the plant journal

SEB Society for Experimental Biology

The Plant Journal (2014) **79,** 243–255 doi: 10.1111/tpj.12557

The Arabidopsis LecRK-VI.2 associates with the pattern-recognition receptor FLS2 and primes *Nicotiana benthamiana* pattern-triggered immunity

Pin-Yao Huang, Yu-Hung Yeh, An-Chi Liu, Chiu-Ping Cheng and Laurent Zimmerli*

Institute of Plant Biology, College of Life Science, National Taiwan University, Taipei, Taiwan

Received 23 January 2014; revised 24 April 2014; accepted 6 May 2014; published online 20 May 2014.
*For correspondence (e-mail lauzim2@ntu.edu.tw).

SUMMARY

Pattern-triggered immunity (PTI) is broad spectrum and manipulation of PTI is believed to represent an attractive way to engineer plants with broad-spectrum disease resistance. PTI is activated upon perception of microbe-associated molecular patterns (MAMPs) by pattern-recognition receptors (PRRs). We have recently demonstrated that the L-type lectin receptor kinase-VI.2 (LecRK-VI.2) positively regulates *Arabidopsis thaliana* PTI. Here we show through *in vitro* pull-down, bimolecular fluorescence complementation and co-immunoprecipitation analyses that LecRK-VI.2 associates with the PRR FLS2. We also demonstrated that LecRK-VI.2 from the cruciferous plant Arabidopsis remains functional after interfamily transfer to the Solanaceous plant *Nicotiana benthamiana.* Wild tobacco plants ectopically expressing *LecRK-VI.2* were indeed more resistant to virulent hemi-biotrophic and necrotrophic bacteria, but not to the fungal pathogen *Botrytis cinerea* suggesting that, as with Arabidopsis, the *LecRK-VI.2* protective effect in *N. benthamiana* is bacteria specific. Ectopic expression of *LecRK-VI.2* in *N. benthamiana* primed PTI-mediated reactive oxygen species production, mitogen-activated protein kinase (MAPK) activity, callose deposition and gene expression upon treatment with the MAMP flagellin. Our findings identified LecRK-VI.2 as a member of the FLS2 receptor complex and suggest that heterologous expression of components of PRR complexes can be used as tools to engineer plant disease resistance to bacteria.

Keywords: ***Arabidopsis thaliana*, *Nicotiana benthamiana*, lectin receptor kinase, priming, innate immunity, pattern-triggered immunity, pattern-recognition receptor, bacteria.**

INTRODUCTION

Plants are constantly exposed and threatened by a variety of pathogenic organisms present in their environment and diseases caused by these pathogens significantly contribute to the overall loss in crop yield worldwide (Oerke, 2007). Crop diseases combined with the threat of global warming that increases extreme weather conditions are important factors destabilizing the global food security. Pesticide applications are generally used to deter pathogens, but more ecologically friendly methods are required. One promising way to increase crop resistance is to boost plant defence capabilities (Wulff *et al.*, 2011). Plants indeed possess defence mechanisms that are activated upon pathogen attack. The first layer of the plant immune response is activated upon recognition of pathogen evolutionarily conserved structures that are called pathogen or microbe-associated molecular patterns (PAMPs or MAMPs). MAMPs recognition by plant pattern-recognition receptors (PRRs) leads to the activation of the pattern-triggered immunity (PTI) (Jones and Dangl, 2006; Boller and Felix, 2009; Tsuda and Katagiri, 2010). Current known examples of bacterial MAMPs/PRRs couples are EF-Tu/EF-Tu RECEPTOR (EFR) (Zipfel *et al.*, 2006), the eubacterial flagellin/FLAGELLIN SENSING2 (FLS2) (Gómez-Gómez and Boller, 2000), and peptidoglycan/LYSIN-MOTIF1 (LYM1) and LYM3 (Willmann *et al.*, 2011). Recently, the RECEPTOR-LIKE PROTEIN1 (RLP1) or RECEPTOR OF eMAX (ReMAX) was shown to detect the MAMP eMAX from *Xanthomonas* bacteria (Jehle *et al.*, 2013). Examples of fungi MAMPs/PRRs defined pairs are the chitin/CHITIN ELICITOR RECEPTOR KINASE1 (CERK1)(Miya *et al.*, 2007; Wan *et al.*, 2008), xylanase/ETHYLENE INDUCING XYLANASE2 (Eix2) (Ron and Avni, 2004), and avirulence gene Ave1/VERTICILIUM1 (Ve1) (de Jonge *et al.*, 2012). Upon elicitation, the PRRs FLS2 and EFR are known to rapidly associate with BRI1-ASSOCIATED RECEPTOR-LIKE KINASE/SOMATIC EMBRYOGENESIS RECEPTOR-LIKE KINASE3 (BAK1/SERK3), forming a

ligand-inducible complex critical for full PTI activation (Chinchilla *et al.*, 2007; Heese *et al.*, 2007). When associated with FLS2, BAK1 acts as a co-receptor by recognizing the C terminus of the FLS2-bound flg22 (Sun *et al.*, 2013). BAK1–FLS2 interaction is repressed by BAK1-interacting receptor kinase2 (BIR2). Upon flg22 perception, BIR2 is removed allowing FLS2–BAK1 association and PTI activation (Halter *et al.*, 2014). In addition to BAK1, other SERKs such as SERK4 also undergo ligand-induced association with FLS2 and EFR (Roux *et al.*, 2011). Beside BAK1 and others SERKs, BOTRYTIS-INDUCED KINASE1 (BIK1) plays a key role in mediating early flagellin signalling from the FLS2/BAK1 receptor complex (Lu *et al.*, 2010a; Zhang *et al.*, 2010). BIK1 physically associates with and phosphorylates the NADPH oxidase RBOHD upon MAMP perception. Importantly, BIK1-dependent phosphorylation of RBOHD is necessary for PTI-mediated reactive oxygen species (ROS) burst and Arabidopsis resistance to bacteria (Kadota *et al.*, 2014; Li *et al.*, 2014). In addition, BRASSINOSTEROID-SIGNALING KINASE1 (BSK1) and DENN (Differentially Expressed in Normal and Neoplastic cells) domain protein STOMATAL CYTOKINESIS-DEFECTIVE1 (SCD1) associate with unstimulated FLS2 (Korasick *et al.*, 2010; Shi *et al.*, 2013). Lectin receptor kinases such as L-type lectin receptor kinases (LecRKs) that possess an extracellular legume-like lectin domain, a transmembrane domain and an intracellular kinase domain (KD)(Bouwmeester and Govers, 2009), are also critical for the PTI response (Singh and Zimmerli, 2013). Notably, AtLecRK-I.9 maintains cell wall-plasma membrane continuum and is essential for resistance to pathogens and for MAMP-triggered callose deposition (Bouwmeester *et al.*, 2011). AtLecRK-V.5 negatively regulates stomatal immunity upstream of ROS biosynthesis (Desclos-Theveniau *et al.*, 2012) and *lecrk-V.5* mutants are more resistant to necrotrophic *Pectobacterium carotovorum* bacteria (Arnaud *et al.*, 2012). Finally, the Arabidopsis LecRK-VI.2 positively modulates early bacterium-mediated PTI upstream of mitogen-activated protein kinases (MAP kinases) signalling (Singh *et al.*, 2012a), but is not critical for Arabidopsis resistance to the fungal pathogen *Botrytis cinerea* (Singh *et al.*, 2012b).

Influx of calcium, the production of ROS, and signalling via mitogen-activated protein kinases (MAPKs) are considered as early PTI responses, while callose deposition and marker gene up-regulation are observed later during PTI activation (Boller and Felix, 2009; Zipfel and Robatzek, 2010; Tena *et al.*, 2011). Successful, virulent pathogens secrete effectors to repress PTI (Deslandes and Rivas, 2012; Feng and Zhou, 2012). The recognition by plants of virulence effectors from pathogens usually through intracellular resistance proteins (R proteins) leads to the activation of a fast and strong defence response generally associated with the development of an hypersensitive response known as effector-triggered immunity (ETI) (Maekawa *et al.*, 2011; Gassmann and Bhattacharjee, 2012). ETI occurs between plant cultivars possessing a specific R gene that recognizes a matching virulence effector from a limited number of pathogenic strains. ETI is largely used in breeding programs to improve crop resistance to diseases. Unfortunately, this type of resistance confers very specific disease resistance and is overcome by rapidly evolving pathogens (Leach *et al.*, 2001; McDonald and Linde, 2002).

Plants can also increase their resistance to disease through the activation of the systemic acquired resistance and induced systemic resistance (Sticher *et al.*, 1997; Van Wees *et al.*, 2008). Such resistance mechanisms rely largely on priming of defence responses. The priming phenomenon is usually associated with a potentiated defence response upon pathogen challenge and consequently priming boosts plants resistance to microbes (Conrath *et al.*, 2006). Priming is often associated with an accumulation of defence signalling components before stress exposure (Beckers *et al.*, 2009; Singh *et al.*, 2012a) and epigenetic modifications (Jaskiewicz *et al.*, 2011; Luna *et al.*, 2012; Rasmann *et al.*, 2012; Po-Wen *et al.*, 2013).

PTI is activated upon plant recognition of MAMPs that are conserved within a class of pathogens and essential for microbe's life. By contrast with ETI that is activated upon recognition of highly specific pathogen virulence effectors and is thus very specific and consequently short lasting, PTI is believed to be long lasting and of broad range (Wulff *et al.*, 2011). PTI manipulation through biotechnological approaches thus looks promising. One recent example of PTI engineering is the successful transfer of the PRR EFR that is restricted to the Brassicaceae family to *Nicotiana benthamiana* and tomato plants from the Solanaceae family that do not possess EFR (Lacombe *et al.*, 2010). Transgenic tobacco and tomato plants ectopically expressing EFR become responsive to the MAMP elf18 and are more resistant to a wide range of bacterial pathogens (Lacombe *et al.*, 2010).

As LecRK-VI.2 positively regulates PTI upstream of MAPK signalling (Singh *et al.*, 2012a), we asked whether LecRK-VI.2 associates with the PRR FLS2. LecRK-VI.2 was found to indeed interact with the PRR FLS2. PTI is repressed by virulent pathogens and thus only marginally participate to plant resistance during a susceptible interaction. Reinforcement of PTI by over-expression of *LecRK-VI.2* however enhances Arabidopsis resistance to virulent bacteria (Singh *et al.*, 2012a). These findings prompted us to test whether heterologous expression of *LecRK-VI.2* in *N. benthamiana* increases its resistance to bacteria. Our data show that interfamily transfer of Arabidopsis *LecRK-VI.2* to the Solanaceous plant *N. benthamiana* confers increased resistance to a broad range of bacteria. Enhanced resistance was correlated with priming of PTI. Our data suggest that *LecRK-VI.2* is a component of the

FLS2 receptor complex and can be used in Solanaceous plants to increase resistance to bacteria.

