in *Arabidopsis* leaves[12,13], it may not elicit a strong or persistent oxidative burst in protoplasts. Thus, the flg22-protoplast system distinguishes between flagellin and oxidative-stress signalling pathways (see below). In addition to the well established parsley protoplast system derived from suspension cultures[7,15,16,20,33], the *Arabidopsis* mesophyll protoplast transient expression system offers a new tool to study defence signalling based on early gene transcription.

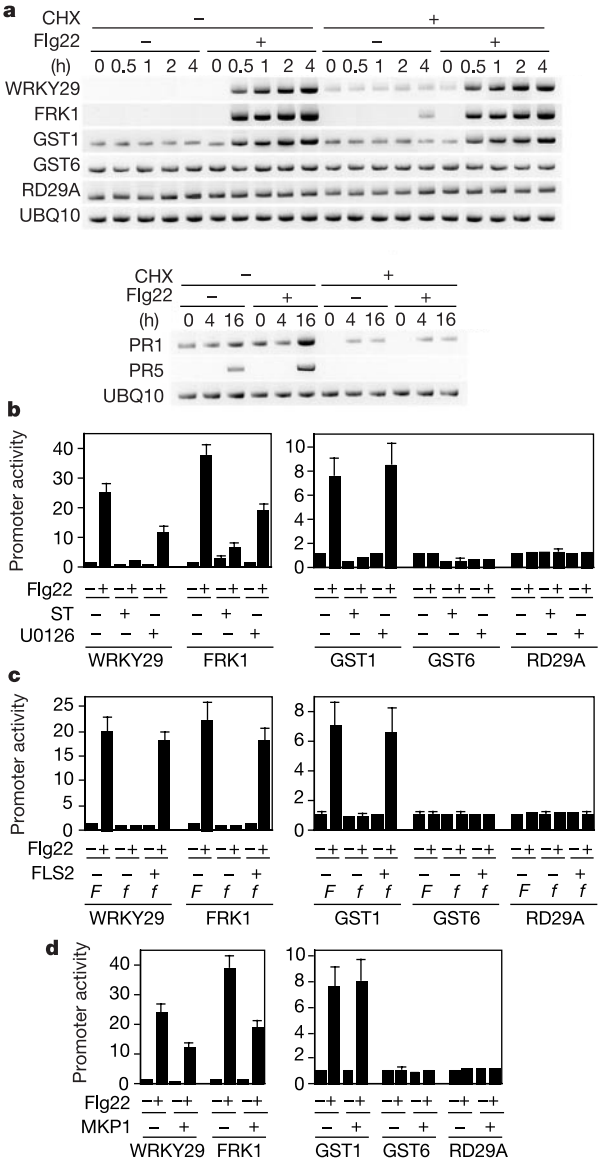

Figure 1 Early-defence gene activation by flg22. **a**, RT-PCR analysis. Protoplasts were preincubated for 1 h without or with 10 μM cycloheximide (CHX), and then treated without or with 100 nM flg22. **b**, Involvement of MAPK. Transfected protoplasts were preincubated for 1 h with 1 μM staurosporine (ST) or with 1 μM U0126 before induction by 100 nM flg22 for 4 h. **c**, FLS2 requirement. Protoplasts were isolated from wild-type (*F*) and *fls2-22* mutant (*f*) leaves. Transfected protoplasts were incubated for 9 h first before incubation without or with 100 nM flg22 for 4 h. **d**, MKP1 suppression.

Flg22 signals through FLS2

To determine whether the *WRKY29*, *FRK1* and *GST1* promoters were activated by flg22 through the FLS2 LRR receptor kinase[12], protoplasts isolated from *fls2* mutant leaves were transiently transfected with the *WRKY29-LUC*, *FRK1-LUC* and *GST1-LUC* reporter constructs. In the *fls2* mutant protoplasts, none of the three promoters could be activated by flg22 (Fig. 1c). We note that transient expression of wild-type FLS2 (ref. 12) restored flg22-inducibility of the promoters in the *fls2* mutant protoplasts (Fig. 1c). Consistent with these results, the broad-spectrum protein kinase inhibitor staurosporine effectively blocked the ability of flg22 to activate the *WRKY29*, *FRK1* and *GST1* promoters (Fig. 1b). These data show that flagellin signalling leading to the expression of *WRKY29*, *FRK1* and *GST1* requires FLS2.

Specific MAPKs in flg22 signalling

The role of MAPK signalling in the flg22-mediated activation of the *WRKY29*, *FRK1* and *GST1* promoters was tested by using the MAPKK inhibitor U0126. As shown in Fig. 1b, U0126 partially blocked transcription of the *WRKY29* and *FRK1* promoters but had no effect on the *GST1* promoter. To further test the involvement of MAPK signalling in the activation of *WRKY29* and *FRK1* promoters, a mouse MAPK phosphatase (MKP1) was transiently expressed in *Arabidopsis* protoplasts[36]. Similarly to U0126, MKP1 partly reduced flg22-induced activation of the *WRKY29* and *FRK1* promoters but had no effect on the *GST1* promoter (Fig. 1d). These results suggested that both MAPK-dependent and MAPK-independent signalling pathways act downstream of FLS2 to activate the *WRKY29* and *FRK1* promoters. Further investigation will be required to test whether calcium signalling plays a role in the MAPK-independent pathway induced by flg22, as suggested by the studies in parsley protoplasts[15].

