Pathogen-secreted proteases activate a novel plant immune pathway

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Mitogen-activated protein kinase (MAPK) pathways play central roles in innate immune signalling networks in plants and animals1-2. In plants, however, the molecular mechanisms of how signal perception is transduced to MAPK activation remain elusive3. Here we report that pathogen-secreted proteases activate a previously unknown signalling pathway in Arabidopsis thaliana involving the Gα, Gβ, and Gγ subunits of heterotrimeric G-protein complexes, which function upstream of an MAPK cascade. In this pathway, receptor for activated C kinase 1 (RACK1) functions as a novel scaffold that binds upstream of an MAPK cascade. The protease–G-protein–RACK1–MAPK thereby linking upstream G-protein signalling to downstream activated C kinase 1 (RACK1) functions as a novel scaffold that binds upstream of an MAPK cascade. The protease–G-protein–RACK1–MAPK cascade modules identified in these studies are distinct from previously described plant immune signalling pathways such as that elicited by bacterial flagellin, in which G proteins function downstream of or in parallel to an MAPK cascade without the involvement of the RACK1 scaffolding protein. The discovery of the new protease-mediated immune signalling pathway described here was facilitated by the use of the broad host range, opportunistic bacterial pathogen Pseudomonas aeruginosa. The ability of P. aeruginosa to infect both plants and animals makes it an excellent model to identify novel immunoregulatory strategies that account for its niche adaptation to diverse host tissues and immune systems.

We found that culture filtrate of P. aeruginosa strain PA14 activates an Arabidopsis β-glucuronidase (GUS) reporter gene under the control of the pathogen-inducible CYP71A12 promoter (CYP71A12pro:GUS). Whereas the well-characterized immune elicitor flg22, a synthetic peptide that corresponds to the active epitope of bacterial flagellin, induces CYP71A12pro:GUS in the root elongation zone3, PA14 culture filtrate activates the reporter in the cotyledons and leaves of both wild-type Arabidopsis Col-0 and fls2 mutant seedlings in which the flagellin receptor is mutated (Fig. 1a).

By screening a collection of 64 P. aeruginosa PA14 regulatory and secretion-related mutants, we found that the induction of the CYP71A12 promoter was dependent on the quorum-sensing gene lasI and on the type II secretion apparatus–encoding genes xcpR, xcpT, xcpW, and xcpZ (Fig. 1a and Extended Data Table 1). Ion-exchange chromatography fractionation (Extended Data Fig. 1a) followed by mass spectrometry (data not shown) identified the elicitor in the PA14 secretome as protease IV, a type II-secreted, PvdS-regulated lysyl class serine protease.

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**Figure 1 | Proteases trigger innate immune responses in Arabidopsis via proteolytic activity.**

- **a** | Activation of CYP71A12pro:GUS in wild-type Arabidopsis Col-0 or fls2 mutant cotyledons by culture filtrates from wild-type P. aeruginosa PA14, from PA14 mutants containing a transposon insertion in lasI or xcpR, or from PA14/ΔprpL. b, Western blot depicting activation of MAPKs by PrpL or flg22. Numbers on the left axis of the blot represent marker size (molecular mass in kilodaltons). c, Chemiluminescence assay showing elicitation of an oxidative burst by PrpL; r.l.u., relative luminescence units. HK: 100 nM ‘heat-killed’ PrpL. d, Callose formation in cotyledons elicited by PrpL or flg22 detected by aniline blue staining. e, Protection of 4-week-old Arabidopsis leaves from P. syringae pv. tomato strain DC3000 infection by pre-infiltrated PrpL; c.f.u., colony-forming units. f, Western blot depicting activation of MPK3 and MPK6 by PrpL and inactive variants of PrpL. The same molecular mass region of the blot is shown as in b. g, Western blot depicting activation of MPK3 and MPK6 by PrpL or ArgC or TLCK-treated PrpL or TLCK-treated ArgC. The same molecular mass region of the blot is shown as in b, h. Growth of X. campestris strains 8004/argC or 8004/vector in 3-week-old B. oleracea leaves. Data represent mean ± s.d.; n = 16 individual seedlings (c) and n = 10 leaves from five plants (e, h); ***, P < 0.001, Student’s t-test. The experiments in a and d were repeated three times with similar results and the representative images shown were selected from at least three images.
encoded by the *P. aeruginosa prpL* gene (PA14_09900). Purified His-tagged PA14 protease IV (referred to as PrpL in the figure legends) activated *CYP71A12pro:GUS* (Extended Data Fig. 1b), whereas culture filtrate from an in-frame deletion mutant of *prpL* (PA14/ΔprpL) did not (Fig. 1a).

Purified protease IV is a very strong elicitor of immune responses in *Arabidopsis*, comparable to flg22 in the activation of MPK3 and MPK6 (but not MPK4) (Fig. 1b), elicitation of an oxidative burst (Fig. 1c), deposition of callose in cotyledons (Fig. 1d), and protection of adult *Arabidopsis* leaves from *Pseudomonas syringae* pathovar (pv.) *tomato* strain DC3000 infection (Fig. 1e). In contrast, trypsin, a well-characterized serine protease, failed to activate MAPK cascades or trigger an oxidative burst (Extended Data Fig. 2a, b). Global transcriptional profiling analysis (Extended Data Fig. 3a), confirmed by quantitative PCR with reverse transcription (RT-qPCR) analysis of selected defence-related genes (Extended Data Fig. 3b), showed a high degree of concordance between the genes activated or repressed by protease IV and genes previously shown to be regulated by flg22 or oligogalacturonides in seedlings (Pearson correlation coefficients of 0.899 and 0.864 for protease-IV-specific burst (Extended Data Fig. 2a, b). Global transcriptional profiling between the genes activated or repressed by protease IV and genes previously shown to be regulated by flg22 or oligogalacturonides in seedlings (Pearson correlation coefficients of 0.899 and 0.864 for protease-IV-activated genes) (E x -P. aeruginosa prpL was reduced about sixfold compared with the 8004/vector control (Extended Data Fig. 4a, d), Treatment of protease IV with the protease inhibitor TLCK (Fig. 1g and Extended Data Fig. 4b, d) or with heat (Fig. 1c and Extended Data Fig. 4c) also resulted in a loss of elicitation ability. The closest homologue of *P. aeruginosa* protease IV in sequenced bacterial genomes is encoded by the argC gene of *Xanthomonas campestris*, a bona fide plant pathogen (Extended Data Fig. 5a). Purified His-tagged argC protease exhibited protease activity in vitro and triggered the activation of MPK3 and MPK6 that is dependent on ArgC protease activity (Fig. 1g).