RESULTS

Arabidopsis LecRK-VI.2 associates with the PRR FLS2 in a ligand-independent manner

LecRK-VI.2 positively regulates FLS2-dependent PTI upstream of MAPK signalling (Singh *et al.*, 2012a), suggesting possible association of LecRK-VI.2 with the PRR FLS2. Interaction was first evaluated by pull-down assays that revealed binding of the KD of LecRK-VI.2 with FLS2 KD (Figure 1a), suggesting that LecRK-VI.2 can directly interact with FLS2 *in vitro.* To further test LecRK-VI.2 and FLS2 possible association, we used bimolecular fluorescence complementation (BiFC) assays (Walter *et al.*, 2004) in Arabidopsis protoplasts. As a first control, we analysed the interaction between BAK1 and FLS2 that is known to occur after flg22 elicitation (Chinchilla *et al.*, 2007; Heese *et al.*, 2007). As expected, YFP fluorescence was detected only after flg22 treatment (Figure 1b), suggesting that our experimental conditions are appropriate. When testing LecRK-VI.2 interaction with FLS2, YFP fluorescence was detected before and after elicitation with flg22 (Figure 1b). Analysis of the association of LecRK-VI.2 with the plasma membrane localized RARE-COLD-INDUCIBLE 2B (RCI2B) (Medina *et al.*, 2007) also known as LTI6B (Cutler *et al.*, 2000) was used to evaluate the specificity of LecRK-VI.2 binding to FLS2. YFP fluorescence was detected when *Arabidopsis* protoplasts were transfected with RCI2B–YFP^N and RCI2B–YFP^C, demonstrating that both constructs were functional. Importantly, no YFP fluorescence was observed

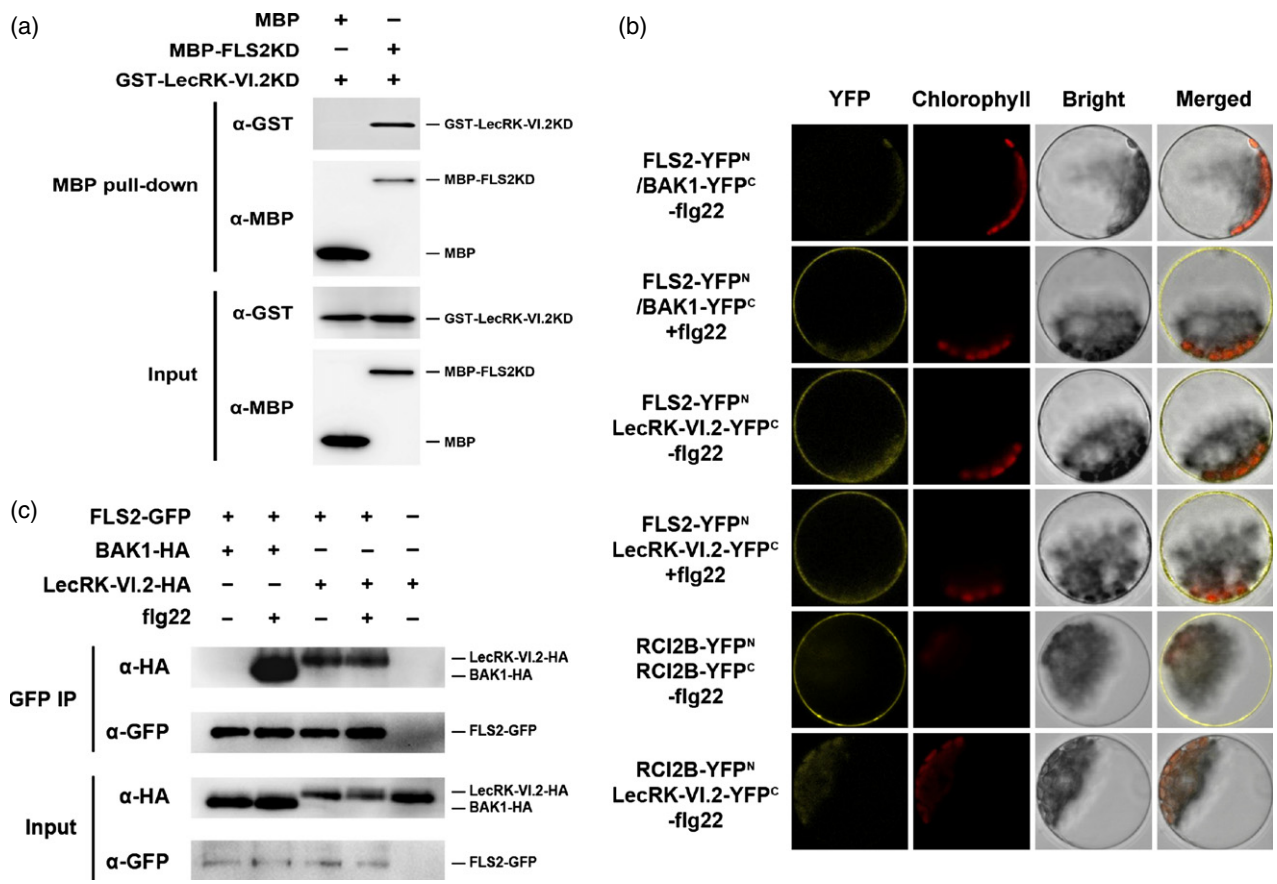

Figure 1. LecRK-VI.2 interacts with FLS2.

(a) *In vitro* maltose-binding protein (MBP) pull-down assay of LecRK-VI.2 interaction with FLS2. *Escherichia coli*-expressed MBP (negative control) or MBP-FLS2KD was incubated with GST-LecRK-VI.2KD and pulled down with amylose resin beads. Input and bead-bound proteins were analysed by western blotting with specific antibodies. Experiments were repeated three times with similar results.

(b) Bimolecular fluorescence complementation (BiFC) analysis. Arabidopsis protoplasts were co-transfected with FLS2–YFP^N and BAK1–YFP^C or LecRK-VI.2–YFP^C, and visualized under a confocal microscope 16 h after transfection. RCI2B–YFP^N and –YFP^C were used for self-association and RCI2B–YFP^N was co-transfected with LecRK-VI.2–YFP^C as the negative control. The protoplasts were treated with (+) or without (–) 1 μM flg22 for 15 min. Experiments were repeated three times with similar results.

(c) Co-immunoprecipitation (Co-IP) analysis of LecRK-VI.2 association with FLS2. Total proteins (input) of *Nicotiana benthamiana* leaves transiently expressing LecRK-VI.2–HA$_3$ or BAK1–HA$_3$ with FLS2–GFP were immunoprecipitated (IP) with anti-GFP antibodies (WB: α-GFP) and then analysed by western blot with an anti-HA antibody (WB: α-HA). Leaves were treated with (+) or without (–) 100 nM flg22 for 5 min. This experiment is one of three independent biological repeats.

when testing LecRK-VI.2 interaction with RCI2B showing that LecRK-VI.2 association with FLS2 observed by BiFC is specific (Figure 1b). Together these results suggest that LecRK-VI.2 associates with the PRR FLS2 in a ligand-independent manner.

To further test possible association of LecRK-VI.2 with FLS2 *in vivo*, we used the co-immunoprecipitation (Co-IP) technique in *Nicotiana benthamiana.* First, equal amounts of FLS2 were pulled down with GFP-Trap beads from *N. benthamiana* transiently co-expressing FLS2–GFP (green fluorescent protein) and HA_3 epitope-tagged BAK1. As expected, detection of BAK1–HA_3 through anti-HA immunoblotting revealed BAK1 only after flg22 elicitation (Chinchilla *et al.*, 2007; Heese *et al.*, 2007) (Figure 1c). We then transiently co-expressed FLS2–GFP with HA_3 epitope-tagged LecRK-VI.2 in *N. benthamiana.* After pulling down equal amounts of FLS2 with GFP-Trap beads and anti-HA immunoblotting, we observed a clear LecRK-VI.2 signal before and after flg22 elicitation (Figure 1c). To ensure that LecRK-VI.2 does not bind non-specifically to anti-GFP agarose beads, we expressed LecRK-VI.2–HA_3 alone. As expected, no signal was observed in the negative control that expressed LecRK-VI.2–HA_3 only (Figure 1c). In addition, the FLS2–GFP signal localized at the cell periphery suggesting a correct FLS2 subcellular localization at the plasma membrane upon transient expression in *N. benthamiana* (Figure S1). Taken together, these experiments strongly suggest that LecRK-VI.2 associates with the PRR FLS2 in a ligand-independent manner.

Arabidopsis LecRK-VI.2 protects *N. benthamiana* against hemi-biotrophic and necrotrophic bacteria, but not against the fungus *Botrytis cinerea*

Over-expression of *LecRK-VI.2* increases Arabidopsis resistance to bacteria (Singh *et al.*, 2012a). To test whether the protective effect of LecRK-VI.2 can be transferred to other plant species, we evaluated the resistance response of *N. benthamiana* transgenics ectopically expressing *LecRK-VI.2* to microbial pathogens. For that purpose, we generated *N. benthamiana* carrying the 35S::*LecRK-VI.2* constructs. *LecRK-VI.2* was also expressed in *N. benthamiana* under the control of the inducible *N. glutinosa* promoter EAS4 (Lin *et al.*, 2014). While inducible in *N. glutinosa*, *N. benthamiana* transgenics expressing *LecRK-VI.2* under the EAS4 promoter demonstrated constitutive expression of *LecRK-VI.2* (Figure S2). Although Arabidopsis lines over-expressing *LecRK-VI.2* develop a stunted phenotype (Singh *et al.*, 2012a), *N. benthamiana* ectopically expressing *LecRK-VI.2* did not demonstrate growth or developmental defects when grown in non-sterile soil over several generations (Figure S3). Five-week-old wild-type (WT) and transgenic *N. benthamiana* were dip-inoculated with hemi-biotrophic bacteria *Pseudomonas syringae* pv. *syringae* (*Pss*) B728a, the causal agent of bacterial brown spot disease on beans also virulent on *N. benthamiana* (Vinatzer *et al.*, 2006), or *Pseudomonas syringae* pv. *tabaci* (*Pta*) 11528 that causes wild-fire disease in soybean and tobacco plants (Ribeiro *et al.*, 1979; Gasson, 1980), and symptoms were evaluated 3 days later. WT and Vector control plants exhibited severe disease symptoms with spreading lesions necrosis, while independent transgenic lines harbouring the 35S::*LecRK-VI.2* or EAS4::*LecRK-VI.2* constructs developed less disease symptoms (Figure 2a, b). In addition, bacterial growth assays were used to quantify bacterial proliferation at 3 h post inoculation (HPI) or at 1, 2, 3 and 4 days post inoculation (DPI). *N. benthamiana* transgenics harboured significantly less *Pss* B728a or *Pta* 11528 then WT and empty Vector controls (Figure 2c, d). *Pta* 11528-mediated symptoms were also evaluated 2 weeks after inoculation. At such a late time point, growth of WT and empty Vector control plants was strongly affected, while transgenic *N. benthamiana* harbouring the 35S::*LecRK-VI.2* or EAS4::*LecRK-VI.2* constructs showed only weak disease symptoms and mock-like flowering (Figure S4). These results suggest that ectopic expression of *LecRK-VI.2* in *N. benthamiana* enhanced resistance to hemi-biotrophic virulent *P. syringae* bacteria. To investigate whether the protective role of Arabidopsis LecRK-VI.2 in *N. benthamiana* applies to necrotrophic microbial pathogens, we inoculated *N. benthamiana* transgenics with *Pectobacterium carotovorum* ssp. *carotovorum* SCC1 (*Pcc* SCC1) bacteria or the fungal pathogen *B. cinerea.* Ectopic expression of *LecRK-VI.2* in *N. benthamiana* increased resistance to *Pcc* SCC1, but not to *B. cinerea.* Typically disease symptoms were less pronounced and lower disease indexes were observed in *N. benthamiana* harbouring the 35S::*LecRK-VI.2* or EAS4::*LecRK-VI.2* constructs, while WT and Vector controls were dramatically damaged by *Pcc* SCC1 (Figure 3a,b). No differences in lesion perimeters were however observed after *B. cinerea* inoculation (Figure S5a, b). Collectively these results suggest that *LecRK-VI.2* protective effect in *N. benthamiana* is bacteria specific.

