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RESEARCH ARTICLE

RECEPTOR-LIKE KINASE 902 Associates with and Phosphorylates BRASSINOSTEROID-SIGNALING KINASE1 to Regulate Plant Immunity

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Running Title: RLK902 regulates plant immunity by phosphorylating BSK1

Short Summary:
In this study, we found that EDR4 and RLK902 positively regulate plant resistance to \textit{Pseudomonas syringae}. EDR4 modulates the subcellular trafficking and accumulation of RLK902 protein. RLK902 associates with BSK1 and transmits immune signals by direct phosphorylation of BSK1.
Abstract

Plants employ receptor-like kinases (RLKs) and receptor-like proteins for rapid recognition of pathogen invasion and RLKs then transmit signals to receptor-like cytoplasmic kinases (RLCKs) to activate immune responses. RLKs are under fine regulation mediated by subcellular trafficking, which contributes to the proper activation of plant immunity. In this study, we show that Arabidopsis thaliana RECEPTOR-LIKE KINASE 902 (RLK902) plays important roles in resistance to the bacterial pathogen Pseudomonas syringae, but not to the fungal powdery mildew pathogen Golovinomyces cichoracearum. RLK902 localizes to the plasma membrane, and associates with ENHANCED DISEASE RESISTANCE 4 (EDR4), a protein involved in clathrin-mediated trafficking pathways. EDR4 and CLATHRIN HEAVY CHAIN 2 (CHC2) regulate the subcellular trafficking and accumulation of RLK902 protein. In addition, RLK902 directly associates with the RLCK BRASSINOSTEROID-SIGNALING KINASE1 (BSK1), a key component of plant immunity, but not with other members of the FLAGELLIN SENSING 2 immune complex. RLK902 phosphorylates BSK1, and Ser-230 of BSK1 is a key phosphorylation site that is critical in RLK902-mediated defense signaling. Taken together, our data indicate that EDR4 regulates plant immunity by modulating the subcellular trafficking and protein accumulation of RLK902 and that RLK902 transmits immune signals by phosphorylating BSK1.

Keywords: RLK902, BSK1, EDR4, Subcellular trafficking, Phosphorylation, Plant immunity
Introduction

Plants employ two parallel pathways to perceive and withstand attack by numerous potential pathogens; together, these pathways constitute the plant innate immunity system (Dangl et al., 2013; Jones and Dangl, 2006; Tang et al., 2017). The first layer of immunity is the recognition of microbe- or host-derived elicitors, also called pathogen/microbe-associated molecular patterns (PAMPs/MAMPs) or damage-associated molecular patterns (DAMPs), in the early stage of invasion (Bigeard et al., 2015; Gouveia et al., 2016). To sense the PAMPs of different invaders, plants arm themselves with cell-surface-localized receptor-like kinases (RLKs) and receptor-like proteins (RLPs) that function as PAMP-recognition receptors (PRRs) (Boller and Felix, 2009; Macho and Zipfel, 2014). RLK- and RLP-type PRRs generally form dynamic complexes with regulatory receptor kinases, such as co-receptors at the plasma membrane, to activate immune signaling and recruit receptor-like cytoplasmic kinases (RLCKs) or other immune components, thereby linking extracellular ligand perception to downstream signaling (Couto and Zipfel, 2016; Liang and Zhou, 2018).

In Arabidopsis, a well-investigated leucine-rich repeat (LRR)-containing RLK called FLAGELLIN SENSING 2 (FLS2) preferentially recognizes bacteria flagellin (or the flagellin epitope flg22) and associates with its co-receptor BRI1-ASSOCIATED RECEPTOR KINASE1 (BAK1) in a ligand-dependent manner (Chinchilla et al., 2007; Sun et al., 2013). BOTRYTIS-INDUCED KINASE1 (BIK1) and BRASSINOSTEROID SIGNALING KINASE 1 (BSK1), which are members of RLCK subfamilies VII and XII, respectively, associate with FLS2 and are crucial for the activation of FLS2-mediated immunity. Upon perception of flg22, BIK1 and BSK1 are activated and dissociate from the FLS2-BAK1 complex to initiate downstream responses (Lu et al., 2010; Shi et al., 2013; Zhang et al., 2010), such as rapid activation of MAP kinase (MPK) cascades (Meng and Zhang, 2013; Yan et al., 2018) and reactive oxygen species (ROS) bursts.
The accumulation and localization of RLKs are under fine regulation in planta. Although RLKs are generally located at the plasma membrane (PM), their subcellular localization is modulated dynamically (Erwig et al., 2017). Mature RLKs are delivered to the PM by secretion and internalized through clathrin-dependent endocytosis. For instance, FLS2 is internalized after sensing flg22, which is sensitive to Brefeldin A (BFA), a vesicle trafficking inhibitor, indicating that FLS2 constitutively undergoes endomembrane trafficking. Subcellular trafficking of FLS2 contributes to the transient recycling of activated PRRs and replenishment of newly synthesized FLS2 (Beck et al., 2012; Robatzek et al., 2006; Smith et al., 2014). Like FLS2 localization, the subcellular trafficking of the chitin receptor components LysM-CONTAINING RECEPTOR KINASE4 (LYK5) and CHITIN ELICITOR RECEPTOR KINASE1 (CERK1) is also under tight control (Erwig et al., 2017).

Increasing evidence shows that components of vesicle trafficking pathways are involved in plant immunity (Gu et al., 2017). For instance, ENHANCED DISEASE RESISTANCE 4 (EDR4) and CLATHRIN HEAVY CHAIN 2 (CHC2) negatively affect plant immunity to powdery mildew. The relocation of EDR1, a negative regulator of plant defense, to the invasion site and its accumulation there rely on EDR4 and CHC2 (Wu et al., 2015). Recent investigations have revealed a positive role of CHC2 in regulating bacteria-induced plant immunity, which is in contrast to its negative role in resistance to powdery mildew. The chc2 mutant displays enhanced susceptibility to *Pseudomonas syringae* and defects of callose deposition and stomatal closure. Moreover, FLS2 undergoes flg22-induced endocytosis in a CHC2-dependent manner (Mbengue et al., 2016).
Although the biological function of EDR4 is not very clear, EDR4 is known to play important roles in subcellular trafficking, contributing to plant defense responses. To further explore the roles of EDR4 in plant immunity, we challenged the edr4 mutant with the bacterial pathogen *P. syringae* and found that EDR4 positively regulates resistance to this bacterium. Furthermore, EDR4 associates with RECEPTOR LIKE KINASE 902 (RLK902) and modulates its subcellular trafficking and protein accumulation, and RLK902 directly interacts with and phosphorylates BSK1, a key component in the FLS2 immune complex. However, we found that RLK902 is not involved in FLS2-mediated immunity. Rather, RLK902 mainly phosphorylated BSK1 at Ser-230, whose phosphorylation is crucial for RLK902-mediated plant resistance to *P. syringae*. We propose that EDR4 positively regulates plant immunity against bacterial pathogens by modulating vesicle trafficking of RLK902, and RLK902 performs its role in plant immunity by phosphorylating BSK1.

Results

The *edr4-1* Mutant Has Enhanced Susceptibility to *P. syringae*

The *edr4-1* mutant shows enhanced resistance to the powdery mildew pathogen *G. cichoracearum* UCSC1 and displays pathogen-induced spontaneous cell death (Wu et al., 2015). To further investigate the role of EDR4 in plant immunity, we inoculated *edr4-1* mutant with the pathogenic bacterium *P. syringae* pv. tomato (*Pto*) DC3000 to assess whether the *edr4-1* mutant conferred resistance to other pathogens. Interestingly, the *edr4-1* mutants were more susceptible than wild-type plants to *Pto* DC3000. At 3 days after inoculation with *Pto* DC3000, bacterial populations in *edr4-1* leaves were significantly greater than those in wild-type plants, and the *EDR4* genomic clone complemented the *edr4-1* phenotype (Figure 1A). We also challenged *edr4-1* mutants with the *Pto* DC3000 *hrcC* strain, which carries a collection of PAMPs but lacks a functional type III secretion system (TTSS) and it therefore has reduced virulence (Zhang
et al., 2010). The edr4-1 mutant displayed enhanced susceptibility to Pto DC3000 hrcC, as for Pto DC3000, indicating that EDR4 acts as a positive regulator in plant immunity to Pto DC3000 (Figure 1B), most likely in PAMP-triggered immunity.

