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A G-type lectin receptor-like kinase in *Nicotiana benthamiana* enhances resistance to the fungal pathogen *Sclerotinia sclerotiorum* by complexing with CERK1/LYK4

Lei Pi[1], Yifan Zhang[1], Jinghao Wang[1], Nan Wang[1], Zhiyuan Yin[1,2*] and Daolong Dou[1,2]

Abstract

Fungal pathogens are among the main destructive microorganisms for crops and ecosystems worldwide, causing substantial agricultural and economic losses. Plant cell surface-localized lysin motif (LysM)-containing receptor-like kinases (RLKs) or receptor-like proteins (RLPs) enhance plant resistance to fungal pathogens via sensing chitin, which is a conserved component of the fungal cell wall. Other types of RLKs also regulate chitin signaling via distinct mechanisms in plants. In this study, we identified a G-type lectin RLK, NbERK1, which positively regulated chitin signaling and resistance to the fungal pathogen *Sclerotinia sclerotiorum* in the model plant *Nicotiana benthamiana*. In addition, the LysM-RLK NbCERK1/NbLYK4 was shown to mediate plant resistance to *S. sclerotiorum* positively. Further, the association of chitin-induced NbCERK1-NbLYK4 was found to be essential for chitin perception and signaling. Importantly, NbERK1 was associated with NbCERK1/NbLYK4 and positively regulated chitin-induced NbCERK1-NbLYK4 association. Moreover, chitin could induce the dissociation of NbERK1 from the NbCERK1-NbLYK4 complex. Also, the kinase activity of NbERK1 was likely essential for this dissociation and plant resistance-enhancing activity of NbERK1. Together, these results suggest that NbERK1 is a novel component of the chitin receptor complex and enhances plant resistance to fungal pathogens via regulating chitin signaling.

Keywords *Sclerotinia sclerotiorum*, Chitin, LysM-RLK, G-type lectin RLK, Pattern-triggered immunity

Background

Plants rely on two layers of the immune system to resist pathogen infection: pattern-triggered immunity (PTI) and effector-triggered immunity (ETI) (Jones and Dangl 2006). Plant surface-localized pattern recognition receptors (PRRs) activate the downstream signaling pathway involved in PTI response after recognizing the conserved pathogen-associated molecular patterns (PAMPs), such as bacterial flg22, fungal chitin, and oomycete necrosis and ethylene-inducing peptide 1-like proteins (NLPs) (Chinchilla et al. 2006; Monaghan and Zipfel 2012; Oome et al. 2014; Shinya et al. 2015). PTI is a key step in priming the plant immune system, and it can successfully counter the invasion of most microorganisms. PRRs are classified into receptor-like kinases (RLKs) and receptor-like proteins (RLPs) (Couto and Zipfel 2016). RLKs contain an extracellular domain (ECD) potentially involved in ligand perception, a transmembrane (TM) domain, and a cytoplasmic kinase domain (CD), while RLPs contain an ECD and TM domains, lacking CD (Shiu and Bleecker 2003;

*Correspondence:
Zhiyuan Yin
zyin@njau.edu.cn
[1] College of Plant Protection, China Agricultural University, Beijing 100193, China
[2] College of Plant Protection, Nanjing Agricultural University, Nanjing 210095, China

Jamieson et al. 2018). Plant RLKs and RLPs can be classified into various types according to their ECD, such as the leucine-rich repeat (LRR) type, lectin motif type, lysine motif (LysM) type, malectin-like domain type, and epidermal growth factor (EGF)-like motif type (Couto and Zipfel 2016; Tang et al. 2017; Saijo et al. 2018).

Fungi are the primary destructive pathogens of terrestrial plants and cause severe damage to crops on a global scale (Fisher et al. 2018). Chitin is an insoluble polymer of β-1,4-linked *N*-acetylglucosamine, a highly conserved structural component of fungal cell walls. It is the best-known PAMP in fungi (Shinya et al. 2015; Bressendorff et al. 2016; Kawasaki et al. 2017). During the fungal invasion, chitin is perceived by LysM-containing RLKs (LysM-RLKs) or RLPs (LysM-RLPs), and subsequently, the immune responses are initiated, ultimately leading to plant resistance to fungi (Tanaka et al. 2013; Gong et al. 2020). In *Arabidopsis*, the LysM-RLKs family has five members (AtCERK1, AtLYK2, AtLYK3, AtLYK4, and AtLYK5) involved in chitin perception and signaling. AtLYK5 is the major chitin receptor, while AtCERK1 is an indispensable chitin coreceptor (Gong et al. 2020). AtLYK5 binds to chitin with a higher affinity than AtCERK1 does. Interestingly, chitin induces the association between AtLYK5 and AtCERK1 (Cao et al. 2014; Xue et al. 2019; Wang et al. 2020). AtLYK4, a minor chitin receptor, bears a certain affinity to chitin and associates with both AtLYK5 and AtCERK1 (Cao et al. 2014). Notably, only AtCERK1 is an active kinase, and chitin-induced phosphorylation and activation of AtCERK1 are essential for chitin signaling (Miya et al. 2007; Cao et al. 2014). In rice, LysM-RLP OsCEBiP is the major chitin receptor with a high affinity to chitin (Kaku et al. 2006; Hayafune et al. 2014). OsCERK1 (ortholog of AtCERK1) is a chitin coreceptor but lacks chitin binding ability. It associates with OsCEBiP upon chitin perception (Shimizu et al. 2010). LysM-RLPs, OsLYP4, and OsLYP6, are minor chitin receptors and bear moderate affinity to chitin (Liu et al. 2012). GhLYK5 is the major chitin receptor in cotton, while GhCERK1 is a chitin coreceptor. Wall-associated kinase GhWAK7A phosphorylates GhLYK5 and regulates the chitin-induced association of GhCERK1 and GhLYK5 (Wang et al. 2020). *Nicotiana benthamiana* is a model plant for studying plant–pathogen interactions. Its CERK1 (NbCERK1) is reported to be a putative chitin receptor and positively regulates chitin signaling (Segonzac et al. 2011). However, other NbLYKs that regulate chitin signaling in *N. benthamiana* are unknown.

The lectin RLKs (LecRLKs) are named due to the presence of the lectin/lectin-like extracellular domain, which can bind to fungal and bacterial cell wall components (Vaid et al. 2013; Sun et al. 2020). Based on the different extracellular lectin domains, LecRLKs can be classified into L-type, G-type, and C-type (Sun et al. 2020). Compared with L-type lectin RLKs, the involvement of G-type lectin RLKs is less investigated in plant disease resistance. In rice, the G-type LecRLK, Pi-d2, was reported to provide resistance against the fungal pathogen *Magnaporthe grisea* (Chen et al. 2006). OsLecRK could activate multiple immune signaling pathways to confer plant defenses against *M. grisea* and bacterial blight pathogen *Xanthomonas oryzae* pv. *oryzae (Xoo)* (Cheng et al. 2013). SDS2 positively regulated plant resistance to *M. grisea* by interacting with the receptor-like cytoplasmic kinases OsRLCK118/176 (Fan et al. 2018). In *N. benthamiana*, NbLRK1 positively regulated plant resistance to *Phytophthora infestans* via interacting with *P. infestans* elicitin INF1 and mediating INF1-induced cell death (Kanzaki et al. 2008). *Nicotiana tobacum* Nt-Sd-RLK modulated plant disease resistance by regulating bacterial lipopolysaccharide (LPS)-induced immune responses (Sanabria et al. 2012). In *Arabidopsis*, LORE was a direct receptor of bacterial LPS, and LPS could induce LORE auto-phosphorylation. LORE enhanced plant resistance to *Pseudomonas syringae* via the perception of LPS (Ranf et al. 2015). *N. benthamiana* ERK1 positively regulated plant resistance to *Phytophthora* spp. by mediating PTI responses induced via *Phytophthora* expansin-like protein PcEXLX1 (Pi et al. 2022). However, the function and mechanism of ERK1 in regulating plant resistance to other pathogens remain unclear.

