The Lectin Receptor Kinase-VI.2 Is Required for Priming and Positively Regulates Arabidopsis Pattern-Triggered Immunity

Prashant Singh,a Yi-Chun Kuo,a,1 Swati Mishra,a,1 Chia-Hong Tsai,a,1,2 Chih-Cheng Chien,a,1
Ching-Wei Chen,a,1 Marie Desclos-Thenieniau,a Po-Wei Chu,a Birgit Schulze,b,3 Delphine Chinchilla,b
Thomas Boller,b and Laurent Zimmerli*a,4

a Department of Life Science and Institute of Plant Biology, National Taiwan University, Taipei 106, Taiwan
b Zurich-Basel Plant Science Center, Botanical Institute, University of Basel, 4056 Basel, Switzerland

Plants face threats from numerous pathogenic microbes and resist attacking pathogens through both constitutive and inducible defenses (Jones and Dangl, 2006). At the site of attack, defense responses are induced through plant membrane proteins teemed pattern recognition receptors (PRRs). PRRs perceive pathogen- or microbe-associated molecular patterns (MAMPs), which are slow-evolving and evolutionarily conserved microbial molecular signatures (Ausubel, 2005; Boller and Felix, 2009). Typical bacterial MAMPs include flagellin (Felix, et al., 1999) and elongation factor Tu (EF-Tu) (Kunze, et al., 2004). MAMP recognition leads to a pattern-triggered immune response called PTI (Jones and Dangl, 2006), which is characterized by accumulation of reactive oxygen species (ROS), activation of mitogen-activated protein kinases (MAPKs), callose deposition, and the expression of defense-related genes (reviewed in Boller and Felix, 2009). Different genetic and molecular approaches have been used to dissect signaling pathways in PTI, yet relatively little is known about the mediators of PTI signaling.

In addition, there are forms of resistance that are initiated by a first challenge and enhance resistance, often systemically, against subsequent pathogen attack. Two distinct types of this form of resistance have been described: systemic acquired resistance and induced systemic resistance (Durrant and Dong, 2005; Zimmerli et al., 2008), nematodes (Oka et al., 1999), insects (Hodge et al., 2005), and microbial pathogens (Jakab et al., 2001; Zimmerli et al., 2001; Cohen, 2002). BABA induces resistance by potentiating the activation of appropriate defense mechanisms upon stress perception, a phenomenon called priming (Prime-A-Plant Group, 2006). Primed plants are in a heightened state of defense and produce a stronger defensive response when challenged. Although priming involves production of defense signaling components before stress exposure (Beckers et al., 2009), the identity of the signaling components involved in priming remains largely elusive.
PTI-induced transcriptional responses are characterized by the upregulation of a large number of genes, including many that encode receptor-like kinases (RLKs), which could be involved in amplifying the PTI response (Navarro et al., 2004; Zipfel et al., 2004; Thimoney et al., 2006). Cell surface receptors with protein kinase activities play a fundamental role in perception and transduction of extra cellular stimuli (De Smet et al., 2009). There are over 600 RLKs in Arabidopsis thaliana; however, the biological functions of most RLKs are unknown (De Smet et al., 2009).

The lectin receptor kinases are a group of RLK proteins characterized by an extracellular lectin motif. Lectin receptor kinases are classified into three types: G, C, and L (Bouwmeester and Govers, 2009). G-type lectin receptor kinases are known as S-domain RLKs because their ectodomains function in self-incompatibility in flowering plants (Kusaba et al., 2001; Sherman-Broyles et al., 2007). C-type (calcium-dependent) lectin receptor kinases mediate innate immune responses by playing a major role in pathogen recognition in mammals (Cambi et al., 2005), but are rare in plants, with only one member of unknown function in Arabidopsis (Bouwmeester and Govers, 2009). There are 42 L-type lectin receptor kinases (LecRKs) in Arabidopsis (Barre et al., 2002; Bouwmeester and Govers, 2009). LecRKs are characterized by an extracellular legume lectin-like domain, a transmembrane domain, and an intracellular kinase domain (Hervé et al., 1996; Bouwmeester and Govers, 2009). LecRKs are involved in biotic and abiotic stress signal transduction. The poplar LecRK gene LPK and the Arabidopsis LecRK gene LecRK-a1/LecRK-V.5 are induced by wounding and senescence, and Arabidopsis LecRK2/LecRK-I.3 is induced by salt stress (Nishiguchi et al., 2002; Riou et al., 2002; He et al., 2004). LecRK members of the Arabidopsis LecRK-VI clade are redundant negative regulators of the ABA response during seed germination (Xin et al., 2009). In addition, LecRK-VI.2 was shown to be a plasma membrane-localized protein (Xin et al., 2009). Moreover, studies in Nicotiana benthamiana showed that LPK1 interacts with Phytophthora infestans INF1 elicitor and mediates INF1-induced cell death (Kanzaki et al., 2001). In Nicotiana attenuata, LecRK1 was shown to suppress insect-mediated inhibition of jasmonic acid-induced defense responses (Gillard et al., 2011). Another LecRK with a potential role in plant defense is the Arabidopsis LecRK79/LecRK-I.9 (Gouget et al., 2006; Bouwmeester et al., 2011).

We used a reverse genetic approach to identify novel components required for priming and Arabidopsis resistance to biotic stress. We thus analyzed T-DNA insertion mutants of genes that are upregulated by the priming agent BABA (Zimmerli et al., 2008). A LecRK-VI.2 T-DNA insertion line demonstrated increased sensitivity to bacterial pathogens. Here, we report gain-of-function analyses, tests with different types of bacteria, and whole-genome microarray studies demonstrating that LecRK-VI.2 is a positive regulator of the Arabidopsis PTI response to bacterial pathogens and a critical modulator of priming.

RESULTS

BABA Primes the Arabidopsis PTI Response

To test whether chemical priming by BABA potentiates the Arabidopsis PTI response, expression levels of PTI-responsive genes were compared in water- and BABA-treated Arabidopsis upon PTI activation. PTI was activated with the type III secretion system-deficient bacterial mutant Pseudomonas syringae pv tomato (Pst) DC3000 hrcA (CB200) that is defective in delivering type-III effectors and, thus, strongly activates the PTI response (Brooks et al., 2004). An alternative treatment with a purified model MAMP, the peptide flg22, which contains the most conserved domain of bacterial flagellin, was also used to induce PTI. WRKY53, a member of the WRKY transcription factor family, FLG22-INDUCED RECEPTOR KINASE1 (FRK1), and NDR1/HIN1-LIKE10 (NLH10) (Xiao et al., 2007; Boudsocq et al., 2010) as well as CAM BINDING PROTEIN 60g (CBP60g), which is involved in MAMP-triggered salicylic acid (SA) signaling (Wang et al., 2009), were used as PTI marker genes. BABA treatment alone slightly upregulated some of the four PTI marker genes tested (Figure 1A). This apparent upregulation of PTI-responsive genes by BABA in the no-PTI activation controls may be due to wounding stress caused by infiltration of buffer. Upon treatment with Pst DC3000 hrcC, BABA-treated Arabidopsis demonstrated potentiated expression levels of WRKY53, FRK1, NLH10, and CBP60g compared with water-treated controls (Figure 1A). BABA-treated plants also had increased upregulation of PTI-responsive genes after treatment with flg22 (Figure 1A).

The accumulation of callose is a classic marker of the PTI response (Gómez-Gómez et al., 1999). We thus compared callose deposition in water- and BABA-treated Arabidopsis after inoculation with Pst DC3000 hrcC or treatment with flg22. Although Arabidopsis plants treated with BABA only demonstrated a slight increase in callose deposits, aniline blue staining and image analysis revealed potentiated accumulation of callose deposits in BABA-treated Arabidopsis upon PTI activation (Figure 1B). Combined with the observation that BABA blocks the coronatine (COR)-dependent reopening of stomata (Tsai et al., 2011), these data suggest that BABA primes the Arabidopsis PTI response.