Pathogens often induce accumulation of defense-related genes (e.g., the PATHOGENESIS-RELATED gene family, or PR genes) after infection. A previous study indicates that transcripts of PR genes accumulated to a greater degree in the edr4-1 mutant than in the wild type after G. cichoracearum infection (Wu et al., 2015). In contrast, the transcript levels of PR1 and PR2 were much lower in edr4-1 plants than in wild-type plants after P. syringae infection (Figure 1C and 1D). Taken together, these data imply that EDR4 plays opposite roles in responses to the fungal powdery mildew G. cichoracearum and the bacterial pathogen P. syringae.

Two RLKs Interact with EDR4 in planta
EDR4 associates with CHC2 to negatively regulate plant resistance to powdery mildew by affecting the accumulation of EDR1 (Wu et al., 2015). As EDR1 also plays a negative role in resistance to Pto DC3000, the positive function of EDR4 in bacterial resistance suggested that EDR4 might regulate the defense response to bacteria by modulating other components of plant immunity.

Previous investigations using the yeast two-hybrid system indicated that two homologous RLKs, RLK902 and RKL1, interact with EDR4. In addition, after infection by P. syringae, the transcript levels of EDR4 and these two RLK genes are suppressed (Tarutani et al., 2004b). However, interactions between EDR4 and the two RLKs were not validated in this earlier study. To test for interactions between the two RLKs and EDR4 in planta, we first performed a split-luciferase complementation imaging (LCI) assay in Nicotiana benthamiana. As shown in Figure 2A, luminescent signals were detected when
EDR4 was co-transformed with RLK902 or RKL1 but not with the empty vector, which was used as a negative control. We quantified the interaction by measuring the luminescence intensity (Figure 2B). Furthermore, we constructed transgenic Arabidopsis plants expressing the three proteins fused to GFP or hemagglutinin (RLK902-GFP, RKL1-GFP and EDR4-HA, respectively). We then performed co-immunoprecipitation (co-IP) experiments using those transgenic plants. As shown in Figure 2C, we confirmed that the RLK902-EDR4 and RKL1-EDR4 interactions also occur in Arabidopsis.

RLK902 and RKL1 belong to the leucine-rich repeat (LRR) III subfamily of RLKs (Figure S1) and share the LRR domain, transmembrane (TM) domain and kinase domain (KD) (Figure S2A). Previous work showed that the coiled-coil (CC) domain of EDR4 is necessary for interaction with EDR1 (Wu et al., 2015). To examine which domains of EDR4 and RLKs, respectively, are required for the interaction, we performed yeast two-hybrid assays using constructs containing truncated fragments of the protein. We found that the KDs of RLK902 and RKL1 were sufficient to bind EDR4, but only full-length EDR4 bound the two RLK proteins (Figure 2D).

**RLK902 Is Required for Immunity against *P. syringae* Infection**

To test whether RLK902 and RKL1 participate in plant defense against pathogenic bacteria, we infected the T-DNA insertion lines RLK902 and RKL1 (Figure S2B-S2D) with *Pto* DC3000 and *Pto* DC3000 *hrcC*. As shown in Figure 3A and 3B, the *rlk902* mutant showed enhanced susceptibility to *Pto* DC3000 and *Pto* DC3000 *hrcC*, similar to that of the *edr4-1* mutant. The genomic DNA of RLK902 rescued the enhanced susceptibility of *rlk902* to *Pto* DC3000 and *Pto* DC3000 *hrcC*. Like the *edr4-1* mutant, the *rlk902* mutant also showed defects in accumulation of PR transcripts (Figure 3C and 3D). To further confirm that these defects were caused by the absence of RLK902, we generated two RNA interference (RNAi) lines that showed significantly reduced
accumulation of *RLK902* transcripts (Figure S2E). Those two RNAi lines supported significantly more growth of both the *Pto* DC3000 and *Pto* DC3000 *hrcC* strains than of the wild-type plant at 3 days post inoculation (dpi) (Figure S2F and S2G), indicating that RLK902 plays positive roles in resistance to bacterial *Pto* DC3000.

Although the amino acid sequences of RLK902 and RKL1 share similarity of more than 75% (Tarutani et al., 2004a), *rkl1* mutants displayed wild-type-like response to *P. syringae* (Figure S3A and S3B), indicating that RKL1 might not be involved in resistance to *Pto* DC3000. To examine whether RLK902 and RKL1 function redundantly in defense responses, we infected an *rlk902 rkl1* double mutant with *Pto* DC3000. The resistance of this double mutant to *Pto* DC3000 was similar to that of the *rlk902* single mutants (Figure S3C). We also generated *edr4-1 rlk902*, *edr4-1 rkl1*, and *edr4-1 rlk902 rkl1* mutants and infected those plants with *Pto* DC3000. As shown in Figure S3D, bacterial growth on those mutants was not significantly different from that on *rlk902* single mutants. Taken together, these data indicate that RLK902, not RKL1, positively regulates plant resistance to *P. syringae*. EDR4 and RLK902 likely function in the same genetic pathway of defense responses to *Pto* DC3000.

To investigate whether RLK902 and RKL1 play roles in powdery mildew resistance, we infected wild-type, *rlk902*, and *rkl1* plants with *G. cichoracearum* UCSC1. Unlike *edr4*, which showed enhanced resistance to powdery mildew, *rlk902* and *rkl1* displayed wild-type-like phenotypes. In addition, loss of one or both RLKs did not affect the powdery mildew resistance phenotype of *edr4-1* (Figure S3E and S3F), indicating that RLK902 and RKL1 may not contribute to powdery mildew resistance. As we did not find any evidence that RKL1 is involved in plant immunity against *Pto* DC3000 and *G. cichoracearum*, we focused on RLK902 in this study.
Overexpression of RLK902 Contributes to Enhancing Plant Resistance

To further investigate the role of RLK902 in resistance to *P. syringae*, we overexpressed RLK902 by introducing a 35S:RLK902-YFP-HA construct into wild-type plants. Phenotyping of two independent overexpression lines, OE-1 and OE-2, indicated that both displayed growth inhibition and spontaneous cell death. After four weeks of growth in short-day condition, OE plants were much smaller than wild-type plants and showed signs of cell death. In contrast, the *rlk902* mutant did not show any growth or developmental defects (Figure S4A and S4B).

To examine whether overexpression of RLK902 caused enhanced resistance, we challenged OE plants with *Pto* DC3000 and found that both OE lines were more resistant to *Pto* DC3000 than the wild type (Figure 4C). We also detected constitutively higher levels of *PR* gene expression in the two OE lines (Figure S4D and S4E). These data provide further support for a positive role of RLK902 in plant immunity.

EDR4 Modulates the Subcellular Trafficking of RLK902

Previously, we showed that EDR4 associates with CHC2 and modulates plant immunity by regulating the relocation of EDR1 (Wu et al., 2015). Since EDR4 associates with RLK902, and both *edr4* and *rlk902* mutants showed similar phenotypes of enhanced susceptibility to *Pto* DC3000, we hypothesized that EDR4 and CHC2 might modulate the subcellular trafficking of RLK902. Consistent with this hypothesis, RLK902 also interacted with CHC2 (Figure S5A-S5C). Both EDR4 and CHC2 interacted with RLK902 at the plasma membrane (Figure S5D). To investigate whether EDR4 and CHC2 contribute to RLK902 localization, we generated transgenic lines by introducing a genomic sequence expressing GFP-tagged RLK902 under the control of its native promoter into the *rlk902* mutant. We then introduced the *RLK902-GFP* transgene into the wild type and the *edr4-1* and *chc2-2* mutants by crossing. As shown in Figure S5F-S5H,
the chc2-2 mutant also displayed enhanced susceptibility to *Pto* DC3000. Next, we observed the epidermal cells of the cotyledons of 7-day-old transgenic seedlings by confocal microscopy. We found that RLK902 was mainly localized at the plasma membrane, as expected for an RLK, and that edr4-1 and chc2-2 mutations did not affect this plasma membrane localization of RLK902 (Figure S5E).

