The Arabidopsis Lectin Receptor Kinase LecRK-V.5 Represses Stomatal Immunity Induced by Pseudomonas syringae pv. tomato DC3000

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Abstract

Stomata play an important role in plant innate immunity by limiting pathogen entry into leaves but molecular mechanisms regulating stomatal closure upon pathogen perception are not well understood. Here we show that the Arabidopsis thaliana L-type lectin receptor kinase-V.5 (LecRK-V.5) negatively regulates stomatal immunity. Loss of LecRK-V.5 function increased resistance to surface inoculation with virulent bacteria Pseudomonas syringae pv tomato DC3000. Levels of resistance were not affected after infiltration-inoculation, suggesting that LecRK-V.5 functions at an early defense stage. By contrast, lines overexpressing LecRK-V.5 were more susceptible to Pst DC3000. Enhanced resistance in lecrk-V.5 mutants was correlated with constitutive stomatal closure, while increased susceptibility phenotypes in overexpression lines were associated with early stomatal reopening. Lines overexpressing LecRK-V.5 also demonstrated a defective stomatal closure after pathogen-associated molecular pattern (PAMP) treatments. LecRK-V.5 is rapidly expressed in stomatal guard cells after bacterial inoculation or treatment with the bacterial PAMP flagellin. In addition, lecrk-V.5 mutants guard cells exhibited constitutive accumulation of reactive oxygen species (ROS) and inhibition of ROS production opened stomata of lecrk-V.5. LecRK-V.5 is also shown to interfere with abscisic acid-mediated stomatal closure signaling upstream of ROS production. These results provide genetic evidences that LecRK-V.5 negatively regulates stomatal immunity upstream of ROS biosynthesis. Our data reveal that plants have evolved mechanisms to reverse bacteria-mediated stomatal closure to prevent long-term effect on CO2 uptake and photosynthesis.

Introduction

Plants are continuously exposed to a variety of microorganisms and have elaborated defense mechanisms to successfully avoid infection by limiting pathogen invasion and multiplication. The earliest event in plant defense response is recognition of microbial molecular signatures called pathogen- or microbe-associated molecular patterns (PAMPs or MAMPs) by pattern recognition receptors (PRRs) located at the plasma membrane [1]. One of the best characterized PRR is the Arabidopsis thaliana receptor kinase Flagellin Insensitive 2 (FLS2) that recognizes and interacts with the peptide flg22, the biologically active epitope of the bacterial PAMP flagellin. PAMPs perception initiates a variety of basal defense response referred to as PAMP-triggered immunity (PTI), which mostly includes reactive oxygen species (ROS) production, increase in Ca2+ influx, activation of mitogen-activated protein kinase (MAPK) cascades, transcriptional activation, callose deposition and stomatal closure [2].

Stomata are microscopic pores surrounded by a pair of guard cells and located at the leaf epidermis. They control CO2 uptake for photosynthesis, water loss during transpiration and play a crucial role in biotic and abiotic stress tolerance [3]. Stomata are critical during the plant innate immune response [4,5]. Bacteria such as Pseudomonas syringae pv tomato (Pst) strain DC3000 induce stomatal closure in Arabidopsis within 1 to 2 h post inoculation. However, Pst DC3000 is able to reopen stomata 3 to 4 h after infection through the action of coronatine (COR) [4]. COR acts downstream of ROS accumulation and reverses the inhibitory effects of flg22 on both K+ (in) currents and stomatal opening [6]. Both salicylic acid (SA) and abscisic acid (ABA) synthesis and signaling pathways are required during bacterial- and PAMP-induced stomatal closure in Arabidopsis [4,7]. Several studies suggest that PAMP-induced stomatal closure share common signaling pathway with the ABA-induced stomatal closure [4,6,8]. PAMP-induced stomatal closure requires the synthesis of H2O2 [9]. In Arabidopsis, ABA- and flg22-induced ROS is dependent on the NADPH oxidase Rboh [10,11,12] and Rboh is specifically required for flg22- and bacterial-induced stomatal closure [13].
Author Summary

During their lifetime, plants face numerous pathogenic microbes. Plants recognize microbial pathogens via plant receptors and recognition leads to the activation of a general defense response. Some foliar pathogens such as bacteria enter plant leaves through natural surface openings such as stomata. To restrict bacterial entry, plants close stomata upon contact with bacteria. A better understanding of stomatal immunity may lead to development of crops with improved disease resistance. Here, we used the model plant Arabidopsis thaliana to study activation of defense responses after infection by Pseudomonas syringae pv. tomato (Pst) DC3000 bacteria. We found that a gene not previously known to function in the defense response, LecRK-V.5 is modulating Arabidopsis resistance. By studying plants with mutations in or overexpressing this gene, we show that LecRK-V.5 negatively regulates plant stomatal immunity to Pst DC3000. In addition, LecRK-V.5 is rapidly expressed at stomata upon activation of the general defense response. Plants with mutations in LecRK-V.5 also demonstrated constitutive accumulation of reactive oxygen species in stomatal guard cells. We conclude that LecRK-V.5 is a protein that negatively regulates closure of stomata upon bacterial infection.

The Arabidopsis lectin receptor kinase LecRK-V.5 (also known as LecRK1 or LecRK-a1) belongs to a multigenic family comprising 45 members [14,15,16]. LecRK-V.5 protein is likely localized at the plasma membrane and the kinase domain can be phosphorylated on serine residues [14,17]. LecRK-V.5 is up-regulated in senescing leaves, and is induced by wounding and oligogalacturonic acid treatments [18]. Although a significant number of LecRKs demonstrate an increased expression upon pathway or elicitor treatments [16], only a few reports demonstrated a function for a LecRK in plant-pathogen interactions [19,20,21]. In this study, we show that lecrk-V.5 is a key regulatory gene in stomatal immunity.