LecRK-VI.2 Is Necessary for Full Resistance to the Virulent Bacterial Pathogen Pst DC3000

A recent microarray analysis (Zimmerli et al., 2008) and quantitative RT-PCR (qRT-PCR) analyses (see Supplemental Figure 1 online) revealed that the Arabidopsis LEGUME-LIKE LECTIN RECEPTOR KINASE-VI.2 (LecRK-VI.2; Bouwmeester and Govers, 2009; formerly LecRK44.1; Xin et al., 2009) is upregulated by the priming agent BABA. Since BABA is known to enhance Arabidopsis resistance against microbial pathogens (Prime-A-Plant Group, 2006), a reverse genetics approach was undertaken to investigate the role of LecRK-VI.2 during pathogenesis. A T-DNA insertion mutant line of LecRK-VI.2, SALK_070801, known as lecrka4.1-1 (Xin et al., 2009) and renamed lecrk-VI.2-1 (Bouwmeester and Govers, 2009), was obtained from the ABRC (http://abrc.osu.edu/). To determine whether the T-DNA insertion affected LecRK-VI.2 expression, we checked LecRK-VI.2 mRNA level in the lecrk-VI.2-1 mutant. RT-PCR analysis indicated that the region containing the T-DNA insertion was not present in transcripts of the homozygotic mutant (Figure 2A), confirming observations by Xin et al. (2009). The mutant lecrk-VI.2-1 thus does not produce a full-length LecRK-VI.2 protein. A cauliflower mosaic virus 35S promoter-LecRK-VI.2 overexpression construct was also introduced into homozygotic
were analyzed by qRT-PCR 90 or 60 min after infiltration of 10^8 Pst DC3000. OC 1 and OC 2 lines were dip inoculated with Pst DC3000 and disease severity was evaluated 3 d later. These two lines exhibited a wild-type level of resistance to Pst DC3000 (Figure 2C). Together, these results indicate that a functional LecRK-VI.2 is required for wild-type levels of Arabidopsis resistance to Pst DC3000 surface inoculation. By contrast, lecrk-VI.2-1 demonstrated a wild-type susceptibility to Pst DC3000 infiltration inoculation (see Supplemental Figures 3A and 3B online). This phenotype is reminiscent of the PTI mutant fts2 (Zipfel et al., 2004), and it indicates that LecRK-VI.2 may play a critical role during Arabidopsis early defense responses.

Bacterial-induced stomatal closure is characteristic of the plant PTI response (Melotto et al., 2006; Zeng et al., 2010). For example, Pst DC3000 bacteria induce stomatal closure in Arabidopsis within 1 h after inoculation. However, after 3 to 4 h, stomata reopen (Melotto et al., 2006). The ability of Pst DC3000 to reopen stomata is dependent on COR (Melotto et al., 2006; Zeng et al., 2010). Wild-type Columbia-0 (Col-0) plants are resistant to surface inoculation with COR-deficient mutants of Pst DC3000 (Pst DC3000 COR−), presumably because they do not reopen stomata upon infection (Melotto et al., 2006). Interestingly, fts2 demonstrates enhanced sensitivity to Pst DC3000 COR− when compared with Col-0 (Zeng and He, 2010). To further evaluate LecRK-VI.2 role during bacterial infection, lecrk-VI.2-1 plants were dip inoculated with Pst DC3000 COR− bacteria and disease development was evaluated 3 d later. The Col-0 wild type was strongly resistant to COR− mutant bacteria, but lecrk-VI.2-1 was highly sensitive to both Pst DC3000 and Pst DC3000 COR− (Figures 2D and 2E). Interestingly, the defective virulence of Pst DC3000 COR− in Col-0 wild type was fully rescued in lecrk-VI.2-1 (Figure 2E).

To further evaluate the involvement of LecRK-VI.2 in the Arabidopsis response to virulent bacteria, we monitored LecRK-VI.2 mRNA expression levels by qRT-PCR after Pst DC3000 inoculation. LecRK-VI.2 transcripts levels were upregulated by Pst DC3000 at 6 h after inoculation (see Supplemental Figure 4A online). LecRK-VI.2 expression levels were also evaluated during Arabidopsis PTI. For that purpose, wild-type Col-0 plants were inoculated with Pst DC3000 hrcC. By 6 h after inoculation, accumulation of LecRK-VI.2 transcripts was observed in Pst DC3000 hrcC-treated Arabidopsis (see Supplemental Figure 4B online). Treatment with purified bacterial MAMPs, such as flg22 and EF-Tu (elf26), or peptidoglycans (PGNs) (Gust et al., 2007) also resulted in accumulation of LecRK-VI.2 transcripts (see Supplemental Figure 4B online). Collectively, these findings suggest LecRK-VI.2 expression is responsive to bacterial inoculation and bacterial MAMP treatments.

**Upregulation of PTI-Responsive Genes and PTI-Mediated Callose Deposition Are Reduced in lecrk-VI.2-1 after Treatment with Pst DC3000 hrcC or Bacterial MAMPs**

To explore whether LecRK-VI.2 is involved in the Arabidopsis PTI response, expression levels of PTI marker genes were monitored.
Upon PTI activation, a time course study revealed that upregulation of the PTI-responsive genes WRKY53, FRK1, NHL10, and CBP60g was reduced in lecrk-VI.2-1 after inoculation with the bacteria Pst DC3000 hrcC (Figure 3A). The response of lecrk-VI.2-1 to purified MAMPs was also evaluated. The lecrk-VI.2-1 mutant demonstrated a decreased response to MAMPs, such as flg22, elf26, PGN, and lipopolysaccharides (LPSs) (Zeidler et al., 2004) (Figure 3B). Ectopic expression of LecRK-VI.2 in the lecrk-VI.2-1 mutant background (OC 1 and OC 2) restored the defective upregulation of WRKY53, FRK1, NHL10, and CBP60g after flg22 treatment (Figure 3B). These data indicate that upregulation of the PTI-responsive WRKY53, FRK1, NHL10, and CBP60g is altered in lecrk-VI.2-1 upon bacteria inoculation and bacterial MAMP treatments. Together, these results suggest that lecrk-VI.2-1 is partially defective in the PTI response.

To further analyze the PTI response in lecrk-VI.2-1, we compared callose deposition in lecrk-VI.2-1 to the wild-type control after inoculation with Pst DC3000 hrcC. Aniline blue staining and image analysis revealed a lower level of callose deposition in lecrk-VI.2-1 at 6 and 9 h after inoculation (Figure 3C). lecrk-VI.2-1 transgensics ectopically expressing LecRK-VI.2 (OC 1 and OC 2) demonstrated a wild-type level of callose deposition after Pst DC3000 hrcC inoculation (Figure 3C). Similarly to bacterial inoculation, lecrk-VI.2-1 accumulated less callose after infiltration with the MAMPs flg22 and elf26 (Figure 3C). These data indicate that LecRK-VI.2 is critical for PTI-mediated callose deposition.