Potentiation of flg22-induced ROS burst in *N. benthamiana* lines expressing *LecRK-VI.2*

As LecRK-VI.2 positively regulates Arabidopsis PTI (Singh *et al.*, 2012a), we hypothesized that *N. benthamiana* transgenics ectopically expressing *LecRK-VI.2* constructs have a reinforced PTI response. We first evaluated whether LecRK-VI.2 modulates PTI-mediated ROS production in *N. benthamiana.* Transient production of ROS such as H_2O_2 is rapidly induced upon MAMP treatment and is considered as an early PTI response (Zipfel and Robatzek, 2010). To test whether LecRK-VI.2 plays a role in MAMP-triggered ROS production in *N. benthamiana*, we analysed H_2O_2 production in WT and transgenic *N. benthamiana* plants in response to 10 nM of the peptide MAMP flg22, which is derived from bacterial flagellin (Felix *et al.*, 1999).

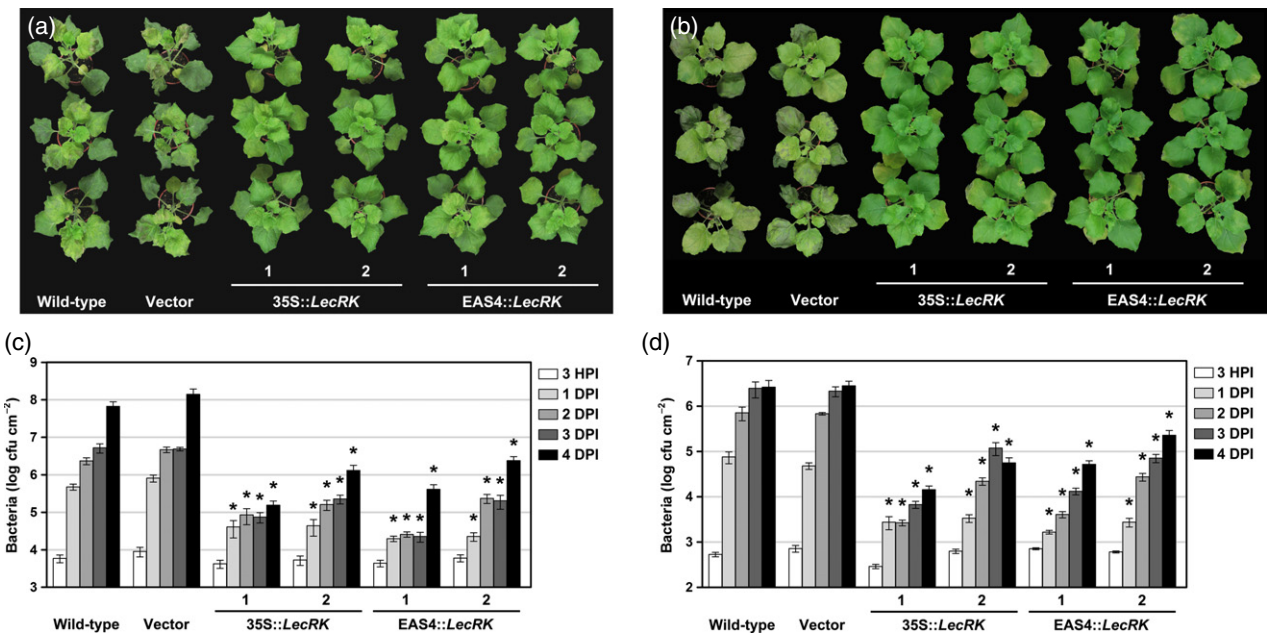

Figure 2. Ectopic expression of *LecRK-VI.2* in *Nicotiana benthamiana* confers resistance to hemi-biotrophic bacteria.
(a, b) Disease phenotypes of *N. benthamiana* transgenics expressing *LecRK-VI.2* under the control of the CaMV 35S promoter (35S) or the *N. glutinosa* promoter EAS4 photographed 4 days after dip-inoculation with 10^6 cfu ml^{-1} *Pss* B728a (a), or *Pta* 11528 (b). Wild-type (WT) and plants carrying the vector pCAMBIA1300 only (Vector) were used as controls. (1) and (2) represent two independent transgenic lines for each constructs. Experiments were repeated at least three times with similar results.
(c, d) Growth of *Pss* B728a (c) and *Pta* 11528 (d) in *N. benthamiana* leaves. Five-week-old plants were dip-inoculated with 10^6 cfu/ml bacteria. Numbers of bacteria in the leaves were quantified at 3 hours post innoculation (HPI), and 1, 2, 3, and 4 days post innoculation (DPI). Data represent the means ± standard error (SE) of three independent experiments each with five biological replicates ($n = 15$). Asterisks denote values significantly different from empty Vector control (*$P < 0.01$, Student's *t*-test). (1) and (2) represent two independent transgenic lines for each constructs.

N. benthamiana transgenics harbouring the 35S::*LecRK-VI.2* or EAS4::*LecRK-VI.2* constructs produced significantly higher levels of ROS within 30 min after flg22 treatment than WT and empty Vector controls (Figure 4a,b). Importantly, ROS production in the mock controls was at WT levels in *N. benthamiana* ectopically expressing *LecRK-VI.2.* Hence, expression of Arabidopsis *LecRK-VI.2* in *N. benthamiana* potentiates the flg22-mediated ROS burst.

Increased MAPK activities in *N. benthamiana* transgenics expressing *LecRK-VI.2*

Arabidopsis treated with MAMPs demonstrate a rapid activation of MAP kinase MPK3 and MPK6 (Nuhse *et al.*, 2000). Similarly, *N. benthamiana* MAPK activity is increased after treatment with flg22 (Segonzac *et al.*, 2011). We thus tested the activities of NbSIPK and NbWIPK, the *N. benthamiana* homologs of respectively MPK3 and MPK6, before and after flg22 elicitation in *N. benthamiana* transgenics harbouring the 35S::*LecRK-VI.2* or EAS4::*LecRK-VI.2* constructs. Ectopic expression of *LecRK-VI.2* in *N. benthamiana* did not affect NbSIPK and NbWIPK activities before PTI elicitation, while a stronger NbSIPK signal was observed in *N. benthamiana* transgenics after flg22 treatment (Figure 5a,b). As already observed (Segonzac *et al.*, 2011), very low NbWIPK signals were visible after flg22 elicitation suggesting that NbWIPK activity is not significantly increased by flg22. *NbSIPK* and *NbWIPK* mRNAs were not up-regulated in *N. benthamiana* transgenics expressing *LecRK-VI.2* (Figure S6). Ectopic expression of *LecRK-VI.2* thus primes *N. benthamiana* MAPK activities upon PTI elicitation.

Ectopic expression of *LecRK-VI.2* in *N. benthamiana* primes PTI-mediated callose deposition

To further investigate the molecular mechanisms underlying the increased resistance phenotypes observed in *N. benthamiana* transgenics, we evaluated whether ectopic expression of *LecRK-VI.2* in *N. benthamiana* affects callose deposition, a typical late PTI response (Gomez-Gomez *et al.*, 1999). PTI-mediated callose deposition in *N. benthamiana* ectopically expressing *LecRK-VI.2* was analysed 24 h after treatment with 40 μM of the peptide MAMP flg22. No statistically significant increase in callose deposition was observed in mock-treated *N. benthamiana* transgenics, suggesting that ectopic expression of *LecRK-VI.2* does not induce constitutive callose deposition (Figure 6). By contrast, callose deposition was potentiated in *N. benthamiana* transgenics after infiltration with flg22 (Figure 6). These results indicate that transgenic *N. benthamiana* lines expressing *LecRK-VI.2* have the ability to accumulate

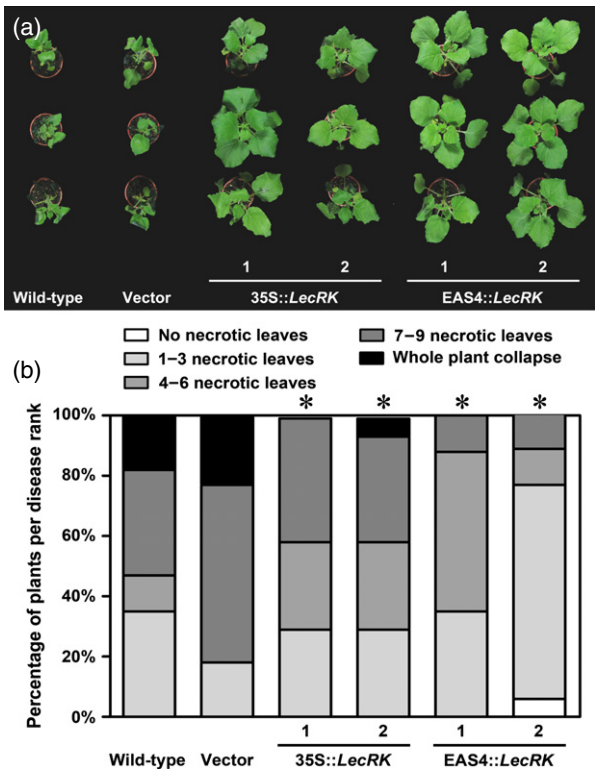

Figure 3. *Nicotiana benthamiana* transgenics ectopically expressing *LecRK-VI.2* are more resistant to necrotrophic *Pcc* SCC1 bacteria.
(a) Pictures of *Pcc* SCC1-mediated symptoms 2 days after vacuum infiltration with 2×10^5 cfu ml^{-1} *Pcc* SCC1 of *N. benthamiana* transgenics expressing *LecRK-VI.2* under the control of the CaMV 35S promoter (35S) or the *N. glutinosa* promoter EAS4. Wild-type (WT) and empty vector (Vector) were used as controls. (1) and (2) represent two independent transgenic lines for each constructs. Experiments were performed four times with similar results.
(b) *N. benthamiana* transgenics were inoculated as in (a) and disease rankings were determined 2 days post innoculation (DPI). Bars represent the percentage of infected plants with the grey-coded disease rank. Data represent three independent experiments each with six biological replicates ($n = 18$). The distribution of the disease rank proportions among the lines was analysed using the chi-squared test. Asterisks denote values significantly different from the empty Vector control ($*P < 0.05$). (1) and (2) represent two independent transgenic lines for each constructs.

callose in a faster and stronger manner than WT and empty Vector controls in response to the MAMP flg22. PTI-mediated callose deposition is thus primed by ectopic expression of *LecRK-VI.2* in *N. benthamiana.*

Increased flg22-responsive gene up-regulation in *N. benthamiana* expressing *LecRK-VI.2*

In Arabidopsis, transgenics with high levels of *LecRK-VI.2* expression exhibit constitutive up-regulation of several PTI marker genes (Singh *et al.*, 2012a). We therefore examined the expression of the *N. benthamiana* PTI marker genes *NbCYP71D20* and *NbACRE132* in transgenic lines ectopically expressing *LecRK-VI.2* (Lacombe *et al.*, 2010; Segonzac *et al.*, 2011). Both *NbCYP71D20* and *NbACRE132* are up-regulated within 30 min of MAMP treatment (Heese *et al.*, 2007; Lacombe *et al.*, 2010). In the mock controls, expression levels of the *NbCYP71D20* and *NbACRE132* PTI-responsive genes were at WT levels in *N. benthamiana* transgenics (Figure 7a,b). By contrast, *NbCYP71D20* and *NbACRE132* expression levels were significantly higher than WT and Vector controls 6 h after treatment with 100 nM flg22 in *N. benthamiana* transgenics harbouring the 35S::*LecRK-VI.2* or EAS4::*LecRK-VI.2* constructs (Figure 7a,b). These data indicate that ectopic expression of *LecRK-VI.2* in *N. benthamiana* potentiates PTI-responsive gene expression.