In particular, our data suggest that LecRK-V.5 negatively regulates bacterial- and PAMP-triggered stomatal closure upstream of ROS production.

Results

LecRK-V.5 negatively regulates disease resistance to bacteria

To identify novel players in the Arabidopsis defense response, a reverse genetic approach was undertaken with the PAMP- and bacteria-responsive LEGUME-LIKE LECTIN RECEPTOR KINASE-V.5 (LecRK-V.5, At3g59700) [16]. Towards this goal, we isolated lecrk-V.5-1, a transcriptional knockout Ds transposon insertion line and lecrk-V.5-2, a T-DNA insertion line producing a truncated transcript (Figure 1A, B). Arabidopsis mutants were dip-inoculated with the virulent bacteria Pst DC3000 and disease progression was evaluated. lecrk-V.5-1 and lecrk-V.5-2 mutants developed less disease symptoms and lower bacterial titers than wild-type (WT) controls (Figure 1C, D). To confirm that the mutation in LecRK-V.5 is responsible for the enhanced Pst DC3000 resistance observed in the lecrk-V.5-1 mutant, 33S::LecRK-V.5 (CL-1 for complemented line 1) and ProLecRK-V.5::LecRK-V.5-HA (CL-2 for complemented line 2) constructs were produced for complementation analysis. LecRK-V.5 was up-regulated about 75 times in CL-1 while CL-2 showed a WT level of LecRK-V.5 expression (Figure S1). The mutant lecrk-V.5-1 transformed with both constructs demonstrated WT susceptibility to Pst DC3000 dip-inoculation (Figure 1C, D). To further ascertain whether LecRK-V.5 is involved in bacterial resistance, we generated transgenic Arabidopsis Col-0 plants harboring the 33S::LecRK-V.5 (OE-1) and 33S::HA-LecRK-V.5 (OE-2) constructs. Both lines demonstrated a strong up-regulation of LecRK-V.5 characterized by expression levels about 250 times higher than WT (Figure S1). Such overexpression lines demonstrated higher Pst DC3000 titer levels than WT controls (Figure 1E). Collectively, these data indicate that LecRK-V.5 negatively regulates Arabidopsis resistance to Pst DC3000.

![Figure 1. LecRK-V.5 negatively regulates disease resistance to bacteria.](https://example.com/figure1.png)

(A) Insertional mutation sites in two lecrk-V.5 mutant lines. Ds transposon (lecrk-V.5-1) and T-DNA (lecrk-V.5-2) insertion sites are shown. Filled box represents exon and arrows denote the different positions of the primers used in the RT-PCR experiments in (B). The relative position of lectin, transmembrane (TM) and kinase domains in LecRK-V.5 predicted protein structure is indicated. (B) RT-PCR analysis of LecRK-V.5 transcripts in WT and lecrk-V.5 mutants. EF-1 was used as a control. (C) Disease symptoms assessed 3 days after dip-inoculation with 1 x 10^7 cfu.ml^-1 Pst DC3000 in WT (Ler), lecrk-V.5-1 and two complemented lines (CL-1 and CL-2). (D) Bacterial growth at 3 days post-inoculation (1 x 10^7 cfu.ml^-1 Pst DC3000 (Pst)) in WT (Col-0 and Ler), lecrk-V.5 mutants and two complemented lines (CL-1 and CL-2). (E) Bacterial titers evaluated 3 days after dip-inoculation with 1 x 10^7 cfu.ml^-1 Pst DC3000 (Pst) in lines overexpressing LecRK-V.5 (OE-1 and OE-2). For (D) and (E), data represent average ± SD. Statistical differences between WT controls and mutants or transgenics are detected with a t test (p<0.01, n=6). All experiments were repeated at least three times with similar results.

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Phenotypes of lecrk-V.5 mutants and lines overexpressing LecRK-V.5 are associated with stomatal immunity

Although leck-V.5-1 and leck-V.5-2 were more resistant to Pst DC3000 after dip-inoculation (Figure 1C, D), both mutants demonstrated WT susceptibility levels after infiltration-inoculation (Figure S2). Since Arabidopsis restricts bacterial invasion through stomatal closure [4,7], we hypothesized that leck-V.5 mutants increased resistance to surface-inoculation with bacteria is due to their ability to prevent bacterial entry inside leaves via stomata. To determine LecRK-V.5 possible function in stomatal immunity, we examined stomatal aperture of leck-V.5 mutants upon Pst DC3000 inoculation. Stomata of buffer-treated epidermal peels of leck-V.5 mutants were closed at levels similar to those observed in WT controls at 1 hpi with bacteria (Figure 2A and Figure S3A). In addition, the COR-dependent stomatal reopening that occurred in WT at 3 hpi was not observed in leck-V.5 mutants (Figure 2A and Figure S3A). Complemented lines demonstrated a WT stomatal aperture in response to Pst DC3000 (Figure 2A), suggesting that mutations in LecRK-V.5 caused the stomatal response phenotype observed in leck-V.5 mutants. We also analyzed stomatal aperture in WT and lines overexpressing LecRK-V.5 at 1, 1.5 and 3 hpi with Pst DC3000. Although stomatal closure in OE-1 and OE-2 was observed at 1 hpi with Pst DC3000, stomata reopened in overexpression lines at 1.5 hpi, a time point where WT stomata were still closed (Figure S3B). An early stomatal reopening may explain the increased susceptibility of OE-1 and OE-2 lines to bacteria.