A Functional LecRK-VI.2 Is Necessary for Stomatal Closure during PTI

To further document the role of LecRK-VI.2 during PTI, stomatal closure was measured after bacterial inoculation or MAMP treatments in lecrk-VI.2-1. As already documented (Melotto et al., 2006), Pst DC3000 induced closure of stomata in the wild-type Col-0 at 1 h after bacterial inoculation, and stomata in lecrk-VI.2-1 upon PTI activation. A time course study revealed that upregulation of the PTI-responsive genes WRKY53, FRK1, NHL10, and CBP60g was reduced in lecrk-VI.2-1 after inoculation with the bacteria Pst DC3000 hrcC (Figure 3A). The response of lecrk-VI.2-1 to purified MAMPs was also evaluated. The lecrk-VI.2-1 mutant demonstrated a decreased response to MAMPs, such as flg22, elf26, PGN, and lipopolysaccharides (LPSs) (Zeidler et al., 2004) (Figure 3B). Ectopic expression of LecRK-VI.2 in the lecrk-VI.2-1 mutant background (OC 1 and OC 2) restored the defective upregulation of WRKY53, FRK1, NHL10, and CBP60g after flg22 treatment (Figure 3B). These data indicate that upregulation of the PTI-responsive WRKY53, FRK1, NHL10, and CBP60g is altered in lecrk-VI.2-1 upon bacteria inoculation and bacterial MAMP treatments. Together, these results suggest that lecrk-VI.2-1 is partially defective in the PTI response.

To further analyze the PTI response in lecrk-VI.2-1, we compared callose deposition in lecrk-VI.2-1 to the wild-type control after inoculation with Pst DC3000 hrcC. Aniline blue staining and image analysis revealed a lower level of callose deposition in lecrk-VI.2-1 at 6 and 9 h after inoculation (Figure 3C). lecrk-VI.2-1 transgensics ectopically expressing LecRK-VI.2 (OC 1 and OC 2) demonstrated a wild-type level of callose deposition after Pst DC3000 hrcC inoculation (Figure 3C). Similarly to bacterial inoculation, lecrk-VI.2-1 accumulated less callose after infiltration with the MAMPs flg22 and elf26 (Figure 3C). These data indicate that LecRK-VI.2 is critical for PTI-mediated callose deposition.

A Functional LecRK-VI.2 Is Necessary for Stomatal Closure during PTI

To further document the role of LecRK-VI.2 during PTI, stomatal closure was measured after bacterial inoculation or MAMP treatments in lecrk-VI.2-1. As already documented (Melotto et al., 2006), Pst DC3000 induced closure of stomata in the wild-type Col-0 at 1 h after bacterial inoculation, and stomata

![Figure 2. A T-DNA Insertion Mutant of the LecRK-VI.2 Gene Demonstrates Enhanced Sensitivity to Pst DC3000 and Pst DC3000 COR- Dip Inoculation.](image)

(A) RT-PCR analysis of LecRK-VI.2 expression in Col-0 wild type, lecrk-

VI.2-1 (lecrk), and four transgenic lines overexpressing LecRK-VI.2 in the lecrk-VI.2-1 mutant background (OC 1 and OC 2 and OH 1 and OH 2). β-Tubulin was used as a loading control.

(B) Phenotype of Pst DC3000–infected Col-0 and lecrk-VI.2-1 (lecrk). Five-week-old Arabidopsis were dip inoculated in a bacterial suspension of 10^7 cfu/mL, and pictures were taken 3 d later. This experiment is one of three independent replicates.

(C) Growth of Pst DC3000. Col-0, lecrk-VI.2-1 (lecrk), OC 1, and OC 2 were dip inoculated in a bacterial suspension of 10^7 cfu/mL, and bacterial titers were evaluated at 2 and 3 d after inoculation (dpi). Values are the means ± SD of three biological replicates (n = 9). Asterisks indicate a significant difference to Col-0 wild-type control based on a t test (P < 0.01).

(D) Phenotype of Pst DC3000 COR–infected Col-0 and lecrk-VI.2-1 (lecrk). Five-week-old Arabidopsis was dipped in a bacterial solution of 5 × 10^6 cfu/mL, and pictures were taken 3 d later. Experiments were repeated three times with similar results.

(E) Growth of Pst DC3000 and Pst DC3000 COR– in Col-0 and lecrk-VI.2-1 (lecrk). Infection conditions as in (D). Values are the means ± SD of three biological replicates (n = 9). Different letters indicate statistically significant differences compared with wild-type Col-0 inoculated with Pst DC3000 (LSD test; P < 0.05).
reopened 2 h later (Figure 3D). Stomata did not close upon bacterial inoculation in lecrk-VI.2-1 (Figure 3D). Ectopic expression of LecRK-VI.2 in the mutant background restored closure of stomata during PTI activation (Figure 3D). Similarly, treatment with the MAMPs flg22 or elf26 did not induce stomatal closure in lecrk-VI.2-1, but the wild-type Col-0 demonstrated a rapid closure of stomata that lasted up to 3 h after MAMP treatment (Figure 3E). Complementation of the stomatal response phenotype was also observed after MAMP treatment (Figure 3E). These data indicate that a functional LecRK-VI.2 is necessary for stomatal closure upon PTI activation in Arabidopsis. Stomatal closure after MAMP treatment is dependent on ABA (Melotto et al., 2006; Zhang et al., 2008). We thus tested lecrk-VI.2-1 sensitivity to ABA-mediated stomatal closure. lecrk-VI.2-1 demonstrated wild-type stomatal closure levels in response to a moderate concentration of ABA (see Supplemental Figure 5 online). This suggests that LecRK-VI.2 acts upstream or independently of ABA signaling during stomatal closure.

MAPK Activation Is Reduced, but Other Early MAMP Responses Are at Wild-Type Levels in flg22-Treated lecrk-VI.2-1

Next, we examined lecrk-VI.2-1 mutant’s early PTI responses, including ROS production, BOTRYTIS-INDUCED KINASE1 (BIK1) phosphorylation, and MAPK activity. But as a very early
PTI response, we first analyzed BRASSINOSTEROID INSENSITIVE1-ASSOCIATED KINASE1 (BAK1)-FLAGELLIN SENSING2 (FLS2) complex formation (Chinchilla et al., 2007). Coimmunoprecipitation experiments revealed that flg22-induced interaction of BAK1 with FLS2 is at a wild-type level in the lecrk-VI.2-1 mutant (Figure 4A). Then, we analyzed ROS production in response to 0.1 μM flg22. Both lecrk-VI.2-1 and the wild type demonstrated a similar production of ROS (Figure 4B). Evaluation of flg22-mediated phosphorylation of BIK1, an integrator of signals downstream of PRRs (Lu et al., 2010; Zhang et al., 2010), revealed wild-type levels of BIK1 phosphorylation in lecrk-VI.2-1 treated for 10 min with flg22 (Figure 4C). The Arabidopsis MITOGEN-ACTIVATED PROTEIN KINASE3 (MPK3) and MPK6 are activated by flg22 treatment (Zipfel et al., 2006). We thus tested MPK3 and MPK6 activity levels in lecrk-VI.2-1 5 and 10 min after flg22 treatment. MAPK activation was reduced in lecrk-VI.2-1 mutant seedlings when compared with the Col-0 wild type (Figure 4D). Thus, a mutation in LecRK-VI.2 alters Arabidopsis MAPK signaling but does not affect early MAMP responses such as ligand-induced BAK1 and FLS2 interaction, production, and BIK1 phosphorylation.

The lecrk-VI.2-1 Mutant Is Compromised in Resistance and PTI Response to Pectobacterium carotovorum subsp carotovorum

The lecrk-VI.2-1 mutants demonstrated enhanced susceptibility to Pst DC3000. To test whether lecrk-VI.2-1 is less resistant to other types of bacteria, we dip inoculated lecrk-VI.2-1 with virulent necrotrophic bacteria P. carotovorum Pcc SCC1 and evaluated the development of necroses 1 d later. In contrast with the hemibiotroph Pst DC3000, which mainly activates the Arabidopsis SA-dependent defense responses, the necrotroph Pcc induces jasmonic acid/ethylene-dependent defense signaling in Arabidopsis (Kazan and Manners, 2008). The lecrk-VI.2-1 mutants developed more water-soaked necroses than the wild-type control and harbored increased bacterial titers upon infection with Pcc SCC1 (Figures 5A and 5B). Transgenic lines ectopically expressing the LecRK-VI.2 in the lecrk-VI.2-1 mutant background demonstrated a normal response to Pcc SCC1 (Figures 5A and 5B). Similarly to Pst DC3000, no differences in symptoms were observed when Pcc SCC1 bacteria were directly infiltrated in the leaves (see Supplemental Figure 6 online).