In this study, we found that NbERK1, NbCERK1, and NbLYK4 were involved in plant resistance to the fungal pathogen *Sclerotinia sclerotiorum* and mediated chitin-induced immune responses in *N. benthamiana* using virus-induced gene silencing (VIGS) assays. NbERK1 complexed with NbCERK1/NbLYK4 and regulated chitin-induced NbCERK1-NbLYK4 association. Furthermore, the predicted kinase catalytic active site (D644) was found to be essential for NbERK1 dissociation from NbCERK1-NbLYK4 complex upon chitin perception and NbERK1-enhanced plant resistance. Our results showed that a G-type lectin RLK, NbERK1, enhances plant resistance to this fungal pathogen via regulating chitin signaling.

Results

NbERK1 regulates plant resistance to *S. sclerotiorum* and chitin-induced immune responses in *N. benthamiana*

Recently, we demonstrated that the G-type lectin RLK, NbERK1, positively regulates plant resistance to the oomycete pathogen *Phytophthora capsici* by mediating the perception of the apoplastic expansin-like protein PcEXLX1 (Pi et al. 2022). The extracellular domains of LecRLKs are similar to lectin proteins, which can bind to fungal and bacterial cell wall components (Vaid et al.

2013). These observations prompted us to examine the involvement of NbERK1 in plant resistance against fungal and bacterial pathogens.

To determine whether NbERK1 is involved in plant resistance against the fungal pathogen *S. sclerotiorum*, two gene silencing constructs (TRV:*NbERK1* and TRV:*NbERK1-1*) were generated targeting different regions of *NbERK1* (Niben101Scf05948g04005.1). Three weeks after agroinfiltration, RT-qPCR analysis confirmed that *NbERK1* was effectively silenced compared with TRV:*GFP*-treated plants (Additional file 1: Figure S1). The agroinfiltrated plants were inoculated with *S. sclerotiorum*, which revealed that plants treated with TRV:*NbERK1* and TRV:*NbERK1-1* exhibited significantly larger lesions than the TRV:*GFP*-treated plants (Fig. 1a, b). This result indicated that NbERK1 should be involved in plant resistance to *S. sclerotiorum*.

Like other PAMPs, chitin can induce some well-characterized immune responses, including the reactive oxygen species (ROS) burst, expression of PTI marker genes, and activation of mitogen-activated protein kinase (MAPK) (Heese et al. 2007; Lloyd et al. 2014; Yu et al. 2017). To determine whether NbERK1 is required for chitin-induced immune responses, we first examined the expression pattern of *NbERK1* upon chitin treatment in *N. benthamiana*. The RT-qPCR analysis showed that *NbERK1* was induced at 1 h post chitin treatment (Fig. 1c). We then measured chitin-induced immune responses in plants treated with TRV:*GFP*, TRV:*NbERK1*, or TRV:*NbERK1-1*. Chitin-induced MAPK activation was compromised in plants treated with TRV:*NbERK1* or TRV:*NbERK1-1* at 5 and 15 min compared with TRV:*GFP*-treated plants (Fig. 1d). Chitin-induced ROS production was also weakened in TRV:*NbERK1*- and TRV:*NbERK1-1*-treated plants compared with the control (Fig. 1e). Consistently, the relative expression of chitin-induced PTI marker genes (*PTI5*, *Acre31*, *WRKY7*, and *WRKY8*) was also reduced in TRV:*NbERK1*- and TRV:*NbERK1-1*-treated plants (Fig. 1f and Additional file 1: Figure S2). Collectively, these findings indicated that NbERK1 regulates chitin-induced immune responses in *N. benthamiana*.

NbERK1 is dispensable for plant resistance to *Pst* DC3000 mutant *ΔhopQ1* and flg22-induced immune responses

To determine whether NbERK1 regulates plant resistance against any bacterial pathogen, we inoculated TRV:*GFP*- and TRV:*NbERK1*-treated plants with *Pseudomonas syringae* pv. *tomato* (*Pst*) DC3000 mutant *ΔhopQ1* (Wei et al. 2007). The result revealed that the bacterial number showed no significant difference in the *GFP*- and *NbERK1*-silenced plants (Additional file 1: Figure S3). Flagelin 22 (flg22), a 22-amino-acid peptide, is the best-studied bacterial PAMP (Felix et al. 1999). The RT-qPCR analysis demonstrated that flg22 did not induce *NbERK1* expression in the early stage (Additional file 1: Figure S3). To verify whether NbERK1 was involved in flg22-induced immune responses, ROS production and the relative expression of PTI marker genes (*PTI5* and *Acre31*) were measured in the *GFP*- and *NbERK1*-silenced plants. Silencing of *NbERK1* did not affect flg22-induced ROS production (Additional file 1: Figure S3) and the upregulation of *PTI5* and *Acre31* (Additional file 1: Figure S3) compared with control plants treated with TRV:*GFP*. The above results suggested that NbERK1 is dispensable for plant resistance to *Pst* DC3000 mutant *ΔhopQ1* and flg22-induced immune responses.

NbCERK1 and NbLYK4 are essential for chitin perception in *N. benthamiana*

To determine which *LYK* gene regulates chitin perception in *N. benthamiana*, we first constructed a phylogenetic tree of all LYK protein family members in *Arabidopsis* and *N. benthamiana* (Additional file 1: Figure S4). Next, the TRV VIGS constructs were generated to silence *NbCERK1*, *NbLYK2*, *NbLYK3*, *NbLYK4*, and *NbLYK5*. The RT-qPCR analyses confirmed that these genes were silenced effectively in TRV-treated plants except *NbLYK2*, and the expression of the other four *NbLYK* homologs was not affected in the corresponding silenced plants (Additional file 1: Figure S5).

The silenced plants were inoculated with *S. sclerotiorum*, revealing that *NbCERK1*- and *NbLYK4*-silenced plants exhibited significantly larger lesions than *GFP*-silenced plants (Fig. 2a, b). To further examine the functions of these *NbLYKs* in regulating *S. sclerotiorum* resistance, GFP, NbCERK1, NbLYK2, NbLYK4, and NbLYK5 each were transiently expressed in *N. benthamiana* (NbLYK3 was not successfully cloned). Subsequently, *S. sclerotiorum* was inoculated at the agroinfiltration sites after 24 h. The results showed that only the transient expression of NbCERK1 enhanced the resistance of plant to *S. sclerotiorum* (Additional file 1: Figure S6).

Chitin-induced ROS production was measured to determine which *NbLYK*-silenced plants showed altered responses to chitin treatment. The results showed that chitin-induced ROS production was remarkably compromised in *NbCERK1*- and *NbLYK4*-silenced plants and partially reduced in *NbLYK3*- and *NbLYK5*-silenced plants compared to TRV:*GFP*-treated control plants (Fig. 2c, d). These findings raised our interest in further exploring the functions of *NbCERK1* and *NbLYK4*. Consistent with the above results, chitin-induced MAPK activation was compromised at 5, 15, and 30 min in *NbCERK1*- and *NbLYK4*-silenced plants compared with TRV:*GFP*-treated plants (Fig. 2e). In addition, the