Because flg22 activation of the *WRKY29* and *FRK1* promoters was partially blocked by U0126 and MKP1, we examined whether flg22 can activate MAPKs in *Arabidopsis* protoplasts. Treatment of protoplasts with flg22 resulted in rapid activation of endogenous protein kinases that phosphorylated myelin basic protein (MBP), a commonly used MAPK substrate, in an in-gel kinase assay (Fig. 2a). These protein kinases were not activated by treatment with flg23RM (not shown), suggesting their importance in pathogen defence. To systematically test the *Arabidopsis* MAPKs (MPKs) to determine which one(s) are involved in flg22 signalling, we chose six representative MPKs corresponding to four out of the five MPK subfamilies that may exhibit distinct functions on the basis of sequence homology analysis[19,37]. These MPKs were tagged with

the haemagglutinin (HA) epitope, transiently expressed in protoplasts, immunoprecipitated with an anti-HA antibody, and tested *in vitro* for MBP kinase activity[35]. The results indicated that MPK3 and MPK6, which belong to the same subfamily, but not the other MPKs, showed strong activation following flg22 treatment (Fig. 2b). The involvement of MPK5 cannot be ruled out, however, owing to its poor expression and/or instability. The result is consistent with the previously reported activation of MPK6 in *Arabidopsis* cultured cells and leaf strips by flg22 (ref. 23). The activation of MPK3 and MPK6 by flg22 did not occur in the *fls2* mutant protoplasts unless functional FLS2, but not a kinase-inactive mutant of FLS2 (FLS2Km), was co-expressed (Fig. 2c). The co-expression of MKP1, which partially blocked flg22-induced activation of *WRKY29* and *FRK1* promoters (Fig. 1d), eliminated MPK3 and MPK6 activation by flg22 (Fig. 2d). These results suggest that flagellin perception by FLS2 leads to the activation of MPK3 and MPK6. The studies also revealed the importance of MAPK-independent pathway in flg22 signalling.

Redundant MAPKKs in flg22 signalling

The *Arabidopsis* genome contains nine MAPKK (MKK) genes that belong to four subfamilies, suggesting at least four distinct functions[19]. Four of these MKKs, MKK1, MKK2, MKK4 and MKK5, belonging to two subfamilies, are expressed in leaf cells[19,37]. Therefore, these four MKK genes were cloned and analysed in the protoplast transient expression assay. To distinguish MKKs from the HA-tagged MPKs in transfected protoplasts, a Myc-epitope tag was fused to each MKK. MAPKKs require phosphorylation to be activated, so gain-of-function mutants of the four MKKs were generated by converting the conserved serine and/or threonine residues in the kinase-activation loop located between subdomains VII and VIII to aspartate or glutamate to mimic phosphorylation. The Myc-tagged wild-type or constitutively active MKKs were co-expressed individually with the six HA-tagged MPKs to systematically determine their regulatory relationships. The results showed that constitutively active MKK4 and MKK5 (MKK4a and MKK5a, respectively) were equally effective at activating MPK3 and MPK6 (Fig. 3a, b). In contrast, constitutively active MKK1 and MKK2 (MKK1a and MKK2a, respectively) were unable to activate these MPKs (Fig. 3b), although MKK1a did activate other MPKs (not shown).

To test directly the role of MKK4 and MKK5 in flg22 signalling, plasmid DNA expressing the constitutively active MKK constructs was cotransfected with various reporter gene constructs into protoplasts[35]. Either MKK4a or MKK5a, but not MKK1a or

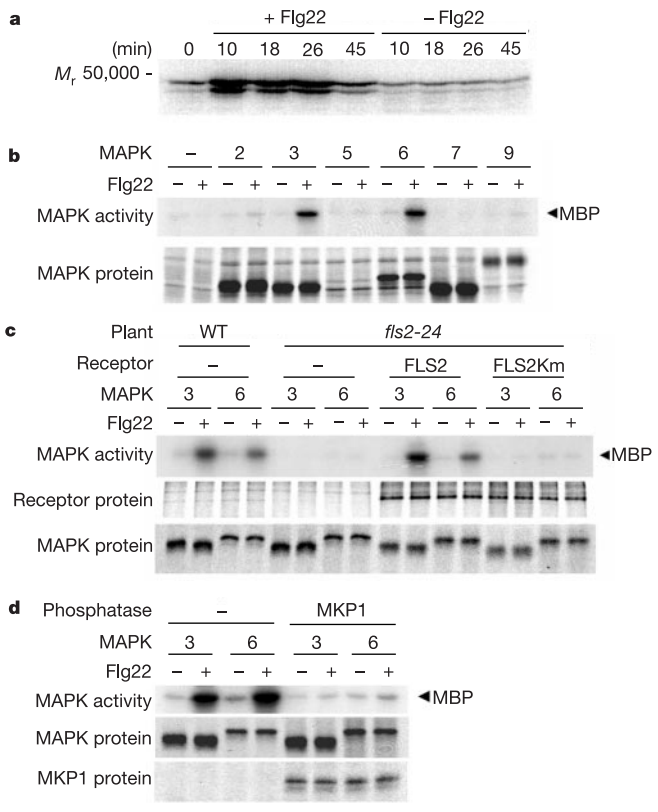

Figure 2 Flg22 activates MPK3 and MPK6 through FLS2. **a**, Flg22 activates endogenous MBP kinases. Protoplasts were treated with or without 1 μM flg22. **b**, Flg22 activates MPKs. MAPK activation with or without 100 nM flg22 for 10 min (top) and expression of each MAPK are shown (bottom). **c**, FLS2 requirement. MPK3 or MPK6 was co-expressed with a wild-type (FLS2) or kinase-inactive (FLS2Km) flg22 receptor in wild-type (WT) and *fls2-24* protoplasts. MAPK activities (top) and FLS2 (middle) and MAPK (bottom) expression are shown. **d**, MKP1 abolishes flg22 activation of MPKs. MAPK activity (top), and MAPK (middle) and MKP1 (bottom) expression are shown.