We noticed that there is a high rate of naturally occurring null mutations in the *Xanthomonas argC* gene (8 out of 22 total alleles in sequenced *Xanthomonas* genomes; Extended Data Fig. 5b–d), suggesting that argC is probably under negative selection. Consistent with the sequence data, the culture filtrate of strain *X. campestris pv. raphani* strain 1946, from which the functional argC gene was cloned, activated the *CYP71A12pro:GUS* reporter, whereas culture filtrates from two *X. campestris pv. campestris* strains (8004 and BP109), which contain presumptive argC null frame shift mutations, failed to activate (Extended Data Fig. 5e). We complemented the null argC mutant in strain 8004 (*Xcc* 8004) with the functional argC gene from strain 1946 (8004/argC) (Extended Data Fig. 5e). Consistent with ArgC-mediated induction of a host immune response during an infection in a mature plant, the growth of 8004/argC in *Brassica oleracea* (broccoli), a natural host of *X. campestris*, was reduced about sixfold compared with the 8004/vector control (Fig. 1h). The expression of haemagglutinin (HA)-tagged ArgC was readily detected in broccoli leaves infected with 8004/argC (Extended Data Fig. 5e), indicating that ArgC is synthesized during infection.

Next, we sought to investigate the mechanism by which protease IV activates an immune response in *Arabidopsis*. Previous studies have shown that G proteins play a role in microbe-associated molecular pattern molecule-mediated responses*. In the case of protease IV, we found reduced expression of defence-related genes in *gβ* or *gβ* mutants (and in a *gβgγ* double mutant), reduced levels of the oxidative burst in a *gβ* mutant and a *gβgγ* double mutant, reduced MPK3 and MPK6 activation, and reduced protection against *P. syringae* infection in a *gβgγ* double mutant (Fig. 2a–c and Extended Data Fig. 6a, b). The induction of *CYP71A12* and activation of MPK3 and MPK6 by *X. campestris* ArgC was also diminished in the G-protein mutants, similar to the pattern observed for protease IV (Fig. 2a, b). In contrast to protease IV and ArgC, in the case of flg22, defence gene expression was only reduced in *gβ* and *gβgγ* double mutants, the oxidative burst was more severely affected in a *gβ* mutant than in a *gγ* mutant, protection against *P. syringae* was only modestly affected in a *gβgγ* double mutant, and the activation of MAPKs was not affected in any of the G-protein mutants (Fig. 2a–c and Extended Data Fig. 6b). These data show that G-protein signalling is required to activate downstream MAPKs in response to protease IV and ArgC, but not flg22 (Fig. 2a), and that G proteins play different roles in canonical microbe-associated molecular pattern molecule and protease-mediated signalling pathways.

In a search of potential signalling components that could link the heterotrimeric G-protein complex to downstream MAPK cascades, we considered the conserved scaffold protein RACK1 (ref. 7). The ration-ale was that RACK1 shares about 25% amino-acid sequence identity with Gβ and like Gβ has a seven-bladed β-propellor structure*, interacts with Gβ in metazoans*, and functions in innate immune signalling in rice*. There are three RACK1 homologues in *Arabidopsis* (RACK1A, 1B, and 1C, which share about 90% amino-acid sequence identity*).

We used three methods to determine whether *Arabidopsis* RACK1 proteins interact with G proteins and MAPKs. In a bimolecular fluorescence complementation (BiFC) assay in *Nicotiana benthamiana* leaves, RACK1A, RACK1B, and RACK1C interacted with Gβ, MEKK1 (K361M), MKK4, MKK5, MKP3, and MPK6, but not Gα or MPK4 (Extended Data Fig. 7a). The kinase-inactive version of MEKK1, MEKK1(K361M), was used in this experiment because the auto-activation of native MEKK1 destabilizes its interaction with RACK1 (data not shown). MEKK1, MKK4/5, and MKP3/6 are the *Arabidopsis* MAPK kinase (MA PKKK), MAPK kinases (MAPKKs), and MAPKs, respectively, that were proposed to constitute an MAPK-signalling cascade in the flg22/FLS2 signalling pathway*. Similar results to those obtained with the BiFC assay in *N. benthamiana* were obtained with BiFC and split firefly luciferase complementation (SFLC) assays for RACK1A interactors in *Arabidopsis* protoplasts (Extended Data Fig. 7b, c). The interaction between RACK1 proteins and MPK3/6, but not MPK4, is consistent with the data in Fig. 1b, showing that MPK6 and MPK3, but not MPK4, are strongly activated after protease IV treatment.

In co-immunoprecipitation experiments in *Arabidopsis* mesophyll protoplasts using Flag-tagged RACK1 proteins as the bait and HA-tagged
mkk1 null mutant complemented with an MEKK1(K361M) construct (mkk1/1–MEKK1/–MEKK1(K361M)) (Extended Data Fig. 8c, d). As previously reported, MEKK1(K361M), which is deficient in kinase activity, rescues the severe growth defect of an mkk1 null mutant22. In contrast to the mkk4,5 knockdown lines, we did not consistently observe a decrease level of protease-IV-triggered MPK3/6 phosphorylation in either of the mkk1 mutants (Extended Data Fig. 8e). One explanation for the partial decrease in WRKY gene induction but not in MPK3/6 phosphorylation in the mkk1 mutants is that multiple MAPKKKs12 function additively to activate MPK3/6 but that the phosphorylation assay is not sensitive enough to detect a partial loss of MAPKKK activity.

Obtaining genetic evidence that RACK1 is required for protease-mediated signalling is challenging because of the functional redundancy of the three RACK1 proteins in Arabidopsis. Transfer-DNA (tDNA) mutants corresponding to insertions in individual rack1 genes did not show any decrease in protease-IV- or flg22-activated MAPK levels (Extended Data Fig. 9a), and only moderate decreases in protease-IV- but not flg22-triggered defence gene induction (Extended Data Fig. 9b). Because rack1a rack1b rack1c triple null mutants have a dwarf phenotype and do not set seeds14, we generated two independent transgenic lines, amiR–rack1–es1 and amiR–rack1–es2, which express a previously described artificial microRNA (amiR–RACK1–4)15 under the control of an oestradiol-inducible promoter. These transgenic lines showed dramatically decreased transcript levels of all three rack1 genes following oestradiol treatment (Extended Data Fig. 9c). Following protease IV or ArgC treatment, amiR–rack1–es1 and amiR–rack1–es2 seedlings that had been induced with oestradiol exhibited markedly decreased levels of activated MPK3 and MPK6 (Fig. 4a). Protoplasts transfected with constitutively expressed amiR–RACK1–4 also showed reduced levels of protease-IV-mediated MPK3 and MPK6 activation (Extended Data Fig. 9d).