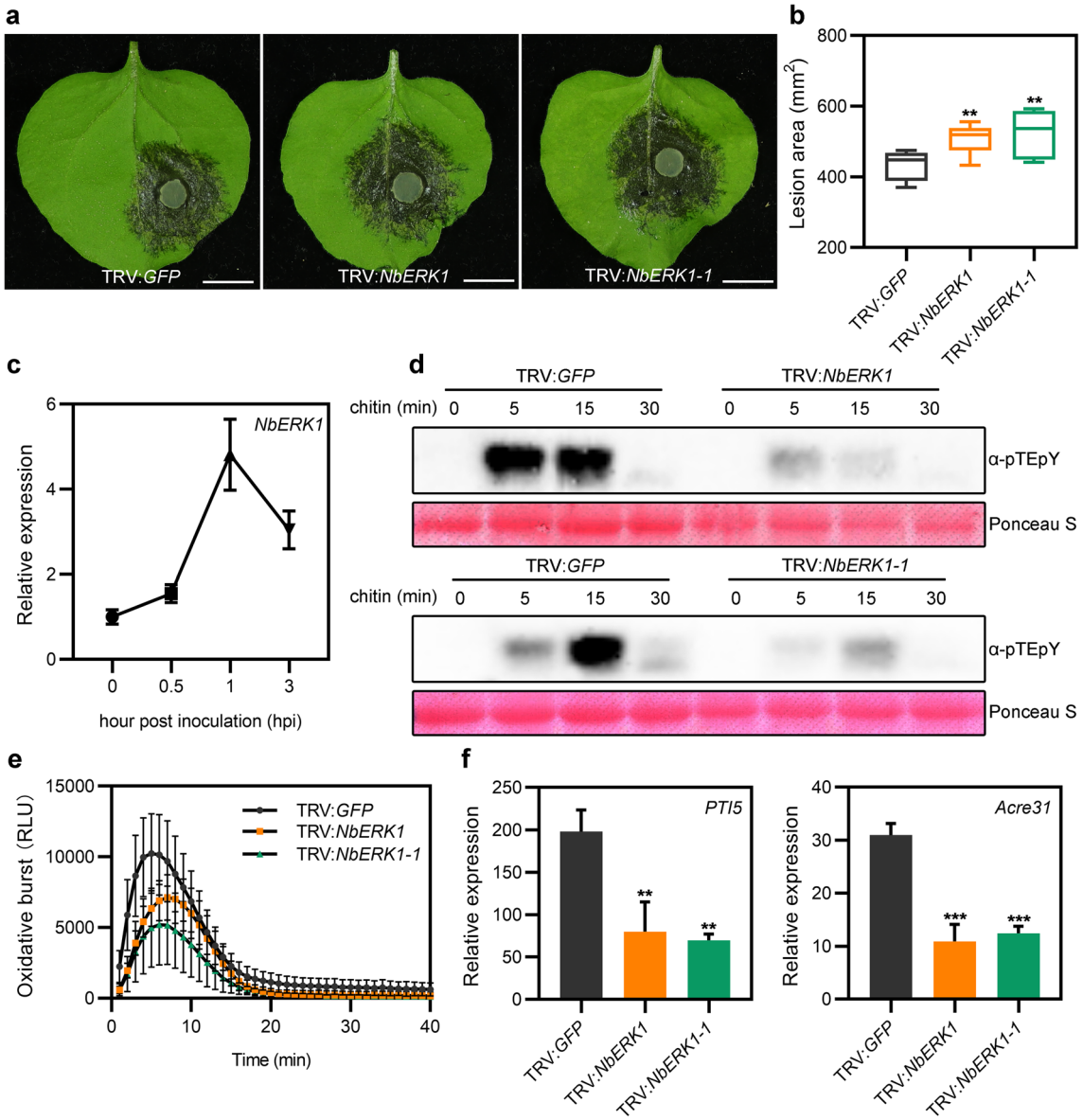


Fig. 1 NbERK1 regulates plant resistance to *Sclerotinia sclerotiorum* and chitin-induced immune responses in *Nicotiana benthamiana*. **a** Representative leaves showing lesions of *N. benthamiana* (pre-treated with TRV:*GFP*, TRV:*NbERK1*, or TRV:*NbERK1-1*) caused by *S. sclerotiorum*. Photos were captured 24 h post-inoculation (hpi). Bars, 1 cm. **b** Lesion areas caused by *S. sclerotiorum* on TRV-treated plants. Six leaves were counted in each replicate. The experiment was repeated three times. Error bars indicate ± *SD* (Student's *t*-test, $^{**}P < 0.01$). **c** The expression of *NbERK1* is induced by chitin treatment in *N. benthamiana*. *N. benthamiana* leaf discs were incubated in a 96-well plate with 200 µL water overnight and treated with 200 µg/mL chitin. The expression of *NbERK1* was analyzed by reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Error bars represent fold changes (± *SD*). **d** MAPK activation of *N. benthamiana* (pre-treated with TRV:*GFP*, TRV:*NbERK1*, or TRV:*NbERK1-1*) treated with 200 µg/mL chitin. α-pTEpY antibody was used to detect MAPK activation. Protein loading was determined by Ponceau S staining. **e** ROS production of *N. benthamiana* (pre-treated with TRV:*GFP*, TRV:*NbERK1*, or TRV:*NbERK1-1*) treated with 200 µg/mL chitin. **f** Relative expression of pattern-triggered immunity (PTI) marker genes (*PTI5* and *Acre31*) in *N. benthamiana* (pre-treated with TRV:*GFP*, TRV:*NbERK1*, or TRV:*NbERK1-1*) induced by 200 µg/mL chitin at 3 hpi. Error bars represent fold changes ± *SD* (Student's *t*-test, $^{**}P < 0.01$; $^{***}P < 0.001$)

expression of chitin-induced PTI marker genes (*PTI5* and *Acre31*) was significantly reduced in *NbCERK1*- and *NbLYK4*-silenced plants (Fig. 2f). Since *NbLYK2* was not effectively silenced in *N. benthamiana*, it is uncertain whether it regulates plant disease resistance and chitin signaling.

In *Arabidopsis*, the association of AtCERK1 and AtLYK5 induced by chitin is essential for triggering

chitin-induced immune signaling (Cao et al. 2014; Erwig et al. 2017). Previously, the association of AtLYK4 with AtCERK1 was found to be chitin-independent (Cao et al. 2014). To determine whether chitin could induce the association of NbCERK1 with NbLYK4, GFP-tagged NbLYK4 (NbD044705.1) was co-expressed with HA-tagged NbCERK1 (NbD008475.1) in *N. benthamiana* for co-immunoprecipitation (Co-IP) assay, which showed that NbLYK4 was associated with NbCERK1 upon chitin treatment (Fig. 2g). Collectively, NbCERK1 and NbLYK4 are essential for chitin perception and plant resistance to the fungal pathogen.

NbERK1 associates with NbCERK1 and NbLYK4

NbERK1 regulates chitin-induced immune responses, and NbCERK1-NbLYK4 complex is essential for chitin perception. Therefore, it is imperative to explore the potential protein–protein interactions between NbERK1 and NbCERK1/NbLYK4. For the Co-IP assay, the NbERK1-HA was co-expressed with NbCERK1-GFP in *N. benthamiana*. NbERK1 was immunoprecipitated with NbCERK1 irrespective of the chitin treatment (Fig. 3a). However, the association between NbERK1 and NbCERK1 was remarkably weakened upon chitin treatment (Fig. 3a). The same association was also observed between NbERK1 and NbLYK4 by Co-IP (Fig. 3b).

To further verify the association of NbERK1 with NbCERK1 and NbLYK4, a luciferase complementation assay was performed in *N. benthamiana*. We observed that NbERK1 was associated with NbLYK4, but not with NbCERK1 or EV, and all proteins were normally expressed (Fig. 3c). These results demonstrated that NbERK1 was associated with NbLYK4 in vivo. Similarly, in the bimolecular fluorescence complementation (BiFC) assay, the YFP signal was detected only in *N. benthamiana* leaves expressing NbERK1-nYFP and cYFP-NbLYK4 (Fig. 3d), but not in leaves expressing NbERK1-nYFP and cYFP-NbCERK1 (Additional file 1: Figure S7). These results indicated that NbERK1 was associated with NbLYK4, but not with NbCERK1 in the BiFC assay. We speculate that the association of NbERK1 and NbCERK1 might be via their extra-cellular domain (ECD). Therefore, we performed a yeast two-hybrid assay using pGADT7/pGBKT7 plasmids containing ECDs or CDs, which showed that NbERK1 was indeed associated with NbCERK1 via ECD (Fig. 3e). Collectively, these data suggest that NbERK1 forms complexes with NbCERK1 and NbLYK4, and it is released from the NbCERK1-NbLYK4 complex upon chitin perception.

NbERK1 is important for the chitin-triggered association of NbCERK1 and NbLYK4

We further investigated the potential role of NbERK1 in the chitin-triggered association of NbCERK1 and NbLYK4. NbCERK1-HA was co-expressed with NbLYK4-GFP in TRV:*GFP*- and TRV:*NbERK1*-treated plants. Silencing of *NbERK1* weakened the chitin-induced association of NbCERK1-NbLYK4 (Fig. 4a), suggesting that NbERK1 is important for the chitin-induced formation of NbCERK1-NbLYK4 complex. To determine the dynamic of NbERK1/NbCERK1/NbLYK4 complex upon chitin induction, NbERK1-HA was co-expressed with NbCERK1-Flag and NbLYK4-GFP in *N. benthamiana*. The result showed that the overexpression of NbERK1 also weakened the chitin-induced NbCERK1-NbLYK4 association (Additional file 1: Figure S8). This result suggests that NbERK1 is possibly also required to promote NbCERK1-NbLYK4 dissociation after the activation of chitin-induced immune responses.