To test the role of LecRK-VI.2 during the PTI response induced by Pcc, expression of LecRK-VI.2 was evaluated after infection with Pcc strain WPP17 that is deficient in several HRP and HRC genes (Yap et al., 2004). Similarly to Pst DC3000 hrcC mutant bacteria (see Supplemental Figure 4B online), LecRK-VI.2 mRNA transcripts accumulated at 6 h after inoculation with Pcc WPP17 (see Supplemental Figure 7 online). In addition, expression levels of the PTI-responsive genes WRKY53, FRK1, NHL10, and CBP60g were reduced in lecrk-VI.2-1 mutant at 6 h after inoculation with Pcc WPP17 (Figure 5C). We also monitored callose deposition in lecrk-VI.2-1 after Pcc WPP17 inoculation. Aniline

![Figure 4](image-url)

**Figure 4.** Unaltered BAK1 and FLS2 Interaction, ROS Accumulation, and BIK1 Phosphorylation, but Reduced MAPK Activation, after flg22 Treatment in lecrk-VI.2-1.

(A) Wild-type flg22-mediated interaction of BAK1 and FLS2 in lecrk-VI.2-1. Wild-type and lecrk-VI.2-1 (lecrk) seedlings were treated with 1 μM flg22 for the indicated time. Proteins were extracted and immunoprecipitated with anti-BAK1 antibodies. Immunoprecipitates were analyzed by immunoblots with anti-FLS2 and anti-BAK1. Ponceau staining is used to estimate equal loading (bottom panel). Experiments were repeated twice with similar results.

(B) Production of ROS of plant leaf discs measured after treatment with flg22 as relative light units (RLUs). Responsiveness of lecrk-VI.2-1 plants (open circles) to 0.1 μM flg22 was not altered compared with Col-0 wild type (closed circles). Bak1-4 (triangles) and fls2 mutant plants (diamonds) were used as control to show no responsiveness. The data represent averages ± SE (n = 6).

(C) Similar flg22-induced BIK1 phosphorylation in Col-0 and lecrk-VI.2-1. Col-0 and lecrk-VI.2-1 protoplasts (lecrk) were concentrated by low-speed centrifugation 6 h after transfection and treated with 1 μM flg22 for 10 min before adding protein sample loading buffer. Experiments were repeated three times with similar results.

(D) Mutant lecrk-VI.2-1 plants showed a reduced flg22-induced MAPK activation. Leaves of 21-d-old Col-0 and lecrk-VI.2-1 (lecrk) were infiltrated with 1 μM flg22 for 5 and 10 min. Immunoblot analysis using phospho-p44/42 MAPK antibody is shown in the top panel. Arrowheads indicate the positions of MPK6 and MPK3. FastBlue staining is used to estimate equal loading in each lane (bottom panel). This experiment is one of three independent replicates.
blue staining and image analysis revealed a lower level of callose deposition in lecrk-VI.2-1 at 9 h after inoculation (Figure 5D). To test whether Pcc induces stomatal closure similarly to Pst DC3000 (Melotto et al., 2006), we analyzed stomatal aperture sizes at 1 h after inoculation with Pcc SCC1. The necrotroph Pcc SCC1 induced stomatal closure in a similar manner as Pst DC3000 (Figure 5E). Like after Pst DC3000 inoculation (Figure 3D), lecrk-VI.2-1 did not demonstrate Pcc SCC1-mediated closure of stomata (Figure 5E). Together, these data indicate that LecRK-VI.2 is critical for Arabidopsis resistance to and PTI activation by the necrotrophic bacteria Pcc.

**High LecRK-VI.2 Expression Levels Induce a Constitutive PTI Response and Resistance to Bacterial Pathogens**

To further characterize the role of LecRK-VI.2 in Arabidopsis resistance to bacteria, we tested the resistance to Pst DC3000 and Pcc SCC1 of the transgenic lines OH 1 and OH 2 that demonstrate high levels of LecRK-VI.2 expression (Figure 2A). OH 1 and OH 2 lines were stunted and highly resistant to Pst DC3000 with OH 1 demonstrating up to 1000 time less bacteria in planta than the Col-0 wild-type control (Figures 6A and 6B). OH 1 and OH 2 transgenics were also more resistant than the wild type to Pcc SCC1 (Figure 6C). In addition, these two overexpression lines demonstrated a constitutive accumulation of PTI-responsive transcripts WRKY53, FRK1, NHL10, and CBP60g (Figure 6D) and constitutive callose deposition (Figure 6E). Treatment with Pst DC3000 hrcC bacteria further increased the level of callose deposition in these two overexpression lines (Figure 6E). Moreover, constitutive closure of stomata was observed in these two lines (Figure 6F). These data indicate that transgenics with high levels of LecRK-VI.2 expression constitutively activate PTI, suggesting that LecRK-VI.2 is a positive regulator of the Arabidopsis PTI response.
Transcriptome Profiling of Arabidopsis Overexpressing LecRK-VI.2 Reveals Significant Overlap with Pathogen-Responsive Genes

To obtain further insight into the molecular mechanism by which LecRK-VI.2 mediates bacterial resistance, we performed genome-wide transcriptome analysis of LecRK-VI.2 overexpressing plants. Statistical analysis showed that the expression of 445 genes was significantly affected (greater than or equal to twofold, \( P < 0.01 \), Benjamini-Hochberg false discovery rate correction) in overexpression plants (OH 1) when compared with wild-type control. Out of these 445 genes, 259 were upregulated (see Supplemental Data Set 1 online) and 186 downregulated in OH 1 (see Supplemental Data Set 2 online). Functional categorization of the upregulated genes by analyzing overrepresented Gene Ontology (GO) Terms of the “biological process” classification using the GO Term Enrichment tool from the GO project (http://amigo.geneontology.org/cgi-bin/amigo/term_enrichment; \( P < 0.01 \)) revealed 30 overrepresented GO biological process terms (see Supplemental Data Set 3 online). Enrichment of GO terms in the overexpression line OH 1 versus the wild type demonstrated a relationship with the GO terms “immune system process” or “response to stimulus” (Figure 7A). Notably, genes such as PENETRATION3, RECOGNITION OF PSEUDomonas PARASITICA5 (RPP5), ENHANCED DISEASE SUSCEPTIBILITY TO ERYsiphe ORONTII16 (EDS16), ACCELERATED CELL DEATH6, CBP60g, RPP4, PHYTOALEXIN DEFICIENT4, BETA-1,3-GLUCANASE2 (BGL2), RESISTANT TO P. SYRINGAE2 (RPS2), ENHANCED DISEASE SUSCEPTIBILITY1, and PATHOGENESIS-RELATED

Figure 6. OH 1 and OH 2 Transgenics with High Ectopic LecRK-VI.2 Expression Levels Demonstrate Strong Resistance to Bacteria and Constitutive PTI.

(A) Pst DC3000 symptoms. Plants were dip inoculated in a bacterial solution of \( 10^7 \) cfu/mL, and symptoms were observed 3 d later. White arrows indicate typical disease symptoms. Experiments were repeated three times with similar results.