Figure 3 | RACK1A interacts with Gβ and MAPKs. a–f, Co-immunoprecipitation (Co-IP) assays in Arabidopsis protoplasts. Protoplasts were treated with 100 nM purified PrpL for 15 min. Target proteins were detected in western blots using anti-HA or anti-Flag antibodies. Numbers on the left axis of blots represent marker size (molecular mass in kilodaltons).

Gβ subunit as the prey, we observed binding between all three Arabidopsis RACK1 proteins and Gβ (Fig. 3a and Extended Data Fig. 7d). In contrast to Gβ, HA-tagged Gz was not pulled down by Flag-tagged RACK1 proteins (Fig. 3b and Extended Data Fig. 7d). In the co-immunoprecipitation experiments, the interaction of Gβ with RACK1A was not dependent on Gz, because the interaction was still present in the gββ double mutant (Extended Data Fig. 7e). Finally, consistent with the BiFC and SFLC assays, HA-tagged MEKK1(K361M), MKK5, MAPK3, and MAPK6 all co-immunoprecipitated with Flag-tagged RACK1A, whereas MAPK4 did not under the same condition (Fig. 3c–f). The amounts of the MAPKK and MAPKs that were pulled down by RACK1A in the co-immunoprecipitation experiments clearly decreased in the presence of protease IV (Fig. 3d–f), suggesting that protease IV releases the activated MAPKs from the RACK1–MAPK cascade complex to execute their downstream cellular functions. In the case of the MAPKK MEKK1, we also identified endogenous RACK1 proteins by mass spectrometric analysis as binding partners of MEKK1(K361M) (Extended Data Fig. 7f) in a transgenic line in which Flag-tagged MEKK1(K361M) is expressed under the control of the 3.9-kilobase (kb) MEKK1 native promoter in a mkk1 null mutant background.

To confirm the physiological relevance of the observed interactions between RACK1 and MAPK cascade components (Fig. 3 and Extended Data Fig. 7), we tested a variety of loss-of-function MAPK mutants and knockdowns. We found that the activation of the defence-related genes WRKY30 and WRKY33 by protease IV was almost completely blocked in two independent mpk3,6-es transgenic lines in which mpk3 is silenced with an oestradiol-inducible MPK3-RNA interference (RNAi) construct in a null mpk6 mutant background (Extended Data Fig. 8a). We also found that both protease-IV-triggered MPK3/6 activation and WRKY30 and WRKY33 gene induction were disrupted in mkk4,5-es transgenic lines (Extended Data Fig. 8a, b), which utilize a single oestradiol-inducible RNAi construct to target both M KK4 and MKK5 messenger RNAs (mRNAs). Finally, we observed a significant decrease in protease-IV-triggered induction of WRKY30 and WRKY33 mRNA accumulation in two mkk1 mutants, an mkk1 null mutant, and the

Figure 4 | Transiently silencing all three rack1 genes abrogates proteases but not flg22-mediated responses. a, Three-day-old wild-type Col-0 and transgenic Arabidopsis seedlings from two independent amir–rack1–es1 lines were treated with oestradiol to activate expression of the artificial microRNA constructs and then 2 days later were treated with PrpL, ArgC or flg22 and harvested for the MAPK phosphorylation assay. The same molecular mass region of the western blot is shown as in Fig. 1b. Seedlings were treated with oestradiol followed by PrpL, ArgC or flg22 as in panel a and then harvested for RT-qPCR analysis of CYP71A12 transcript levels. Water-treated Col-0 was used as a normalization control. c, Protection of 4-week-old wild-type Col-0 and transgenic amir–rack1–es1 or amir–rack1–es2 plants from P. syringae pv. tomato strain DC3000 infection mediated by PrpL or flg22 24 h after treatment with oestradiol. Data represent mean ± s.d.; n = 3 biological replicates with each experiment containing 12 seedlings (b) and n = 10 leaves from five plants (c); **p < 0.01; ***p < 0.001. Student’s t-test versus Col-0 (b) and versus mock (c). d, A model of protease-activated novel innate immune signalling pathway in Arabidopsis.
Fig. 9d, e). Similarly, knockdown of the *rack1* genes blocked protease-IV- or ArgC-mediated defence gene induction (Fig. 4b) and protease-IV-mediated activation of MAPKs or defence gene expression or protection against *P. syringae* were not affected by knockdown of the *rack1* genes (Fig. 4a–c and Extended Data Fig. 9e). These data are consistent with the conclusion that RACK1 proteins function in the protease IV and ArgC signalling pathway but not the flg22 pathway.

The RACK1 proteins studied here are the first MAPK cascade scaffolding proteins discovered for the large family of plant genes encoding MAPK cascade components. In yeast, the scaffolding protein Ste5 links an MAPK cascade to G-protein signalling in the mating pathway that is mediated by G-protein-coupled receptor stimulation by yeast pheromone<sup>16</sup>. In mammals, the scaffolding protein β-arrestin 2 brings MAPK cascade activity under the control of upstream G-protein-coupled receptors<sup>16</sup>. However, since plants do not have canonical G-protein-coupled receptors or orthologues of Ste5 and β-arrestin<sup>6,16</sup>, our data suggest that the linkage of G-proteins to MAPKs via RACK1 is mechanistically distinct from G-protein signalling in metazoans and yeast.

The protease-activated signalling pathway is summarized in the model shown in Fig. 4d. It remains to be determined whether the cleavage of protein targets by protease IV directly or indirectly activates downstream responses. In the latter possibility, pathogen-secreted proteases could release host polypeptides that function as damage-associated molecular patterns which are subsequently recognized by corresponding immune receptors. In either case, an evolutionary and physiological interpretation of our findings is that plants evolved a new surveillance system to recognize and respond to pathogen-encoded proteases that disrupt host homeostasis via their proteolytic activity.

**Online Content** Methods, along with any additional Extended Data display items and Source Data, are available in the online version of the paper; references unique to these sections appear only in the online paper.