WT Col-0 Arabidopsis are resistant to surface inoculation with COR-deficient mutants of Pst DC3000 (Pst DC3000 COR−), presumably because their stomata do not reopen upon infection [4,22]. To further assess the possible role of LecRK-V.5 in stomatal immunity, lines overexpressing LecRK-V.5 (OE-1 and OE-2) were dip-inoculated with Pst DC3000 COR− bacteria and disease development was evaluated 3 days later. The defective virulence of Pst DC3000 COR− observed in Col-0 was almost fully rescued in lines overexpressing LecRK-V.5 (Figure 2B). We also analyzed stomatal aperture in WT and overexpression lines after Pst DC3000 COR− inoculation. Lines overexpressing LecRK-V.5 reopened stomata as early as 4 hpi (Figure 2C). Since Arabidopsis WT stomata do not reopen upon infection with Pst DC3000 COR− (Figure 2C) [4,22], stomatal reopening in OE-1 and OE-2 lines may explain their increased susceptibility to Pst DC3000 COR− (Figure 2B). Taken together these data suggest a role for LecRK-V.5 in mediating stomatal movement in response to Pst DC3000 bacteria in Arabidopsis.

A negative role for LecRK-V.5 in PAMP-induced stomatal closure

To further define the role of LecRK-V.5 during stomatal immunity, we tested the ability of lines overexpressing LecRK-V.5 to respond to PAMP-mediated stomatal closure. Epidermal peels were incubated with the PAMPs flg22, the active elongation factor Tu epitope elf26 and lipopolysaccharides (LPS), which promote stomatal closure [4,9]. Stomatal closure was strongly reduced in OE-1 and OE-2 lines after PAMP treatments at concentrations that are usually used to induce stomatal closure in WT controls (Figure 3A) [4]. Treatments with higher concentrations of flg22 or elf26 partially restored OE-1 and OE-2 sensitivity to these PAMPs (Figure S4A, B). By contrast, these transgenics demonstrated a defective stomatal response to LPS concentrations up to 200 ng.mL−1 (Figure S4C). Surprisingly, stomata of lines overexpressing LecRK-V.5 responded similarly to WT control to low concentrations of these PAMPs when all 3 PAMPs were applied together (Figure 3B). This observation may explain the observed stomatal closure of OE-1 and OE-2 after Pst DC3000 inoculation (Figure S3B).

lecrk-V.5 mutants demonstrate normal apoplastic PTI responses

Stomatal closure is only one aspect of the PTI response. To determine whether apoplastic PTI responses were constitutively elevated in leck-V.5 mutants, we analyzed the production of H2O2.
as an early response to PAMPs in lecrk-V.5-1 and lecrk-V.5-2 leaves in response to flg22 [23]. ROS production after flg22 treatment was at a WT level in both lecrk-V.5 mutants (Figure 4A). We also examined callose deposition [24] and accumulation of the PTI-responsive FRK1, N菲尔/10 and CIPP1P1 [25] after inoculation with the Type III secretion system deficient bacterial mutant Pst DC3000 hrcC (CB200) [26]. Callose deposition and PTI-gene expression levels were not constitutively elevated in lecrk-V.5 mutants and inoculation with Pst DC3000 hrcC induced WT callose deposition (Figure 4B) and PTI-gene expression levels (Figure 4C, D). These data suggest that enhanced resistance of lecrk-V.5 mutants to Pst DC3000 is not correlated with a constitutively activated apoplastic PTI or boosted ROS production, increased callose deposition or PTI-responsive gene expression levels upon PTI activation in the leaves.

LecRK-V.5 is localized at the plasma membrane and expressed in stomatal guard cells

To determine the subcellular localization of LecRK-V.5, the LecRK-V.5-GFP fusion protein driven by the cauliflower mosaic virus 35S promoter was transiently expressed in Arabidopsis mesophyll protoplasts. Confocal imaging indicates that the fluorescence signal is confined to a ring external to the chloroplast signal, while the control protoplasts expressing GFP alone showed a nuclear and cytoplasmic GFP localization (Figure 3A-D). Since LecRK-V.5 was previously detected in plasma membrane fraction [17], our data confirm that LecRK-V.5 is localized at the plasma membrane.

We then asked whether LecRK-V.5 expression is localized at stomatal guard cells upon stomatal immunity activation. LecRK-V.5 promoter GUS analyses indicated that LecRK-V.5 is induced specifically in stomatal guard cells 15 min after Pst DC3000 inoculation or flg22 treatment (Figure 5E). This expression pattern suggests a role for LecRK-V.5 in stomatal movement upon pathogen infection.

LecRK-V.5 regulates stomatal closure upstream of COR site of action

COR counteracts PAMP-induced stomatal closure and reverses flg22 inhibition of inward K+ channels downstream of ROS production [4,6]. To determine whether LecRK-V.5 regulates stomatal closure upstream or downstream of COR site of action, we evaluated stomatal apertures in epidermal peels of WT plants treated with COR. Treatments with COR only opened closed stomata in lecrk-V.5-1 and lecrk-V.5-2 (Figure 6A, B), suggesting that LecRK-V.5 negatively regulates PAMP-induced stomatal closure upstream of COR site of action. Although Pst DC3000 bacteria produce COR [4], inoculation with Pst DC3000 did not open stomata of lecrk-V.5 mutants (Figure 2A). Epidermal peels were then treated with flg22 alone or flg22 together with COR. The PAMP flg22 induced stomatal closure in WT plants and further closure in both lecrk-V.5 mutants (Figure 6A, B). However, COR did not counteract flg22-mediated stomatal closure in lecrk-V.5 mutants while stomata of flg22-treated WT controls did reopen after COR treatment (Figure 6A, B). Together, these data suggest that mutations in LecRK-V.5 inhibit the COR-dependent reopening of stomata during PAMP-induced stomatal closure.