(B) Pst DC3000 growth. Titers were determined 3 d after dip inoculation with \( 10^7 \) cfu/mL bacteria. Values are the means ± SD of three biological replicates (n = 9). Asterisks indicate a significant difference to Col-0 wild-type control based on a t test (\( P < 0.01 \)).

(C) Pcc SCC1 symptoms. Symptoms were observed 2 d after dip inoculation with \( 2 \times 10^5 \) cfu/mL Pcc SCC1. White arrows indicate Pcc SCC1-mediated water soaked necroses. Bars = 1 cm.

(D) Constitutive PTI-responsive transcript accumulation. Gene expression levels relative to Col-0 (defined value of 1) were analyzed by qRT-PCR. EF-1 and UBQ10 were used for normalization. The values are the means ± SD of three biological replicates (n = 9).

(E) Constitutive callose deposition. Leaves were syringe infiltrated with buffer or Pst DC3000 hrcC (1 \( \times \) 10^6 cfu/mL), and samples were collected 6 h later. Numbers in the pictures are the average and SD of the number of callose deposits per square mm from three biological replicates (n = 27). Bar = 200 μm.

(F) Constitutive stomatal closure. Stomatal apertures in epidermal peels exposed to buffer or bacteria (1 \( \times \) 10^6 cfu/mL) were analyzed at 1 and 3 h after inoculation. Results are shown as mean (n > 60 stomata) ± SE. Asterisks indicate a significant difference to buffer control based on a t test analysis (\( P < 0.001 \)). Experiments were repeated three times with similar results.

[See online article for color version of this figure.]
GENE5 (PR5), which are associated with the GO term “innate immune response,” were upregulated in OH 1 (Kunkel and Brooks, 2002; Wang et al., 2009) (see Supplemental Data Set 3 online). In addition, analyses of 147 upregulated transcripts from Supplemen
tal Data Set 1 online (greater than or equal to fourfold) by comparison with published microarray data sets of genes induced by treatment with the MAMP flg22, the SA functional analog benzo(1)thiadiazole (BTH), and during infection with virulent (Pst DC3000) or avirulent (Pst DC3000 avrRpm1) bacteria revealed numerous coregulated genes (Figure 7B; see Supplemental Data Set 4 online).

The GO terms “protein autophosphorylation,” “protein phosphorylation,” “phosphorylation,” “phosphate metabolic process,” and “phosphorus metabolic process” were overrepresented in the GO “biological process” category (see Supplemental Data Set 3 online). Similarly, GO terms such as “protein kinase activity” and “kinase activity” with relationship with the GO term “catalytic activity” were overrepresented in the GO “molecular function” category (Figure 7C; see Supplemental Data Set 3 online). These data suggest that many genes with kinase activity are upregulated in OH 1. For example, 12 Cys-rich RLKs were significantly upregulated in OH 1. Several Cys-rich RLKs are pathogens responsive and participate in the regulation of defense reactions (Acharya et al., 2007; Wrzaczek et al., 2010).

Analysis of the downregulated genes from Supplemental Data Set 2 online revealed overrepresentation of the GO terms “Plant-type cell wall loosening” and “Plant-type cell wall modification” in the “biological process” classification (see Supplemental Data Set 5 online). Genes from the α-expansin family EXPA11, EXPA10, EXPA1, EXPA5, and EXPA3 were associated with these two GO terms (see Supplemental Data Set 5 online). Expansins are catalysts of cell wall enlargement (Cosgrove, 2000), and repression of their expression may explain the OH 1 stunted phenotype.

**BABA Priming and BABA-Induced Resistance Are Altered in lecRK-VI.2-1**

In this study, BABA was shown to prime the PTI response (Figure 1), and the BABA-responsive LecRK-VI.2 was demonstrated to act as a positive regulator of Arabidopsis PTI. We then asked whether LecRK-VI.2 is critical for BABA priming of PTI. BABA potentiation of WRKY33, FRK1, NHL10, and CBP60g expression levels was reduced in lecRK-VI.2-1 after Pst DC3000 hrcC and flg22 exposure (Figure 8A). In addition, priming of PTI-mediated callose deposition was also strongly diminished in lecRK-VI.2-1 (Figure 8B). Together, these data indicate that LecRK-VI.2 is required for BABA priming of the Arabidopsis PTI response. Chemical priming by BABA is associated with enhanced resistance to pathogens (Zimmerli et al., 2000; Prime-A-Plant Group, 2006). We thus evaluated BABA-induced resistance to the virulent Pst DC3000 in lecRK-VI.2-1. Confirming earlier studies (Zimmerli et al., 2000; Ton et al., 2005), BABA-treated Arabidopsis harbored ~30 times less bacteria than the water-treated controls (Figure 8C). By contrast, BABA protective effect was ~10 times less efficient in lecRK-VI.2-1 (Figure 8C). This observation suggests that a functional LecRK-VI.2 is necessary for complete BABA-induced resistance to Pst DC3000.

**DISCUSSION**

The plant innate immune system detects and arrests invasion of potentially dangerous microbes (Boller and He, 2009). However, successful, virulent pathogens can suppress the plant innate immunity (Metraux et al., 2009). Here, we characterize the role of an Arabidopsis L-type lectin receptor kinase, LecRK-VI.2, in priming and innate immunity by T-DNA insertion mutant and gain-of-function analyses. Our data support the following conclusions.
were syringe infiltrated with hrcC DC3000 Arabidopsis BABA priming of PTI-responsive genes is defective in (A) Resistance. LecRK-VI.2 Is Critical for BABA Priming and BABA-Induced Figure 8.

10 of 15 The Plant Cell BABA-treated Arabidopsis expression ratios of BABA- to water-treated (defined value of 1). Data before RNA extraction for qRT-PCR analyses. Values are relative gene based on a Asterisks indicate a significant difference to Col-0 wild-type control (B) each with three technical replicates (n = 9). Asterisks indicate a significant difference to wild-type Col-0 based on a t test (P < 0.01).

(B) BABA priming of callose deposition is altered in lecrk-VI.2-1. Leaves were syringe infiltrated with Pst DC3000 hrcC (1 × 10^6 cfu/mL) or flg22 (200 nM) and samples were collected 9 h later for evaluation of callose deposits. Values are ratio of callose deposits per square mm of BABA- to water-treated Arabidopsis ± SD from three independent biological replicates (n = 9). Asterisks indicate a significant difference to Col-0 wild-type control based on a t test (P < 0.01).

(C) BABA-induced resistance is defective in lecrk-VI.2-1. Bacterial titers were analyzed on wild-type Col-0 and lecrk-VI.2-1 two days post dip-inoculation with Pst DC3000 (1 × 10^6 cfu/mL). Values are ratio of water- to BABA-treated Arabidopsis ± SD of 3 biological replicates (n = 9). Asterisks indicate a significant difference to Col-0 wild-type control based on a t test (P < 0.01).