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1. Tena, G., Boudsocq, M. & Sheen, J. Protein kinase signaling networks in plant innate immunity. *Curr. Opin. Plant Biol.* **14**, 519–529 (2011).
2. Arthur, J. S. & Ley, S. C. Mitogen-activated protein kinases in innate immunity. *Nature Rev. Immunol.* **13**, 679–692 (2013).
3. Millet, Y. A. et al. Innate immune responses activated in Arabidopsis roots by microbe-associated molecular patterns. *Plant Cell* **22**, 973–990 (2010).
4. Denoux, C. et al. Activation of defense response pathways by OGS and flg22 elicitors in Arabidopsis seedlings. *Mol. Plant* **1**, 423–445 (2008).
5. Traidej, M., Marquart, M. E., Caballero, A. R., Thibodeaux, B. A. & O’Callaghan, R. J. Identification of the active site residues of *Pseudomonas aeruginosa* protease IV. *J. Biol. Chem.* **278**, 2549–2553 (2003).
6. Urano, D., Chen, J.-G., Botella, J. R. & A.M. Heterotrimeric G protein signalling in the plant kingdom. *Open Biol.* **3**, 120186 (2013).
7. Ullah, H. et al. Structure of a signal transduction regulator, RACK1, from Arabidopsis thaliana. *Protein Sci.* **17**, 1771–1780 (2008).
8. Dell, E. J. et al. The jk subunit of heterotrimeric G proteins interacts with RACK1 and two other WD repeat proteins. *J. Biol. Chem.* **277**, 49886–49895 (2002).
9. Nakashima, A. et al. RACK1 functions in rice innate immunity by interacting with the Rac1 immune complex. *Plant Cell* **20**, 2265–2279 (2008).
10. Chen, J.-G. et al. RACK1 mediates multiple hormone responsiveness and developmental processes in Arabidopsis. *J. Exp. Bot.* **57**, 2697–2708 (2006).
11. Asai, T. et al. MAP kinase signalling cascade in Arabidopsis innate immunity. *Nature* **415**, 977–983 (2002).
12. Suarez-Rodriguez, M. C. et al. MEKK1 is required for flg22-induced MPK4 activation in Arabidopsis plants. *Plant Physiol.* **143**, 661–669 (2007).
13. MAPK Group (Ichimura, K. et al.). Mitogen-activated protein kinase cascades in plants: a new nomenclature. *Trends Plant Sci.* **7**, 301–308 (2002).
14. Guo, J. & Chen, J.-G. RACK1 genes regulate plant development with unequal genetic redundancy in Arabidopsis. *BMC Plant Biol.* **8**, 108 (2008).
15. Li, J.-F. et al. Comprehensive protein-based artificial microRNA screens for effective gene silencing in plants. *Plant Cell* **25**, 1507–1522 (2013).
16. Witzel, F., Maddison, L. & Bühlgen, N. How scaffolds shape MAPK signaling: what we know and opportunities for systems approaches. *Front. Physiol.* **3**, 475 (2012).
was inserted between the oestradiol-inducible promoter and the Escherichia coli in plasmid pEX18-g. The genomic fragment was used to complement a transgenic mekk1/pMEKK1::MEKK1(K361M) mutation in MEKK1 to disrupt MEKK1 kinase activity, and a double Flag-tag start codon, an ‘AAGG’ to ‘ATGG’ mutation in exon 2 (corresponding to K361M) pFGC–EST–RACK1 was used to generate transgenic into Xanthomonas campestris. The fractionation was performed at 4 uC overnight, followed by centrifugation at 20,000 g. The pellet was resuspended in 30 ml buffer A (20 mM Tris, pH 8.8), concentrated again into 10 ml buffer A, and loaded onto a 1-mL DEAE anion-exchange chromatography column (GE Healthcare) that was eluted with buffer B (20 mM Tris, pH 8.8, 1 M NaCl) and equilibrated with buffer A. The protein sample was loaded onto the column. The eluted protease IV was concentrated to 150 μl using Centricon Plus-70 filter (Millipore) to remove the excess ammonium sulfate, and diluted again into 10 ml buffer A. The protein sample was loaded onto a Superdex 200 gel filtration column (GE Healthcare). Purified protease IV was exchanged into M9 minimal medium and filter-sterilized using a 0.22 μm low-protein-binding filter (Millipore). The concentration of the purified protease IV was adjusted to 20 μM, aliquoted, and stored at −80 °C before being used for plant treatments. X. campestris protease ArgC was purified using the same protocol. Protease assay. The protease activity assay of protease IV and its homologue ArgC was determined as previously described. Protease IV and ArgC were inactivated by TLCK as previously described.

**Plant growth.** Seeds were sterilized in 20% bleach for 2 min and washed three times with sterile water. Seedlings were grown in liquid MS medium (Murashige and Skoog basal medium with vitamins from PhytoTechnology Laboratories supplemented with 0.5 g l−1 MES hydrate and 0.5% sucrose at pH 5.7) in either 24-well assay plates (BD Falcon) (eight seeds and 0.5 ml medium per well) for MAPK assays, microarray and RT-qPCR analysis, callose induction and GUS expression, or 96-well plates (Greiner Bio-One) (one seed and 0.2 ml medium per well) for oxidative burst measurements. Plates were sealed with Micropore tape and placed on grid-like shelves over water trays on a FloraLight cart in a plant growth chamber for 10 days at 21 °C with 75% relative humidity under 16 h of daylight (65–70 μE m−2 s−1). The media in 24-well plates was exchanged for fresh media on day 8, whereas the media in 96-well plates was exchanged for sterile water on day 9.

**Elaborate treatments.** The synthetic peptide flg22 was synthesized by Genscript. Experimentally determined optimal concentrations of protease IV were as follows: 20 nM for oxidative burst measurements, microarray and RT-qPCR analyses; 40 nM for MAPK activation; 100 nM for GUS expression; 500 nM for callose elicitation and the infection protection assay. For direct comparison, the same concentrations of flg22 and protease IV or ArgC were used in the same assays. Ten-day-old seedlings were treated with different elicitors for the following except otherwise specified: 6 h for GUS assays in reporter line CYP71A12pro:GUS; 10 min for MAPK activation assays; 1 h or 6 h for RT-qPCR analysis of selected genes; and 18 h for callose induction.

**Transient silencing of MAPK or MAPKK genes in transgenic plants.** In two independent npk3,6,es transgenic lines, MAPK3 was silenced with an oestradiol-inducible MPK3-RNAi construct in a null npk6 mutant (Salk_002471) background. In two mpk3,4,es transgenic lines, a single oestradiol-inducible RNAi construct was used to target both MPK4 and MPK5 mRNAs. Details of the construction of the transgenic and control plants were cloned into the EcoRI and the XhoI sites of pETP30 (ref. 24), creating plasmids pETP-prl3, pETP-prl4; and rifampicin 100 μg ml−1. Antibiotics were supplemented as needed: ampicillin or carbenicillin, 50 μg ml−1 for E. coli or 300 μg ml−1 for P. aeruginosa; kanamycin 50 μg ml−1 for E. coli and Xanthomonas campestris or 200 μg ml−1 for P. aeruginosa; and rifampicin 100 μg ml−1 for Arabidopsis. Purification of P. aeruginosa protease IV and Xanthomonas campestris ArgC.