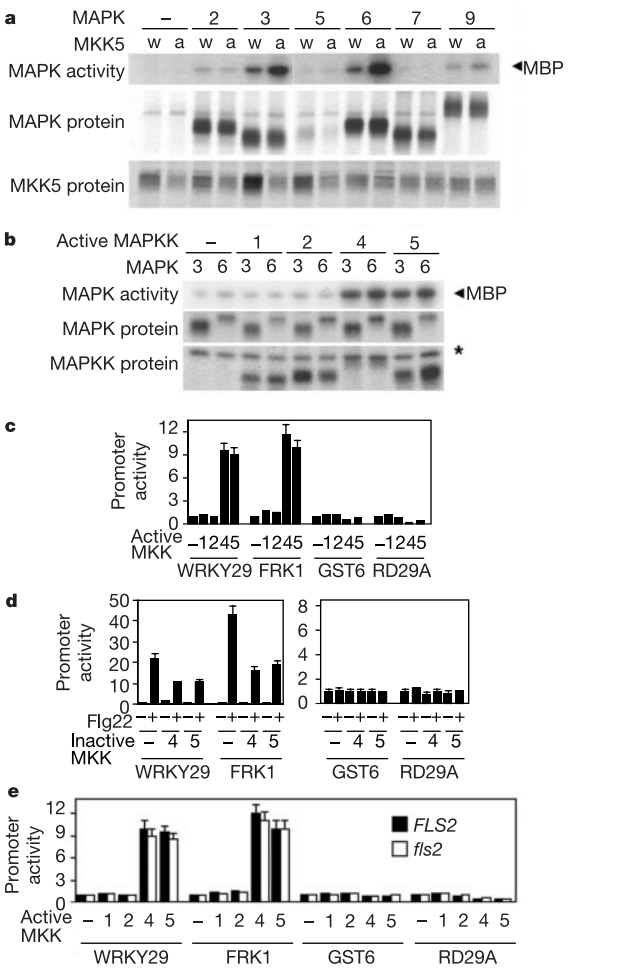

Figure 3 MKK4 and MKK5 activate MPK3/MPK6 and early-defence genes. **a**, MKK5 activates MPK3 and MPK6. Protoplasts were expressing MAPK and wild-type (w) or constitutively active MKK5 mutant (a). MAPK activity (top) and MAPK (middle) and MAPKK (bottom) expression are shown. **b**, MKK4 and MKK5 redundancy. Protoplasts were expressing MPK3 or MKP6 and one constitutively active MKK. MAPK activity (top) and MAPK (middle) and MAPKK (bottom) expression are shown. Asterisk indicates a background band. MBP, myelin basic protein. **c**, MKK4a and MKK5a activate *WRKY29* and *FRK1* promoters. Protoplasts were expressing GFP or a constitutively active MKK. **d**, Dominant-negative MKK4 and MKK5 partially inhibit early-defence gene promoters. protoplasts were expressing GFP or a kinase-inactive MKK4 or MKK5. **e**, MKK4 and MKK5 act downstream of FLS2. Wild-type (*FLS2*) and *fls2-24* (*fls2*) protoplasts were expressing GFP or a constitutively active MKK.

MKK2a, was sufficient to activate specifically the *WRKY29* and *FRK1* promoters (Fig. 3c). To test further the importance of MKK4 and MKK5 in the early-defence response, corresponding dominant-negative mutants were generated by replacing the conserved lysine residue in the ATP-binding site with methionine. As shown in Fig. 3d, dominant-negative MKK4 or MKK5 but not MKK1 (not shown) could partially block flg22 activation of the *WRKY29* and *FRK1* promoters similarly to U0126 or MKP1 (Fig. 1b, d). In addition, MKK4a and MKK5a, but not MKK1a and MKK2a, could bypass the requirement of FLS2 in flg22 signalling, demonstrated by their ability to activate the *WRKY29* and *FRK1* promoters in *fls2* mutant protoplasts (Fig. 3e).

Thus, on the basis of both gain-of-function and loss-of-function analyses, it appears that flagellin signalling activates MKK4 and MKK5, which in turn phosphorylate and activate MPK3 and MPK6, leading to the expression of early-defence response genes. The functions of MKK4 and MKK5 in flg22 signalling are probably redundant in *Arabidopsis* leaf cells (see below). It has been proposed that MPK3 and/or MPK6 mediate expression of *GST6* in *Arabidopsis* protoplasts treated with H_2O_2 (ref. 35). A MAPKK functioning in this oxidative-stress signalling pathway has not been identified. Although MKK4 and MKK5 can activate MPK3 and MPK6, neither MKK4a nor MKK5a was able to induce the expression of *GST6*, a reporter gene in the oxidative-stress signalling pathway (Fig. 3c, e). This result suggests that flagellin and H_2O_2 may activate different MAPK signalling cascades.