LecRK-VI.2 Positively Regulates the Arabidopsis PTI Response

The mutant lecrk-VI.2-1 was more susceptible to both hemibiotrophic Pst DC3000 and necrotrophic Pcc bacteria that are known to activate different Arabidopsis downstream defense signaling cascades (Kazan and Manners, 2008). In addition, enhanced susceptibility was observed after surface inoculation, but not after infiltration inoculation, a phenotype that is reminiscent of the PTI-defective flagellin receptor mutant fis2 (Zipfel et al., 2004; Zeng and He, 2010). These two observations suggest that LecRK-VI.2 affects Arabidopsis defense responses at an early stage, possibly the PTI. Increased susceptibility in lecrk-VI.2-1 was correlated with a defective bacterial- and MAMP-induced stomatal closure, MPK3 and MPK6 phosphorylation, PTI-responsive gene expression, and callose deposition. Transgenic complementation lines overexpressing LecRK-VI.2 restored the aberrant PTI response of the mutant lecrk-VI.2-1, indicating that the observed alterations in PTI responses are genuine effects of LecRK-VI.2 mutation. In addition, transgenics overexpressing LecRK-VI.2 at high levels demonstrated an active PTI response characterized by constitutive PTI marker gene upregulation, constitutive callose deposition, and closed stomata. Transcriptome analysis of a LecRK-VI.2 overexpression line revealed upregulation of numerous genes responsive to virulent or avirulent bacteria, the MAMP flg22, or to the SA functional analog BTH. PTI and effector-triggered immunity transcriptomes are known to significantly overlap (Schwessinger and Zipfel, 2008), and PTI-mediated SA-responsive gene induction is well acknowledged (Tsuda et al., 2008). Therefore, data from analysis of a T-DNA insertion mutant, gain-of-function approaches, and transcriptome analysis converge to indicate that LecRK-VI.2 positively regulates the Arabidopsis PTI response. Transgenic lines with high LecRK-VI.2 expression levels were stunted. It is known that plants with constitutive active defense responses are smaller than normal. For example, cup1 (constitutive expressor of PR genes) is smaller than the wild type and highly resistant to pathogens (Bowling et al., 1994). Since LecRK-VI.2 overexpression and PTI activation levels correlate, our data suggest that LecRK-VI.2 modulates Arabidopsis PTI in a dose-dependent manner.

LecRK-VI.2 is a plasma membrane–localized protein (Xin et al., 2009), and its structural features (an extracellular lectin motif–containing domain, a transmembrane domain, and an intracellular kinase domain) suggest that this receptor kinase functions as a PRR. The mutant lecrk-VI.2-1 was not totally unresponsive and still showed a reduced response to distinct bacterial MAMPs. LecRK-VI.2 may therefore not specifically recognize one particular MAMP. Indeed, there are examples where mutations in a receptor for a specific MAMP lead to a total absence of response to one particular MAMP and a normal response to other MAMPs. This was demonstrated in fis2 and efr mutants after treatments with flagellin and EF-Tu, respectively (Zipfel et al., 2004, 2006). These observations suggest that LecRK-VI.2 does not function as the sole PRR for a MAMP.

FLS2 and BAK1 complex formation upon flg22 treatment occurs rapidly upon PTI activation (Chinchilla et al., 2007). The mutant bik1 is compromised in defense responses induced by
fig22, and BIK1 plays a critical role in the integration of signals downstream of PRRs (Lu et al., 2010). Both ligand-mediated BAK1-FLS2 complex formation and BIK1 phosphorylation were at wild-type levels in lecrk-VI.2-1. In addition, ROS production that is also considered as an early PTI response (Zipfel and Robatzek, 2010) was not compromised in lecrk-VI.2-1. Collectively, these data suggest that LecRK-VI.2 works downstream of FLS2. However, MPK3 and MPK6 activation by fig22 was reduced in lecrk-VI.2-1. LecRK-VI.2 may thus perceive a yet unknown signaling component to induce a positive feedback mechanism on the PTI signaling cascade upstream of MAPK signaling and downstream of MAMP perception. For example, such a positive feedback mechanism could be triggered by plant cell endogenous factors, such as damage-associated molecular patterns produced in response to pathogen attack, as it has been proposed for the Arabidopsis peptide Pep1 (Yamaguchi et al., 2006; Ryan et al., 2007).

**LecRK-VI.2 Plays a Critical Positive Role during Stomatal Immunity**

The mutant lecrk-VI.2-1 displayed a complete loss of stomatal closure upon bacterial inoculation or MAMP treatments. In addition, 
Pst DC3000 COR-deficient mutant multiplied at high wild-type levels in lecrk-VI.2-1 (Figure 2E). These observations are partially similar to the mutant fls2 (Zeng and He, 2010). However, stomatal closure upon elf26 treatment is also defective in lecrk-VI.2-1, suggesting a positive role of LecRK-VI.2 during stomatal immunity activation at a signaling node downstream of MAMP perceptions. High overexpression levels of LecRK-VI.2 induced a constitutive closure of stomata, further suggesting a positive role of LecRK-VI.2 in stomatal immunity. Overexpression of the Arabidopsis \( \alpha \)-expansin gene EXPA1 accelerates stomatal opening (Zhang et al., 2011). Repression of the expression of genes from \( \alpha \)-expansin family EXPA11, EXPA10, EXPA1, EXPA5, and EXPA3 in OH 1 may explain closed stomata in this transgenic line. The normal stomatal response of lecrk-VI.2-1 to ABA treatment indicates that LecRK-VI.2 acts upstream or independently of ABA-mediated regulation of stomatal closure. Loss of stomatal closure upon bacterial attack per se may largely explain lecrk-VI.2-1-enhanced susceptibility to surface inoculation with bacteria and to the COR- 
Pst DC3000 mutant. Little is known about how PTI activation arrests pathogen ingress (Zipfel and Robatzek, 2010). Efficient stomatal closure upon bacterial attack may represent one possible mechanism that can be used by plants to stop bacterial invasion and concomitant proliferation.

**LecRK-VI.2 Is Key for Priming of the Arabidopsis PTI Response**

Since BABA upregulated LecRK-VI.2 (see Supplemental Figure 1 online; Zimmerli et al., 2008) and the mutant lecrk-VI.2-1 demonstrated a defective response to BABA, we propose that LecRK-VI.2 is a key mediator of BABA priming and BABA-induced resistance. Accumulation of positive regulators of defense, such as MPK3 and MPK6, is critical for priming (Beckers et al., 2009). Our data suggest that LecRK-VI.2 positively modulates the PTI signaling upstream of MPK3 and MPK6, thus confirming the implication of MAPK signaling in priming and further demonstrating that accumulation of defense signaling components prior stress challenge is essential for priming (Beckers et al., 2009). BABA priming and BABA-induced resistance were not completely abolished in lecrk-VI.2-1. This could be explained by redundancy as BABA probably signals through more than one priming modulator. BABA indeed upregulates numerous defense-related genes (Zimmerli et al., 2008).

Lectin receptors, such as dectin-1, play important roles in vertebrate innate immunity (Saijo et al., 2010). Our data suggest that plant lectin receptor kinases, like animal lectin receptor kinases, are key modulators of the first layer of defense against microbial pathogens. In addition, we show that LecRK-VI.2 is a critical signaling component for priming in Arabidopsis.

**METHODS**

**Biological Materials and Growth Conditions**

*Arabidopsis thaliana* Col-0 plants were grown in commercial potting soil/perlite (3:2) at 22 to 24°C day and 17 to 19°C night temperature under a 9-h-light/15-h-dark photoperiod. The lighting was supplied at an intensity of \( \sim 100 \text{ \mu E\,m}^{-2}\text{s}^{-1} \) by fluorescence tubes. T-DNA insertion mutants of LecRK-VI.2 (known as lecrkA4.2-1) and LecRK-VI.4 (known as lecrkA4.3-1) are a gift from Z.L. Zheng (City University of New York) (Xin et al., 2009). BIK1 construct (pHBT-BIK1-HA) under the control of a constitutive cauliflower mosaic virus 3SS with an HA epitope tag was obtained from P. He (Lu et al., 2010). Bacterial strains 
Pst DC 3000, 
Pst DC 3000 COR- (DB29), and the 
Pst DC 3000 hrcC Mutant (CB200) were provided by B.N. Kunkel (Washington University, St. Louis, MO). 
Pst bacteria were cultivated at 28°C and 340 rpm in King’s B medium containing rifampicin ( 
Pst DC3000), rifampicin, spectinomycin, and kanamycin (DB29) or rifampicin and kanamycin (CB200). We obtained Pcc hp- and hrc-deficient strain WPP17 (Yap et al., 2004) from A.O. Charkowski (University of Wisconsin, Madison, WI), and Pcc SCC1 (Kariola et al., 2003) expressing the green fluorescent protein (Kwon et al., 2009) were a gift from O.K. Park (Korea University, Seoul, Korea). 
Pcc bacteria were cultivated at 28°C and 340 rpm in Luria-Bertani medium (Bioman Scientific) for 16 to 18 h without selection (Pcc WPP17) or with ampicillin (Pcc SCC1).