DISCUSSION

LecRK-VI.2 positively regulates Arabidopsis PTI upstream of MAPK signalling (Singh *et al.*, 2012a). We thus asked whether LecRK-VI.2 belongs to PRR complexes. LecRK-VI.2 indeed was found to associate with the PRR FLS2 that recognizes the bacterial MAMP flagellin (Gómez-Gómez and Boller, 2000; Chinchilla *et al.*, 2006). However, LecRK-VI.2 is not critical for early PTI signalling events such as ROS accumulation, ligand-mediated BAK1-FLS2 complex formation and BIK1 phosphorylation (Singh *et al.*, 2012a). LecRK-VI.2 may thus function in the PRR FLS2 complex independently of these early PTI events. BIK1 and the closely related PBL1 are not critical for flg22-induced MAPK activation (Feng *et al.*, 2012). Thus, a BIK1-independent signalling cascade should regulate, possibly through other receptor-like cytoplasmic kinases, flg22-mediated MAPK activation (Lu *et al.*, 2010b). LecRK-VI.2-dependent responses may thus signal through this BIK1-independent pathway. BIK1-dependent phosphorylation of the NADPH oxidase RBOHD is critical for the ROS burst observed after flg22 treatment (Kadota *et al.*, 2014; Li *et al.*, 2014). LecRK-VI.2 independency towards BIK1 may thus explain WT levels of PTI-mediated ROS accumulation in the *lecrk-VI.2* mutant (Singh *et al.*, 2012a). In agreement with the idea that LecRK-VI.2 functions independently of BIK1, *bik1* and *lecrk-VI.2* mutants behave differently towards *Pst* DC3000. The mutant *bik1* is indeed more resistant (Veronese *et al.*, 2006), while *lecrk-VI.2* is more sensitive to *Pst* DC3000 bacteria (Singh *et al.*, 2012a). Alternatively, other LecRKs may play a redundant role in ROS production, ligand-induced BAK1-FLS2 complex formation and BIK1 phosphorylation, thus masking in the *lecrk-VI.2* mutant a possible role for LecRK-VI.2 in these early PTI responses. Redundancy was recently shown in *bkk1* mutant that demonstrates WT ROS accumulation after treatment with flg22 or elf18 (Roux *et al.*, 2011). The double *bak1-5 bkk1-1* mutant however shows a stronger reduction of ROS production than each single mutant (Roux *et al.*, 2011). The observed interaction between LecRK-VI.2 and FLS2 has important implication for our understanding of PRR complexes, notably emphasizing that kinases not belonging to the leucine-rich

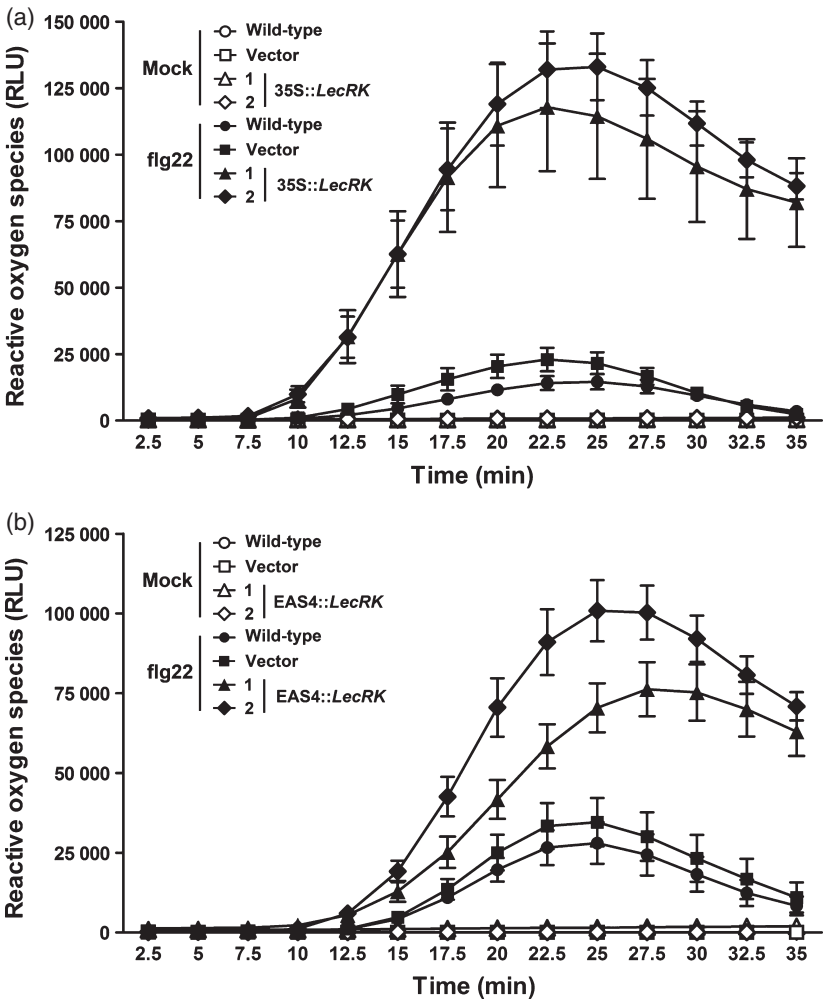

Figure 4. Priming of ROS production after flg22 elicitation in *Nicotiana benthamiana* transgenics.

(a, b) Leaf discs from *N. benthamiana* transgenic lines harbouring the 35S::*LecRK-VI.2* (a), or EAS4::*LecRK-VI.2* (b) constructs and wild-type (WT) or empty vector (Vector) controls were treated with 10 nM flg22. Production of ROS is expressed as relative light units (RLU) for a period of 35 min after elicitation. Values are expressed as means ± standard error (SE) of six leave discs ($n = 6$). (1) and (2) represent two independent transgenic lines for each constructs. Each experiment is one of three independent biological replicates.

repeat receptor-like kinase (LRR-RLK) family may participate in the regulation of PRRs.

Transgenic expression of *LecRK-VI.2* in *N. benthamiana* conferred strong resistance to two strains of hemi-biotrophic bacteria. Surprisingly, *N. benthamiana* resistance to *Pcc* SCC1 necrotrophic bacteria was weaker. During infection, necrotrophs generate an arsenal of tissue macerating enzymes that could overcome a strengthened PTI explaining why *N. benthamiana* transgenics ectopically expressing *LecRK-VI.2* were only moderately resistant to *Pcc* SCC1 infection (Kim *et al.*, 2011; Davidsson *et al.*, 2013). However, resistance of *N. benthamiana* to the necrotrophic fungal pathogen *B. cinerea* was not increased by ectopic expression of *LecRK-VI.2.* Compared to bacteria, fungal pathogens generate different MAMPs that are recognized by other PRRs (Newman *et al.*, 2013). For example, the fungal pathogen *B. cinerea* does not generate the MAMP flg22, but produces among others, the MAMP chitin that is recognized by the LysM-domain RLK CERK1 (Miya *et al.*, 2007; Wan *et al.*, 2008). Thus, ectopic expression of *LecRK-VI.2* in *N. benthamiana* may not affect fungal PTI, explaining why *N. benthamiana* transgenics expressing *LecRK-VI.2* did not demonstrate increased resistance to *B. cinerea.* Overall, these results show that interfamily transfer of Arabidopsis *LecRK-VI.2* to the Solanaceous plant *N. benthamiana* confers increased resistance to adapted foliar phytopathogenic bacteria.

To date, only a few examples of successful interfamily transfer of resistance genes have been reported (Wulff *et al.*, 2011). Notably, Maekawa *et al.* (2012) showed that the MLA1 immune receptor from the monocotyledonous barley protects immunocompromised dicotyledonous Arabidopsis against the barley powdery mildew fungus, *Blumeria graminis* f. sp. *hordei.* Similarly, ectopic expression of the tomato resistance gene *Ve1* in Arabidopsis increases Arabidopsis resistance to the fungal pathogens *Verticillium dahliae* and *Verticillium albo-atrum* (Fradin *et al.*, 2011). In addition, transgenic expression of the Arabidopsis LecRK-I.9 in the Solanaceous plants *N. benthamiana* and potato increases resistance to the oomycete pathogen *Phytophthora infestans* (Bouwmeester *et al.*, 2014). The successful interfamily transfer of the Arabidopsis

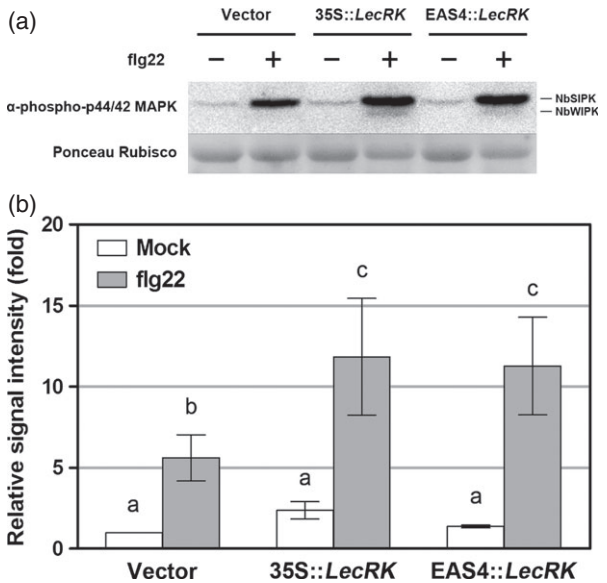

Figure 5. Augmented MAPK activities in *Nicotiana benthamiana* expressing *LecRK-VI.2.*

(a) Leaves of 5-week-old *N. benthamiana* transgenic lines harbouring the 35S::*LecRK-VI.2* (35S::*LecRK*) or EAS4::*LecRK-VI.2* (EAS4::*LecRK*) were infiltrated with either 50 nM flg22 (+) or mock-treated with water (–) for 10 min. Extracted proteins were subjected to immunoblot analysis using an anti-phospho-p44/42-MAPK antibody, which cross-reacts with phosphorylated NbSIPK and NbWIPK, the homologs of Arabidopsis MPK3 and MPK6, respectively (top panel). Ponceau staining of Rubisco is shown to assess equal loading in each lane (bottom panel). Experiments were performed three times independently. Transgenic lines (1) were used for this assay.

(b) Relative band intensities of phosphorylated NbSIPK and NbWIPK were analysed using Image J software. Relative band intensities were compared to the mock-treated empty vector control (Vector) (defined value of 1). Values represent the averages ± standard error (SE) from three independent experiments ($n = 3$). $^{a–c}$Bars with different letters are significantly different from each other (two-way analysis of variance (ANOVA), $P < 0.05$).