**Puriﬁcation of P. aeruginosa protease IV and X. campestris ArgC.** The P. aeruginosa protease IV(X. campestris strain 1946 argC) gene was cloned into the BamHI and HindIII sites of pEX18-gm. The resulting plasmids were transformed into P. aeruginosa PAO ADD1976 by electroporation to generate the strains ADD/pETP-prl3 and ADD/pETP-prl4 for puriﬁcation of His-tagged protease IV or His-tagged ArgC, respectively. For argC complementation in Xanthomonas, the X. campestris strain 1946 argC gene was cloned into the BamHI site of pVS61 (ref. 26), creating plasmid pVS61-argC. An HA-tag was incorporated at the carboxy (C)-terminal of the argC gene to detect the complemented protein. The resulting plasmid and empty pVS61 vector were transformed into Xanthomonas campestris strain 8004 by triparental conjugation.

**Antibiotics were supplemented as needed: ampicillin or carbenicillin, 50 μg ml−1 for E. coli or 300 μg ml−1 for P. aeruginosa; kanamycin 50 μg ml−1 for E. coli and Xanthomonas campestris or 200 μg ml−1 for P. aeruginosa; and rifampicin 100 μg ml−1 for Arabidopsis.** Purification of P. aeruginosa protease IV and Xanthomonas campestris ArgC.

**Construcion of Arabidopsis transgenic lines.** Construction of amiR-rack1-b was performed as follows: the X. campestris strain 8004 was transformed with pVS61-argC. The resulting plasmid was introduced into Agrobacterium tumefaciens GV3101 cells by electroporation, and GV3101/pVS61-argC EST–RACK1 was used to generate transgenic Arabidopsis plants with an in-frame deletion mutant was constructed using a method described above for 4 days. Then the medium was changed to MS medium containing 10 μM oestradiol (Sigma, 100 mM stock in dimethylsulphoxide (DMSO)). After exposure to oestradiol for 3 days, the seedlings were treated with water and 40 nM purified protease IV for 10 min (for MAPK assays) or 20 nM purified protease IV for 1 h (for RT-qPCR assays).

**Transient silencing of rac1 genes in protoplasts and transgenic plants.** Mesophyll protoplasts isolated from leaves of 4-week-old Arabidopsis plants (4 × 106 cells in 200 μl) were transfected with 40 μg (20 μl) of amir–RACK1 construct to generate transgenic Arabidopsis plants with an in-frame deletion upstream of the start codon, an ‘AAGG’ to ‘ATGG’ mutation in exon 2 (corresponding to K361M mutation in MEKK1) to disrupt MEKK1 kinase activity, and a double Flag-tag coding sequence upstream of the stop codon.

**Fractionation of the PA14 secrome.** One litre of PA14 cells grown in M9 minimal medium (6.8 g l−1 NaH2PO4, 3 g l−1 KH2PO4, 0.5 g l−1 NaCl, 1 g l−1 NH4Cl, 2 mM MgSO4·7 H2O, 0.1 mM CaCl2, 10 μM FeCl3, 0.4% glucose, 10 mg ml−1 thiamine) was centrifuged at 20,000g for 4 h. The pellet was resuspended in 30 ml buffer A (20 mM Tris, pH 8.8, concentrated) was loaded onto a 1-mL DEAE anion-exchange chromatography column (GE Healthcare) that was washed with buffer B (20 mM Tris, pH 8.8, 1 M NaCl) and equilibrated with buffer A. Proteins were separated into 1 ml fractions with a linear gradient of buffer B (0–60% within 20-column volumes). The fractionation was performed at 4 °C with a flow rate of 1 ml min−1.

**Puriﬁcation of P. aeruginosa protease IV and X. campestris ArgC.** Secreted proteins from ADD1976/pETP-prl3 were precipitated as described above and resuspended in lysis buffer (50 mM Na2HPO4·7 H2O, 300 mM NaCl, 10 mM imidazole, pH 8.0). The sample was loaded onto a 5-mL His-Bind II affinity column (GE Healthcare) and the 6× His-tagged PA14 protease IV was purified according to the manufacturer’s instructions. The eluted protease IV was concentrated to 150 μl and immediately subjected to a Superdex 200 gel filtration column (GE Healthcare). Purified protease IV was exchanged into M9 minimal medium and filter-sterilized using a 0.22 μm low-protein-binding filter (Millipore). The concentration of the purified protease IV was monitored. The eluted protease IV was concentrated to 150 μl and immediately subjected to a Superdex 200 gel filtration column (GE Healthcare). Purified protease IV was exchanged into M9 minimal medium and filter-sterilized using a 0.22 μm low-protein-binding filter (Millipore). The concentration of the purified protease IV was monitored. The eluted protease IV was concentrated to 150 μl and immediately subjected to a Superdex 200 gel filtration column (GE Healthcare). Purified protease IV was exchanged into M9 minimal medium and filter-sterilized using a 0.22 μm low-protein-binding filter (Millipore). The concentration of the purified protease IV was monitored. The eluted protease IV was concentrated to 150 μl and immediately subjected to a Superdex 200 gel filtration column (GE Healthcare). Purified protease IV was exchanged into M9 minimal medium and filter-sterilized using a 0.22 μm low-protein-binding filter (Millipore). The concentration of the purified protease IV was monitored. The eluted protease IV was concentrated to 150 μl and immediately subjected to a Superdex 200 gel filtration column (GE Healthcare). Purified protease IV was exchanged into M9 minimal medium and filter-sterilized using a 0.22 μm low-protein-binding filter (Millipore). The concentration of the purified protease IV was monitored. The eluted protease IV was concentrated to 150 μl and immediately subjected to a Superdex 200 gel filtration column (GE Healthcare). Purified protease IV was exchanged into M9 minimal medium and filter-sterilized using a 0.22 μm low-protein-binding filter (Millipore). The concentration of the purified protease IV was monitored. The eluted protease IV was concentrated to 150 μl and immediately subjected to a Superdex 200 gel filtration column (GE Healthcare). Purified protease IV was exchanged into M9 minimal medium and filter-sterilized using a 0.22 μm low-protein-binding filter (Millipore). The concentration of the purified protease IV was monitored.
MAPK activity. Total proteins in seedling or protoplast lysates were resolved on a 10% SDS–polyacrylamide gel electrophoresis (SDS–PAGE) gel and transferred to a polyvinylidene difluoride membrane. Western blot analysis was conducted by using anti-phospho ERK antibodies (Cell Signaling) as the primary antibody at 1:1,000 dilution in 5% BSA and horseradish peroxidase (HRP)-conjugated anti-rabbit antibodies as the secondary antibody at 1:10,000 dilution in 5% non-fat milk. The immunoblots signal was visualized with a SuperSignal West Femto kit (Thermo Scientific).