To examine whether vesicle trafficking of RLK902 was altered in edr4-1 and chc2-2 mutants, we treated the aforementioned transgenic plants with Brefeldin A (BFA), a fungal metabolite that inhibits vesicle trafficking and leads to endomembrane aggregates (Brandizzi et al., 2002; Ichimura et al., 2002; Nebenführ et al., 2002; Robinson et al., 2008). After 30 minutes of BFA treatment, we observed that RLK902-GFP in the wild-type background formed aggregates in the cytoplasm. However, lack of EDR4 or CHC2 affected the production of BFA bodies (Figure 4A). We also measured the fluorescence intensity of root tip cells, and used the ratio of intracellular fluorescence intensity (\(F_{\text{internal}}\)) to total fluorescence intensity of the whole cell (including the cell membrane; \(F_{\text{total}}\)) to analyze the subcellular trafficking of RLK902 quantitatively. Consistent with the confocal imaging results, BFA caused intracellular aggregation of RLK902 in the wild type, but not in the edr4-1 and chc2 mutants, as indicated by the value of \(F_{\text{internal}}/F_{\text{total}}\) (Figure 4B). BFA treatment leads to inhibition of vesicle trafficking and formation of BFA bodies. RLK902-GFP was observed in BFA bodies in WT plants indicates that the localization of RLK902 is involved in subcellular trafficking processes. The reduced production of BFA bodies in the edr4-1 and chc2-2 mutants indicate that the subcellular trafficking of RLK902 was defected in those mutants, therefore, the subcellular trafficking of RLK902 is dependent on EDR4 and CHC2.

To further study the relationship between EDR4 and RLK902, we challenged 4-week-old wild-type, edr4-1, and chc2-2 plants with *Pto* DC3000, and then measured the abundance
of *RLK902* transcripts by quantitative reverse-transcription PCR and the abundance of *RLK902* protein by western blot. The transcript level of *RLK902* did not differ significantly between wild-type and mutant plants (Figure 4C). In addition, we examined the protein level of *RLK902* before and after inoculation of *Pto* DC3000. As shown in Figure 4D and 4E, the *RLK902* protein is increased both in wild-type and mutant plants in the early stage of pathogen inoculation, and *Pto* DC3000 treatment could partially restore the compromised accumulation of *RLK902* in the *edr4* and *chc2* mutants. However, the protein levels of *RLK902* were much lower in the *edr4* or *chc2* mutant background than in the wild type both before and after inoculation, indicating that EDR4 and CHC2 contribute to the accumulation of the *RLK902* protein.

**RLK902 Physically Associates with BSK1**

Receptor-like kinases often transduce signals to downstream RLCKs (Liang and Zhou, 2018; Tang et al., 2017). BSK1 and BIK1 are two RLCKs associated with PRRs that play important roles in PTI (Lu et al., 2010; Shi et al., 2013; Zhang et al., 2010). Because *RLK902* was indispensable for plant immunity, we first tested whether *RLK902* could form a complex with BSK1 or BIK1.

As shown in Figure 5A and 5B, we detected an interaction between *RLK902* and BSK1 in an LCI assay. We also performed a yeast two-hybrid assay to examine the interactions *in vitro*. Our data indicated that *RLK902* could bind BSK1 in yeast, which implied that *RLK902* and BSK1 directly associate (Figure 5C). To further confirm the interaction between *RLK902* and BSK1, we generated transgenic Arabidopsis plants expressing *RLK902*-GFP and BSK1-MYC, and performed a Co-IP assay. As shown in Figure 5D, *RLK902* interacted with BSK1 in Arabidopsis which further confirmed their association *in planta*. The interaction between *RLK902* and BSK1 was no significant changed after inoculated with *Pto* DC3000 *hrcC* (Figure 5D). In contrast to those observations,
however, we did not detect any interaction between RLK902 and BIK1 either \textit{in vitro} or \textit{in planta} (Figure S6A-S6C).

BSK1 has previously been shown to interact with FLS2 and modulate flg22-induced immunity responses, including the ROS burst (Shi et al., 2013). To investigate whether RLK902 forms a complex with FLS2, we examined whether RLK902 associates with other components of the FLS2 immune complex, including BAK1 and FLS2. However, we did not detect any interactions between RLK902 and FLS2 or BAK1 by either LCI or yeast two-hybrid assays (Figure S6A-S6C). Consistent with this observation, the \textit{rlk902} mutant did not show any defects in ROS burst after being treated with flg22 (Figure S6D). Therefore, RLK902 might not participate in defense response elicited by flg22 recognition but may instead function independently of FLS2.

\textbf{RLK902 Phosphorylates BSK1 Directly \textit{in vitro}}

As RLK902 directly interacts with BSK1, we hypothesized that RLK902 activates BSK1 by direct phosphorylation. To determine whether RLK902 could phosphorylate BSK1, we generated a kinase-deficient form of BSK1 (BSK1\textsuperscript{K104E}) and expressed it in \textit{Escherichia coli} to allow us to carry out \textit{in vitro} kinase assays. As shown in Figure 6A, RLK902-KD and BSK1, but not BSK1\textsuperscript{K104E}, showed autophosphorylation activity, and BSK1\textsuperscript{K104E} could be phosphorylated by RLK902-KD.

A previous study showed that the Ser-230 residue of BSK1 is the major phosphorylation site for the brassinosteroid receptor BRASSINOSTEROID INSENSITIVE 1 (BRI1) (Tang et al., 2008). We therefore examined whether the Ser-230 of BSK1 is also the phosphorylation site for RLK902. We expressed and purified BSK1\textsuperscript{K104E S230A-His} in \textit{E. coli} for an \textit{in vitro} kinase assay. No phosphorylation of BSK1\textsuperscript{K104E} by RLK902-KD was detected when Ser-230 was mutated to alanine (Figure 6A), indicating that Ser-230 is the
main phosphorylation site modified by RLK902.

**Phosphorylated Ser-230 of BSK1 Contributes to Plant Immunity**

Based on the results of the *in vitro* kinase assays described above, we hypothesized that RLK902 transmits signals to BSK1 by phosphorylation. To test this hypothesis and assess the role of Ser-230 phosphorylation in plant immunity, we examined whether the phosphomimetic mutation of Ser-230 of BSK1 could rescue the susceptibility of the *rlk902* mutant to *Pto* DC3000. First, we introduced the BSK1<sub>S230D</sub> and BSK1<sub>S230A</sub> variants into the *rlk902 bsk1-1* double mutant and selected two individual transgenic lines of both BSK1<sub>S230D</sub>/*rlk902 bsk1-1* and BSK1<sub>S230A</sub>/*rlk902 bsk1-1*, which accumulated similar protein level of BSK1 variants for pathogen inoculation (Figure 6B). Then, we inoculated wild-type, *rlk902, rlk902 bsk1-1, BSK1<sub>S230D</sub>/rlk902 bsk1-1* and BSK1<sub>S230A</sub>/rlk902 bsk1-1* plants with *Pto* DC3000. At 3 days after inoculation, the population of the pathogen in transgenic lines of BSK1<sub>S230D</sub>/rlk902 bsk1-1, but not BSK1<sub>S230A</sub>/rlk902 bsk1-1, was significantly lower than that in the *rlk902* mutant, but greater than in the wild type, indicating that the phosphomimetic mutation of BSK1 could partially rescue the defects of *rlk902* mutants in defense against *P. syringae* (Figure 6C). This observation indicated that the Ser-230 residue of BSK1 plays a critical role in resistance to bacterial pathogens, and phosphorylation of Ser-230 by RLK902 contributes to plant immunity.