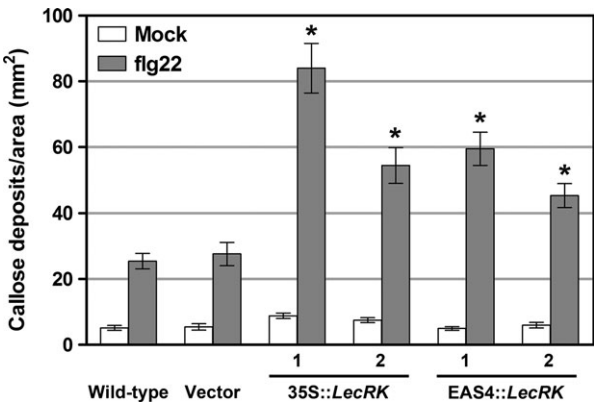

Figure 6. Potentiation of flg22-mediated callose deposition in *Nicotiana benthamiana* ectopically expressing *LecRK-VI.2.*

Leaves of 5-week-old plants were infiltrated with 40 μM flg22 or water (Mock) and samples were collected 24 h later for aniline blue staining. Data represent the average number of callose deposits per square mm ± standard error (SE) from three independent experiments each with 16 biological repeats ($n = 48$). Asterisks denote values significantly different from the empty vector control (Vector) (*$P < 0.01$, Student's *t*-test). (1) and (2) represent two independent transgenic lines for each constructs.

PRR EFR to the Solanaceous plants *N. benthamiana* and tomato represents the only known example of engineered disease resistance through the use of PTI (Lacombe *et al.*, 2010). Our results show that ectopic expression of *LecRK-VI.2*, a positive regulator of Arabidopsis PTI that associates with the PRR FLS2 enhances resistance of the Solanaceous plant *N. benthamiana* to phytopathogenic bacteria. These data demonstrate the feasibility of interfamily transfer of not only PRRs, but also components of PRR complexes. A frequent problem associated with heterologous expression of defence-related genes is that it often results in spontaneous necrosis and/or a dwarf phenotype and constitutive activation of plant defence responses (Hammond-Kosack and Parker, 2003; Gurr and Rushton, 2005). Similarly to transgenic expression of *EFR* (Lacombe *et al.*, 2010), *N. benthamiana* transgenics expressing *LecRK-VI.2* did not demonstrate constitutive PTI activation and harboured a WT growth pattern under laboratory conditions. By contrast, over-expression of *LecRK-VI.2* in Arabidopsis leads to a constitutive activation of the PTI response and a dwarf phenotype (Singh *et al.*, 2012a). Downstream PTI signalling cascades may only be partially conserved between Arabidopsis and *N. benthamiana* resulting in a weakened PTI signalling in *N. benthamiana.* For example, LecRK-VI.2 is known to be critical for Arabidopsis FLS2- and EFR-dependent PTI activation (Singh *et al.*, 2012a). As EFR is absent in Solanaceous plants such as *N. benthamiana* (Kunze *et al.*, 2004), ectopic expression of *LecRK-VI.2* in *N. benthamiana* may thus only affect the FLS2-dependent PTI signalling while both FLS2- and EFR-dependent PTI responses are activated in Arabidopsis over-expressing *LecRK-VI.2.* This may result in less impact in *N. benthamiana* than in Arabidopsis that possesses FLS2 and EFR PRRs (Zipfel *et al.*, 2004, 2006; Singh *et al.*, 2012a). Upon elicitation with the MAMP flagellin, potentiation of ROS accumulation, MAPK activities, callose deposition and gene expression were observed in transgenic *N. benthamiana* expressing *LecRK-VI.2* indicating priming of PTI. A faster activation of the PTI response upon flg22 elicitation in *N. benthamiana* transgenics may explain the observed increased resistance to bacteria.

Our data show that a component of a PRR complex can be transferred from one plant family to another to engineer increased resistance to bacteria. Current knowledge suggests that LecRK-VI.2 is specific for resistance to bacteria (Singh *et al.*, 2012a,b), possibly by targeted association with bacterial PRRs. Engineering crops with a combination of different positive regulator of PTI that are efficient against different type of pathogens may generate crop plants with broad-spectrum resistance to multiple genera of pathogens. The discovery of proteins associated with and/or modulating PRRs is thus critical for the future

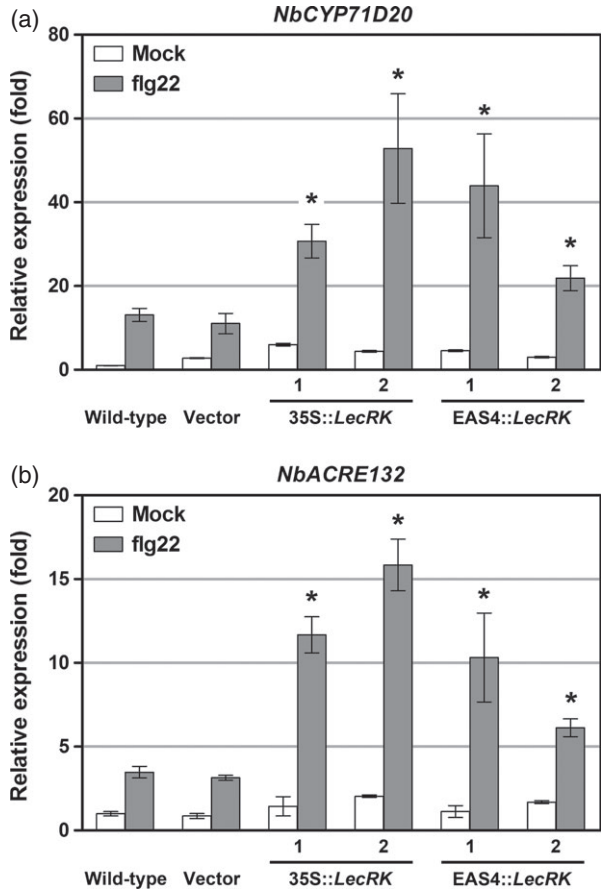

Figure 7. *Nicotiana benthamiana* transgenics demonstrate increased flg22-mediated up-regulation of PTI marker genes.
Expression of PTI marker genes *NbCYP71D20* (a) and *NbACRE132* (b) were analysed in *N. benthamiana* leaves 6 h after infiltration with 100 nM flg22 or water (Mock). *ELONGATION FACTOR–1 ALPHA* (*NbEF-1α*) was used for normalization. Relative gene expression levels were compared to the mock-treated empty vector control (Vector) (defined value of 1). Values represent the averages ± standard error (SE) from three independent experiments each with three biological repeats ($n = 9$). Asterisks indicate a significant difference to the empty vector control (Vector) based on a *t*-test (*$P < 0.01$). (1) and (2) represent two independent transgenic lines for each constructs.

development of novel biotechnological approaches to produce pathogen resistant crops.

EXPERIMENTAL PROCEDURES

Biological materials and growth conditions

Arabidopsis thaliana (L. Heyhn.) plants ecotype Columbia (Col-0) were grown in commercial potting soil/perlite (3:2) at 22°C day and 19°C night temperature under a 9-h-light period. The lighting was supplied at an intensity of ~100 μE m^{-2} sec^{-1} by fluorescence tubes. *N. benthamiana* plants were grown in a controlled-environment growth chamber at 25°C under 16 h of light. Bacterial strain *Pss* B728a and *Pta* 11528 were a kind gift from N.C. Lin (National Taiwan University, Taipei, Taiwan). We obtained *Pcc* SCC1 bacteria (Kariola *et al.*, 2005) expressing the GFP (Kwon *et al.*, 2009) from O. K. Park (Korea University, Seoul, Korea). To prepare the bacterial inoculum, *Pseudomonas* or *Pcc* SCC1 bacteria were cultivated overnight at 28°C with constant shaking (340 rpm) in King's B medium with 100 mg/L rifampicin or Luria-Bertani medium (Bioman Scientific Co. Ltd, http://www.bioman.com.tw/) with 100 mg/L ampicillin, respectively. Bacteria were washed twice and resuspended in 10 mM $MgSO_4$ at an OD_{600} of 0.2 (*Pseudomonas*) or 0.25 (*Pcc* SCC1) corresponding to 1×10^8 cfu ml^{-1}. The fungus *B. cinerea* obtained from C.Y. Chen (National Taiwan University, Taipei, Taiwan) was grown at room temperature (18–25°C) on potato dextrose broth-agar plates as described (Zimmerli *et al.*, 2001).

Pathogen infection assays

Disease assays with *Pss* B728a and *Pta* 11528 were as previously described (Vinatzer *et al.*, 2006). Briefly, 5-week-old *N. benthamiana* plants were dipped in bacterial solutions of 10^6 cfu/ml containing 0.01% Silwet L-77 (Bioman Scientific Co., Ltd.) for 5 min. The infected plants were kept at 100% relative humidity for 1 day, and symptoms were photographed 3 days later (i.e. 4 days after inoculation). To assess bacterial populations, three leaves per plant were marked before inoculation and 15 leaf discs from the marked leaves (0.2827 cm^2/disc) were harvested from five different plants 3 HPI, or 1, 2, 3 or 4 days after dip-inoculation. Leaf discs were washed twice with sterile distilled water and homogenized in 10 mM $MgSO_4$. Four 10-μl droplets of appropriate dilutions were applied on King's B plates containing 100 mg L^{-1} of rifampicin. Bacterial colonies were counted after 48 h at 28°C. For infections with *Pcc* SCC1, 5-week-old *N. benthamiana* plants were vacuum infiltrated with a bacterial solution of 2×10^5 cfu ml^{-1} containing 0.005% Silwet L-77. The infected plants were kept at 100% relative humidity for 1 day, and disease development was scored at 2 DPI using a disease rating scale. Six plants were used in each experimental replicates. For infections with *B. cinerea*, 5 μl droplets of spore suspension (10^5 spores ml^{-1} in 1/4 PDB) were deposited on each side of the leaf midvein on three leaves per 5-week-old plants (six droplets per plants). Leaves of same age were used for droplet-inoculation. The infected plants were kept at 100% relative humidity throughout the assay. Disease symptoms and lesion perimeters were determined at 3 DPI. Perimeters were measured in pixels with IMAGEJ software (http://rsb.info.nih.gov/ij/), by the freehand selection tool and calibrated to centimeters using a ruler in the photo.

Transgene construction

Phusion High-Fidelity DNA Polymerase (New England BioLabs, https://www.neb.com/) was used for all PCR reactions. To generate ectopic expression construct for stable *N. benthamiana* transgenics, 35S promoter and CDS of *LecRK-VI.2* amplified from pEarleyGate 100 gateway clone containing *LecRK-VI.2* (Singh *et al.*, 2012a) and NOS terminator amplified from p35SN were cloned into pGEM-T vector (Promega). Fragment of 35S::*LecRK-VI.2*-NOS was then cloned into T-DNA binary vector pCAMBIA1300 (http://www.cambia.org/daisy/cambia/home.html) using *Xba*I/*Pst*I digestion. In parallel, EAS4 promoter amplified from pHGWFS7-EAS4 construct (Lin *et al.*, 2014) was first cloned into pCAMBIA1300 using *Eco*RI/*Xba*I restriction enzyme. LecRK-VI.2-NOS fragment was then introduced into this vector using *Xba*I/*Pst*I to create EAS4::LecRK-VI.2-NOS construct.