Oxidative burst measurement. 
H2O2 was detected using a luminol–HRP-based chemiluminescence assay. A 10 mg ml−1 500× HRP (Sigma–Aldrich) stock solution was prepared by dissolving 10 mg HRP in water. A 20 mg ml−1 500× luminol (Sigma–Aldrich) stock solution was prepared by dissolving 20 mg luminol in 100 mM KOH. For each elictor, a master reaction mixture was prepared by diluting individual elictor, HRP, and luminol stocks with water. The plates were kept in the dark for 1 h before elicitation. The following procedures were performed in the dark. Liquid was removed at the end of the 1-h pre-treatment and 200 µl of master reaction mixture was added into each well. Plates were placed into a 96-well scintillation reader immediately and light emission was monitored using a 96-well scintillation counter (1450 Microbeta Wallac TriLux Scintillation/Luminescence counter). Every plate was read for about 30 cycles. Kinetics of 
H2O2 production was determined by plotting the average chemiluminescence counts from all the seedlings under the same condition over the reading period. Every time point is the mean value of 16 seedlings.

RNA isolation and microarray and RT-qPCR analysis. Total RNA was isolated according to the manufacturer’s instructions using a RNaseasy Plant Mini Kit (Qiagen). DNA was removed using the DNA-free kit (Ambion), and reverse transcription reactions were performed using an Script CDNA synthesis kit (Bio-Rad). Complementary DNA (cDNA) concentrations were measured using a Nano-drop instrument. Microarray hybridizations and scanning were performed according to protocols previously described. 

Fold expression was calculated using log2-transformed expression values by subtracting the mean of control samples from the mean of treated samples. Microarray CEL accession number GSE58518.

For microarray analysis, RNA quality was assessed by checking the integrity of RNA on an Agilent 2100 Bioanalyzer (Agilent Technologies). Target labelling was elicitor treatment.

5 h after elicitation, whereas as implemented in Bioconductor’s ‘affy’ package, version 1.36.1 (refs 32, 33). Fold expression was calculated using log2-transformed expression values by subtracting the mean of control samples from the mean of treated samples. Microarray CEL accession number GSE58518.

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the growth medium. Leaves of 4- to 5-week-old *N. benthamiana* plants were infiltrated with agrobacteria (final attenuation, $D_{600\text{nm}} = 0.01$) containing constructs expressing the mCherry/N fragment fused to GPA1, AGB1, or MAPKs and the mCherryC fragment fused to RACK1A/B/C. The agroinfiltration experiment was performed as described previously.37

*Arabidopsis* protoplasts 18 h after transfection and *N. benthamiana* leaf pieces 2 days after agroinfiltration were imaged using a Leica DM-6000B upright fluorescence microscope with phase and differential interference contrast equipped with a Leica FW4000 digital image-acquisition and processing system.

**SFLC.** For plasmids used in the SFLC assay, the genes for protein–protein interaction tests were inserted into the Xbali/BamHI-digested pcFLucN or pcFLucC vectors after digestion of their PCR products with XbaI (or SpeI, NheI if the XbaI site was present in the gene) at the 5’ end and with BamHI (or BglII if the BamHI site was present in the gene) at the 3’ end, allowing the expression of a chimaeric gene of interest with the coding sequence of FLucN or FLucC at the 3’ end. SFLC experiments performed in protoplasts were performed as described previously.37 Briefly, 10 μg (5 μl) of PREY plasmids were used to co-transfect 10 μg (5 μl) of BAIT plasmids. One microgram of UBQ10::GUS plasmid was used in each transfection as an internal normalization control. After 6 h to allow for protein expression, the luminescence of each sample was recorded by a GloMax-Multi microplate multi-mode reader (Promega) with the integration time set as 1 s.

17. Rahme, L. G. et al. Common virulence factors for bacterial pathogenicity in plants and animals. *Science* **268**, 1899–1902 (1995).
18. Liberati, N. T. et al. An ordered, nonredundant library of *Pseudomonas aeruginosa* strain PA14 transposon insertion mutants. *Proc. Natl Acad. Sci. USA* **103**, 2833–2838 (2006).
19. Motley, S. T. & Lory, S. Functional characterization of a serine/threonine protein kinase of *Pseudomonas aeruginosa*. *Infect. Immun.* **67**, 5386–5394 (1999).
20. Parker, J. E., Barber, C. E., Mi-jiao, F. & Daniels, M. J. Interaction of Xanthomonas campestris with *Arabidopsis thaliana*: characterization of a gene from *X. c. pv. raphani* that confers avirulence to most *A. thaliana* accessions. *Mol. Plant Microbe Interact.* **6**, 216–224 (1993).
21. Djonovitc, S. et al. Trehalose biosynthesis promotes *Pseudomonas aeruginosa* pathogenicity in plants. *PLoS Pathog.* **9**, e1003217 (2013).
22. Prentki, P. & Krisch, H. M. In vitro insertional mutagenesis with a selectable DNA fragment. *Gene* **29**, 303–313 (1984).
23. Hirsch, A. M. et al. *Rhizobium mellioti* nodulation genes allow *Agrobacterium tumefaciens* and *Escherichia coli* to form pseudonodules on alfalfa. *J. Bacteriol.* **158**, 1133–1143 (1984).
24. Cheng, Z., Duan, J., Hao, Y., McConkey, B. J. & Glick, B. R. Identification of bacterial proteins mediating the interactions between *Pseudomonas putida* UW4 and *Brassica napus* (canola). *Mol. Plant Microbe Interact.* **22**, 686–694 (2009).
25. Smith, A. W. & Iglewski, B. H. Transformation of *Pseudomonas aeruginosa* by electroporation. *Nucleic Acids Res.* **17**, 10509 (1989).
26. Kim, J.-G. et al. Xanthomonas T3S effector XopH suppresses PAMP-triggered immunity and interacts with a tomato atypical receptor-like kinase and TFF1. *Plant Cell* **21**, 1305–1323 (2009).
27. Curtis, M. D. & Grossniklaus, U. A gateway cloning vector set for high-throughput functional analysis of genes in plants. *Plant Physiol.* **133**, 462–469 (2003).
28. Lee, L.-Y., Fang, M.-J., Kuang, L.-Y. & Gelvin, S. B. Vectors for multi-color bimolecular fluorescence complementation to investigate protein-protein interactions in living plant cells. *Plant Methods* **4**, 24 (2008).
29. Zuo, J., Niu, Q. W. & Chua, N. H. Technical advance: an estrogen receptor-based transactivator XVE mediates highly inducible gene expression in transgenic plants. *Plant J.* **24**, 265–273 (2000).
30. Clough, S. J. & Bent, A. F. Floral dip: a simplified method for Agrobacterium-mediated transformation of *Arabidopsis thaliana*. *Plant J.* **16**, 735–743 (1998).
31. Engel, L. S., Hill, J. M., Caballero, A. R., Green, L. C. & O’Callaghan, R. J. Protease IV, a unique extracellular protease and virulence factor from *Pseudomonas aeruginosa*. *J. Biol. Chem.* **273**, 16792–16797 (1998).
32. Irizarry, R. A. et al. Exploration, normalization, and summaries of high density oligonucleotide array probe level data. *Biostatistics* **4**, 249–264 (2003).
33. Gentleman, R. C. et al. Bioconductor: open software development for computational biology and bioinformatics. *Genome Biol.* **5**, R80 (2004).
34. 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).
35. Meyer, D., Lauber, E., Roby, D., Arlat, M. & Kroj, T. Optimization of pathogenicity assays to study the *Arabidopsis thaliana–Xanthomonas campestris* pathosystem. *Mol. Plant Pathol.* **6**, 327–333 (2005).
36. Yoo, S. D., Cho, Y. H. & Sheen, J. Xanthomonas mesophyll protoplasts: a versatile cell system for transient gene expression analysis. *Nature Protocols* **2**, 1565–1572 (2007).
37. Li, J.-F., Bush, J., Xiong, Y., Li, L. & McCormack, M. Large-scale protein–protein interaction analysis in *Arabidopsis* mesophyll protoplasts by split firefly luciferase complementation. *PLoS ONE* **6**, e27364 (2011).
38. Li, J.-F., Park, E., von Arnim, A. G. & Nebenfuhr, A. The FAST technique: a simplified Agrobacterium-based transformation method for transient gene expression analysis in seedlings of *Arabidopsis* and other plant species. *Plant Methods* **5**, 6 (2009).
39. King, S. R. F. et al. *Phytophthora infestans* RXLR effector PexRD2 interacts with host MAPKKKc to suppress plant immune signaling. *Plant Cell* **26**, 1345–1359 (2014).
Extended Data Figure 1 | Protease IV-triggered GUS staining in CYP71A12pro:GUS transgenic Arabidopsis seedlings. a, Activation of CYP71A12pro:GUS by a DEAE fraction of the PA14 secretome (left) and purification of the eliciting activity by DEAE chromatography (right). b, Activation of CYP71A12pro:GUS in 10-day-old seedlings by 100 nM purified PrpL. The experiments in a and b were repeated three times with similar results.
Extended Data Figure 2 | Trypsin does not activate MAPK cascade or elicit an oxidative burst in *Arabidopsis*. a, Western blot depicting activation of MAPKs by 40 nM flg22, or 40 nM purified PrpL, or trypsin in 10-day-old seedlings. The same molecular mass region of the western blot is shown as in Fig. 1b. b, Chemiluminescence assay showing elicitation of an oxidative burst in 10-day-old seedlings by 20 nM purified PrpL or trypsin. Error bars, s.d.; n = 16 individual seedlings.
Extended Data Figure 3 | Transcriptional analysis of purified protease IV.