To further clarify the role of NbERK1 kinase activity in chitin-induced NbCERK1-NbLYK4 complex formation, we first examined the kinase activity of NbERK1 using a prokaryotic expression system. However, the CD of NbERK1 was found to be toxic when expressed in *Escherichia coli*, which prevents recombinant protein production. Therefore, we generated the potential NbERK1 kinase-inactive mutant (NbERK1-D644N) by mutating the Asp residue at position 644 (D644). D644 is an essential residue in the catalytic loop, which was identified by sequence comparison with *Arabidopsis* LecRK-IX.1 and LecRK-IX.2 (Wang et al. 2015). Next, we determined the importance of NbERK1 kinase activity for NbERK1

(See figure on next page.)

Fig. 2 NbCERK1 and NbLYK4 are essential for plant resistance to *Sclerotinia sclerotiorum* and chitin perception. **a** Representative leaves showing lesions of *Nicotiana benthamiana* (pre-treated with TRV:*GFP*, TRV:*NbCERK1*, TRV:*NbLYK2*, TRV:*NbLYK3*, TRV:*NbLYK4*, or TRV:*NbLYK5*) caused by *S. sclerotiorum*. Photos were captured at 24 h post-inoculation (hpi). Bars, 1 cm. **b** Lesion areas caused by *S. sclerotiorum* on TRV-treated plants. Six leaves were counted in each replicate. The experiment was repeated three times. Error bars indicate ± *SD* (Student's *t*-test, $^{**}P < 0.01$; $^{***}P < 0.001$). **c** ROS production of *N. benthamiana* (pre-treated with TRV:*GFP*, TRV:*NbCERK1*, TRV:*NbLYK2*, TRV:*NbLYK3*, TRV:*NbLYK4*, or TRV:*NbLYK5*) treated with 200 μg/mL chitin. **d** The total RLU was the sum of the RLUs for the first 30 min. Error bars indicate ± *SD* (Student's *t*-test, $^{***}P < 0.001$). **e** MAPK activation of *N. benthamiana* (pre-treated with TRV:*GFP*, TRV:*NbCERK1*, or TRV:*NbLYK4*) treated with 200 μg/mL chitin. α-pTEpY antibody was used to detect the MAPK activation. Protein loading was determined by Ponceau S staining. **f** Relative expression of PTI marker genes (*PTI5* and *Acre31*) in *N. benthamiana* (pre-treated with TRV:*GFP*, TRV:*NbCERK1*, or TRV:*NbLYK4*) induced by 200 μg/mL chitin at 3 hpi. Error bars represent fold changes ± *SD* (Student's *t*-test, $^{**}P < 0.01$). **g** NbLYK4 associates with NbCERK1 upon chitin treatment in *N. benthamiana*. GFP-tagged NbLYK4 and HA-tagged NbCERK1 were co-expressed in *N. benthamiana* for 36–48 h. Samples were treated with 200 μg/mL chitin for 15 min before harvesting. Protein associations were detected by Co-IP assays

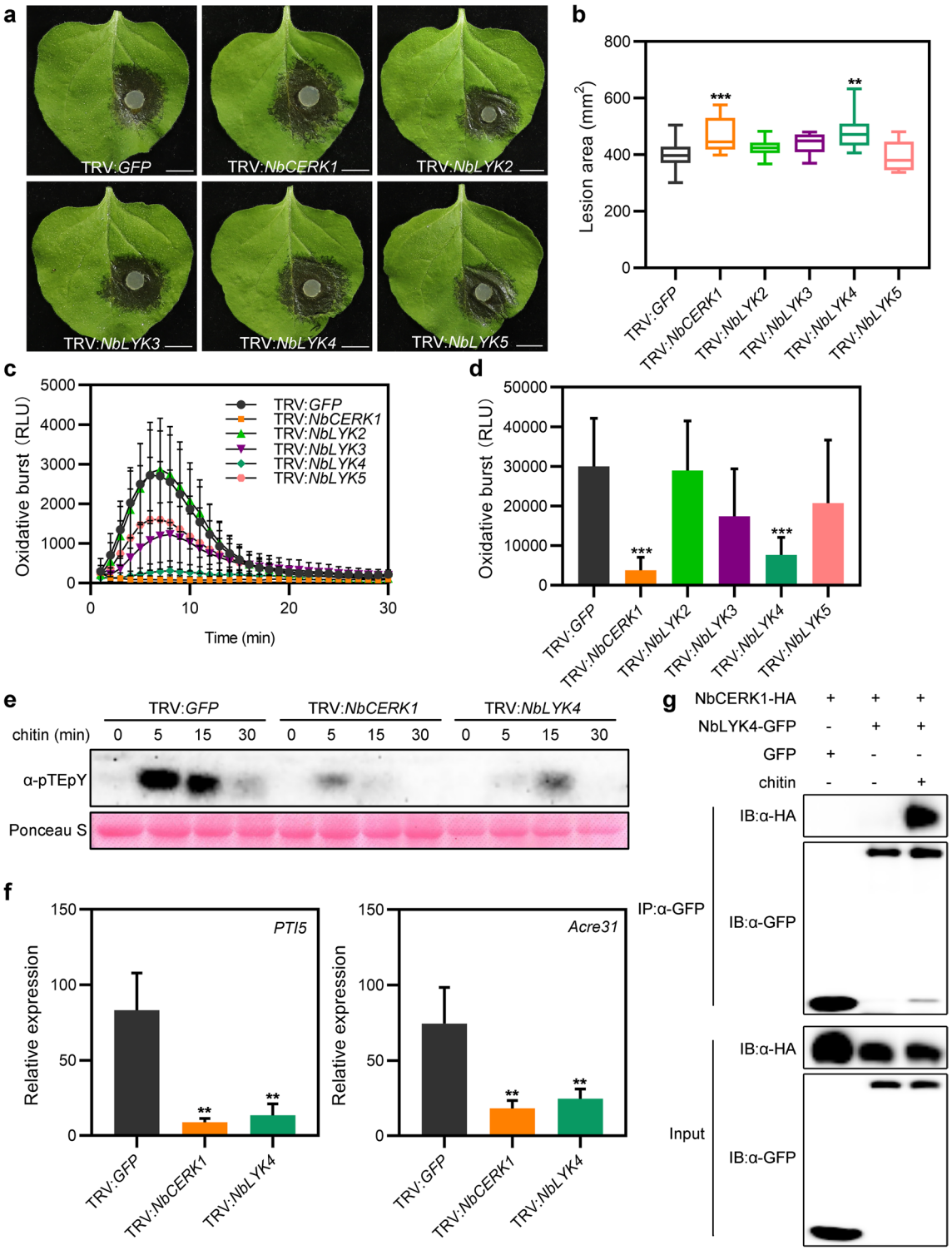


Fig. 2 (See legend on previous page.)

dissociation from NbCERK1-NbLYK4 complex upon chitin perception. For the Co-IP assay, NbERK1-D644N-HA or NbERK1-HA was co-expressed with NbCERK1-GFP in *N. benthamiana*. We observed no change in the association between NbERK1-D644N and NbCERK1 upon chitin treatment (Fig. 4b). Similarly, we found no change

in the association between NbERK1-D644N and NbLYK4 upon chitin treatment (Fig. 4c), suggesting that the kinase catalytic active site (D644) of NbERK1 is important for NbERK1 dissociation from NbCERK1-NbLYK4 complex upon chitin perception.

The kinase catalytic active site (D644) of NbERK1 is essential for plant resistance-enhancing activity and its protein stability

Programmed cell death (PCD) is critical in plant immunity (Fan et al. 2018). Activation of cell-surface receptors may lead to plant cell death (Gao et al. 2009). To determine whether NbERK1 has cell death-inducing activity, NbERK1-HA was transiently expressed in *N. benthamiana*. We observed NbERK1-induced cell death in *N. benthamiana* at 3 days post-infiltration (Fig. 5a). To determine the importance of the kinase catalytic active site for NbERK1-induced cell death activity, NbERK1-D644N-HA was also transiently expressed in *N. benthamiana*. The kinase-null mutant (NbERK1-D644N-HA) expression failed to induce cell death in *N. benthamiana*, which was confirmed by quantifying ion leakage (Fig. 5a, b). Immunoblotting analysis confirmed protein expression (Fig. 5c). These results indicated that the NbERK1 kinase catalytic active site (D644) is essential for the cell death-inducing activity of NbERK1.