Generation of *N. benthamiana* transgenics

To create transgenic lines, seeds of *N. benthamiana* were surface sterilized by incubation in 20% of household bleach for 10 min followed by five washes with sterile distilled water. The surface sterilized seeds were sowed on Murashige and Skoog (MS) medium (#M0222, Duchefa Biochemie, http://www.duchefa-biochemie.com/)

plates containing 1% sucrose and grown for 1 month. Colonies of *A. tumefaciens* strain LBA4404 carrying either the constructs or the empty vector were grown into 50 ml liquid YEP medium (10 g L^{-1} yeast extract, 10 g L^{-1} bacto peptone, 5 g L^{-1} NaCl, pH 7.0) containing 100 mg L^{-1} rifampicin and 50 mg L^{-1} kanamycin at 28°C with constant shaking (340 rpm) overnight. After centrifugation, the bacterial pellet was suspended to a final OD_{600} of 0.6 to 0.8 in co-culture medium (MS with vitamins, 3% sucrose, 0.1 mg L^{-1} 1-naphthaleneacetic acid, 1 mg L^{-1} benzoic acid, 100 μM acetosyringone). For plant transformation, the upper leaves of sterile *N. benthamiana* were excised and cut into pieces of 1 cm^2 in area by avoiding leaf margins and midveins. The explants were immersed in the prepared *Agrobacterium* inoculum on a Petri plate for 1 min. After inoculation, the explants were blotted dried on sterile tissue paper and placed, the adaxial side up, on to co-culture plates (co-culture medium containing 0.2% phytagel). The explants were co-cultivated in the dark for 2 days. After co-cultivation, the explants were transferred to shooting medium (MS with vitamins, 3% sucrose, 0.1 mg L^{-1} 1-naphthaleneacetic acid, 1 mg L^{-1} benzoic acid, 200 mg L^{-1} timentin, 15 mg L^{-1} hygromycin, 0.2% phytagel) for transformant selection. The regenerating explants were transferred to fresh shooting medium every 2 weeks. Shoots were excised once they reached a length of 5–10 mm and transferred to rooting medium (MS with vitamins, 3% sucrose, 200 mg L^{-1} timentin, 0.2% phytagel). Two independent single insertion lines per construct were further screened for homozygous progenies using segregation analyses of hygromycin resistance. Homozygous T3 plants were used for the assays presented.

Expression and purification of recombinant proteins

A maltose-binding protein (MBP)-tagged FLS2 kinase domain (MBP-FLS2KD) construct was generated as described by Schwessinger *et al.* (2011). Glutathione S-transferase (GST)-tagged LecRK-VI.2 kinase domain (GST-LecRK-VI.2KD) construct was produced as described in Singh *et al.* (2012b). MBP was expressed from pMAL™-c5X (New England BioLabs). *E. coli* strain BL21 (DE3) pLysS (Millipore, http://www.emdmillipore.com/life-science-research/novagen/c_YTKb.s1OFbwAAAEjSGVXhFCX) was used to express MBP and MBP-FLS2KD, and GST-LecRK-VI.2KD was expressed in BL21 (DE3) strain (Novagen). For protein expression, cells were grown in Luria-Bertani medium containing 0.4% glucose and either 100 mg L^{-1} ampicillin and 34 mg L^{-1} chloramphenicol for BL21 (DE3) pLysS strain or 100 mg L^{-1} ampicillin for BL21 (DE3) strain at 28°C. When OD_{600} was around 2, isopropyl β-D-1-thiogalactopyranoside (IPTG) was added to a final concentration of 0.4 mM to induce protein expression. The bacterial cultures were then incubated at 16°C for 16 h (MBP), 2 h (MBP-FLS2KD), or 8 h (GST-LecRK-VI.2KD). MBP-tagged and GST-tagged proteins were purified following manufacturer's instructions using MBP-Trap™ HP or GSTTrap™ HP (GE Healthcare, http://www3.gehealthcare.com/en), respectively. All proteins were dialyzed against dialysis buffer (20 mM Tris–HCl, 200 mM NaCl, 1 mM EDTA, pH 7.4).

In vitro pull-down assay

One microgram of MBP or MBP-FLS2KD was incubated with 2 μg of GST-LecRK-VI.2KD in a binding buffer (20 mM Tris–HCl, 200 mM NaCl, 1 mM EDTA, pH 7.4) under agitation at 4°C. After 2 h, 50 μl of amylose resin beads (GE Healthcare) were added, and the incubation continued for another 2 h. The beads were then washed five times with the washing buffer (20 mM Tris–HCl, 200 mM NaCl, 1 mM EDTA, 0.6% Triton X-100, pH 7.4). Input and pulled-down proteins were resolved by 8% SDS-PAGE and detected by western blotting. Anti-MBP (#M6295, SIGMA) and anti-GST (#sc-138 HRP, Santa Cruz Biotechnology Inc., http://www.scbt.com/) antibodies were used to detect MBP and GST fusion proteins, respectively.

BiFC assay

Full-length coding sequences (CDS) of *FLS2*, *BAK1*, and *LecRK-VI.2* without stop codon amplified from cDNA of Arabidopsis Col-0 were inserted into entry vector pCR8/GW/TOPO, and subcloned into vectors containing N-terminal (YFPN) or C-terminal (YFPC) YFP (Lu *et al.*, 2010c) through an LR reaction (Invitrogen). The constructs were transformed into Arabidopsis protoplasts by polyethylene glycol (Sigma, http://www.sigmaaldrich.com/) for transient expression (Yoo *et al.*, 2007). Transfected cells were imaged using a TCS SP5 confocal spectral microscope imaging system (Leica).

Co-IP assay in *N. benthamiana*

Entry clones containing CDS of *FLS2*, *BAK1*, and *LecRK-VI.2* without stop codon were subcloned into pEarleyGate 103 destination vector with 35S promoter and C-terminal GFP fusion (Earley *et al.*, 2006) or into the pGWB14 vector with C-terminal triple HA fusion (Nakagawa *et al.*, 2007) using LR reaction. The constructs were electroporated into *A. tumefaciens* competent cells strain GV3101. Transient expression and Co-IP were performed as previously described (Roux *et al.*, 2011).

FLS2–GFP localization

FLS2–GFP transient expression in *N. benthamiana* was performed as described (Roux *et al.*, 2011). The GFP signal was observed with a Zeiss LSM 780 confocal microscope (http://www.zeiss.com/corporate/en_de/home.html).

ROS evaluation

Detection of ROS production was monitored by a luminol-based assay on leaf disc samples (Keppler *et al.*, 1989). Leaf discs (3 mm in diameter) from fully expanded leaves of 5-week-old *N. benthamiana* were incubated in 100 μl distilled water in a 96-well plate overnight. Before measurement, the water was removed and 100 μl of assay solution (100 μM luminol, 1 μM ml^{-1} peroxidase, and 10 nM flg22) was added to the wells. Luminescence was measured using CentroLIApc LB 692 plate luminometer (Berthold Technologies, https://www.berthold.com/) with a measuring time of 2.5 sec on each well at indicated time points.

MAPK activation assay

Fully expanded leaves of 5-week-old *N. benthamiana* were syringe-infiltrated with 50 nM flg22 or mock-treated with water for 10 min. Leaf samples were flash frozen in liquid N_2 and ground to fine powder. Total proteins were extracted from 300 mg of frozen samples in 300 μl modified Lacus buffer (Bogre *et al.*, 1999) (50 mM Tris–HCl, pH 7.5, 10 mM $MgCl_2$, 15 mM EGTA, 100 mM NaCl, 2 mM dithiothreitol, 1 mM NaF, 0.5 mM Na_3VO_4, 0.1% Triton-X100, and 0.5 mM phenylmethylsulfonyl fluoride) supplemented with protease inhibitor cocktail (Roche, http://www.roche.com/index.htm) and were quantified with the Bradford assay (Bio-Rad, http://www.bio-rad.com/). Forty micrograms of proteins were used per lane in western blots. Phosphorylated MAPK were detected with anti-phospho-p44/42 MAPK primary antibodies (#9101, Cell Signaling Technology, http://www.cellsignal.com/) following the manufacturer's instructions. Western blot band intensities were quantified using IMAGEJ software (http://imagej.nih.gov/ij/).

Callose deposition assays

Callose deposition assays for *N. benthamiana* were modified from Nguyen *et al.* (2010). Fully expanded leaves of 5-week-old *N. benthamiana* were infiltrated with 40 μM of flg22 dissolved in water with a needleless syringe. Control plants were infiltrated with water only. After 24 h, leaf disks from infiltrated areas were excised with a cork borer (0.2827 cm^2 in area). The collected leaf disks were incubated in 95% ethanol at 37°C until the leaf discs were cleared of chlorophyll, with the ethanol being replaced until the clearing was complete. The cleared leaf disks were washed two times with 70% ethanol and then three times with distilled water. The leaf disks were then vacuum-infiltrated with 1% aniline blue in 150 mM K_2HPO_4 (pH 9.5/KOH) and incubated in the dark overnight. Callose deposits were visualized under UV illumination using a Nikon Optiphot-2 microscope. Callose deposits were counted using the 'analyse particles' function of IMAGEJ.

RNA extraction and gene expression analysis

Total RNA was extracted and purified using the MaestroZol reagent according to the manufacturer's instructions (Omics Biotechnology Co., Ltd, http://www.omicsbio.com/) with the addition of PLUS reagent for polysaccharides and proteoglycans elimination. Genomic DNA contaminations were removed using Qiagen RNase-free DNase Set (http://www.qiagen.com/). For cDNA synthesis, 2 μg of total RNA were prepared in a volume of 22 μl DEPC and denatured at 65°C for 5 min. Eighteen ml of master mix (16 M-MLV buffer, 1 mM dNTP, 5 mM oligo(dT), 100 U M-MLV reverse transcriptase, [Invitrogen]) was added into each tube and then incubated at 37°C for 1 h followed by 70°C for 10 min. RT-PCR amplification was done with 1 μl of the first-strand cDNA as template, 10 μl of Taq PLUS PCR MasterMix (#KT205, Tiangen Biotech, http://www.tiangen.com/en/), 1 μl of 10 mM each forward and reverse primers in a total volume of 20 μL. The cycling conditions were 94°C for 5 min for one initial step followed by 94°C for 30 sec, 58°C for 30 sec and 72°C for 1 min, for 35 cycles. The PCR was terminated with one extra step at 72°C for 10 min. Real-time PCRs were conducted on a CFX Real-Time PCR Detection System (Bio-Rad). SYBR Green fast qPCR master mix (Kapa Biosystems; 1 μl of cDNA, 9 μl SYBR Green supermix, 6 μl deionized water, 1 μl of 10 mM forward primer, 1 μl of 10 mM reverse primer, in a total volume of 18 μl per well) was employed for the analysis. The cycling conditions were composed of an initial 3 min denaturation step at 95°C, followed by 40 cycles of 95°C for 3 sec, 60°C for 30 sec. Melting curves were run from 65°C to 95°C with 0.5-sec time interval. Data were analysed using the Bio-Rad CFX manager software. *N. benthamiana ELONGATION FACTOR-1 ALPHA* (*NbEF-1α*) was used as reference genes for normalization. The details of primers used are summarized in Table S1.