a, Genome-wide transcriptomic profiles obtained with Affymetrix Arabidopsis ATH1 GeneChips of 10-day-old seedlings treated with 20 nM purified PrpL and comparison with published flg22 and oligogalacturonide responses. A Venn diagram shows the similarity of expression behaviour ( | fold change | > 2) in response to the three treatments. b, Defence gene induction levels measured by RT-qPCR in 10-day-old Col-0 seedlings treated with 20 nM purified PrpL or 20 nM flg22 for 1 h (WRKY29, 30, and 33) or 6 h (GST6, ERF1, and CYP71A12). Data represent mean ± s.d.; n = 3 biological replicates, each containing eight seedlings.
Extended Data Figure 4 | Protease IV-triggered responses are dependent on proteolytic activity. a, Induction of defence-related genes by 20 nM purified PrpL or inactive variants of PrpL measured by RT-qPCR. b, Induction of defence-related genes by 20 nM purified PrpL or 20 nM flg22, or 20 nM TLCK-treated PrpL or 20 nM TLCK-treated flg22 measured by RT-qPCR. c, Induction of defence-related genes by 20 nM PrpL or 20 nM heat-treated PrpL or 20 nM flg22 or 20 nM heat-treated flg22 measured by RT-qPCR. d, Chemiluminescence assay showing elicitation of an oxidative burst by 20 nM purified PrpL, 20 nM inactive variants of PrpL, or 20 nM TLCK-treated PrpL. Data represent mean ± s.d.; n = 3 biological replicates with each experiment contains eight seedlings (a–c) and n = 16 individual seedlings (d).
a

Extended Data Figure 5 | Sequence analyses of Xanthomonas argC genes. a, The protein sequence alignment between P. aeruginosa PA14 PrpL and X. campestris pv. raphani strain 1946 ArgC (Xcr ArgC). b–d, Three independent presumptive null mutations in the Xanthomonas argC gene: an insertion of G, a single nucleotide mutation, and a deletion. The extra G is highlighted in black in b; the single nucleotide substitution is indicated by an arrow in c; and the single base deletion is highlighted in red in d. The resulting premature stop codons are highlighted in red. Sequences were aligned to the argC allele in X. campestris pv. raphani strain 1946 (Xcr-1946), from which the argC gene was cloned. X. campestris pv. campestris strains 8004 (Xcc-8004); X. campestris pv. vesicatoria (Xcv).

e. Activation of CYP71A12pro:GUS in 10-day-old seedlings by culture filtrate from X. campestris strain Xcr-1946, Xcc-8004, or Xcc-BP109, and X. campestris strain 8004 complemented with a functional argC gene (8004/argC) or transformed with empty vector (8004/vector). Detection of HA-ArgC with an anti-HA antibody. The GUS staining was repeated three times with similar results and the representative images shown were selected from at least three images.

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Extended Data Figure 6 | G proteins are required for protease IV response.