To gain more insights into the relationship between RLK902 and BSK1, we infected wild-type, *rlk902, bsk1-1* and *rlk902 bsk1-1* plants with *Pto* DC3000, and we found that the *rlk902 bsk1-1* double mutant displayed similar susceptibility to the *bsk1-1* mutant (Figure 6D), indicating that BSK1 and RLK902 are likely to be in the same genetic pathway. In addition, we crossed RLK902 overexpression line with the *bsk1-1* mutant and found the *bsk1-1* mutation could partially rescue the growth inhibition and enhanced...
resistance to \textit{Pto} DC3000 in the RLK902 overexpression lines (Figure S7A-S7B). Taken together, these observations indicate that RLK902 acts upstream of BSK1, and that RLK902 transmits immune signals to BSK1 through phosphorylation of BSK1 Ser-230.

**Discussion**

In this study, we found that EDR4 and RLK902 play important roles in resistance to the bacterial pathogen \textit{P. syringae}. We showed that EDR4 physically associates with RLK902 and thereby regulates its intracellular trafficking and accumulation. In addition, RLK902 associates with BSK1, a key component in plant immunity, and transmits immune signals to BSK1 by phosphorylating its Ser-230 residue. Although the biological function of EDR4 is not clear, it may contribute to clathrin-mediated vesicle trafficking, as an adaptor protein helping to transport the associated cargo to its destination (Wu et al., 2015).

Vesicle trafficking plays important roles in plant immunity. For instance, internalization of FLS2 by vesicle trafficking is induced by flg22, and clathrin-dependent endocytosis and subcellular compartmentalization of FLS2 are critical to FLS2-mediated immune responses (Beck et al., 2012; Chinchilla et al., 2007; Mbengue et al., 2016; Robatzek et al., 2006). Previously, we showed that EDR4 negatively regulates the defense response to powdery mildew by modulating the relocation of EDR1. Moreover, the loss-of-function mutants of \textit{EDR1} and \textit{EDR4} show very similar phenotypes, including enhanced resistance to \textit{G. cichoracearum} and mildew-induced cell death (Wu et al., 2015).

Intriguingly, however, we found in this study that edr4-1 mutants showed enhanced susceptibility to the bacterial pathogen \textit{Pto} DC3000, unlike edr1 mutants, which are resistant to \textit{Pto} DC3000. It is interesting that EDR4 plays opposite roles with respect to bacterial \textit{Pto} DC3000 and the powdery mildew pathogen. One explanation for these observations is that the cargo transported by EDR4 has opposite effects in bacterial and
fungal resistance. Powdery mildew resistance in edr4 mutants results from the mislocalization of EDR1, a negative regulator of defense responses; in contrast, the defect in bacterial resistance results from the mislocalization of RLK902, a positive regulator of bacterial resistance. Consistent with this scenario, the chc2 mutant is also more resistant to powdery mildew (Wu et al., 2015), but more susceptible to Pto DC3000, than the wild type (Mbengue et al., 2016). In addition, loss of function of EDR4 and CHC2 affects not only RLK902 trafficking but also the accumulation of RLK902 protein. The defects in protein accumulation may be caused by improper vesicle trafficking, in a pattern similar to that seen for FLS2 accumulation (Smith et al., 2014). Interestingly, Pto DC3000 treatment could partially restore the compromised accumulation of RLK902 in edr4 and chc2 mutants. It is likely that plants employ an unknown mechanism to accumulate more RLK902 after pathogen invasion, besides EDR4 and CHC2-mediated pathway. In this scenario, in the edr4 and chc2 mutants, improper trafficking lead to less RLK902 proteins, but on the other hand, plants may accumulate more RLK902 for activating defense responses in an EDR4 and CHC2-independent manner. However, the total protein level of RLK902 in the edr4 or chc2 mutants is much lower than that in WT plants before or after infectin which weakens the resistance to Pseudomonas syringae. Plants employ numerous RLKs and RLPs to allow rapid recognition of various pathogen infections. RLKs can be classified into different subfamilies according to motifs in their ectodomains (ECDs). Members of LRR-RLK subfamily contain different number of LRRs. The number of LRRs is often associated with roles as receptors, co-receptors or signaling proteins in ligand recognition (Sun et al., 2013; Tang et al., 2017). For instance, a number of proteins in the LRR-RLK family, including FLS2, EFR, PEPR1, PEPR2, BAK1, SOBIR1, BIR1 and BIR2, play important parts in plant immunity. Among these, FLS2 (28 LRRs), EFR (21 LRRs) and PEPR1/2 (28 LRRs/26 LRRs) contain large number of LRRs and function as receptor to sense PAMPs or danger signals; BAK1 (4
LRRs) and SOBIR1 (5 LRRs) contain smaller numbers of LRRs and function as co-receptors; and BIR1 and BIR2 (4 LRRs each) function as regulatory proteins in the immune complex. RLK902 belongs to the LRR-RLK subfamily, with only 5 LRRs, a transmembrane domain and an intracellular kinase domain. Because RLK902 contains only a few LRRs, it is unlikely that it is a receptor; rather, it presumably functions as a co-receptor or as a signaling protein that helps transmit the immune signal to downstream components by phosphorylation.

The Arabidopsis Columbia genome encodes 225 LRR-RLKs. Recently, an extracellular network of Arabidopsis LRR-RLKs was established by examining the interactions of 200 LRR-RLKs with ECDs. More than 2,500 interactions, including 567 high-confidence interactions, were identified. Among the high-confidence interactions, several LRR-RLKs were identified as interacting with the ECD of RLK902, including SOBIR1 and PSKR1, which are known components in plant immunity, as well as some LRR-RLKs that have not been functionally characterized (Smakowska-Luzan et al., 2018). SOBIR1 is a co-receptor for many receptor-like proteins (Albert et al., 2015; Gust and Felix, 2014), and PSKR1 is a receptor for PSK, which is involved in plant immunity (Mosher and Kemmerling, 2013). It would be interesting to study how RLK902 is involved in SOBIR1- and PSKR1-mediated defense, and whether other RLK902-interacting LRR-RLKs contribute to plant immunity. Further characterization of RLK902-interacting proteins should shed new light on the role of RLK902 in plant immunity.

In this study, we showed that RLK902 associates with and phosphorylates BSK1 at Ser-230. Phosphorylation of BSK1 Ser-230 appears to be critical for RLK902-mediated resistance, as the BSK1<sup>S230D</sup> variant could partially rescue the enhanced susceptibility phenotype of the <i>rlk902</i> mutant. However, the inability of the BSK1<sup>S230D</sup> variant to fully
rescue the *rlk902* phenotype suggests that RLK902 may also transduce signals to other RLCKs or proteins that contribute to bacterial resistance independent of BSK1. Alternatively, RLK902 might phosphorylate other sites of BSK1 that were not identified in this study. This possibility was not supported by our *in vitro* kinase assay, as RLK902 did not phosphorylate the BSK1$^{S230A}$ variant. However, we could not rule out the possibility that RLK902 might phosphorylate BSK1 at other sites under certain specific conditions *in planta*.

Interestingly, Ser-230 of BSK1 is also the major phosphorylation site for BRI1 (Tang et al., 2008), an LRR-RK that perceives brassinosteroid plant hormones and plays essential roles in diverse growth and development processes (Li and Chory, 1997). How plants use the same component differently in plant immunity and development is not well understood. One explanation is that different RLKs may localize to distinct microdomains. Recently, it was shown that BRI1 and FLS2 localize at distinct plasma membrane nanodomains, suggesting that signaling specificity may result from the spatial separation of FLS2 and BRI1 in the plasma membrane (Bücherl et al., 2017). Both RLK902 and BRI1 associate with BSK1, but these two complexes may localize to different nanodomains and play distinct roles. In this scenario, even though RLK902 and BRI1 phosphorylate the same site of BSK1, they would transmit signals to downstream components to regulate different biological processes.