ACKNOWLEDGEMENTS

We are grateful to N.C. Lin, C.Y. Chen and O.K. Park for the pathogens. We thank the staff of Technology Commons, College of Life Science, National Taiwan University for their help in microscopy and for qRT-PCR equipment. We also thank Y.S. Cheng and members of his lab for help with protein purification. We acknowledge members of Zimmerli's laboratory for critical comments. This work was supported by the National Science Council of Taiwan grants 99-2628-B-002-053-MY3 and 102-2628-B-002-011-MY3 (to L.Z.) and grant 102-2311-B-002-026 (to C.P.C.), and the Frontier and Innovative Research grant of the National Taiwan University code numbers 101R7846 and 102R7846 (to L.Z.).

SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article.

Figure S1. Subcellular localization of transiently expressed FLS2-GFP in *N. benthamiana* leaves.

Figure S2. Transgenic expression of *LecRK-VI.2* in *N. benthamiana.*

Figure S3. Growth phenotype of *N. benthamiana* ectopically expressing *LecRK-VI.2.*

Figure S4. *Pta* 11528-mediated disease symptoms 2 weeks after inoculation.

Figure S5. WT resistance levels of *N. benthamiana* transgenics ectopically expressing *LecRK-VI.2* to *B. cinerea* infection.

Figure S6. Ectopic expression of *LecRK-VI.2* in *N. benthamiana* does not up-regulate *NbSIPK* and *NbWIPK.*

Table S1. Primers used in this study.

REFERENCES

Arnaud, D., Desclos-Theveniau, M. and Zimmerli, L. (2012) Disease resistance to *Pectobacterium carotovorum* is negatively modulated by the *Arabidopsis* lectin receptor kinase LecRK-V.5. *Plant Signal. Behav.* **7**, 1070–1072.

Beckers, G.J., Jaskiewicz, M., Liu, Y., Underwood, W.R., He, S.Y., Zhang, S. and Conrath, U. (2009) Mitogen-activated protein kinases 3 and 6 are required for full priming of stress responses in *Arabidopsis thaliana*. *Plant Cell*, **21**, 944–953.

Bogre, L., Calderini, O., Binarova, P. *et al.* (1999) A MAP kinase is activated late in plant mitosis and becomes localized to the plane of cell division. *Plant Cell*, **11**, 101–113.

Boller, T. and Felix, G. (2009) A renaissance of elicitors: perception of microbe-associated molecular patterns and danger signals by pattern-recognition receptors. *Annu. Rev. Plant Biol.* **60**, 379–406.

Bouwmeester, K. and Govers, F. (2009) *Arabidopsis* L-type lectin receptor kinases: phylogeny, classification, and expression profiles. *J. Exp. Bot.* **60**, 4383–4396.

Bouwmeester, K., de Sain, M., Weide, R., Gouget, A., Klamer, S., Canut, H. and Govers, F. (2011) The lectin receptor kinase LecRK-I.9 is a novel *Phytophthora* resistance component and a potential host target for a RXLR effector. *PLoS Pathog.* **7**, e1001327.

Bouwmeester, K., Han, M., Blanco-Portales, R., Song, W., Weide, R., Guo, L.-Y., van der Vossen, E.A.G. and Govers, F. (2014) The *Arabidopsis* lectin receptor kinase LecRK-I.9 enhances resistance to *Phytophthora infestans* in Solanaceous plants. *Plant Biotechnol. J.* **12**, 10–16.

Chinchilla, D., Bauer, Z., Regenass, M., Boller, T. and Felix, G. (2006) The *Arabidopsis* receptor kinase FLS2 binds flg22 and determines the specificity of flagellin perception. *Plant Cell*, **18**, 465–476.

Chinchilla, D., Zipfel, C., Robatzek, S., Kemmerling, B., Nurnberger, T., Jones, J.D., Felix, G. and Boller, T. (2007) A flagellin-induced complex of the receptor FLS2 and BAK1 initiates plant defence. *Nature*, **448**, 497–500.

Conrath, U., Beckers, G.J.M., Flors, V. *et al.* (2006) Priming: getting ready for battle. *Mol. Plant Microbe Interact.* **19**, 1062–1071.

Cutler, S.R., Ehrhardt, D.W., Griffitts, J.S. and Somerville, C.R. (2000) Random GFP:cDNA fusions enable visualization of subcellular structures in cells of Arabidopsis at a high frequency. *Proc. Natl Acad. Sci. USA*, **97**, 3718–3723.

Davidsson, P.R., Kariola, T., Niemi, O. and Palva, E.T. (2013) Pathogenicity of and plant immunity to soft rot pectobacteria. *Front. Plant Sci.* **4**, 191.

Desclos-Theveniau, M., Arnaud, D., Huang, T.-Y., Lin, G.J.-C., Chen, W.-Y., Lin, Y.-C. and Zimmerli, L. (2012) The Arabidopsis lectin receptor kinase LecRK-V.5 represses stomatal immunity induced by *Pseudomonas syringae* pv. *tomato* DC3000. *PLoS Pathog.* **8**, e1002513.

Deslandes, L. and Rivas, S. (2012) Catch me if you can: bacterial effectors and plant targets. *Trends Plant Sci.* **17**, 644–655.

Earley, K.W., Haag, J.R., Pontes, O., Opper, K., Juehne, T., Song, K. and Pikaard, C.S. (2006) Gateway-compatible vectors for plant functional genomics and proteomics. *Plant J.* **45**, 616–629.

Felix, G., Duran, J.D., Volko, S. and Boller, T. (1999) Plants have a sensitive perception system for the most conserved domain of bacterial flagellin. *Plant J.* **18**, 265–276.

Feng, F. and Zhou, J.-M. (2012) Plant–bacterial pathogen interactions mediated by type III effectors. *Curr. Opin. Plant Biol.* **15**, 469–476.

Feng, F., Yang, F., Rong, W., Wu, X., Zhang, J., Chen, S., He, C. and Zhou, J.M. (2012) A *Xanthomonas* uridine 5′-monophosphate transferase inhibits plant immune kinases. *Nature*, **485**, 114–118.

Fradin, E.F., Abd-El-Haliem, A., Masini, L., van den Berg, G.C., Joosten, M.H. and Thomma, B.P. (2011) Interfamily transfer of tomato *Ve1* mediates *Verticillium* resistance in Arabidopsis. *Plant Physiol.* **156**, 2255–2265.

Gassmann, W. and Bhattacharjee, S. (2012) Effector-triggered immunity signaling: from gene-for-gene pathways to protein–protein interaction networks. *Mol. Plant Microbe Interact.* **25**, 862–868.

Gasson, M.J. (1980) Indicator technique for antimetabolic toxin production by phytopathogenic species of *Pseudomonas*. *Appl. Environ. Microbiol.* **39**, 25–29.

Gómez-Gómez, L. and Boller, T. (2000) FLS2: An LRR receptor–like kinase involved in the perception of the bacterial elicitor flagellin in *Arabidopsis*. *Mol. Cell*, **5**, 1003–1011.

Gomez-Gomez, L., Felix, G. and Boller, T. (1999) A single locus determines sensitivity to bacterial flagellin in *Arabidopsis thaliana*. *Plant J.* **18**, 277–284.

Gurr, S.J. and Rushton, P.J. (2005) Engineering plants with increased disease resistance: what are we going to express? *Trends Biotechnol.* **23**, 275–282.

Halter, T., Imkampe, J., Mazzotta, S. *et al.* (2014) The leucine-rich repeat receptor kinase BIR2 is a negative regulator of BAK1 in plant immunity. *Curr. Biol.* **24**, 134–143.

Hammond-Kosack, K.E. and Parker, J.E. (2003) Deciphering plant-pathogen communication: fresh perspectives for molecular resistance breeding. *Curr. Opin. Plant Biol.* **14**, 177–193.

Heese, A., Hann, D.R., Gimenez-Ibanez, S., Jones, A.M.E., He, K., Li, J., Schroeder, J.I., Peck, S.C. and Rathjen, J.P. (2007) The receptor-like kinase SERK3/BAK1 is a central regulator of innate immunity in plants. *Proc. Natl Acad. Sci. USA*, **104**, 12217–12222.

Jaskiewicz, M., Conrath, U. and Peterhansel, C. (2011) Chromatin modification acts as a memory for systemic acquired resistance in the plant stress response. *EMBO Rep.* **12**, 50–55.

Jehle, A.K., Lipschis, M., Albert, M., Fallahzadeh-Mamaghani, V., Furst, U., Mueller, K. and Felix, G. (2013) The receptor-like protein ReMAX of *Arabidopsis* detects the microbe-associated molecular pattern eMax from *Xanthomonas*. *Plant Cell*, **25**, 2330–2340.

Jones, J.D.G. and Dangl, J.L. (2006) The plant immune system. *Nature*, **444**, 323–329.

de Jonge, R., van Esse, H.P., Maruthachalam, K. *et al.* (2012) Tomato immune receptor Ve1 recognizes effector of multiple fungal pathogens uncovered by genome and RNA sequencing. *Proc. Natl Acad. Sci. USA*, **109**, 5110–5115.

Kadota, Y., Sklenar, J., Derbyshire, P. *et al.* (2014) Direct regulation of the NADPH oxidase RBOHD by the PRR-associated kinase BIK1 during plant immunity. *Mol. Cell*, **54**, 1–13.

Kariola, T., Brader, G., Li, J. and Palva, E.T. (2005) Chlorophyllase 1, a damage control enzyme, affects the balance between defense pathways in plants. *Plant Cell*, **17**, 282–294.

Keppler, L.D., Baker, C.J. and Atkinson, M.M. (1989) Active oxygen production during a bacteria-induced hypersensitive reaction in tobacco suspension cells. *Phytopathology*, **79**, 974–978.

Kim, H.S., Thammarat, P., Lommel, S.A., Hogan, C.S. and Charkowski, A.O. (2011) *Pectobacterium carotovorum* elicits plant cell death with DspE/F but the *P. carotovorum* DspE does not suppress callose or induce expression of plant genes early in plant-microbe interactions. *Mol. Plant Microbe Interact.* **24**, 773–786.

Korasick, D.A., McMichael, C., Walker, K.A., Anderson, J.C., Bednarek, S.Y. and Heese, A. (2010) Novel functions of Stomatal Cytokinesis-Defective 1 (SCD1) in innate immune responses against bacteria. *J. Biol. Chem.* **285**, 23340–23348.

Kunze, G., Zipfel, C., Robatzek, S., Niehaus, K., Boller, T. and Felix, G. (2004) The N terminus of bacterial elongation factor Tu elicits innate immunity in *Arabidopsis* plants. *Plant Cell*, **16**, 3496–3507.

Kwon, S.J., Jin, H.C., Lee, S., Nam, M.H., Chung, J.H., Kwon, S.I., Ryu, C.M. and Park, O.K. (2009) GDSL lipase-like 1 regulates systemic resistance associated with ethylene signaling in Arabidopsis. *Plant J.* **58**, 235–245.

Lacombe, S., Rougon-Cardoso, A., Sherwood, E. *et al.* (2010) Interfamily transfer of a plant pattern-recognition receptor confers broad-spectrum bacterial resistance. *Nat. Biotechnol.* **28**, 365–369.

Leach, J.E., Vera Cruz, C.M., Bai, J. and Leung, H. (2001) Pathogen fitness penalty as a predictor of durability of disease resistance genes. *Annu. Rev. Phytopathol.* **39**, 187–224.

Li, L., Li, M., Yu, L. *et al.* (2014) The FLS2-associated kinase BIK1 directly phosphorylates the NADPH oxidase RBOHD to control plant immunity. *Cell Host Microbe*, **15**, 329–338.