Next, GFP and NbERK1 were transiently expressed in *N. benthamiana* to determine the plant resistance-enhancing activity of NbERK1. Subsequently, *S. sclerotiorum* was inoculated at the agroinfiltration sites after 24 h. We found that pretreatment of plants with NbERK1 exhibited significantly enhanced resistance to *S. sclerotiorum* compared with the control GFP (Fig. 5d). NbERK1-D644N-HA was also transiently expressed in *N. benthamiana* to determine its function in enhancing plant resistance. As expected, the expression of NbERK1-D644N-HA did not affect plant resistance compared with GFP (Fig. 5e). Collectively, these findings indicate that the kinase catalytic active site (D644) of NbERK1 is essential for plant resistance-enhancing activity.

The protein abundance of kinase-null mutant (NbERK1-D644N-HA) in Fig. 5c was significantly less than that of NbERK1, which led us to explore the importance of the NbERK1 kinase catalytic active site for the NbERK1 protein stability. NbERK1-HA and NbERK1-D644N-HA were transiently expressed in *N. benthamiana* for 2 d, and leaves were treated with 50 μM cycloheximide (CHX, a protein synthesis inhibitor) for 0, 2, and 4 h before harvesting. We found that the protein abundance of NbERK1-D644N-HA was less than NbERK1 before and after CHX treatment (Fig. 5f). These results suggest that the kinase catalytic active site (D644) of NbERK1 is also essential for its protein stability.

Discussion

Plant pathogenic fungi often cause severe plant diseases leading to significant economic losses. *S. sclerotiorum* is an important fungus with a broad host range, which can cause yield reduction in many crops, such as soybeans (Bolton et al. 2006). Chitin is a fungal cell wall component that can activate PTI response, including ROS burst, expression of marker genes, and activation of MAPK (Heese et al. 2007; Lloyd et al. 2014; Yu et al. 2017). Previously, we found that NbERK1 positively regulated plant resistance to the oomycete pathogen *P. capsici* by mediating the perception of the apoplastic expansin-like protein PcEXLX1. In this study, we showed that NbERK1 enhances plant resistance to the fungal pathogen *S. sclerotiorum* by regulating chitin signaling, though it is not involved in plant resistance to the bacterial pathogen *Pst* DC3000 mutant *ΔhopQ1*. NbERK1 was associated with chitin sensory receptors NbCERK1-NbLYK4 and was found to be significant for chitin-induced NbCERK1 and NbLYK4 association. The results further indicated that the kinase catalytic active site (D644) of NbERK1 was essential for NbERK1 dissociation from NbCERK1-NbLYK4 complex upon chitin perception and enhancing plant resistance through NbERK1 in *N. benthamiana*.

LysM-RLKs function in chitin perception, signaling, and plant resistance to fungal pathogens (Kawasaki et al. 2017). The major chitin receptor of *Arabidopsis* and cotton is LYK5 (AtLYK5 and GhLYK5). Chitin-induced AtCERK1-AtLYK5/ GhCERK1-GhLYK5 complex formation is essential for chitin signaling in *Arabidopsis* and cotton (Wan et al. 2012; Cao et al. 2014; Wang et al.

(See figure on next page.)

Fig. 3 NbERK1 associates with NbCERK1 and NbLYK4. **a** NbERK1 associates with NbCERK1 in *Nicotiana benthamiana*. GFP-tagged NbCERK1 and HA-tagged NbERK1 were co-expressed in *N. benthamiana* for 36–48 h. Samples were treated with 200 μg/mL chitin for 15 min before harvesting. Protein associations were detected by Co-IP assays. **b** NbERK1 associates with NbLYK4 in *N. benthamiana*. GFP-tagged NbLYK4 and HA-tagged NbERK1 were co-expressed in *N. benthamiana* for 36–48 h. Samples were treated with 200 μg/mL chitin for 15 min before harvesting. Protein associations were detected by Co-IP assays. **c** NbERK1 associates with NbLYK4 by a split-luciferase assay. NbERK1-Nluc was co-expressed with the indicated Cluc constructs in *N. benthamiana*. The intensity of protein interaction was shown by the relative luminescence unit (RLU), and protein expression was shown by immunoblotting (mean ± *SD*, n ≥ 6). **d** NbERK1 associates with NbLYK4 in the BiFC assay. NbERK1 and NbLYK4 were fused to the N terminus or C terminus of the YFP fragment, respectively. YFP fluorescence was detected by confocal microscopy. Bars, 25 μm. **e** NbERK1 associates with NbCERK1 in the yeast two-hybrid assay. Yeast cells with corresponding vectors were grown on SD-Leu/-Trp or SD-Ade/-His/-Leu/-Trp medium. AD and BD represent empty plasmids. ECD, extracellular domain; CD, cytosolic domain

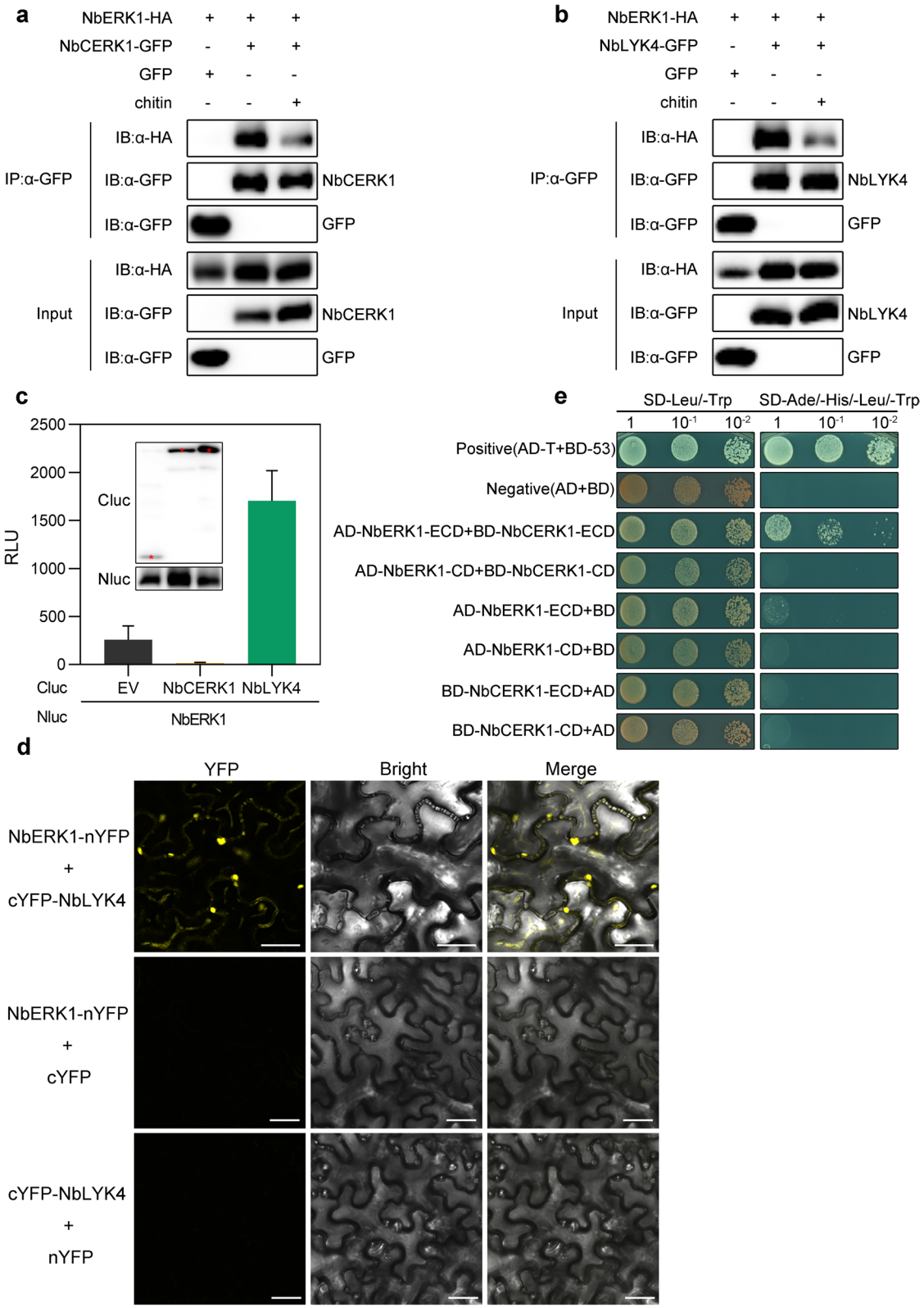
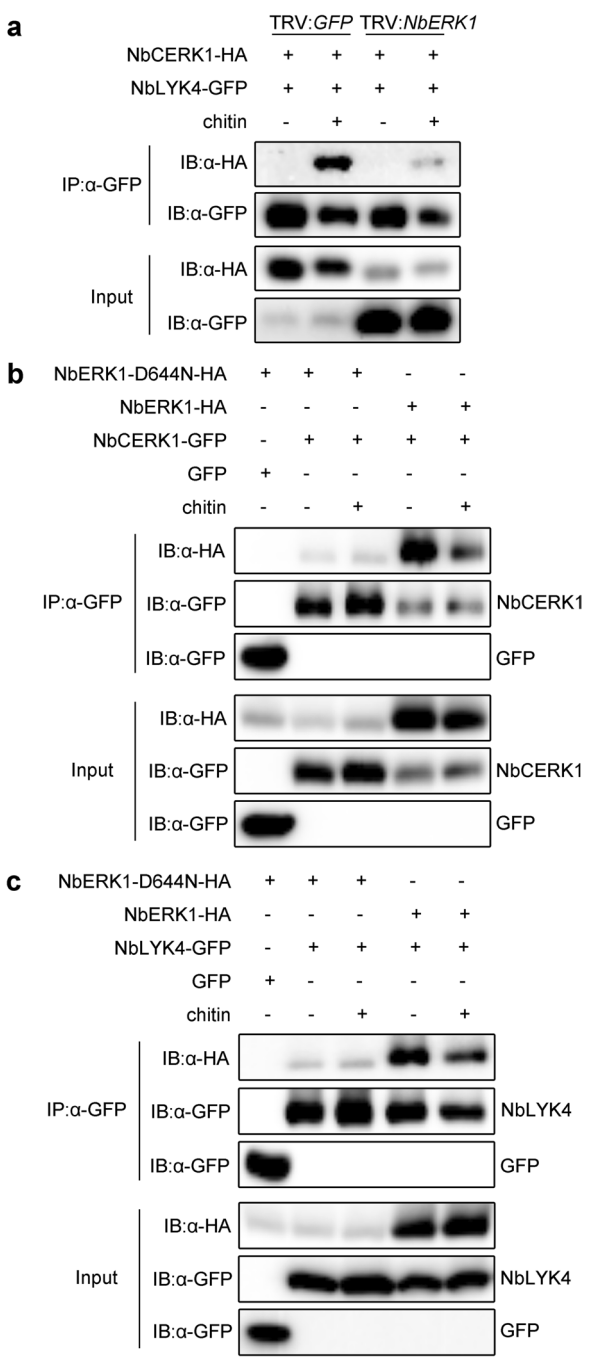