A specific MAPKKK in flg22 signalling

On the basis of sequence homology in the kinase domains to mammalian MAPKKKs, there are at least 25 putative *Arabidopsis* MAPKKKs that can be divided into six subgroups[19]. The sequence similarity among the subgroups is low, especially in the regulatory domains, indicating that the subgroups have distinct functions[19,38]. We analysed four *Arabidopsis* MAPKKKs (CTR1, EDR1, MEKK1 and ANP1; refs 19, 38) from three subgroups expressed in leaf cells, to determine whether any of them could activate MKK5. Constitutively active derivatives of these four MAPKKKs were constructed by deleting the putative regulatory domain[35]. The four constitutively active MAPKKKs were fused to a HA-epitope tag and co-expressed individually with Myc-epitope-tagged wild-type MKK5 in protoplasts. To determine whether MKK5 had been phosphorylated and activated *in vivo*, MKK5 was immunoprecipitated using an anti-Myc antibody, and its activity was determined *in vitro* using a purified kinase-inactive MPK6 fused to glutathione S-transferase (GST–MPK6Km) as a substrate. As shown in Fig. 4a, MKK5 was specifically activated by constitutively active MEKK1 (ΔMEKK1) and could phosphorylate MPK6. Constitutively active ANP1 (ΔANP1), which can mimic the H_2O_2 signal and induce the *GST6* promoter[35], activated MKK5 only marginally despite a high level of protein expression (Fig. 4a), suggesting that flagellin and H_2O_2 activate different MAPK pathways. A control experiment using a kinase-inactive mutant of MKK5 ruled out the possibility of a direct phosphorylation of GST–MPK6Km by MEKK1 (not shown).

Consistent with the above results, ΔMEKK1 activated the *WRKY29* and *FRK1* promoters in the absence of flg22 in both wild-type and *fls2* mutant protoplasts (Fig. 4b, c) but not the activity of the H_2O_2- or ΔANP1-inducible *GST6* promoter (Fig. 4b). Constitutively active CTR1 (ΔCTR1), which did not activate MKK5, had no effect on the *WRKY29* and *FRK1* promoter activities (Fig. 4b, c). To test further the involvement of MEKK1 in flg22 signalling, a dominant-negative mutant was generated by altering the ATP-binding site but maintaining the regulatory and protein–protein interaction domains of the full-length MEKK1. The dominant-negative mutant (MEKK1in) partially suppressed flg22 activation of *WRKY29* and *FRK1* promoters (Fig. 4d), similarly to U0126 treatment, MKP1, and dominant-negative MKK4 or MKK5 (Figs 1b, d and 3d). Dominant-negative CTR1 did not have an effect (not shown). Taken together, the results described so far strongly suggest that flagellin perception by FLS2 leads to the induction of *WRKY29* and *FRK1* transcription through the activation of a MAPK signalling cascade consisting of MEKK1, MKK4/MKK5, and MPK3/MPK6.

Positive feedback control by WRKYs

WRKY proteins have been shown to bind to W-box DNA elements (containing a TGAC core sequence) that are found in the promoters of many defence-related genes including *WRKY29* (10 W boxes) and *FRK1* (15 W boxes)[28,32–34]. In parsley protoplasts, a fungal pathogen-inducible WRKY1 protein, with two WRKY domains, is targeted to the nucleus and activates its own promoter by binding to multiple W boxes[33]. *Arabidopsis* WRKY29, with a single WRKY domain, is not the orthologue of parsley WRKY1 (ref. 33). WRKY29-GFP, a WRKY29 and green fluorescent protein fusion, was constitutively localized in the nucleus in the presence or absence of flg22 (Fig. 5a). Transient expression of WRKY29 activated its own promoter in the absence of flg22 (Fig. 5b), indicating a positive feedback control[33]. WRKY29 also strongly activated the *FRK1* promoter, but suppressed the basal activities of the *GST6* and *RD29A* promoters (Fig. 5b). In the *fls2* mutant protoplasts, transient expression of WRKY29 activated the *WRKY29* and *FRK1*, but not *RD29A*, promoters, suggesting that WRKY29 acts downstream of the flagellin receptor (Fig. 5c). Treatment of WRKY29-expressing protoplasts with flg22 did not further enhance the activity of the *WRKY29* and *FRK1* promoters (Fig. 5b). These results are consistent