Both lecrk-V.5 mutants accumulate high levels of ROS in guard cells

To further clarify the role of LecRK-V.5 in the stomatal response, ROS levels in lecrk-V.5-1 and lecrk-V.5-2 guard cells were analyzed with the fluorescent dyes 2',7'-dichlorofluorescein diacetate (H2DCFDA) [27,28]. Microscopy and fluorescence emission analyses revealed a higher level of ROS in guard cells of both lecrk-V.5 mutants (Figure 7A). WT controls and complemented lines demonstrated similar levels of ROS production (Figure 7A), suggesting that mutations in LecRK-V.5 caused ROS accumulation observed in lecrk-V.5 mutants. Diphenylene iodum chloride (DPI), an inhibitor of NADPH oxidases known to inhibit ABA-induced stomatal closure [28], was used to test a possible role for NADPH oxidases in lecrk-V.5 stomatal phenotype. DPI treatments induced stomatal opening in lecrk-V.5-1 and lecrk-V.5-2 (Figure 7B). To further evaluate the role of ROS, plants were treated with ascorbic acid (ASC), a chemical known to reduce ROS levels [27]. Treatment with ASC also opened constitutively closed stomata in lecrk-V.5 mutants (Figure 7B). These results suggest that over-accumulation of ROS is responsible for the constitutive stomatal closure observed in lecrk-V.5 mutants. Treatments with PAMPs such as flg22, elf26 or LPS boosted guard cell ROS production at WT levels in lecrk-V.5-1 (Figure S5). By contrast, treatments with these PAMPs increased guard cell ROS levels in WT, but no increase of ROS production was observed in Arabidopsis overexpressing LecRK-V.5 (Figure 7C). Furthermore, H2O2-induced stomatal closure was normal in overexpression lines (Figure 7D) suggesting that LecRK-V.5 does not influence stomatal closure signaling downstream of ROS biosynthesis. LecRK-V.5 may thus function upstream of ROS production in guard cell movement.
LecRK-V.5 role in stomatal immunity is mechanistically linked to ABA signaling

Innate immunity-mediated stomatal closure depends on ABA signaling [4,6,22]. To evaluate whether LecRK-V.5 plays a role in the ABA-mediated stomatal closure, stomatal apertures after treatment with ABA were assessed in lines overexpressing LecRK-V.5. Stomata of such lines were greatly compromised in their ability to respond to ABA (Figure 8A). Furthermore, overexpression lines demonstrated reduced ROS production after ABA treatment (Figure 8B). COR inhibits ABA-induced stomatal closure [4,6]. We thus evaluated the possibility that lecrk-V.5 mutants were resistant to COR-inhibition of stomatal closure upon ABA treatments. Similarly to PAMP-induced stomatal closure (Figure 4A, B), COR treatments did not open ABA-treated lecrk-V.5-1 and lecrk-V.5-2 stomata while reopening in WT controls was observed (Figure 8B, D). To further evaluate the role of LecRK-V.5 in ABA-mediated stomatal closure, we manipulated the ABA-mediated regulation of guard cell pH. Butyrate which causes an acidification of cytoplasm, and K292a, a protein kinase inhibitor, are able to suppress ABA-induced alkalinization occurring upstream of ROS production in guard cells [29]. Both compounds opened constitutively closed lecrk-V.5-1 and lecrk-V.5-2 stomata (Figure 8E, F). Double mutants generated by crossing lecrk-V.5-1 with ABA-insensitive mutants abi1-1D and abi2-1D [30,31] demonstrated a WT stomatal aperture further indicating that LecRK-V.5 functions in ABA signaling (Figure 8G). In addition to ABA, the plant hormones jasmonate (JA) and SA play a positive role in stomatal closure [7,29,32]. Both OE-1 and OE-2 lines demonstrated WT levels of stomatal closure in response to these two phytohormones (Figure S6). Collectively, these results suggest that LecRK-V.5 interferes with ABA- but not with JA- or SA-mediated stomatal closure signaling.

Discussion

As a part of the plant innate immune system, stomata play an active role in limiting bacterial entry into plant tissues and subsequent disease symptoms [4,5]. A rapid stomatal closure occurs upon bacterial challenge and some pathogens have evolved
rapid stomatal closure and the negatively regulates Arabidopsis resistance to bacteria through LecRK-V.5. Taken together these data suggest that LecRK-V.5 plant stomatal reopening mechanism positively modulated by susceptible to surface-inoculation with in PAMP-induced stomatal closure described so far are highly immunity [4,9]. Similarly to the induced stomatal closure implying the PTI response in stomatal expression lines were more susceptible than WT plants to bacteria, differences between surface- and infiltration-inoculation were also observed in Arabidopsis mutants defective in bacteria-induced stomatal closure [4,22,35,36]. Since lecrk-V.5 mutants demonstrated WT apoplastic PTI (Figure 4), closed stomata and inhibition of COR-dependent stomatal reopening is the most straightforward explanation for the enhanced resistance phenotype of lecrk-V.5 mutants to Pst DC3000 surface inoculation. Leckrk-V.5 overexpression lines were more susceptible than WT plants to bacteria, notably to Pst DC3000 COR− dip-inoculation (Figure 2B). Enhanced susceptibility to WT Pst DC3000 was correlated with an earlier reopening of stomata, further pointing for a role of Leckrk-V.5 in stomatal immunity. The reopening of stomata after inoculation with Pst DC3000 COR− suggests the existence of a plant stomatal reopening mechanism positively modulated by Leckrk-V.5. Taken together these data suggest that Leckrk-V.5 negatively regulates Arabidopsis resistance to bacteria through fine-tuning of stomatal immunity.