Fig. 3 (See legend on previous page.)



◀ **Fig. 4** NbERK1 is important for the chitin-triggered association of NbCERK1 and NbLYK4. **a** Silencing of *NbERK1* reduces the chitin-triggered association of NbCERK1 and NbLYK4 in *Nicotiana benthamiana*. GFP-tagged NbLYK4 and HA-tagged NbCERK1 were co-expressed in *N. benthamiana* (pre-treated with TRV:*GFP* or TRV:*NbERK1*) for 36–48 h. Samples were treated with 200 μg/mL chitin for 15 min before harvesting. Protein associations were detected by Co-IP assays. **b** Chitin does not induce dissociation of NbERK1-D644N from NbCERK1. GFP-tagged NbCERK1 was co-expressed with HA-tagged NbERK1-D644N or NbERK1 in *N. benthamiana* for 36–48 h. Samples were treated with 200 μg/mL chitin for 15 min before harvesting. Protein associations were detected by Co-IP assays. **c** Chitin does not induce dissociation of NbERK1-D644N from NbLYK4. GFP-tagged NbLYK4 was co-expressed with HA-tagged NbERK1-D644N or NbERK1 in *N. benthamiana* for 36–48 h. Samples were treated with 200 μg/mL chitin for 15 min before harvesting. Protein associations were detected by Co-IP assays

2020). However, our results showed that *N. benthamiana* NbLYK5 exerted an insignificant effect on plant resistance to *S. sclerotiorum* and chitin signaling (Fig. 2). Conversely, silencing *NbLYK4* significantly reduced chitin-induced immune responses and plant resistance to *S. sclerotiorum* (Fig. 2). Importantly, chitin could induce the association of NbLYK4 with NbCERK1 (Fig. 2), which was different from the association of AtLYK4-AtCERK1 (Cao et al. 2014), suggesting that NbLYK4 might be the major chitin receptor of *N. benthamiana*. These observations indicate that chitin signaling is different among different species.

Evidence suggests that additional RLKs in the chitin receptor complex regulate chitin signaling. For instance, the malectin-like domain-containing RLK, IOS1, from *Arabidopsis* regulate BAK1-dependent and -independent PTI responses. IOS1 positively modulates chitin signaling by associating with AtCERK1, which is BAK1-independent (Yeh et al. 2016). Similarly, *Arabidopsis* malectin-like RLK FERONIA promotes chitin signaling by associating with AtCERK1 (Stegmann et al. 2017). Here, we found that NbERK1 also regulated chitin-induced immune responses by associating with NbCERK1 (Figs. 1, 3). In cotton, a wall-associated RLK, GhWAK7A, functions in chitin signaling via regulating chitin-induced GhCERK1-GhLYK5 association (Wang et al. 2020). Likewise, our results revealed that NbERK1 positively mediated chitin signaling by regulated chitin-induced NbCERK1-NbLYK4 (chitin sensory receptors) association (Figs. 1, 4). Additionally, the LRR-RLK LIK1 is a negative chitin response regulator by interacting with AtCERK1. The *lik1* mutant plants were found to be more susceptible to *S. sclerotiorum* (Le et al. 2014). However, we observed that NbERK1 is a positive regulator of chitin signaling, and *NbERK1*-silenced plants were also more susceptible to *S. sclerotiorum* (Fig. 1). In addition to the above examples of association with chitin sensory receptors, a few RLKs regulate chitin signaling via interacting with RLCK. For example, the G-type lectin RLK SDS2 positively regulates chitin signaling by interacting with OsRLCK118/176 in rice (Fan et al. 2018). In summary, our results highlight a new mechanism by which G-type lectin RLK regulates chitin signaling. Furthermore, it was established as a novel component in the chitin receptor complex.