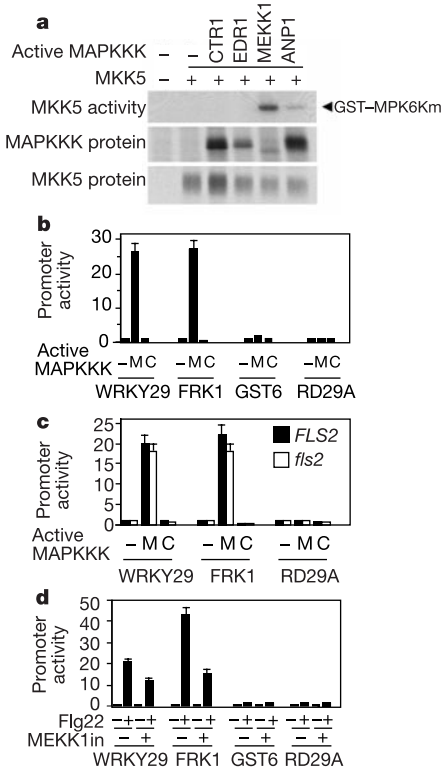

Figure 4 MEKK1 initiates the flg22 MAPK cascade. **a**, Constitutively active MEKK1 activates MKK5. Protoplasts were expressing MKK5 and one constitutively active MAPKKK. MKK5 activity using GST–MPK6Km as a substrate (top) and MAPKKK (middle) and MKK5 (bottom) expression are shown. **b**, Constitutively active MEKK1 induces the *WRKY29* and *FRK1* promoters. Protoplasts were expressing GFP or constitutively active MEKK1 (M) or CTR1 (C). **c**, MEKK1 acts downstream of FLS2. Protoplasts were isolated from wild-type (*FLS2*) and mutant (*fls2*) leaves. **d**, Dominant-negative MEKK1 inhibits flg22 activation of the *WRKY29* and *FRK1* promoters. Protoplasts were expressing a kinase-inactive MEKK1 (MEKK1in).

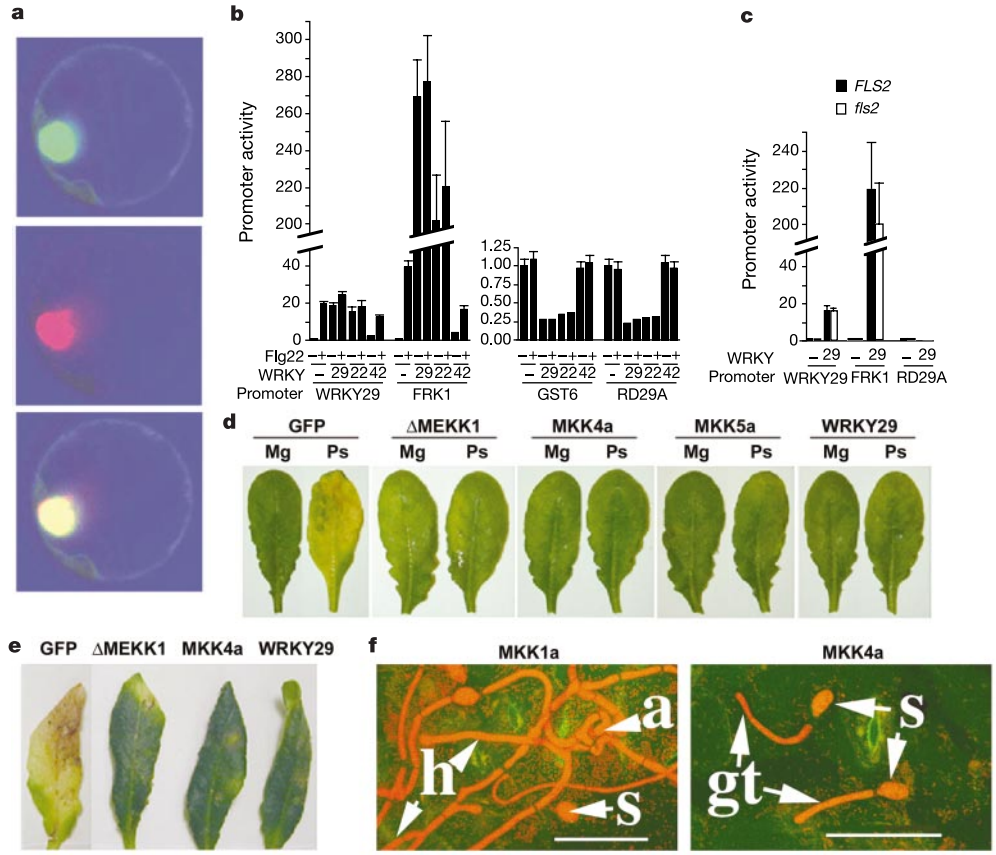

Figure 5 The flg22 MAPK cascade and specific WRKYs are important for *Arabidopsis* defence. **a**, Nuclear localization of WRKY29-GFP. Visualization of protoplasts expressing WRKY29-GFP (top), a red-fluorescent nuclear marker (middle), and super-imposition (bottom) without flg22. **b**, WRKY22 and WRKY29 activate early-defence genes. Protoplasts were expressing GFP, WRKY29 (29), WRKY22 (22) or WRKY42 (42). **c**, WRKY29 acts downstream of FLS2. Protoplasts were isolated from wild-type (*FLS2*) and mutant (*fls2*) leaves. **d**, Leaves expressing ΔMEKK1, MKK4a, MKK5a or WRKY29 exhibit reduced disease symptoms after *P. syringae* (Ps) infection. Control was infiltrated with 10 mM $MgSO_4$ (Mg). **e**, Leaves expressing ΔMEKK1, MKK4a or WRKY29 exhibit reduced disease symptoms after *B. cinerea* infection on the right half of each leaf. **f**, The early stage of *B. cinerea* development was inhibited on leaves expressing MKK4a. On MKK1a-expressing leaves (MKK1a), germinated spores (s) of *B. cinerea* formed superficial hyphae (h) and branched appressoria (a) 2 days after infection. The fungal spores (s) formed only germ tubes (gt) on MKK4a-expressing leaves (MKK4a). Scale bars, 50 μm.

with the idea that unmodified wild-type WRKY29 can act as a transcriptional activator. In contrast to WRKY29, a different *Arabidopsis* WRKY protein, WRKY42 (At4g04450), did not activate the *WRKY29* and *FRK1* promoters, but instead slightly inhibited the flg22 activation of these promoters (Fig. 5b). Moreover, unlike WRKY29, WRKY42 did not suppress basal activities of the *GST6* and *RD29A* promoters (Fig. 5b). Thus, we suggest that WRKY29 is a key transcriptional activator involved in the expression of defence genes in *Arabidopsis* innate immune responses. Similarly to MKK and MPK gene families, the large WRKY family also has members that are highly homologous to WRKY29 (ref. 32). The homologous WRKY22 (At4g01250) in the same WRKY subgroup[32] was cloned and tested in the protoplast transient assay. WRKY22 regulated these promoters similarly to WRKY29 (Fig. 5b), suggesting that WRKY29 and WRKY22 may be functionally redundant.