Previously, we showed that BSK1 associates with FLS2 and acts as a key component in the FLS2 immune complex, thereby transmitting immune signals upon flg22 treatment (Shi et al., 2013). However, the *rlk902* mutant did not show any defects in ROS burst when treated with flg22, indicating that RLK902 is not involved in this defense response as BSK1. Although RLK902 did not interact with either FLS2 or the co-receptor BAK1, we could not rule out the possibility that RLK902 interact with FLS2 or BAK1 in a...
ligand-induced manner to regulate FLS2-mediated defense responses other than ROS burst. It is equally possible that there is an RLK902-associated immune complex may sense undefined PAMPs and then transduce a signal to BSK1 to activate resistance. In addition, BSK1 is associated with FLS2 and plays key roles in FLS2-activated defense; however, it remains to be determined whether FLS2 or BAK1 phosphorylates BSK1, and whether the Ser-230 residue is the site of BSK1 phosphorylation by FLS2 or BAK1.

RLK902 and RKL1 share protein sequence similarity of more than 75% (Tarutani et al., 2004a); however, it seems that only RLK902 is involved in plant immunity. The \textit{rkl1} mutant, unlike the \textit{rlk902} mutant, showed a phenotype similar to that of wild-type plants when infected by bacterial pathogens. The \textit{rlk902 rkl1} double mutant did not display enhanced susceptibility compared with the \textit{rlk902} single mutant. Our study suggests that RLK902 and RKL1 are not functionally redundant in plant immunity. Consistent with this view, \textit{RLK902} and \textit{RKL1} have different expression patterns. \textit{RKL1} is mainly expressed in vascular tissues of roots, young rosette leaves, and floral organ abscission zones, whereas \textit{RLK902} displays a more global expression pattern (Wu et al., 2016). Their different expression patterns indicate the functional differentiation of these two RLKs. In addition, RLK902 and RKL1 interact with different LRR-RLKs (Smakowska-Luzan et al., 2018), also indicating distinct roles for RLK902 and RKL1.

In conclusion, we identified RLK902 as a key component of plant immunity. Our findings support a model in which EDR4 regulates vesicle trafficking and accumulation of RLK902. An RLK902-associated immune complex perceives signal from pathogens and transduces the immune signal to BSK1 by phosphorylation of Ser-230 of BSK1 (Figure 7).

\section*{Methods}
Plant Materials and Plant Growth Condition
The wild-type *Arabidopsis thaliana* used in this study is Col-0, and all mutants were in the Col-0 background. The *rlk902* (CS410869) and *rkl1* (Sail_525D09) mutants were ordered from the Arabidopsis Biological Resource Center. The *bsk1-1*, *edr4-1*, and *chc2-2* mutants were described previously (Shi et al., 2013; Wu et al., 2015). *A. thaliana* and *Nicotiana benthamiana* plants were grown in the growth room at 23°C with photoperiods of 9-h-day/15-h-night for phenotyping and 16-h-day/8-h-night for seed setting.

Pathogen Inoculation
*Pseudomonas syringae* strains were cultured on the King’s B medium containing rifampicin. Leaves from four-week-old plants were infiltrated with suspensions of *P. syringae* strains (OD$_{600}$ = 0.0005 in 10 mM MgCl$_2$ for leaf infiltration and OD$_{600}$ = 0.1 for spray inoculation). *Pto* DC3000 inoculation was performed as described (Djoumovic et al., 2013; Mengiste et al., 2003).

Real-time PCR Analysis
Total RNA extraction was performed with Ultrapure RNA Kit (CWBIO). First-strand cDNA from 2 µg of total RNA was synthesized using FastQuant RT Kit (TIANGEN). Realtime PCR assays were performed as previously described (Nie et al., 2012).

Yeast Two-hybrid Assays
The indicated vectors were co-transformed into yeast strain Y190, and positive clones were selected on synthetic defined (SD)/–Leu/–Trp medium. Clones selected randomly were cultured on SD/–Leu/–Trp/–His/–Ade medium and stained with 40 mg/mL X-Gal (Clontech) to determine β-galactosidase activity. Coding sequences (CDS) of genes were amplified and ligated into pGADT7 or pGBKT7. Plasmids carrying *EDR4* and its
truncated forms were described previously (Wu et al., 2015).

**Plant Protein Extraction and Co-IP Assay**

Leaves of *N. benthamiana* or four-week-old transgenic *Arabidopsis* were collected and ground in liquid nitrogen. Total proteins were extracted using native extraction buffer (50 mM Tris-MES (pH 8.0), 0.5 M sucrose, 1 mM MgCl₂, 10 mM EDTA, 5 mM DTT, 1% (v/v) NP-40 and 1% (w/v) protease inhibitor cocktail S8830 (Sigma)). For co-IP assays, 25 µL GFP-Trap Magnetic Agarose (Chromotek) was added to 1 mL of total protein extract for a 2-hour incubation. After incubation, magnetic agarose was washed 3 times with cold PBS buffer containing 0.1% (v/v) Triton X-100. The agarose beads were resuspended in 100 µL PBS buffer with SDS loading buffer for immunoblotting. Immunoblotting was performed as described previously (Zhao et al., 2014).

**Split-Luciferase Complementation Assay**

*Agrobacterium tumefaciens* strains GV3101 containing the indicated plasmids was infiltrated into leaves of *N. benthamiana* and incubated in the growth room for 72 hours. 1 mM luciferin was sprayed onto the leaves for CCD imaging. The details of this assay have been described previously (Chen et al., 2008).

**Bimolecular Fluorescence Complementation (BiFC)**

The coding sequences of corresponding genes were cloned into two intermediate vectors, pSY736 and pSY735, in-frame with the N-terminus and C-terminus of the yellow fluorescent protein (YFP). Then, the fusion sequences were amplified and cloned into the binary vector pMDC32 for expressing the fusion protein *in planta*. *Agrobacterium* strain GV3101 containing the respective plasmids were injected into 4-week-old *N. benthamiana* leaves. YFP signal were detected by confocal microscopy at 3 days after injection.
Confocal Microscopy

Seven-day-old seedlings and leaves from four-week-old plants of transgenic *Arabidopsis* were used in confocal imaging. Seedlings were pretreated with 2 µg/mL BFA or mock solution (1/2x Murashige and Skoog (MS) liquid medium) for 30 minutes and stained by FM4-64 (Invitrogen) for a few seconds before observation. The fluorescence signal was observed and photographed with a Zeiss LSM 710 NLO confocal microscope. Quantification of the ratio of fluorescence values was performed as described previously (Larson et al., 2017).

ROS Burst Measurement

ROS burst measurement was performed as described previously (Shi et al., 2013; Zhang et al., 2010). The luminescence was measured with a GLOMAX96 Luminometer (Promega). Leaves of 4-week-old plants were treated with 100 nM flg22 for ROS measurement.

Site-Directed Mutagenesis

PCR-based site-directed mutagenesis was used in this study. Primers for mutagenesis contained altered codons (Table S1). Constructs containing CDS or genomic sequence of genes of interest were used as amplified templates. Amplification products were transformed into *Trans-2* blue Chemically Competent Cells (Transgen Biotech).

Protein Purification

CDS of RLK902-KD and BSK1 were PCR-amplified and cloned into the pGEX-4T vector for GST fusions or into pET28a for His fusions. Plasmids were transformed into *E. coli* (BL21) and the cultures were grown at 37 °C grown to OD600 = 0.4-0.6. Next, 1 mM IPTG was added and the cultures were grown overnight at 16 °C for expression of
recombinant proteins. Recombinant proteins were purified according to the instructions of the manufacturers of His-tag affinity resin (Ni-NTA Agarose, QIAGEN) and GST-tag affinity resin (Glutathione Sepharose 4B, GE Healthcare).