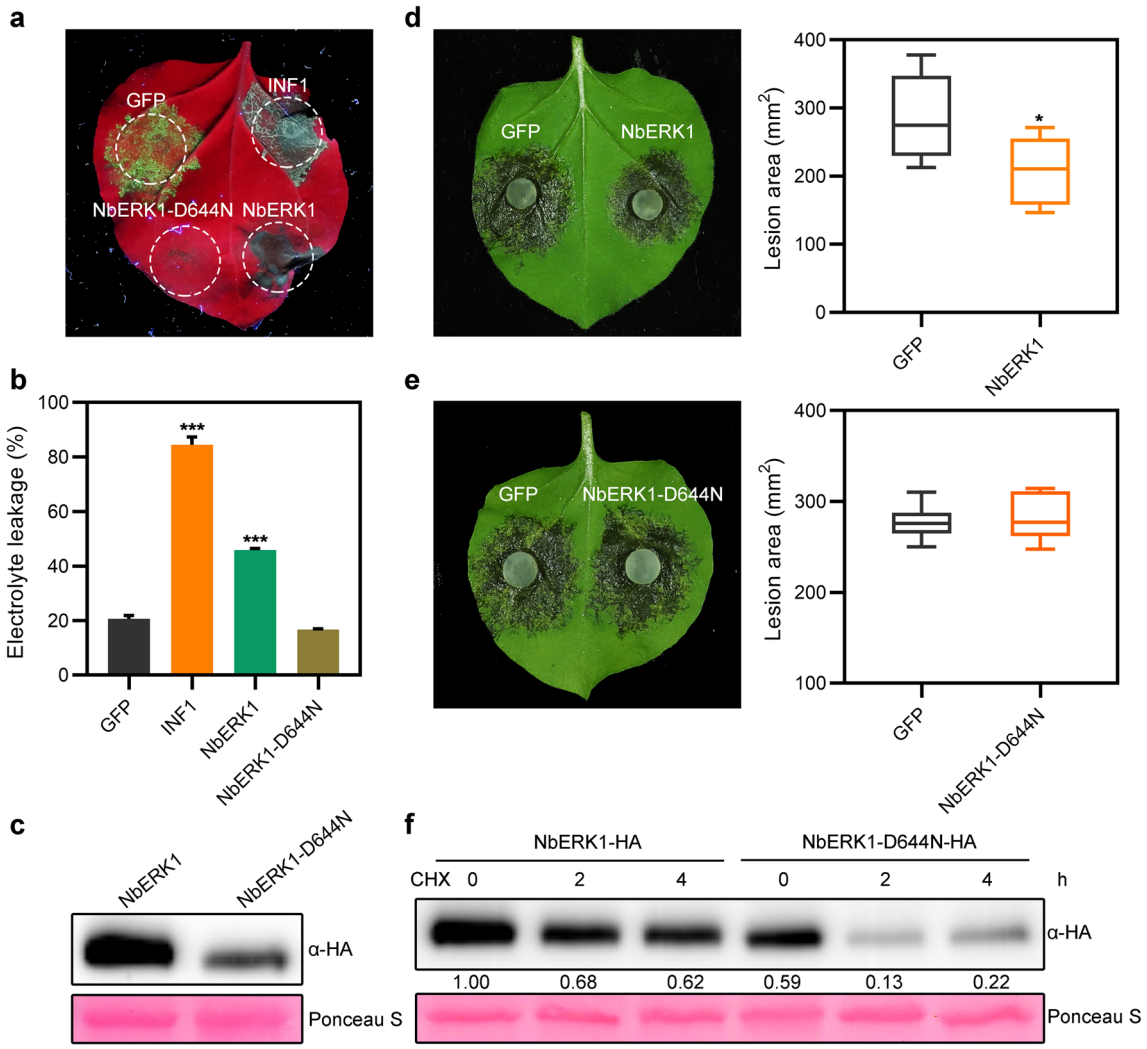


Fig. 5 The kinase catalytic active site of NbERK1 is essential for cell death-inducing/plant resistance-enhancing activity and protein stability. **a** The kinase catalytic active site of NbERK1 affects its cell death-inducing activity. Transient expression of NbERK1 and NbERK1-D644N in *Nicotiana benthamiana* by agroinfiltration. INF1 and green fluorescent protein (GFP) were used as controls. Photos were captured under UV light. **b** Cell death was quantified by measuring electrolyte leakage. Error bars indicate ± *SD* (Student's *t*-test, ***$P < 0.001$). **c** Immunoblotting analysis of NbERK1 and NbERK1-D644N expressed in *N. benthamiana*. **d**, **e** The kinase catalytic active site of NbERK1 affects its plant resistance-enhancing activity. Representative leaves showing lesions of *N. benthamiana* caused by *Sclerotinia sclerotiorum*. Leaves were infiltrated by *Agrobacterium tumefaciens* strains harboring GFP, NbERK1, or NbERK1-D644N 24 h before *S. sclerotiorum* inoculation. Photos were captured 24 hpi. Lesion areas were counted by ImageJ. Error bars indicate ± *SD* (Student's *t*-test, *$P < 0.05$). **f** The kinase catalytic active site of NbERK1 affects protein stability. NbERK1-HA and NbERK1-D644N-HA were transiently expressed in *N. benthamiana* for 2 d. Leaves were treated with 50 μM CHX for 0, 2, and 4 h before harvesting. Protein abundances were detected by immunoblotting using anti-HA antibodies

Overexpression of *LecRLKs* can induce plant cell death and disease resistance. For example, overexpression of G-type lectin RLK *SDS2* from *O. sativa* induces plant programmed cell death and resistance to *M. grisea* (Fan et al. 2018). The L-type lectin RLK *LecRK-IX.2* induces SA accumulation, which leads to cell death in *Arabidopsis* (Luo et al. 2017). In addition, overexpression of L-type lectin RLKs *LecRK-IX.1* and *LecRK-IX.2* from *Arabidopsis* can enhance plant resistance to *Phytophthora* and induce plant cell death. Moreover, the cell death-inducing/plant resistance-enhancing activity of LecRK-IX.1 and LecRK-IX.2 rely on their kinase catalytic active site (Wang et al. 2015). Similar to our results, the NbERK1 kinase catalytic active site was also essential for the cell death-inducing/plant resistance-enhancing activity of NbERK1 (Fig. 5). In addition, our results showed that the kinase catalytic active site of NbERK1 was important for NbERK1 dissociation from NbCERK1-NbLYK4 complex upon

chitin perception (Fig. 4b, c). These findings suggest that NbERK1 modulates chitin sensory complex NbCERK1-NbLYK4 to enhance plant resistance in a kinase-dependent manner.

Conclusions

In this study, we identified a G-type lectin RLK, NbERK1, which enhances plant resistance to the fungal pathogen *S. sclerotiorum* via regulating chitin signaling. NbERK1 associates with the chitin receptor complex in *N. benthamiana* and likely modulates the chitin sensory complex NbCERK1-NbLYK4 to enhance plant resistance in a kinase-dependent manner. Collectively, these results provide a novel regulator of chitin-induced plant immunity.

Methods

Plant material and strain cultures

Nicotiana benthamiana plants used in this study were grown in a glasshouse (25°C, 16 h photoperiod, and 60% relative humidity). *Sclerotinia sclerotiorum* was grown at 25°C on potato dextrose agar (PDA) medium (boiled extracts from 200 g of fresh potato, 20 g of glucose, and 15 g agar per liter). *Escherichia coli* strain DH5α was cultured on Luria–Bertani (LB) medium at 37°C and *Agrobacterium tumefaciens* strain GV3101 was cultured on LB medium at 28°C.

Plasmid construction

For virus-induced gene silencing (VIGS) in *N. benthamiana*, the gene fragments were amplified from the cDNA of *N. benthamiana* and ligated into the pTRV2 vector (Liu et al. 2002). For co-immunoprecipitation (Co-IP) in *N. benthamiana*, constructs were generated using the pCAMBIA1300-GFP, pCAMBIA1300-HA, or pCAMBIA1300-Flag vectors. For transient expression in *N. benthamiana*, constructs were generated by the pCAMBIA1300-HA or pSuper-HA vectors. For split-luciferase complementation assays in *N. benthamiana*, constructs were generated by the pCAMBIA1300-Cluc-3×Flag or pCAMBIA1300-Nluc-HA vectors. For bimolecular fluorescence complementation assays in *N. benthamiana*, constructs were generated by using the pSuper-HA-cYFP or pSuper-cMyc-nYFP vectors. For the yeast two-hybrid assays, constructs were generated using the pGADT7 or pGBKT7 vectors. Primers used in this study are listed in Additional file 2: Table S1.

Agrobacterium-mediated transient expression and VIGS in *N. benthamiana*

Plasmids were transformed into *A. tumefaciens* strain GV3101 by electroporation. *A. tumefaciens* carrying the plasmid were pelleted and resuspended in infiltration solution (10 mM magnesium chloride ($MgCl_2$), 10 mM 2-(N-morpholino) ethanesulfonic acid (MES) pH 5.7, and 200 μM acetosyringone). The OD_{600} was adjusted to 0.6 and infiltrated into six-week-old plant leaves.

For VIGS assay, *A. tumefaciens* cultures harboring pTRV2 constructs were mixed with *A. tumefaciens* cultures harboring pTRV1 construct in a 1:1 ratio to a final OD_{600} of 0.8 before infiltration into leaves of four-leaf-stage *N. benthamiana*. The efficiency of gene silencing was determined by RT-qPCR and TRV:*GFP* was used as a control.

RNA isolation and RT-qPCR

N. benthamiana total RNAs were extracted from leaves using the RNA kit (ZomanBio) and 500 ng of total RNA were used as templates for first-strand cDNA synthesis using the reverse transcription kit (ZomanBio). RT-qPCR was performed on the ABI QuantStudio 6 Flex system (Thermo Fisher) using 2×HQ SYBR qPCR Mix (Low ROX) (ZomanBio). The primers used for RT-qPCR are listed in Additional file 2: Table S1.