a, Induction of CYP71A12 and GST6 gene expression by 20 nM purified PrpL in 10-day-old wild-type Col-0, g7 single mutants (agg1-1c and agg2-1), or a g7/2 double mutant measured by RT-qPCR. b, Chemiluminescence assay showing elicitation of an oxidative burst by 20 nM purified PrpL or 20 nM flg22 in wild-type Col-0 or G-protein tDNA mutants. Data represent mean ± s.d.; n = 3 biological replicates with each containing eight seedlings (a) and n = 16 individual seedlings (b). **P < 0.01, Student’s t-test.
Extended Data Figure 7 | Interactions between RACK1 and Gβ or MAPKs. a, Split-mCherry assay in 4-week-old Agrobacterium-infiltrated N. benthamiana leaves. Images were pseudocoloured for visualization. Scale bar, 100 μm. RACK1A, B, C proteins were fused with the C-terminal half of mCherry and the potential interaction partner proteins were fused with the N-terminal half of mCherry. b, Split-mCherry assay in Arabidopsis protoplasts. RACK1A protein was fused with the C-terminal half of mCherry and the potential interaction partner proteins were fused with the N-terminal half of mCherry. Green fluorescent protein (GFP) was included in each experiment to serve as a transfection control. Images were pseudocoloured for visualization. Scale bar, 10 μm. c, Relative interaction intensity between RACK1A and G proteins or MAPKs measured by SFLC. RACK1A protein was fused with the FlucN or FlucC to pair with G proteins or MAPKs fused with the other half of firefly luciferase. Both constructs were co-expressed in protoplasts for 6 h and the complemented luciferase activity was used to relatively quantify protein–protein interactions. UBQ10::GUS was included in each experiment to serve as a transfection normalization control. Data represent mean ± s.d.; n = 3 technical replicate samples. d, Protoplasts were co-transfected with GPA1-HA or AGB1-HA and RACK1B/C-Flag or a control vector. Co-immunoprecipitation was performed with an anti-Flag antibody. Top: the expression of GPA1 or AGB1 protein. Middle: AGB1, but not GPA1, co-immunoprecipitates with RACK1 proteins. Bottom: pulldown of RACK1 proteins by anti-Flag antibody. Protoplasts were treated with 100 nM purified PrpL for 15 min. e, Co-immunoprecipitation between GPA1 or AGB1 and RACK1A was performed in wild-type Col-0 or gαβ mutant Arabidopsis mesophyll protoplasts. Numbers on the left of blots represent marker size in kilodaltons. f, Mass spectrophotometric analysis of endogenous proteins pulled down by Flag-tagged MEKK1(K361M). A peptide conserved in all three RACK1 proteins is shown. The experiments in a and b were repeated three times with similar results.
Extended Data Figure 8 | Protease IV-triggered defence responses in wild-type Col-0 and MAPK mutants. a, Induction of WRKY30 and WRKY33 gene expression by 20 nM purified PrpL in 7-day-old seedlings of wild-type Col-0 and transgenic mkk4/5-es1/2 and mkk4.5-es1/2 plants in the absence or presence of oestradiol. b, Western blot depicting activation of MPK3 and MPK6 by 40 nM purified PrpL in 7-day-old seedlings of wild-type Col-0 and transgenic mkk4.5-es1 plants in the absence or presence of oestradiol. The same molecular mass region of the western blot is shown as in Fig. 1b. c, Induction of WRKY30 and WRKY33 gene expression by 20 nM purified PrpL in 10-day-old wild-type Col-0 and mkk1/pMEKK1::MEKK1(K361M) mutant seedlings. d, Induction of WRKY30 and WRKY33 gene expression by 20 nM purified PrpL in 4-day-old wild-type Col-0 and mkk1 null mutant seedlings. e, Western blot depicting activation of MPK3 and MPK6 by 40 nM purified PrpL in 10-day-old wild-type Col-0 and mkk1/pMEKK1::MEKK1(K361M) mutant seedlings or 4-day-old wild-type Col-0 and mkk1 null mutant seedlings. The same molecular mass region of the western blot is shown as in Fig. 1b. Data represent mean ± s.d.; n = 3 biological replicates with each containing eight seedlings (a, c, d); **P < 0.01; ***P < 0.001, Student’s t-test versus Col-0 controls.
Extended Data Figure 9 | RACK1 proteins are required for protease IV response. a, Western blot depicting activation of MAPKs by 40 nM purified PrpL or 40 nM flg22 in 5-day-old seedlings of wild-type Col-0 and individual rack1::tDNA insertion mutants. The same molecular mass region of the western blot is shown as in Fig. 1b. b, Induction of CYP71A12 by 20 nM purified PrpL or 20 nM flg22 in 5-day-old seedlings of wild-type Col-0 and individual rack1::tDNA insertion mutants. c, RT-qPCR analysis of rack1a, rack1b, and rack1c transcript levels in the 5-day-old Col-0 or amiR–rack1–es1 and amiR–rack1–es2 seedlings. d, RT-qPCR analysis of rack1a, rack1b, and rack1c transcript levels in Arabidopsis protoplasts transfected with amiR–RACK1–4 or artificial microRNA control. e, Western blot depicting activation of MAPKs by 40 nM purified PrpL or 40 nM flg22 in Arabidopsis protoplasts transfected with amiR–RACK1–4 or artificial microRNA control. The same molecular mass region of the western blot is shown as in Fig. 1b. Data represent mean ± s.d.; n = 3 biological replicates (b–d); *P < 0.05; **P < 0.01, Student’s t-test.
Extended Data Table 1  | P. aeruginosa PA14 transposon mutants screened for activation of CYP71A12pro:GUS

| gene names | gene IDs | type* |
|------------|----------|-------|
| aprA       | 865      | 1     |
| aprD       | 7385     | 1     |
| aprE       | 1317     | 1     |
| aprF       | 922      | 1     |
| aprI       | 4760     | 1     |
| aprX       | 1421     | 1     |
| hasAp      | 3774     | 1     |
| hasF       | 1253     | 1     |
| cbpD       | 1394     | 2     |
| cupB5      | 75       | 2     |
| lasA       | 1299     | 2     |
| lasB       | 759      | 2     |
| lipA       | 2386     | 2     |
| lipC       | 2417     | 2     |
| pepB       | 629      | 2     |
| phoA       | 914      | 2     |
| phoB       | 3473     | 2     |
| phoR       | 1112     | 2     |
| plcB       | 1956     | 2     |
| plcH       | 210      | 2     |
| plcN       | 267      | 2     |
| pmpA       | 93       | 2     |

| gene names | gene IDs | type* |
|------------|----------|-------|
| toxA       | 399      | 2     |
| xcpP       | 3450     | 2     |
| xcpQ       | 417      | 2     |
| xcpR       | 812      | 2     |
| xcpT       | 4498     | 2     |
| xcpW       | 3292     | 2     |
| xcpZ       | 4249     | 2     |
| xplhA      | 4239     | 2     |
| xqhA       | 246      | 2     |
| exoT       | 7001     | 3     |
| exoU       | 339      | 3     |
| exoY       | 7430     | 3     |
| pscD       | 1303     | 3     |
| aeaA       | 375      | 5     |
| eprS       | 69       | 5     |
| estA       | 354      | 5     |
| lopA       | 631      | 5     |
| tps1       | 556      | 5     |
| tps2       | 600      | 5     |
| tps3       | 555      | 5     |
| tps5       | 545      | 5     |
| motA       | 2879     | S     |
| motB       | 1935     | S     |
| pilA       | 7353     | S     |
| pilD       | 2579     | S     |
| tadZ       | 1689     | S     |

* Numbers represent the type of secretion system. For example, ‘2’ means type II secreted protein or type II secretion machinery protein. R, regulatory proteins; S, surface proteins.