Treatments with PAMPs such as flg22, elf26, or LPS induce rapid stomatal closure and the β2 mutant is defective in flg22-induced stomatal closure implying the PTI response in stomatal immunity [4,9]. Similarly to the β2 mutant, all mutants defective in PAMP-induced stomatal closure described so far are highly susceptible to surface-inoculation with Pst DC3000 or Pst DC3000 COR-deficient mutant bacteria most likely because of a defect in bacteria-induced stomatal closure [4,22,35,36]. Lines overexpressing Leckrk-V.5 were defective in PAMP-induced stomatal closure, suggesting a negative role for Leckrk-V.5 during stomatal immunity. Treatments of over-expression lines with low concentrations of elf26, LPS and flg22 that did not induce stomatal closure when applied individually, triggered stomatal closure when applied all together (Figure 3B). This observation suggests that different PAMPs additively activate the stomatal immunity response modulated by Leckrk-V.5. It likely explains why over-expression lines exhibit a WT bacterium-induced stomatal closure. Other aspects of the Arabidopsis PTI response such as the flg22-triggered oxidative burst, Pst DC3000 hrcC-mediated callose deposition and up-regulation of PTI marker genes were not affected in lecrk-V.5 mutants. These apoplastic PTI responses are mostly mediated by mesophyll cells [1,22,36]. The recently isolated scord5 mutant also shows a defective stomatal immunity but exhibits WT apoplastic immunity [36]. Our data therefore confirm recent findings indicating that stomatal immunity can be distinguished from the general PTI response [36]. Localized expression of Leckrk-V.5 upon PTI activation at stomatal guard
cells may explain the specific role of LecRK-V.5 in stomatal immunity.

The signaling pathways leading to bacteria and PAMP-induced stomatal closure downstream of PRRs (e.g. FLS2) remains unclear. Analyses of SA-deficient nahG transgenics, SA-biosynthetic mutant sid2/eds16 and ABA-deficient mutant aba3 indicate that SA and ABA biosynthesis are required for PAMP-induced stomatal closure [4]. The ABA signaling components OST1, ABI1, GPA1 and OST2 are also required for bacteria- and PAMP-induced stomatal closure [4,6,34,35,36]. These studies illustrate the complexity of hormonal crosstalks involved in stomatal immunity. LecRK-V.5 overexpression lines were defective in ABA-mediated stomatal closure but not in SA- and JA-mediated stomatal closure. ABA-induced ROS production was also affected in lines over-expressing LecRK-V.5 [4,6,34,35,36]. These studies illustrate the complexity of hormonal crosstalks involved in stomatal immunity. LecRK-V.5 overexpression lines were defective in ABA-mediated stomatal closure but not in SA- and JA-mediated stomatal closure. ABA-induced ROS production was also affected in lines over-expressing LecRK-V.5. As it was proposed for ABI1, OST1, GPA1 and OST2 are also required for bacteria- and PAMP-induced stomatal closure [4,6,34,35,36]. LecRK-V.5 appears to specifically function in guard cell ABA signaling pathway downstream of PAMP perception. LecRK-V.5 may thus act at a specific branch involving ABA for the control of stomatal immunity.

During stomatal closure, ABA induces ROS accumulation, which activates plasma membrane calcium channels, induces increase in cytosolic Ca\(^{2+}\), and triggers stomatal closure [28,37,38]. In this study, constitutive high levels of ROS in guard cells were correlated with constitutively closed stomata in lecrk-V.5 mutants. This phenotype was reverted by treatments with an inhibitor of NADPH oxidase (DPI) [28] or ASC, a chemical that reduces ROS levels [27]. Increase in ROS levels and stomatal closure after PAMP and ABA treatments were impaired in lines overexpressing LecRK-V.5. In addition, suppression of ABA-induced alkalinization that takes place upstream of ROS production [29] opened closed stomata of lecrk-V.5 mutants. Recent studies suggest that constitutive ROS accumulation does not induce stomatal closure, but ROS accumulation mediated by ABA does [39,40]. Collectively these observations suggest a role for LecRK-V.5 upstream of ROS production in the ABA-mediated stomatal closure signaling. Importantly, H\(_2\)O\(_2\)-induced stomatal closure in LecRK-V.5 overexpression lines was not affected. Thus LecRK-V.5 probably does not disrupt the pathway downstream of ROS biosynthesis. Since generation of H\(_2\)O\(_2\) in guard cells and stomatal closure in response to ABA occurs via NADPH oxidases Rboh [10], LecRK-V.5 may act upstream of Atrboh in PAMP- and bacteria-mediated ROS production. By preventing guard cell ROS accumulation, LecRK-V.5 may function in ROS homeostasis to regulate H\(_2\)O\(_2\) content in guard cells. Plants likely have evolved a mechanism to reverse PAMP- or ABA-triggered stomatal closure to prevent long-term detrimental effects on CO\(_2\) uptake and photosynthesis. LecRK-V.5 may be one component of this protective mechanism.

In this study, the L-type lectin receptor kinase LecRK-V.5 was identified as a key player in stomatal immunity. We propose a model in which LecRK-V.5 negatively regulates the signaling pathway leading to PTI-mediated stomatal closure downstream of ABA and upstream of NADPH oxidase to fine tune ROS accumulation in stomatal guard cells (Figure 9).
SnRK2E/SnRK2.6 protein kinase involved in PAMP-induced stomatal closure [4] was initially identified as a positive regulator upstream of ROS in guard cell ABA signaling [41]. A recent study showed that OST1 interacts with and phosphorylates AtrbohF [42]. Further studies are required to identify protein partners of LecRK-V.5 and implicated in PTI-mediated stomatal closure signaling upstream of ROS production.