Resistance to bacterial and fungal pathogens

On the basis of the results obtained with the protoplast transient expression assays, we tested whether *Arabidopsis* plants exhibited enhanced resistance to bacterial pathogens when the flagellin MAPK cascade is constitutively activated or WRKY29 is expressed by means of *Agrobacterium*-mediated transient transformation[39]. When infected by the virulent bacterial pathogen *Pseudomonas syringae*, transiently transformed control leaves expressing GFP developed chlorotic lesions (Fig. 5d). Similar results were obtained with untransformed leaves and leaves expressing ΔCTR1, MKK1a or wild-type WRKY42 as controls (not shown). In contrast, transformed leaves expressing ΔMEKK1, MKK4a, MKK5a or wild-type WRKY29 displayed enhanced resistance to *P. syringae* (Fig. 5d). The observation that both MKK4a and MKK5a can confer pathogen resistance is consistent with their functional redundancy. We note that development of soft-rot symptoms caused by infection with the fungal pathogen *Botrytis cinerea* was also effectively suppressed when ΔMEKK1, MKK4a or WRKY29, but not GFP, was transiently expressed in leaves (Fig. 5e). Microscopic analysis indicated that in *Arabidopsis* leaves expressing MKK1a as a control, fungal spores germinated and formed superficial mycelium and branched appressoria filled with intensely stained cytoplasm within two days after *B. cinerea* infection (Fig. 5f). In contrast, only germ tubes were formed during the same period of time in leaves expressing MKK4a. Similar results were obtained with ΔMEKK1, MKK5a and WRKY29 (not shown). These results suggest that defence responses activated by the flagellin MAPK cascade or WRKY29 are effective against both fungal and bacterial pathogens.

Discussion

We have demonstrated that *Arabidopsis* mesophyll protoplast transient expression assays can be combined with genetic and genomic information to provide a powerful tool for the analysis of MAPK signalling involved in plant innate immunity. Because wild-type WRKY22 or WRKY29 is sufficient to mimic flg22 and MAPK signalling, we suggest that a specific WRKY inhibitor may be

phosphorylated and inactivated by the flagellin MAPK cascade upon pathogen infection. As summarized in the model shown in Fig. 6, this would make the flg22 signalling pathway partially analogous to signalling pathways in insects and mammals, in which Toll and TLRs, respectively, initiate a cascade of signalling events leading to phosphorylation and degradation of an inhibitor of a Rel-like transcription factor[2,4]. It will be interesting to test whether the flg22- and FLS2-dependent phosphorylation of the ankyrin-containing protein AtPhos43, recently identified by a proteomic approach[40], is MAPK dependent and can control WRKY22 and WRKY29 subcellular localization and/or activity.

The flagellin MAPK cascade leading to the activation of *Arabidopsis* MPK3 and MPK6 is reminiscent of the activation of the tobacco orthologues of MPK3 and MPK6, WIPK and SIPK, respectively, by fungal elicitin, as well as during Avr9-Cf-9 and TMV-N gene-for-gene interactions[14,21,22]. Recently, Yang *et al.*[41] identified a tobacco MAPKK, NtMEK2, which can activate WIPK and SIPK. Expression of a constitutively active mutant of NtMEK2 in tobacco leaves led to the induction of hypersensitive cell death and the expression of defence genes in the absence of pathogens. The results suggest that a MAPK cascade containing NtMEK2, WIPK and SIPK is involved in the expression of fungal pathogen defence responses in tobacco[41]. The *Arabidopsis* orthologues of NtMEK2 are MKK4 and MKK5[19,41], further suggesting the importance of the flagellin MAPK cascade in pathogen defence. Moreover, the data in Fig. 5 suggest that the *Arabidopsis* flagellin MAPK cascade may also be involved in expression of fungal defence responses without eliciting extensive hypersensitive cell death as in the case of NtMEK2 in tobacco. This, in turn, suggests that signalling events initiated by diverse pathogens converge into a conserved MAPK cascade (shown in Fig. 6)[7,14,17–19,23,41]. However, additional quantitative analyses in both transiently and stably transformed plants are required to confirm the proposed role of the flg22 MAPK defence-response pathway.