**In Vitro Kinase Assays**

For the *in vitro* kinase assay, 2 µg BSK1/BSK1<sub>K104E</sub> and 4 µg RLK902 were used. Kinases and substrates were incubated at 30°C in 30 µL reaction buffer containing 25 mM Tris-HCl (pH 7.5), 10 mM MgCl<sub>2</sub>, 10 mM CaCl<sub>2</sub>, 10 mM MnCl<sub>2</sub> and 1 mM DTT, and 100 µM ATP (or 10 µCi [γ<sup>32</sup>P]ATP for autoradiogram detection) was added and the reactions further incubated for 30 min at 30 °C. The phosphorylated proteins were detected by autoradiography.

**Statistical Analysis**

Statistical comparison of quantitative assays of three independent experiments was performed using a mixed-effects model for nested ANOVA, implemented in R (Liu et al., 2016; Shen et al., 2017). The resulting ANOVA *P*-values were used to indicate significant differences between multiple groups.

**Primers used in this study**

The primers used in this study are listed in Supplemental Table 1.

**Accession Numbers**

Sequence data from this article can be found in The Arabidopsis Information Resource (TAIR) or GenBank/EMBL databases under the following accession numbers: *RLK902* (AT3G17840), *RLK1* (AT1G48480), *EDR4* (AT5G05190), *CHC2* (AT3G08530), *BSK1* (AT4G35230), *PR1* (AT2G14610), *PR2* (AT3G57260) and *ACTIN2* (AT3G18780).
Author contributions
D.T. and Y.Z. designed the experiments. Y.Z. performed most of the experiments. G.W. and H.S. assisted in materials preparation. D.T. and Y.Z. wrote the article.

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References


Figure Legends

Figure 1: EDR4 Positively Regulates Plant Resistance to Pseudomonas syringae.
(A-B) Bacterial growth in leaf samples measured at 3 hours (0 DPI) and 3 days post inoculation (3 DPI) in four-week-old plants of the indicated genotypes that were inoculated with a suspension (OD$_{600} = 0.0005$) of the virulent _P. syringae_ strain _Pto_ DC3000 (A) or the TTSS-deficient (avirulent) strain _Pto_ DC3000 _hrcC_ (B). WT: wild type. Results represent the mean and standard deviation (SD) in three independents experiments. cfu: colony-forming units. Lower-case letters indicate statistically significant differences ($P < 0.05; n = 9; ANOVA$).

(C-D) Transcript accumulation of _PR1_ and _PR2_ detected by quantitative reverse transcription PCR at 0 and 3 days after spray inoculation with _Pto_ DC3000 (OD$_{600} = 0.1$). _ACTIN2_ was used as an internal control. Two asterisks indicate statistically significant differences ($P < 0.01, n = 3, ANOVA$).

Figure 2: EDR4 Interacts with the Homologous Receptor-like Kinases RLK902 and RKL1 in planta.

(A) Images from a split-luciferase complementation imaging (LCI) assay of _N. benthamiana_ leaves 3 days after the plants were transformed with constructs consisting of EDR4 fused with N-terminal fragment of firefly luciferase (Nluc) and either RLK902 or RKL1 fused with a C-terminal (Cluc). The indicated constructs were transiently co-expressed. EV: empty vector.

(B) Luminescence (in relative luminescence units, RLU) in the same plants measured 3 days later for quantitative interaction determination. Results represent the mean and SD in three independents experiments. Two asterisks indicate statistically significant differences ($P < 0.01, ANOVA, n \geq 8$).

(C) RLK902 and RKL1 interact with EDR4 in Arabidopsis. Top two rows show results of immunoprecipitation (IP) and co-immunoprecipitation (co-IP) assays of leaf samples from transgenic plants expressing or co-expressing RLK902-GFP, RKL1-GFP and EDR4-HA. Bottom two rows show protein detection by western blot assay (WB) with
α-GFP or α-HA antibodies, respectively.

(D) RLK902 and RKL1 interact with EDR4 in yeast two-hybrid assays. Yeast cells (OD$_{600}$ = 0.5) containing the indicated plasmids were spotted onto SD/–Trp/–Leu or SD/–Trp/–Leu/–His/–Ade/X-α-Gal medium, as indicated. KD: kinase domain. CC: coiled-coil domain. LCR: low-complexity regions. AD and BD stand for pGADT7 and pGBK7.

Figure 3: RLK902 Contributes to Plant Resistance to *Pseudomonas syringae*.

(A-B) Bacterial density in leaf samples taken at 3 hours (0 DPI) and 3 days post inoculation (3 DPI) in four-week-old plants of the indicated genotypes that were inoculated with a suspension (OD$_{600}$ = 0.0005) of the virulent *P. syringae* strain *Pto* DC3000 (A) or the TTSS-deficient strain *Pto* DC3000 *hrcC* (B). WT: wild type. Results represent the mean and SD in three independents experiments. cfu: colony-forming units. Lower-case letters indicate statistically significant differences ($P < 0.05$; $n = 9$; ANOVA).

(C-D) Transcriptional accumulation of *PR1* (C) and *PR2* (D) detected by quantitative realtime PCR at 0 and 3 DPI with *Pto* DC3000 (OD$_{600}$ = 0.1). *ACTIN2* was used as an internal control. Two asterisks represent statistically significant differences ($P < 0.01$, $n = 3$, ANOVA).

Figure 4: EDR4 Modulates the Subcellular Trafficking and Protein Accumulation of RLK902.

(A) The intercellular trafficking of RLK902 is dependent on EDR4 and CHC2. Images obtained by confocal microscopy of 7-day-old transgenic seedlings of the indicated genotypes are shown. Transgenic plants of different backgrounds from that expressing RLK902-GFP were used in this assay. Single optical sections (GFP signals and plasma membrane stained by FM4-64) and merge images are shown. Scale bar = 20 µm. The magnified images of the boxed areas are shown at top right and scale bar of these images
is 5 μm.

(B) Quantitative analysis of trafficking interference caused by mutation of EDR4 and CHC2. The bars showed the proportion of intracellular GFP fluorescence intensity ($F_{\text{internal}}$) in the entire cell ($F_{\text{total}}$; total GFP intensity of cells including the cell membrane and cytoplasm). Lower-case letters indicate statistically significant differences ($P < 0.05$, $n = 50$, ANOVA). CK: transgenic seedlings without BFA treatment, BFA: transgenic seedlings treated with BFA.

(C) Transcript levels of $RLK902$ detected by quantitative reverse transcription PCR. $ACTIN2$ was used as an internal control. Lower-case letters indicate statistically significant differences ($P < 0.05$; $n = 3$; ANOVA). ns: nonsignificant. HPI: hours post inoculation.

(D) Protein levels of RLK902 detected by western blot assay with α-GFP antibody. Plant actin detected by α-ACTIN antibody is shown as a protein loading control.

(E) Quantitative analysis of protein accumulation by band intensity measurement. The data are shown as the mean ± SD (from three repeated experiments).

Figure 5: RLK902 Physically Associates with BSK1.

(A) Image from a split-luciferase complementation imaging (LCI) assay of $N. benthamiana$ leaves 3 days after the plant was transformed with constructs consisting of BSK1 and RLK902 fused with the N- and C-terminal fragments of firefly luciferase (Nluc and Cluc), respectively. The indicated constructs were transiently co-expressed.

(B) Luminescence (in relative luminescence units, RLU) in the same plants measured 3 days later for quantitative interaction determination (B). Two asterisks indicate statistically significant differences ($P < 0.01$, ANOVA, $n \geq 8$).