Materials and Methods

Biological materials and growth conditions

Arabidopsis thaliana (L. Heynh.) ecotypes Columbia (Col-0) and Landsberg erecta (Ler), and derived mutant lines were grown as previously described [43]. The lecrk-V.5-1 (GT12539) Ds transposon insertion line (Ler) is from the Cold Spring Harbor Laboratory (http://genetrap.cshl.org/) and the lecrk-V.5-2 (GK-623G01) T-DNA insertion line (Col-0), abi1-1D and abi2-1D were obtained from the Nottingham Arabidopsis Stock Centre (NASC, http://arabidopsis.info/). Details about PCR analyses performed to screen for homozygous mutants are described under “Gene Expression Studies”. Bacterial strains Pst DC3000, Pst DC3000 COR (DB29) and Pst DC3000 hetC mutant (CB200) were provided by B.N. Kunkel (Washington University, St. Louis, USA) [26]. Pst bacteria were cultivated at 28°C, 340 rpm in King’s B medium containing rifampicin (Pst DC3000), rifampicin, spectinomycin and kanamycin (DB29) or rifampicin and kanamycin (CB200).

Bacterial infection assays

Bacterial disease assays were conducted as previously described [44]. For surface inoculation, plants were dipped in a bacterial solution of 1×10^7 cfu.ml^-1 containing 0.01% Silwet L-77 (Biomax Scientific Co., Ltd.). Alternatively, three fully expanded leaves per plant were infiltrated on the abaxial surface with Pst DC3000 at a concentration of 1×10^7 cfu.ml^-1 using a needleless 1 ml syringe.

Plasmid constructions and generation of transgenic plants

All cloning experiments were performed using the genomic clone AF001168 kindly provided by Christine Hervé (INRA Toulouse, France). According to [19], 707 pb of the LeRK-V.5 promoter was PCR amplified using the GW-pRK-F (5’-CTCTGCAACAATTGGGAGGAGGGGG-3’) and GW-pRK-R (5’-GTTCAGGAGACTTGTTGGGTG-3’) primers. The coding sequence (CDS) of LeRK-V.5 was PCR amplified using the GW-RK-F (5’-ATGTCCTCGTGAAACTTATTATTCTGCAACAATTGGGAGGAGGGGG-3’) and GW-RK-R (5’-TCA-AGGACCCTGGAGGAGGAGAACAATTGGGAGGAGGGGG-3’) primers. The resulting PCR products were directly cloned by the TOPO cloning reaction into the pCRBl/GW/TOPO vector (Invitrogen) following manufacturer’s instructions. The promoter and CDS (without stop codon) of LecRK-V.5 was PCR amplified with the GW-attB1-RK-F (5’-GGGGACAAGTTTGTACAAAAAAGCAGGCTCTGCAACAATTGGGAGGAGGGGG-3’) and GW-attB2-RK-R (5’-GGGGACCATTTTGTTACAGAAAGCTGGGTGCGGCGGCGGAGGAGAAAAGG3’) primers and recombined by the BP reaction into the pDONR221 vector (Invitrogen). The LeRK-V.5 promoter was subcloned by LR reaction in the pMD163 vector [45] to produce the ProLeRK-V.5::GUS construct. The LeRK-V.5 CDS and promoter plus CDS were then introduced into the plasmids pEarley-
Figure 9. Model of LecRK-V.5 role in PAMP-induced stomatal closure. This model is based on the information provided in this study and references cited in the Discussion. PAMPs are perceived by pattern recognition receptors (PRRs) in the guard cell. PAMPs perception is mechanistically linked to ABA-regulated stomatal closure via ROS production by NADPH oxidase. The virulence factor COR is secreted by Pst DC3000 to interfere with stomatal closure by reverting flg22-inhibition. LecRK-V.5 negatively regulates PAMP-mediated stomatal closure downstream of ABA but upstream of ROS production.

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Generation of double mutants

The leek-V.5-1 abi1-1D and leek-V.5-1 abi2-1D double mutants were selected in the F2 progeny of crosses between the two corresponding homozygous parents. Verifications of genotypes involving abi1-1 and abi2-1 crosses were performed as described [51].