Lin, Y.M., Shih, S.L., Lin, W.C., Wu, J.W., Chen, Y.T., Hsieh, C.Y., Guan, L.C., Lin, L. and Cheng, C.P. (2014) Phytoalexin biosynthesis genes are regulated and involved in plant response to *Ralstonia solanacearum* infection. *Plant Science*, **224**, 86–94.

Lu, D., Wu, S., Gao, X., Zhang, Y., Shan, L. and He, P. (2010a) A receptor-like cytoplasmic kinase, BIK1, associates with a flagellin receptor complex to initiate plant innate immunity. *Proc. Natl Acad. Sci. USA*, **107**, 496–501.

Lu, D., Wu, S., He, P. and Shan, L. (2010b) Phosphorylation of receptor-like cytoplasmic kinases by bacterial flagellin. *Plant Signal. Behav.* **5**, 598–600.

Lu, Q., Tang, X., Tian, G. *et al.* (2010c) *Arabidopsis* homolog of the yeast TREX-2 mRNA export complex: components and anchoring nucleoporin. *Plant J.* **61**, 259–270.

Luna, E., Bruce, T.J.A., Roberts, M.R., Flors, V. and Ton, J. (2012) Next-generation systemic acquired resistance. *Plant Physiol.* **158**, 844–853.

Maekawa, T., Kufer, T.A. and Schulze-Lefert, P. (2011) NLR functions in plant and animal immune systems: so far and yet so close. *Nat. Immunol.* **12**, 817–826.

Maekawa, T., Kracher, B., Vernaldi, S., Loren, V., van Themaat, E. and Schulze-Lefert, P. (2012) Conservation of NLR-triggered immunity across plant lineages. *Proc. Natl Acad. Sci. USA*, **109**, 20119–20123.

McDonald, B.A. and Linde, C. (2002) Pathogen population genetics, evolutionary potential, and durable resistance. *Annu. Rev. Phytopathol.* **40**, 349–379.

Medina, J., Ballesteros, M.L. and Salinas, J. (2007) Phylogenetic and functional analysis of *Arabidopsis* RCI2 genes. *J. Exp. Bot.* **58**, 4333–4346.

Miya, A., Albert, P., Shinya, T., Desaki, Y., Ichimura, K., Shirasu, K., Narusaka, Y., Kawakami, N., Kaku, H. and Shibuya, N. (2007) CERK1, a LysM receptor kinase, is essential for chitin elicitor signaling in *Arabidopsis*. *Proc. Natl Acad. Sci. USA*, **104**, 19613–19618.

Nakagawa, T., Kurose, T., Hino, T., Tanaka, K., Kawamukai, M., Niwa, Y., Toyooka, K., Matsuoka, K., Jinbo, T. and Kimura, T. (2007) Development of series of gateway binary vectors, pGWBs, for realizing efficient construction of fusion genes for plant transformation. *J. Biosci. Bioeng.* **104**, 34–41.

Newman, M.A., Sundelin, T., Nielsen, J.T. and Erbs, G. (2013) MAMP (microbe-associated molecular pattern) triggered immunity in plants. *Front. Plant Sci.* **4**, 139.

Nguyen, H.P., Chakravarthy, S., Velásquez, A.C., McLane, H.L., Zeng, L., Nakayashiki, H., Park, D.-H., Collmer, A. and Martin, G.B. (2010) Methods to study PAMP-triggered immunity using tomato and *Nicotiana benthamiana*. *Mol. Plant Microbe Interact.* **23**, 991–999.

Nuhse, T.S., Peck, S.C., Hirt, H. and Boller, T. (2000) Microbial elicitors induce activation and dual phosphorylation of the *Arabidopsis thaliana* MAPK 6. *J. Biol. Chem.* **275**, 7521–7526.

Oerke, E.C. (2007) Crop losses to animal pests, plant pathogens, and weeds. In *Encyclopedia of Pest Management*, Volume **II** (Pimentel, D., ed.). UK: CRC Press, pp. 116–120.

Po-Wen, C., Singh, P. and Zimmerli, L. (2013) Priming of the *Arabidopsis* pattern-triggered immunity response upon infection by necrotrophic *Pectobacterium carotovorum* bacteria. *Mol. Plant Pathol.* **14**, 58–70.

Rasmann, S., De Vos, M., Casteel, C.L., Tian, D., Halitschke, R., Sun, J.Y., Agrawal, A.A., Felton, G.W. and Jander, G. (2012) Herbivory in the previous generation primes plants for enhanced insect resistance. *Plant Physiol.* **158**, 854–863.

Ribeiro, R.D.L.D., Hagedorn, D.J., Durbin, R.D. and Uchytil, T.F. (1979) Characterization of the bacterium inciting bean wildfire in Brazil. *Phytopathology*, **69**, 208–212.

Ron, M. and Avni, A. (2004) The receptor for the fungal elicitor ethylene-inducing xylanase is a member of a resistance-like gene family in tomato. *Plant Cell*, **16**, 1604–1615.

Roux, M., Schwessinger, B., Albrecht, C., Chinchilla, D., Jones, A., Holton, N., Malinovsky, F.G., Tor, M., de Vries, S. and Zipfel, C. (2011) The *Arabidopsis* leucine-rich repeat receptor-like kinases BAK1/SERK3 and BKK1/SERK4 are required for innate immunity to hemibiotrophic and biotrophic pathogens. *Plant Cell*, **23**, 2440–2455.

Schwessinger, B., Roux, M., Kadota, Y., Ntoukakis, V., Sklenar, J., Jones, A. and Zipfel, C. (2011) Phosphorylation-dependent differential regulation of plant growth, cell death, and innate immunity by the regulatory receptor-like kinase BAK1. *PLoS Genet.* **7**, e1002046.

Segonzac, C., Feike, D., Gimenez-Ibanez, S., Hann, D.R., Zipfel, C. and Rathjen, J.P. (2011) Hierarchy and roles of pathogen-associated molecular pattern-induced responses in *Nicotiana benthamiana*. *Plant Physiol.* **156**, 687–699.

Shi, H., Shen, Q., Qi, Y., Yan, H., Nie, H., Chen, Y., Zhao, T., Katagiri, F. and Tang, D. (2013) BR-SIGNALING KINASE1 physically associates with FLAGELLIN SENSING2 and regulates plant innate immunity in *Arabidopsis*. *Plant Cell*, **25**, 1143–1157.

Singh, P. and Zimmerli, L. (2013) Lectin receptor kinases in plant innate immunity. *Front. Plant Sci.* **4**, 124.

Singh, P., Kuo, Y.C., Mishra, S. *et al.* (2012a) The lectin receptor kinase-VI.2 is required for priming and positively regulates *Arabidopsis* pattern-triggered immunity. *Plant Cell*, **24**, 1256–1270.

Singh, P., Chien, C.-C., Mishra, S., Tsai, C.-H. and Zimmerli, L. (2012b) The *Arabidopsis* LECTIN RECEPTOR KINASE-VI.2 is a functional protein kinase and is dispensable for basal resistance to *Botrytis cinerea*. *Plant Signal. Behav.* **8**, e22611.

Sticher, L., Mauch-Mani, B. and Metraux, J.P. (1997) Systemic acquired resistance. *Annu. Rev. Phytopathol.* **35**, 235–270.

Sun, Y.D., Li, L., Macho, A.P., Han, Z.F., Hu, Z.H., Zipfel, C., Zhou, J.M. and Chai, J.J. (2013) Structural basis for flg22-induced activation of the *Arabidopsis* FLS2-BAK1 immune complex. *Science*, **342**, 624–628.

Tena, G., Boudsocq, M. and Sheen, J. (2011) Protein kinase signaling networks in plant innate immunity. *Curr. Opin. Plant Biol.* **14**, 519–529.

Tsuda, K. and Katagiri, F. (2010) Comparing signaling mechanisms engaged in pattern-triggered and effector-triggered immunity. *Curr. Opin. Plant Biol.* **13**, 459–465.

Van Wees, S.C., Van der Ent, S. and Pieterse, C.M. (2008) Plant immune responses triggered by beneficial microbes. *Curr. Opin. Plant Biol.* **11**, 443–448.

Veronese, P., Nakagami, H., Bluhm, B., Abuqamar, S., Chen, X., Salmeron, J., Dietrich, R.A., Hirt, H. and Mengiste, T. (2006) The membrane-anchored *BOTRYTIS-INDUCED KINASE1* plays distinct roles in *Arabidopsis* resistance to necrotrophic and biotrophic pathogens. *Plant Cell*, **18**, 257–273.

Vinatzer, B.A., Teitzel, G.M., Lee, M.-W., Jelenska, J., Hotton, S., Fairfax, K., Jenrette, J. and Greenberg, J.T. (2006) The type III effector repertoire of *Pseudomonas syringae* pv. *syringae* B728a and its role in survival and disease on host and non-host plants. *Mol. Microbiol.* **62**, 26–44.

Walter, M., Chaban, C., Schütze, K. *et al.* (2004) Visualization of protein interactions in living plant cells using bimolecular fluorescence complementation. *Plant J.* **40**, 428–438.

Wan, J., Zhang, X.C., Neece, D., Ramonell, K.M., Clough, S., Kim, S.Y., Stacey, M.G. and Stacey, G. (2008) A LysM receptor-like kinase plays a critical role in chitin signaling and fungal resistance in *Arabidopsis*. *Plant Cell*, **20**, 471–481.

Willmann, R., Lajunen, H.M., Erbs, G. *et al.* (2011) *Arabidopsis* lysin-motif proteins LYM1 LYM3 CERK1 mediate bacterial peptidoglycan sensing and immunity to bacterial infection. *Proc. Natl Acad. Sci. USA*, **108**, 19824–19829.

Wulff, B.B.H., Horvath, D.M. and Ward, E.R. (2011) Improving immunity in crops: new tactics in an old game. *Curr. Opin. Plant Biol.* **14**, 468–476.

Yoo, S.D., Cho, Y.H. and Sheen, J. (2007) *Arabidopsis* mesophyll protoplasts: a versatile cell system for transient gene expression analysis. *Nat. Protoc.* **2**, 1565–1572.

Zhang, J., Li, W., Xiang, T.T. *et al.* (2010) Receptor-like Cytoplasmic kinases integrate signaling from multiple plant immune receptors and are targeted by a *Pseudomonas syringae* effector. *Cell Host Microbe*, **7**, 290–301.

Zimmerli, L., Metraux, J.P. and Mauch-Mani, B. (2001) beta-aminobutyric acid-induced protection of *Arabidopsis* against the necrotrophic fungus *Botrytis cinerea*. *Plant Physiol.* **126**, 517–523.

Zipfel, C. and Robatzek, S. (2010) Pathogen-associated molecular pattern-triggered immunity: Veni, Vidi. ? *Plant Physiol.* **154**, 551–554.

Zipfel, C., Robatzek, S., Navarro, L., Oakeley, E.J., Jones, J.D.G., Felix, G. and Boller, T. (2004) Bacterial disease resistance in *Arabidopsis* through flagellin perception. *Nature*, **428**, 764–767.

Zipfel, C., Kunze, G., Chinchilla, D., Caniard, A., Jones, J.D.G., Boller, T. and Felix, G. (2006) Perception of the bacterial PAMP EF-Tu by the receptor EFR restricts *Agrobacterium*-mediated transformation. *Cell*, **125**, 749–760.