(C) RLK902 interacts with BSK1 in yeast two-hybrid assays. Yeast cells (OD$_{600} = 0.5$) containing the indicated plasmids were spotted onto the indicated medium. AD and BD stand for pGADT7 and pGBK7.
(D) RLK902 interacts with BSK1 in *Arabidopsis*. Top two rows show results of immunoprecipitation (IP) and co-immunoprecipitation (co-IP) assays of leaf samples from transgenic plants expressing or co-expressing the constructs RLK902-GFP or BSK1-MYC in *Arabidopsis thaliana*. Bottom two rows show protein detection by western blot assay (WB) with α-GFP or α-MYC antibodies, respectively. *Pto** DC3000 hrcC* treatment was performed by spray inoculation with bacteria suspension (OD$_{600}$ = 0.1) and sampled 3 hours after inoculation.

**Figure 6: RLK902 Phosphorylates BSK1.**

(A) RLK902 phosphorylates BSK1 *in vitro*. Above, autoradiogram showing phosphorylation of the RLK902-KD-GST, BSK1-His and mutated BSK1 recombinant proteins, expressed in *E. coli* strain BL21 (DE3), that were incubated in an *in vitro* kinase assay using radioactive isotopes. Below, input protein visualized by Coomassie brilliant blue staining (CBB).

(B) BSK1$^{S230A}$-GFP and BSK1$^{S230D}$-GFP fusion proteins of transgenic plants were detected by immunoblot analysis with α-GFP antibody. Plant actin detected by α-ACTIN antibody is showed as a protein loading control and dm stands for the *rlk902 bsk1-1* double mutant.

(C) Ser-230 of BSK1 is required for RLK902-mediated resistance to *Pto** DC3000. The graph shows bacterial growth in leaf samples taken at 3 days post inoculation (DPI) from plants injected with a suspension of *Pto** DC3000 (OD$_{600}$ = 0.0005). Lower-case letters indicate statistically significant differences (P < 0.05; n = 9; ANOVA). dm stands for the *rlk902 bsk1-1* double mutant.

(D) The *rlk902 bsk1-1* mutant displayed similar susceptibility to the *bsk1-1* mutant in response to *Pto** DC3000. The graph shows bacterial density in leaf samples taken at 3 dpi from plants injected with a suspension of *Pto** DC3000 (OD$_{600}$ = 0.0005). Lower-case letters indicate statistically significant differences (P < 0.05; n = 9; ANOVA). dm stands
for the *rlk902 bsk1-1* double mutant.

**Figure 7: Working Model**

A working model summarizing the main results of this work. In wild-type plants, EDR4 regulates subcellular trafficking and accumulation of RLK902. RLK902 transmits immune signals to BSK1 by direct phosphorylation. In the *edr4* mutant, subcellular trafficking and accumulation of RLK902 are affected, and the plants display weakened immune responses.

**Supplemental Information**

**Figure S1: Phylogenetic Tree Analysis Based on Amino Acid Sequence of RLK902.**

The phylogenetic tree was drawn using the neighbor-joining method (using MEGA software). The scale bar and branch length values indicate the average number of amino acid substitutions per site.

**Figure S2: RLK902 RNA Interference Lines Display Defects in Resistance to *Pseudomonas syringae*.**

(A) Diagrams of protein structure showing the main domains of RLK902 and RKL1. LRR: Leucine-rich repeat domain. TM: transmembrane domain. KD: kinase domain.

(B) Gene structure diagrams of *RLK902* and *RKL1*. T-DNA insertions used in this study are marked.

(C-D) Semi-quantitative PCR. *ACTIN2* was used as internal control.

(E) Two RNAi lines show significant decreases in *RLK902* expression. Relative transcript levels of *RLK902* were detected by quantitative realtime PCR. *ACTIN2* was used as internal control. Lower-case letters indicate statistically significant differences (*P* < 0.05; *n* = 3; ANOVA).

(F-G) Four-week-old plants were inoculated with *Pto* DC3000 (F) and *Pto* DC3000 *hrcC*
(G) Leaves were injected with bacteria suspension (OD$_{600} = 0.0005$) and sampled at 3 hours (0 DPI) and 3 days past inoculation (3 DPI). Data are shown as mean ± SD. cfu: colony-forming units. Lower-case letters indicate statistically significant differences ($P < 0.05$; $n = 9$; ANOVA).

Figure S3: RLK902 and RKL1 Do Not Show Functional Redundancy in Resistance to Pto DC3000 and Powdery Mildew.

(A-D) Bacterial density in leaf samples taken 3 hours (0 DPI) and 3 days post inoculation (3 DPI) in four-week-old plants of the indicated genotypes that were inoculated with a suspension (OD$_{600} = 0.0005$) of Pto DC3000 (A, C and D) or Pto DC3000 hrcC (B). Genotypes were wild-type (WT, Col-0 ecotype), single mutants (rkl1, rlk902 edr4-1), double mutants (rlk902 rkl1, edr4-1 rlk902, edr4-1 rkl1) and triple mutants (edr4-1 rlk902 rkl1). Lower-case letters indicate statistically significant differences ($P < 0.05$; $n = 9$; ANOVA).

(E) Four-week-old Arabidopsis plants of the indicated WT and mutant strains infected with G. cichoraceous. The infected leaves were photographed (scale bars = 1 cm) and stained with trypan blue for fungal structure observation (scale bars = 100 µm) 8 days after infection.

(F) Growth quantification of powdery mildew. Leaves were collected 5 days after infection. The number of conidiophores per colony was counted. Data are displayed as mean ± SD. Lower-case letters indicate statistically significant differences ($P < 0.05$; $n = 25$; ANOVA).

Figure S4: Overexpression of RLK902 Contributes to Enhancing Plant Resistance.

(A) Uninfected four-week-old plants grown under short-day conditions were photographed. Scale bar = 1 cm.
(B) The transcript levels of *RLK902* were detected by quantitative realtime PCR. *ACTIN2* was used as an internal control. OE-1 and OE-2 refer to two RLK902 overexpression lines. Lower-case letters indicate statistically significant differences ($P < 0.05$, $n = 3$, ANOVA).

(C) Bacterial density in leaf samples taken at 3 days post inoculation with a suspension of *Pto DC3000* ($OD_{600} = 0.0005$). Lower-case letters indicate statistically significant differences ($P < 0.05$, $n = 9$, ANOVA).

(D-E) Transcript levels of *PR1* (C) and *PR2* (D) detected by quantitative realtime PCR in extracts from uninfected plant leaves. *ACTIN2* was used as an internal control. Lower-case letters indicate statistically significant differences ($P < 0.01$, $n = 3$, ANOVA).

Figure S5: RLK902 Interacts with CHC2, but EDR4 and CHC2 Do Not Affect the Localization of RLK902.

(A-B) RLK902 interacts with CHC2 in *Nicotiana benthamiana*. (A) Split-luciferase complementation imaging (LCI) assay of leaves 3 days after the plants were transformed with constructs expressing full-length proteins fused with Nluc or Cluc. The indicated constructs were transiently co-expressed in *N. benthamiana*. EV: empty vector. (B) Luminescence (in relative luminescence units, RLU) in the same plants was measured 3 days later for quantitative interaction determination. Asterisks indicate statistically significant differences ($P < 0.01$, ANOVA, $n \geq 8$).

(C) RLK902 interacts with CHC2 in a co-IP assay. The constructs, RLK902-GFP and CHC2-N-MYC, were transiently expressed or co-expressed in *N. benthamiana*. Protein was detected by western blot assay (WB) with $\alpha$-GFP or $\alpha$-MYC antibodies, respectively.

(D) The interactions between EDR4, CHC2 and RLK902 were examined with the BiFC assay in *N. benthamiana*. Proteins were fused to the N-terminal or C-terminal of YFP (YN and YC) respectively. The *N. benthamiana* leaves were infiltrated with *A. tumefaciens* strain GV3101 containing the indicated constructs pairs. YFP signal was
detected by confocal microscopy. Bar = 20 µm.

(F) Leaves of 7-day-old transgenic seedlings that expressed RLK902-GFP were imaged by confocal microscopy. Plasma membrane was stained with FM4-64. Both single optical and merged images are shown. Scale bar = 20 µm.