Gene expression studies

Semi-quantitative PCR and qRT-PCR were as described [43], with some modifications. Total RNA was extracted and purified using Qiagen RNeasy plant Mini Kit with additional genomic DNA cleanup using Qiagen RNase-Free DNase Set. For cDNA synthesis, RNA was first diluted to 2 μg in a total volume of 22 μL DEPC water and denatured at 65°C for 5 min. Eighteen point five μL of master mix (1× M-MLV buffer, 1 mM dNTP, 5 μM OligoT, 100 U M-MLV reverse transcriptase, [Invitrogen]) was added into each tube and then incubated at 37°C for 1 hr, 70°C for 10 min. cDNA was diluted 5-fold before real-time PCR or semi-quantitative PCR. PCR amplification was done with 2 μL of the first-strand cDNA as template, 1 unit of Taq DNA polymerase (Viogene), 1.56 μM dNTP and 0.5 μM of primers in a total volume of 20 μL. Primers used for screening of homozygous mutants were GT12539-F (5'-TCGGGCTTCAAGGTATCTTC-3') and GT12539-R (5'-CGATGGAAAGCCTCATTACG-3') for the leek-V.5-1 mutant (P1) and Salk_083045-F (5'-ATGGGTGTTGTTAGTTAATGG-3') and Salk_083045-R (5'-CCTCGGATTTCTCATTTGTCA-3') for the leek-V.5-2 mutant (P2). The cycling conditions were 94°C for 5 min for one initial step followed by 94°C for 30 s, 60°C for 30 s and 72°C for 1 min, for 40 cycles. The PCR was terminated with one extra step at 72°C for 10 min. iQ SYBR Green supermix [Bio-RAD] (2 μL of cDNA, 9 μL SYBR Green supermix, 5 μL filtered water, 1 μL of 10 μM forward primer, 1 μL of 10 μM reverse primer, in a total volume of 18 μL per well) was employed for real-time PCR analysis. The cycling conditions were composed of an initial 3 min denaturation step at 95°C, followed by 40 cycles of 95°C for 30 s, 54°C for 35 s, 72°C for 35 s (iQ Real-Time PCR Detection System, Bio-RAD). Melting curve was run from 55°C to 95°C with 10-second time interval to ensure the specificity of product. Data were analyzed using Bio-Rad iQ5 software (version 2.0). Elongation factor 1 (EF-1) and ubiquitin (UBQ10) were used as reference genes for normalization of gene expression levels in all samples. The WT without any treatment or mock treatment were considered as controls (expression level = 1) in each experiment. qRT-PCR forward and reverse primers of each gene were as follows: 5'-GAATTTGGAGTTTCACAGTACA-3' and 5'-GT-CGGTTAATCCATGACTCT-3' for LecRK-V.5 expression in complemented lines (CL-1 and CL-2); 5'-TCATGGGCACTCTCCGCTCTAC-3' and 5'-CTATGCAAGGTTGTAGTAC-3' for LecRK-V.5 expression in overexpression lines (OE-1 and OE-2); 5'-AAA TGG AGA GAG CAA CAC AAT G-3' and 5'-ATC GCC CAT TCC AAC AAT GTT AC-3' for GmEF-1F2 (At5g57220); 5'-TTCGTCGTACC-3' and 5'-GCTACGAGAGGTTGTAGTAC-3' for LecRK-V.5 expression in overexpression lines (OE-1 and OE-2); 5'-AAA TGG AGA GAG CAA CAC AAT G-3' and 5'-ATC GCC CAT TCC AAC AAT GTT AC-3' for GmEF-1F2 (At5g57220); 5'-TTCGTCGTACC-3' and 5'-GCTACGAGAGGTTGTAGTAC-3' for LecRK-V.5 expression in overexpression lines (OE-1 and OE-2); 5'-AAA TGG AGA GAG CAA CAC AAT G-3' and 5'-ATC GCC CAT TCC AAC AAT GTT AC-3' for GmEF-1F2 (At5g57220); 5'-TTCGTCGTACC-3' and 5'-GCTACGAGAGGTTGTAGTAC-3' for LecRK-V.5 expression in overexpression lines (OE-1 and OE-2); 5'-AAA TGG AGA GAG CAA CAC AAT G-3' and 5'-ATC GCC CAT TCC AAC AAT GTT AC-3' for GmEF-1F2 (At5g57220); 5'-TTCGTCGTACC-3' and 5'-GCTACGAGAGGTTGTAGTAC-3' for LecRK-V.5 expression in overexpression lines (OE-1 and OE-2); 5'-AAA TGG AGA GAG CAA CAC AAT G-3' and 5'-ATC GCC CAT TCC AAC AAT GTT AC-3' for GmEF-1F2 (At5g57220); 5'-TTCGTCGTACC-3' and 5'-GCTACGAGAGGTTGTAGTAC-3' for LecRK-V.5 expression in overexpression lines (OE-1 and OE-2);

Subcellular localization in protoplast

For transient expression of the GFP fusion proteins, constructs expressing 35S:LecRK-V.5-GFP and vector alone were co-transfected into Arabidopsis mesophyll protoplasts according to a previously described protocol [48]. Briefly, leaves from 5-week-old plants were digested in an enzyme solution containing 1.5% cellulose R10 (Yakult Pharmaceutical Ind. Co.) and 0.3% macerozyme R10 (Yakult Pharmaceutical Ind. Co.). Transfected protoplasts were incubated overnight under light at room temperature. Confocal laser scanning microscopy with excitation at 488 nm and emission at 500–530 nm (Leica TCS SP5 Confocal, Leica, Wetzlar, Germany) was carried out to visualize subcellular localization of LecRK-V.5-GFP. Autofluorescence was monitored at 488 nm, and transmission images were collected in parallel.
Stomatal experiments

Leaf peels were collected from the abaxial side of fully expanded leaves and floated in stomatal buffer (10 mM MES-KOH, 30 mM KCl, pH 6.15) for 2.5 h under light (100 μmol m⁻² s⁻¹) to ensure that most of stomata were opened before treatments [4]. Purified chemical lipopolysaccharide (LPS from P. aeruginosa, Sigma), flg22 peptide (Biomer Technology, CA), elf26 (Biomer Technology, CA) or COR (Sigma) were used at indicated concentrations. flg22 and elf26 were diluted in 10 mM MgSO₄. COR and LPS were respectively diluted in milliQ water and in MES buffer containing 0.25 mM MgCl₂ and 0.1 mM CaCl₂ [5]. ABA, methyl jasmonate (MeJA) and SA used at indicated concentrations were dissolved in 10% ethanol (Sigma). Diphenyleneiodonium chloride (DPI, Sigma) and K252a (Sigma) were dissolved in dimethylsulfoxide (DMSO). Ascorbic acid (ASC) and sodium butyrate (Butyrate, Sigma) were prepared in milliQ water. Mock controls were MES buffer containing 0.1% ethanol for MeJA, ABA and SA, 0.1% DMSO for DPI and K252a, and milliQ water for ASC and Butyrate. Bacterial concentration used was 1×10⁶ cfu·ml⁻¹ in 10 mM MgSO₄. Stomatal apertures were measured as described [49].