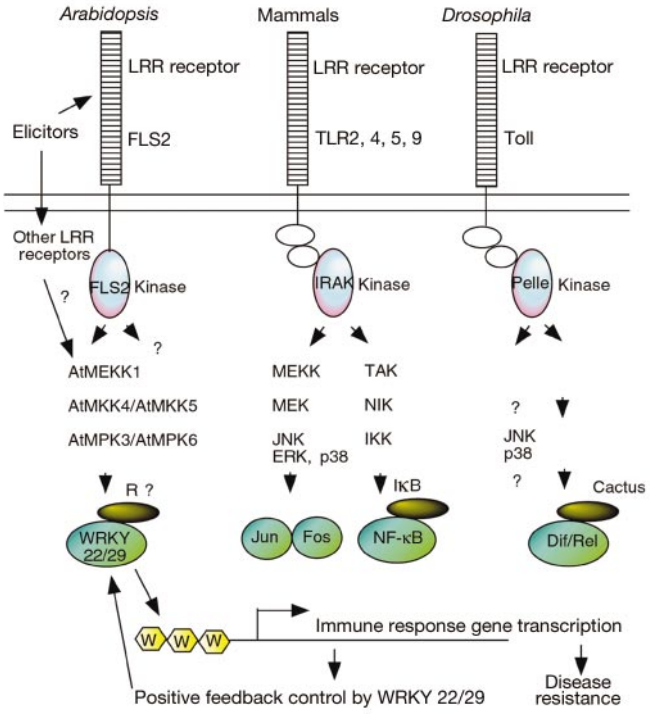

Figure 6 Model of innate immune signalling activated by LRR receptors in *Arabidopsis*, mammals and *Drosophila*. A putative repressor (R) could control WRKY22 and WRKY29 activity because their overexpression bypasses the requirement of elicitors. The conserved signalling pathways for innate immune responses in animals are summarized on the basis of recent reviews on mammals[2] and *Drosophila*[4].

As shown in Figs 1b, d, 3d and 4d, our data also reveal the previously unexpected existence of a MAPK-independent pathway in flg22 signalling. Characterization of this new pathway, which appears to be as important as the MAPK pathway for the induction of defence-related genes, will probably uncover additional complexity in the signalling pathways that control the expression of early-defence-related genes. Future work may also allow these MAPK-dependent and MAPK-independent pathways to be linked with other defence signalling components, such as NDR1, EDS1, PAD4, EDR1, MPK4, PBS1 and PBS2, defined by *Arabidopsis* mutants[8,31,42–44].

The lack of MAPK cascade mutations in the flg22 signalling pathway is most probably due to functional redundancy in the pathway, as suggested by the identification of the MKK4/MKK5 and MPK3/MPK6 pairs in our study. The WRKY22 and WRKY29 transcription factors in the flg22 signalling pathway may also provide redundant functions. Furthermore, similar to the yeast MAPKKK STE11 (ref. 45), some plant MAPK signalling components could be involved in more than one pathway[19]. The H_2O_2-activated MAPK cascade shares MPK3 and MPK6 with the flg22 pathway but activates different target genes[35]. The MAPKKK of the flagellin cascade, MEKK1, may also initiate a different MAPK cascade. On the basis of two-hybrid analyses and mutant complementation assays in yeast, an *Arabidopsis* MAPK cascade (MEKK1-MKK1/MKK2-MPK4) has been proposed[37]. In addition, we note that, besides the flagellin MAPK cascade, other pathways using untested MAPK, MAPKK and MAPKKK genes could also be important in *Arabidopsis* innate immunity. Finally, plants transiently expressing ΔMEKK1, MKK4a or WRKY29 exhibited enhanced resistance to both bacterial and fungal pathogens (Fig. 5), suggesting that other pathogen-derived signals in addition to flg22 may activate the MEKK1, MKK4/MKK5, MPK3/MPK6, WRKY22/WRKY29 signalling pathway through distinct receptors (Fig. 6)[40]. Given this complexity, a combination of genetic, genomic, cellular and biochemical approaches including the construction of knockout mutants and expression of signalling components in transgenic plants will be required to untangle the intertwined signalling webs mediated by MAPK cascades in plants.

Note added in proof: Transient expression of MKK4a in *Arabidopsis* leaves, as in Fig. 5d, inhibited the growth of *P. syringae maculicola* ES4326 15-fold one day after infiltration, and over 100-fold four days after infiltration. The results are consistent with the leaf phenotype shown in Fig. 5d. □

Methods

Reporter constructs

To identify early-response genes, a subtracted cDNA library that represented mRNA species induced by the elicitor flg22 in *Arabidopsis* mesophyll protoplasts was generated as previously described[30]. The DNA regions immediately upstream from the translation start sites of the *WRKY29*, *FRK1* and *GST1* genes were amplified by PCR from *Arabidopsis* (Col-0) genomic DNA. The sizes of the amplified fragments were 2.6 kilobases (kb), 2.8 kb, and 0.9 kb, respectively. The promoters were fused to a luciferase reporter gene to generate *WRKY29-LUC*, *FRK1-LUC* and *GST1-LUC*[26,35].

Effector constructs

The *Arabidopsis* cDNAs, encoding MAPKs, were: MPK2 (Atlg59580), MPK3 (At3g45640), MPK5 (At4g11330), MPK6 (At2g43790), MPK7 (At2g18170), MPK9 (At3g18040). The MAPKKs were: MKK1 (At4g26070), MKK2 (At4g29810), MKK4 (Atlg51660), MKK5 (At3g21220). The MAPKKKs were: CTR1 (At5g03730), EDR1 (At1g08720), MEKK1 (At4g08500), ANP1 (At1g09000). Others were receptor FLS2 (At5g46330), transcription factors WRKY22 (At4g01250), WRKY29 (At4g23550) and WRKY42 (At4g04450). All cDNAs were obtained by PCR from an *Arabidopsis* cDNA library[26,35] and verified by sequencing. Mouse MKP1 was a gift from H. Sun and N. Tonks[36]. PCR products were fused to the double HA (MAPK, MAPKKK, receptor and WRKY) or Myc (MAPKK and MKP1) epitope tag sequence and inserted into a plant expression vector or pCB302 containing the *35SC4PPDK* promoter and the *NOS* terminator[35,36,39].