(G) Relative expression levels of PRI and PR2 were detected by quantitative realtime PCR. ACTIN2 was used as internal control. Asterisk indicates statistically significant differences ($P < 0.05; n = 3; ANOVA$).

(H) Four-week-old plants were inoculated with Pto DC3000 (bacteria suspension, OD$_{600}$ = 0.0005) and sampled at 3 days past inoculation (3 DPI). Data are shown as mean ± SD. cfu: colony-forming units. Asterisk indicates statistically significant differences ($P < 0.05; n = 9; ANOVA$).

Figure S6: RLK902 Do not Interact with FLS2, BAK1 or BIK1.

(A) LCI assay to detect interaction between RLK902 and components of the FLS2 PRR complex. The indicated constructs were transiently co-expressed in N. benthamiana, and the LCI assay was carried out 3 days later. EV: empty vector.

(B) Luminescence (in relative luminescence units, RLU) was measured 3 days later for quantitative interaction determination. Asterisks indicate statistically significant differences ($P < 0.01, ANOVA, n \geq 8$).

(C) Yeast two-hybrid assay to detect interaction between RLK902 and FLS2, BAK1 or BIK1. Yeast cells (OD$_{600}$ = 0.5) containing the indicated plasmids were spotted onto the indicated medium. Photographs were taken 5 days after incubation. AD and BD stand for pGADT7 and pGBK7.

(D) Measurement of flg22-induced reactive oxygen species (ROS) burst. Total photon counts during 30 min of flg22 treatment are presented to indicate ROS production. Lower-case letters indicate statistically significant differences ($P < 0.01, n = 12, ANOVA$).
Figure S7: The Enhanced Resistance Induced by *RLK902* Overexpression Is Partially Dependent on BSK1.

(A) Uninfected four-week-old plants grown under short-day conditions were photographed. Scale bar = 1 cm.

(B) The number of bacteria was counted at 3 days post inoculation with a suspension of *Pto* DC3000 (OD$_{600} = 0.0005$). Lower-case letters indicate statistically significant differences ($P < 0.05, n = 9$, ANOVA).

Table S1: Primers used in this study.
Figure 1

A

\[
\text{Log}_{10}(\text{cfu/cm}^2)
\]

\[0\ \text{DPI} \quad 3\ \text{DPI}\]

\(\text{Pto DC3000}\)

B

\[
\text{Log}_{10}(\text{cfu/cm}^2)
\]

\[0\ \text{DPI} \quad 3\ \text{DPI}\]

\(\text{Pto DC3000 hrc}^-\)

C

\[
\text{Relative Expression}
\]

\[0\ \text{DPI} \quad 3\ \text{DPI}\]

\(\text{PR1/ACTIN2}\)

D

\[
\text{Relative Expression}
\]

\[0\ \text{DPI} \quad 3\ \text{DPI}\]

\(\text{PR2/ACTIN2}\)
Figure 2

A

RLK902    EV    RKL1    EV

EDR4-Nluc

B

Relative Luminescence

[Graph showing luminescence levels for RLK902, RKL1, EV, and EDR4-Nluc.]

C

RLK902-GFP  -  +  -  +  -
RKL1-GFP    -  -  +  -  +
EDR4-HA     +  +  +  -  -

Co-IP: GFP
IP: HA
WB: GFP
WB: HA

D

RLK902    RKL1    RLK902-KD    RKL1-KD

EDR4    CC    LCR    Dur3133    BD

SD/-Leu/-Trp

EDR4    CC    LCR    Dur3133    BD

SD/-Leu/-Trp/-His/-Ade/X-α-Gal
Figure 3

(A) Pto DC3000

(B) Pto DC3000 hrcC−

(C) PR1/ACTIN2

(D) PR2/ACTIN2
Figure 4

A

<table>
<thead>
<tr>
<th>RLK902-GFP</th>
<th>FM4-64</th>
<th>Merge</th>
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</thead>
<tbody>
<tr>
<td>WT</td>
<td>CK</td>
<td></td>
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<tr>
<td>edr4-1</td>
<td>BFA</td>
<td></td>
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<tr>
<td>chc2-2</td>
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</tbody>
</table>

B

![Graph showing relative expression](image)

C

![Relative Expression](image)

D

<table>
<thead>
<tr>
<th>Pto DC3000</th>
<th>0</th>
<th>3 HPI</th>
<th>24 HPI</th>
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<tbody>
<tr>
<td>WT</td>
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<td></td>
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<tr>
<td>edr4-1</td>
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</tr>
<tr>
<td>chc2-2</td>
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</tr>
</tbody>
</table>

E

![Band Intensity](image)
Figure 5

A

RLK902 EV
BSK1-Nluc

B

Relative Luminescence

2500
2000
1500
1000
500
0

RLK902 Cluc
EV BSK1

C

BSK1 BD
SD/-LW
BSK1 BD
SD/-LWH/-Ade
/X-α-Gal

D

RLK902-GFP
BSK1-MYC
Pto DC3000 hrcC*

co-IP: MYC
IP: GFP
Input
WB: MYC
Input
WB: GFP
**Figure 6**

A

<table>
<thead>
<tr>
<th>Treatment</th>
<th>RLK902-KD</th>
<th>BSK1</th>
<th>BSK1&lt;sub&gt;K104E&lt;/sub&gt;</th>
<th>BSK1&lt;sub&gt;K104E5230A&lt;/sub&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>RLK902-KD</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>BSK1</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>BSK1&lt;sub&gt;K104E&lt;/sub&gt;</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>BSK1&lt;sub&gt;K104E5230A&lt;/sub&gt;</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

**Autoradiogram**

- pRLK902-KD
- pBSK1

**Input CBB**

- RLK902-KD
- BSK1/mBSK1

B

<table>
<thead>
<tr>
<th>Treatment</th>
<th>BSK1&lt;sub&gt;K104E&lt;/sub&gt;/dm L1</th>
<th>BSK1&lt;sub&gt;K104E&lt;/sub&gt;/dm L2</th>
<th>BSK1&lt;sub&gt;K104E5230A&lt;/sub&gt;/dm L1</th>
<th>BSK1&lt;sub&gt;K104E5230A&lt;/sub&gt;/dm L2</th>
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</thead>
<tbody>
<tr>
<td>α-GFP</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>α-ACTIN</td>
<td>+</td>
<td>+</td>
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<td>+</td>
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C

**Pto DC3000 3DPI**

<table>
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<tr>
<th>Treatment</th>
<th>Log&lt;sub&gt;10&lt;/sub&gt; (cfu/cm&lt;sup&gt;2&lt;/sup&gt;)</th>
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</thead>
<tbody>
<tr>
<td>WT</td>
<td>a</td>
</tr>
<tr>
<td>rlk902</td>
<td>b</td>
</tr>
<tr>
<td>dm</td>
<td>c</td>
</tr>
<tr>
<td>BSK1&lt;sub&gt;K104E&lt;/sub&gt;/dm L1</td>
<td>d</td>
</tr>
<tr>
<td>BSK1&lt;sub&gt;K104E5230A&lt;/sub&gt;/dm L1</td>
<td>d</td>
</tr>
<tr>
<td>BSK1&lt;sub&gt;K104E5230A&lt;/sub&gt;/dm L2</td>
<td>d</td>
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</table>

D

**Pto DC3000 3DPI**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Log&lt;sub&gt;10&lt;/sub&gt; (cfu/cm&lt;sup&gt;2&lt;/sup&gt;)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>a</td>
</tr>
<tr>
<td>bsk1-1</td>
<td>b</td>
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<td>rlk902</td>
<td>c</td>
</tr>
<tr>
<td>dm</td>
<td>b</td>
</tr>
</tbody>
</table>
Figure 7

Wild type

edr4 Mutants

Subcellular Trafficking

Normal Immune Responses

Weakened Immune Responses

EDR4

RLK902

BSK1

Mislocalization

Immune signal

Immune signal

Subcellular Trafficking

EDR4

BSK1

RLK902