Monitoring ROS in guard cells

2′,7′-dichlorodihydrofluorescein diacetate (H₂DCFDA) fluorescence analysis were performed essentially as described [49]. After 2.5 hrs incubation in stomatal buffer, epidermal peels were transferred to 50 mM H₂DCFDA in 10 mM Tris-HCl pH 7.2 for 15 min. Excess H₂DCFDA was then removed by washing 3 times in 10 mM Tris-HCl pH 7.2. Then, PAMPS (5 μM flg22, 5 μM elf26 or 100 ng·μL⁻¹ LPS) or 10 μM ABA were added to the incubation buffer for 10 min. H₂DCFDA fluorescence was observed with an Olympus BX51 fluorescence microscope with an excitation at 460–490 nm and emission at 495–540 nm. Images were captured with an Olympus DP2-BSW digital camera linked to the Olympus DP2-BSW software. Fluorescence was analyzed using ImageJ 1.42 software.

Apoplastic oxidative burst evaluation

ROS released by leaf tissue was assayed as described [23]. Leaf of 5-week-old Arabidopsis plants were cut in 2 mm² pieces and floated overnight in water. ROS production was triggered with 1 μM flg22 applied together with 100 μM luminol (Sigma) and 1 μg/mL of horseradish peroxidase (Sigma). Luminescence was measured by a Centro LB 962 microplate luminometer (Berthold Technologies) for 20 min after addition of flg22.

Gus staining

Surface-sterilized seeds were sown on 1/2 MS agar plates and cold-treated at 4°C in the dark for 3 days. The plates were then moved to germination conditions 22–24°C day, 17–19°C night temperature under a 15-h-light/9-h-dark photoperiod. Ten-day-old seedlings were transferred to 24 well plates. Three seedlings per well were soaked in 200 μL liquid 1/2 MS containing 1% sucrose. After incubation overnight, seedlings were treated with 1×10⁶ cfu·ml⁻¹ Pst DC3000 hrcC mutant (CB200) for 1 hr and 5 hrs.

Accesion numbers

Sequence data from this article can be found in the Arabidopsis Genome Initiative under accession number(s): LeCRK-V.5 (At5g57220), FRK1 (At2g19190), NH110 (At2g35980), CP81f2 (At2g37220), EF-1 (At1g07920), UBQ10 (At4g05320), ABI1 (At1g26080) and ABI2 (At3g57050).

Supporting Information

Figure S1 LeCRK-V.5 expression levels in transgenic lines. Relative expression levels in WT (Ler) and two complemented lines (CL-1 and CL-2) and WT (Col-0) and two overexpression lines (OE-1 and OE-2). Transcript levels were determined by qRT-PCR and normalized to both EF-1 and UBQ10. Expression levels were compared to WT controls with a defined expression value of 1. Bars indicate SD (n = 6). Experiments were repeated 3 times with similar results. (TIF)

Figure S2 Susceptibility of lecrk-V.5 mutants to Pst DC3000 infiltration-inoculation. Bacterial growth (colony forming units (cfu) per cm leaf area) was determined in Ler, Col-0 and lecrk-V.5 mutants infiltrated with 1×10⁶ cfu·ml⁻¹ Pst DC3000 (Ps6). Data represent average ±SD. Means were not significantly different between WT and mutants when evaluated by a t-test (P<0.01, n = 9). dpi, day post inoculation. Experiments were repeated 3 times with similar results. (TIF)

Figure S3 Stomatal aperture in lecrk-V.5-2 mutant and overexpression lines after bacterial inoculation. (A) Stomatal aperture of WT Col-0 and lecrk-V.5-2 after a 1 hr and 3 hr incubation time with MES buffer (Control) or 1×10⁶ cfu·ml⁻¹ Pst DC3000 (Ps6). (B) Stomatal aperture in WT Col-0 and overexpression lines (OE-1 and OE-2) after 1, 1.5 and 3 hrs incubation in MES buffer (Control) or 1×10⁶ cfu·ml⁻¹ Pst DC3000 (Ps6). Results are shown as mean of ≥60 stomata ± SE. Asterisks indicate significant differences between WT and mutant/OE based on a t test (P<0.001). All experiments were repeated at least three times with similar results. hpi, hour post inoculation. (TIF)

Figure S4 Altered PAMP-induced stomatal closure in lines overexpressing LecRK-V.5. The stomatal response of lines overexpressing LeCRK-V.5 (OE-1 and OE-2) to different concentrations of flg22 (A), elf26 (B) and LPS (C). Results are shown as mean of ≥60 stomata ± SE. Asterisks indicate significant differences between WT Col-0 and OE lines based on a t test (P<0.001). All experiments were repeated at least three times with similar results. hpi, hour post inoculation. (TIF)
on a t test (P<0.001). All experiments were repeated at least three times with similar results.

(TIF)

Figure S5 ROS production upon PAMPs treatments. ROS detected by H2DCFDA fluorescence in guard cells of WT Ler and *lecRK-V-5* mutant after treatments with MES buffer (Control), 5 mM flg22, 5 mM etr26 and 100 ng mL−1 LPS. Results are shown as mean ± SE. Asterisks indicate significant differences to WT control based on a t test analysis (n=60; P<0.001). Experiment was repeated at least three times with similar results. (TIF)

Figure S6 Lines overexpressing *LecRK-V.5* demonstrate a WT stomatal response to MeJa and SA. Effect of MeJa (A) and SA (B) on stomatal aperture in WT Col-0 and overexpression lines OE-1 and OE-2. Results are shown as mean ± SE. No significant differences between Col-0 and OE lines were observed based on a t test (P<0.001). All experiments were repeated at least three times with similar results. (TIF)

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Author Contributions

Conceived and designed the experiments: DA MDT LZ. Performed the experiments: DA MDY GHJ WYC YCL. Analyzed the data: DA MDT LZ. Wrote the paper: DA MDT LZ.
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