The constitutively active forms of MAPKKs were generated by site-specific mutation, replacing the serine or threonine residues in the activation loop domain [S/T]XXXXX[S/T] by acidic amino acids glutamate or aspartate: MKK1(T218ES224D), MKK2(T220DT226E), MKK4(T224DS230E) and MKK5(T215ES221E). The active forms of MAPKKK were generated by keeping the catalytic domain only: ΔCTR1(544–799),

ΔEDR1(662–921), ΔMEKK1(326–592), ΔANP1(57–338). The inactive forms of all kinases were generated by site-specific mutation, replacing the conserved lysine residues in the kinase ATP-binding loop by a methionine: FLS2(K898M), MKK4(K108M), MKK5(K99M), MPK6(K92M,K93M), MEKK1(K361M). All these mutations were verified by sequencing.

Arabidopsis mesophyll protoplast transient expression assay

Protoplast transient expression assay was carried out as described previously[26,35]. Promoter activities are represented by LUC/GUS activities, and normalized to the value obtained with protoplasts without the treatments. In case of protoplasts prepared from *fls2* mutant plants, promoter activities are normalized to the value obtained with wild-type protoplasts transfected with a control GFP plasmid. Constitutively active MAPKKKs, immunoprecipitated from transfected protoplasts, phosphorylated casein *in vitro*[36], indicating that they are active kinases. GFP fluorescence was observed by either Nikon TE200 fluorescent microscopy or with Leica TCS NT Confocal Spectrophotometer (Germany). Co-expression of a nuclear-targeted red-fluorescent protein was used as a control[46].

RT-PCR

Samples were taken at indicated time points after treatment and total RNA was prepared as described[30]. RT-PCR was performed with 1 ng of total RNA and 0.6 μM of each primer using OneStep RT-PCR Kit (Qiagen). PCR was run for 35 cycles. Constitutively expressed *UBQ10* (AT4g05320) or *ACTIN-1* (At2g37620) mRNA was always co-amplified with each mRNA and used as an internal standard.

Protein kinase assays

The MAPK in-gel kinase assay was carried out as described[35]. For immuno-complex assays, tagged kinases were immunoprecipitated from lysates of transfected protoplasts with the corresponding antibody and analysed with a known substrate as indicated. The GST–MPK6Km fusion protein was generated by subcloning the coding sequence into pGEX-4T-1 vector (Amersham Pharmacia Biotech) in frame with the GST coding sequence. The fusion protein was purified according to the manufacturer's procedure and used as a substrate for *in vitro* phosphorylation assay. Except for the kinase-inactive mutants that were used as controls, all protein kinases showed significant auto-phosphorylation activity and were able to phosphorylate an exogenous substrate, indicating that they are active kinases (not shown).

Agrobacterium-mediated transient transformation

To analyse plant susceptibility to a bacterial pathogen, five-week-old *Arabidopsis* plants were infiltrated with a suspension (containing 100 μM acetosyringone) of *A. tumefaciens* carrying a binary vector pCB302 (ref. 39) expressing GFP, ΔMEKK1, MKK4a, MKK5a or WRKY29. The plants were incubated for three days under the conditions described[30]. Infiltrated leaves were then inoculated with 10 mM $MgSO_4$ or the same solution with suspended *P. syringae maculicola* ES4326 (10^4 colony-forming units, CFU, per cm^2) at the location of transient gene expression. The leaves were photographed three days later.

To analyse plant susceptibility to a fungal pathogen, a suspension (containing 100 μM acetosyringone and 0.01% Silwett L-77) of *A. tumefaciens* expressing GFP, ΔMEKK1, MKK4a, MKK1a or WRKY29 was first applied to the lower surface of a leaf of six-week-old *Arabidopsis*. The leaves were incubated for 24 h and then the upper surface of transformed leaves was infected with *B. cinerea* sclerotia. The infected leaves were photographed five days later after removing the fungal sclerotia. To observe the early stage of *B. cinerea* development, 5-μl drops of a 5×10^5 spores ml^{-1} suspension were placed on the upper surface of an *Arabidopsis* leaf. Two days after infection, fungal structures were visualized by trypan blue lactophenol staining and observed with Confocal Spectrophotometer TCS NT (Leica, Germany). Untransformed and GFP-expressing control leaves developed similar lesions to those on the MKK1a-expressing leaves.

Received 28 August; accepted 24 December 2001.

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Acknowledgements

We thank C. Xiang for the pCB302 minibinary vector, O. R. Patharkar and J. Cushman for the DsRed-CSP1 plasmid; S. Volko and J. Stone for sharing unpublished information; S.-H. Cheng, B. Moore and I. Hwang for technical advice; W.-C. Chen for help in bioinformatics; and S. Ramu and F. Rolland for critical reading of the manuscript. This work was supported by a NSF plant genome project grant to J.S. and F.M.A., a USDA grant to J.S., and a NIH grant to F.M.A. T.A. was supported in part by fellowships from the Toyobo Biotechnology Foundation and the Uehara Memorial Foundation.

Correspondence and requests for materials should be addressed to J.S. (e-mail: sheen@molbio.mgh.harvard.edu).