**Insertion Mutant Isolation**

Seeds of the lecrk-VI.2-1 (SALK_070801, genetic background Col-0) mutant were ordered at the ABRC (http://abrc.osu.edu/). Two PCRs were performed to determine the homozygous plants as suggested at the SIGnAL website (http://signal.salk.edu/tdnaprimers.2.html).

**Complementation**

To complement lecrk-VI.2-1, we used the Gateway entry clone G12088 containing the full-length cDNA LecRK-VI.2 ordered at Riken (http://www. brc.riken.jp/lab/epd/Eng/). After confirmation by sequencing, it was recombined into the Gateway-compatible destination vector pEarlyGate 100 (ordered at ABRC) containing the 3SS promoter through the LR reaction (Invitrogen). The final construct was electroporated into *Agrobacterium tumefaciens* competent cells strain GV3101. Agrobacterium containing Pro<sub>3SS</sub>LecRK-VI.2 was then used to transform the homozygotic lecrk-VI.2-1 via floral dipping (Clough and Bent, 1998). Multiple transgenic lines were obtained and raised to homozygotic T3 lines.
Disease Assays

Disease assays with Pst DC3000 strains were conducted as previously described (Zimmerli et al., 2000). Briefly, five-week-old Arabidopsis were doped in a bacterial suspension of 10^7 colony-forming units (cfu)/mL Pst DC3000, 10^6 cfu/mL Pst DC3000 hrcC, or, as indicated, in 10 mM MgSO_4 containing 0.01% Silwet L-77 (Lehle Seeds) for 15 min. For inoculation by infiltration, leaves were syringe infiltrated with the indicated bacterial concentrations. For Pcc inoculations, cells were collected by centrifugation and resuspended in 10 mM MgSO_4 at A_{600} = 0.25, corresponding to a concentration of 10^8 cfu/mL. Bacteria were then diluted to 2 × 10^7 for (Pcc SCC1) or 10^6 cfu/mL for Pcc WPP17 in 10 mM MgSO_4 containing 0.01% Silwet L-77 (Lehle Seeds). Five-week-old Arabidopsis were dip inoculated or syringe infiltrated with Pcc SCC1 or Pcc WPP17, respectively. After inoculation, plants were kept at 100% relative humidity, and symptoms were evaluated 1 or 2 d later. Bacterial titers were determined as previously described for Pst DC3000 (Zimmerli et al., 2000).

Quantitative RT-PCR

For RNA extraction, whole Arabidopsis rosettes were flash frozen in liquid nitrogen and subsequently ground to powder. Total RNA extraction and purification and PCRs were performed as described (Wu et al., 2010). Normalization of gene expression across different samples was performed with EF-1α and UBQ10 as internal controls. The reactions were conducted on an iCycler sequence detection system (Bio-Rad) and SYBR Green fast qPCR master mix (Kapa Biosystems). Primers used are in Supplemental Table 1 online. The melting curve was used to verify single amplicons. The fold changes relative to the gene expression in the control sample were determined using the Bio-Rad qIQ optical system software.

RT-PCR

Total RNA extraction and cDNA biosynthesis were as described in the section above. One microliter of cDNA was used as template, and standard PCR conditions were applied using Ampliqon III Taq polymerase. The thermal cycling program was 94°C for 2 min, followed by 31 cycles at 94°C for 30 s, 60°C for 30 s, and 72°C for 1 min and 30 s followed by one cycle at 72°C for 15 min.

Chemical Treatments

Two days before bacterial inoculation or MAMP treatments, plants were soil drenched with BABA (Fluka) at a final concentration of 200 μM or with water (control). The peptides representing the MAMPs flagellin (flg22) and EF-Tu (elf26) were synthesized by Biomer Technology, flg22 or elf26 (200 mM), 50 ng/mL of Staphylococcus aureus–soluble PGN fragments (Fluka), or 1 μg/mL LPS (Sigma-Aldrich) was dissolved in 10 mM MgSO_4 and syringe infiltrated into leaves of 5-week-old Arabidopsis. Buffer MgSO_4 only was used as a control. MAMP-treated leaves were flash frozen in liquid nitrogen 30 or 60 min later, and the tissue was used for further gene expression analyses. Three leaves per plant from four different plants (12 leaves in total) were used for each analysis.

Assays for generation of ROS were performed as previously described (Kunze et al. 2004). Leaf pieces of 4-week-old Arabidopsis plants were cut in 2-mm² pieces and floated overnight in water. One piece per well was transferred into a 96-well plate (LIA White; Greiner Bio-One) containing 1 mg of horseradish peroxidase (Sigma-Aldrich) and 100 nM luminol (Sigma-Aldrich). Leaves were treated with 0.1 μM flg22, and luminescence was measured in a plate reader (MicroLumat LB96P; Berthold Technologies) for 30 min after addition of flg22.

Callose Staining

Leaves of 5-week-old Arabidopsis were syringe infiltrated with either 10^8 cfu/mL Pst DC3000 hrcC, 5 × 10^6 Pcc WPP17, 1 μM flg22, or 1 μM elf26 in 10 mM MgSO_4. Control plants were infiltrated with 10 mM MgSO_4 only. After infiltration, nine leaf discs from three different plants were selected for analyses. Harvested leaf samples were cleared overnight by incubation in 95% ethanol at room temperature and then washed three times (2 h for each washing) with sterilized water. Cleared leaves were stained with 0.01% aniline blue in 0.15 M phosphate buffer, pH 9.5, for 24 h. Callose deposits were visualized under UV illumination using a Nikon Optiphot-2 microscope. Callose deposits were counted using the “analyze particles” function of ImageJ (http://rsb.info.nih.gov/ij/).

Stomatal Assay

Plants were kept under light (100 μmol m⁻² s⁻¹) for at least 3 h to open stomata before the beginning of the experiments. Epidermis of three fully expanded leaves from three different plants was peeled off and placed on glass slides with the cuticle side in contact with either MES buffer (25 mM MES-KOH, pH 6.15, and 10 mM KCl) or MAMPs (5 μM flg22 or 5 μM elf26) in MES buffer or with 10 mM MgSO_4 or bacterial suspensions (1 × 10^8 cfu/mL Pst DC3000 or 5 × 10^8 cfu/mL Pcc SCC1) in 10 mM MgSO_4. At various time points, pictures were taken of random regions using an Olympus BX51 microscope digital camera and application software DP2-BSW. The width of the stomatal aperture was measured using the “measure” function of ImageJ (http://rsb.info.nih.gov/ij/).

Co-immunoprecipitation Experiments

Wild-type and lec1-6-2–1 mutant seedlings were grown in liquid Murashige and Skoog (Duchefa) with 1% Suc for 3 weeks under continuous light and treated with 1 μM flg22 for the indicated time. Proteins were extracted and immunoprecipitated with anti-BAK1 antibodies (Schulze et al., 2010). Immunoprecipitates were analyzed by immunoblot with anti-PDS2 (Chinchilla et al., 2007) and anti-BAK1.

Protoplast Transplant and BIK1 Phosphorylation Assays

Protoplast transient assays were performed as described (Shan et al., 2008). For BIK1 phosphorylation assays, 0.1 mL protoplasts at a density of 2 × 106/mL were transfected with 20 μg of plasmid DNA containing BIK1 construct under the control of a constitutive cauliflower mosaic virus 35S with an HA epitope tag (pHB-T-BIK1-HA). The protoplasts were concentrated by a low-speed centrifuge 6 h after transfection and treated with 1 μM flg22 for 10 min. BIK1 mobility shift (BIK1 phosphorylation) was analyzed by immunoblot with an anti-HA antibody (Lu et al., 2010).

Microarray Experiments and Data Analysis

Total RNA was isolated using TRIzol reagent from three independent biological replicates of wild-type (Col-0) and overexpression line (OH 1) plants (six samples). Each sample represents six plants grown for 5 weeks without any treatment. Microarray experiments were performed at the Affymetrix Gene Expression Service Lab at Academia Sinica (Taipei, Taiwan). Ten micrograms of total RNA were used for cDNA synthesis, labeled by in vitro transcription followed by fragmentation according to the manufacturer’s suggestions (GeneChip Expression Analysis Technical Manual rev5; Affymetrix). Eleven microarrays of labeled samples were hybridized to the GeneChip Arabidopsis ATH1 at 45°C for 16.5 h. Washing and staining were performed using the Fluidic Station-450. GeneChip was scanned with Affymetrix GeneChip Scanner 7G.
entities with signal probe intensity levels lower than 100 and 9954 out of 22,810 entities passed this first filtering. Unpaired t tests were then performed to compare wild-type and OH1 samples (P value < 0.01), and the Benjamini Hochberg method was applied to control the false discovery rate. This way, 619 entities out of 9954 showed significant changes between wild-type and OH1 samples. Finally, the data were further filtered by fold changes. Four hundred-fifty out of 619 statistically significant values demonstrated a fold change ≥2. Annotations of Arabidopsis genes based on the probe set identifiers were obtained from BAR (http://bbc.botany.utoronto.ca/). Genes of Figure 7B were clustered using Cluster (genes were correlated through absolute correlation centered and complete linkage) and TreeView software (Eisen et al., 1998).

MAPK Assay

MAPK activities were determined in crude protein extracts from leaves of 3-week-old plants infiltrated with 1 μM flg22 for 15 min and prepared as described (Romeis et al., 1999). The protein concentration was determined using the BCA protein assay kit (Pierce) with BSA as a standard. Crude extracts (10 μg total protein per lane) were separated on an 11.25% SDS gel, and proteins were transferred onto nitrocellulose (Amersham) by wet electroblotting (Mini-Protean II system; Bio-Rad). Activated MAPKs were detected by overnight incubation with anti-p42/44 MAPK primary antibodies (1:1000; Cell Signaling Technology), followed by incubation with anti-rabbit-HRP secondary antibodies (Sigma-Aldrich) for 1 h. The signals were visualized using an enhanced chemiluminescence system (Western Lightning Plus-ECL kit; Perkin-Elmer) following the manufacturer’s instructions.

Accession Numbers

Sequence data from this article can be found in the Arabidopsis Genome Initiative under the following accession numbers: LecRK-VI.2 (At1g07920), FRK1 (At2g19190), LecRK-VI.3 (At5g01550), LecRK-VI.4 (At5g01560), NHL10 (At1g35980), FRK1 (At2g19190), WRKY53 (At2g23810), CBP60g (At5g26920), EF-1 (At1g07920), UBO10 (At4G03520), and TUB2 (At5g62690). Microarray data are deposited at the Gene Expression Omnibus under accession number GSE26646 (http://www.ncbi.nlm.nih.gov/geo/).

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure 1. BABA Upregulates LecRK-VI.2.

Supplemental Figure 2. Pst DC3000-Induced Symptoms in lecrk-VI Mutants of LecRK-VI Clade.

Supplemental Figure 3. Lecrk-VI.2-1 Is Normally Resistant to Infiltration Inoculation with Pst DC3000.

Supplemental Figure 4. Lecrk-VI.2 Is Upregulated by Virulent Bacteria and during the PTI Response.

Supplemental Figure 5. Normal Response of lecrk-VI.2-1 to ABA-Mediated Stomatal Closure.

Supplemental Figure 6. The Mutant lecrk-VI.2-1 Is Normally Sensitive to Pcc SCC1 after Syringe Infiltration.

Supplemental Figure 7. Lecrk-VI.2 Expression Is Upregulated by Pcc WPP17.

Supplemental Table 1. Sequences of the Primers Used in This Study.

Supplemental Data Set 1. Upregulated Transcripts (Fold Change ≥2; P < 0.01, t Test, n = 3 per Group) in Leaves from OH 1 Plants versus Leaves from Col-0 Control Plants.

Supplemental Data Set 2. Downregulated Transcripts (Fold Change ≥2; P < 0.01, t Test, n = 3 per Group) in Leaves from OH 1 Plants versus Leaves from Col-0 Control Plants.

Supplemental Data Set 3. Overrepresented Gene Ontology Terms of the “Biological Process” and “Molecular Function” Classifications Using the “GO Term Enrichment” Tool from the GO Project.

Supplemental Data Set 4. Comparison of (OH 1/Col-0) Upregulated Genes from Supplemental Data Set 1 Online (Greater Than or Equal to Fourfold) with Published Microarray Data Sets of Genes Activated by the MAMP flg22, the Chemical Benzothiadiazole, Virulent (Pst DC3000), or Avirulent (Pst DC3000 avrRpm1) Bacteria.

Supplemental Data Set 5. Overrepresented Gene Ontology Terms of the “Biological Process” and “Cellular Component” Classifications Using the “GO Term Enrichment” Tool from the GO Project.

ACKNOWLEDGMENTS

We thank Zhi-Liang Zheng and the ABRC for providing seeds and Barbara Kunkel, Amy Charikowski, and Okhmea Park for the pathogens. We also thank Jeng-Feng Chang and Shashi Kant Singh for their assistance in MAPK and BIK1 phosphorylation assay. We thank Ping He for providing the BIK1 construct. The Affymetrix Gene Expression Service Lab (http://ipmb.sinica.edu.tw/affy/), supported by Academia Sinica, is acknowledged for performing the Affymetrix GeneChip assay. We thank the staff of Technology Commons, College of Life Science, National Taiwan University for their help with microscopy and microarray data analysis and for qRT-PCR equipment. We also thank Johann Weber and Wen-Dar Lin for help with microarray analysis and Thorsten Hamann and members of Zimmerli’s laboratory for critical comments. This work was supported by the National Science Council of Taiwan Grants 96-2628-B-002-112-MY3 and 99-2628-B-002-053-MY3 (to L.Z.), the Frontier and Innovative Research Grant of the National Taiwan University (code number 99R70436 to L.Z.), Deutsche Akademie der Naturforscher Leopoldina Grant BMBF-LPD 9901-152 (to B.S.), and Swiss National Science Foundation Grants 3100A3-120655 (to D.C.) and 3100A3-105852 (to T.B.).

AUTHOR CONTRIBUTIONS


Received January 12, 2012; revised February 9, 2012; accepted March 2, 2012; published March 16, 2012.

REFERENCES


Beckers, G.J., Jaskiewicz, M., Liu, Y., Underwood, W.R., He, S.Y.,


The Lectin Receptor Kinase-VL2 Is Required for Priming and Positively Regulates Arabidopsis Pattern-Triggered Immunity

Prashant Singh, Yi-Chun Kuo, Swati Mishra, Chia-Hong Tsai, Chih-Cheng Chien, Ching-Wei Chen, Marie Desclos-Theveniau, Po-Wei Chu, Birgit Schulze, Delphine Chinchilla, Thomas Boller and Laurent Zimmerli

Plant Cell; originally published online March 16, 2012;
DOI 10.1105/tpc.112.095778

This information is current as of July 1, 2017