ROS burst assay

N. benthamiana leaf discs (Ø 0.5 cm) were taken using a cork-borer set (Sigma-Aldrich) and incubated in a 96-well plate with 200 μL water for 12 h. To perform the flg22-induced ROS production assay, the water was replaced with the reaction buffer containing 1 μM flg22 (Sangon), 20 μM luminol (Sigma-Aldrich), and 20 μg/mL horseradish peroxidase (Sigma-Aldrich). To perform the chitin-induced ROS production assay, the water was replaced with the reaction buffer containing 200 μg/mL chitin (Sangon), 5 μM L-012 (Waco), and 20 μg/mL horseradish peroxidase (Sigma-Aldrich). Luminescence was measured using the luminometer (Tecan F200).

S. sclerotiorum and bacterial infection assays

For *S. sclerotiorum* infection assay, it was grown on PDA plates for 3 days at 25°C in the dark. The mycelial plug of *S. sclerotiorum* was taken using a cork-borer set and then inoculated onto the *N. benthamiana* leaves (pre-treated with TRV or pre-infiltrated with *A. tumefaciens* cultures). The inoculated plants were stored in boxes with high humidity for 24 h in the dark. The lesion areas were measured by ImageJ.

For bacterial infection assay, *N. benthamiana* leaves (pre-treated with TRV) were infiltrated with *Pst* DC3000 *ΔhopQ1*. Leaf bacterial number was determined 3 days post inoculation (Zhang et al. 2010).

MAPK activation assay

N. benthamiana leaf discs (Ø 0.5 cm) (pre-treated with TRV) were taken using a cork-borer set and incubated in a 96-well plate with 200 μL ddH_2O for 12 h. To perform

the chitin-induced MAPK activation assay, ddH_2O was replaced with the reaction buffer containing 200 μg/mL chitin for 0, 5, 15, and 30 min. For each time point, three leaf discs were collected and total proteins were extracted by RIPA buffer (150 mmol/L sodium chloride (NaCl), 50 mmol/L Tris pH 7.5, 1% Triton X-100, 1% SDS, 1% deoxycholate, 0.5 mmol/L EDTA, 1 × phenylmethanesulfonyl fluoride (PMSF), and 1 × protease inhibitor cocktail). Total proteins were separated by SDS-PAGE gels. MAPK signals were detected by anti-Phospho-p44/p42 MAPK antibody (anti-pTEpY) (Cell Signaling Technology) and stained by Ponceau S for protein loading.

Western blotting and Co-IP assay

To determine the expression of the protein in *N. benthamiana* leaves, total proteins were extracted using RIPA buffer and then separated by SDS-PAGE gels. Western blotting was detected with corresponding antibodies. For the Co-IP assay, total proteins were extracted using Lysis buffer (150 mmol/L NaCl, 10 mmol/L Tris pH 7.5, 0.5% Triton X-100, 0.5 mmol/L EDTA, 2% (w/v) polyvinylpolypyrrolidone, 10% glycerol, 1 × PMSF, 1 × protease inhibitor cocktail, and 1 × Ps341), incubated with GFP-Nanoab-Agarose (Lablead) for 2 h and washed four times with Wash buffer (10 mmol/L Tris pH 7.5, 150 mmol/L NaCl, 0.5 mmol/L EDTA). Immunoprecipitations were detected using anti-HA (Cwbio), anti-GFP (Proteintech), or anti-FLAG (Proteintech) antibodies.

Chitin treatment method

For the Co-IP and PTI marker gene assays, chitin treatment means injecting chitin solution (200 μg/mL) into the leaves before harvesting. For other assays, chitin treatment means incubating the leaves in chitin solution (200 μg/mL) before harvesting.

Luciferase complementation assay

The indicated Nluc and Cluc constructs were expressed in *N. benthamiana* by agroinfiltration for 2 days. Leaf discs (Ø 0.5 cm) were collected and into a 96-well plate with 1 mM luciferin (Biovision). The luciferase activity was measured with the luminometer (Tecan F200).

Bimolecular fluorescence complementation assay

The indicated nYFP and cYFP constructs were expressed in *N. benthamiana* by agroinfiltration for 2 days. YFP signal was detected by confocal microscopy (Leica SP8). The excitation and emission wavelengths for YFP were 514 nm and bright field was used to observe the cell contour of epidermis of *N. benthamiana*.

Yeast two-hybrid assay

The indicated plasmids were co-transformed into yeast strain AH109. Select positive clones on synthetic defined (SD) medium without Leu and Trp. Protein–protein interactions were tested by transferring transformants to the SD medium without Ade, His, Leu, and Trp.

Electrolyte leakage assay

Five *N. benthamiana* leaf discs (1.0 cm in diameter) were collected 72 h after agro-infiltration and floated on 5 mL ddH_2O for 3 h at room temperature. The electrolyte leakage value A was measured by the conductivity meter (Mettler Toledo, LE703). Then the leaf discs were boiled for 20 min. Value B was measured with the conductivity meter when the solution was restored to room temperature. Relative electrolyte leakage was calculated by comparing value A and value B.

Protein stability assay in *N. benthamiana*

To detect the effect of the kinase catalytic active site on protein stability, NbERK1 and NbERK1-D644N were transiently expressed in *N. benthamiana* for 2 days. Leaves were treated with 50 μM protein synthesis inhibitor cycloheximide (CHX) for 0, 2, and 4 h before harvesting. NbERK1 and NbERK1-D644N abundances were detected by immunoblotting with anti-HA (Cwbio).

Abbreviations

CD	Cytoplasmic kinase domain
CHX	Cycloheximide
Co-IP	Co-immunoprecipitation
ECD	Extracellular domain
EGF	Epidermal growth factor
ETI	Effector-triggered immunity
GFP	Green fluorescent protein
hpi	Hours post-inoculation
LB	Luria–bertani
LecRLKs	Lectin RLKs
LPS	Lipopolysaccharide
LRR	Leucine-rich repeat
LysM	Lysin motif
MAPK	Mitogen-activated protein kinase
MES	2-(N-morpholino) ethanesulfonic acid
$MgCl_2$	Magnesium chloride
NLPs	Necrosis and ethylene-inducing peptide 1-like proteins
PAMPs	Pathogen-associated molecular patterns
PCD	Programmed cell death
PDA	Potato dextrose agar
PMSF	Phenylmethanesulfonyl fluoride
PRRs	Pattern recognition receptors
Pst	*Pseudomonas syringae* Pv. *tomato*
PTI	Pattern-triggered immunity
RLCK	Receptor-like cytoplasmic kinase
RLKs	Receptor-like kinases
RLPs	Receptor-like proteins
ROS	Reactive oxygen species
SA	Salicylic acid
SD	Synthetic defined
TM	Transmembrane
VIGS	Virus-induced gene silencing
Xoo	*Xanthomonas oryzae* Pv. *oryzae*

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s42483-023-00182-0.

Additional file 1: **Figure S1**. Detection of the silencing efficiency of the *NbERK1* gene in *N. benthamiana*. **Figure S2**. Relative expression of chitin-induced *WRKY7* and *WRKY8* in *N. benthamiana*. **Figure S3**. NbERK1 is not involved in plant resistance to the bacterial pathogen and flg22-induced immune responses. **Figure S4**. Phylogenetic analysis of LYK family proteins from *N. benthamiana* and *Arabidopsis*. **Figure S5**. Detection of the silencing efficiency of *NbLYK* genes in *N. benthamiana*. **Figure S6**. The transient expression of NbCERK1 enhances the plant resistance to *S. sclerotiorum*. **Figure S7**. NbERK1 does not associate with NbCERK1 in the BiFC assay. **Figure S8**. The overexpression of NbERK1 weakens the chitin-induced NbCERK1-NbLYK4 association

Additional file 2: **Table S1**. Primers used in this study

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Not applicable.

Authors' contributions
ZY, LP, and DD designed the experiments. LP, YZ, JW, and NW performed the experiments. ZY performed the bioinformatics analyses. LP, ZY, and DD wrote the manuscript. All authors read and approved the final manuscript.

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Availability of data and materials
An improved NbD version of *N. benthamiana* proteome can be obtained from the Oxford Research Archive at https://ora.ox.ac.uk/objects/uuid:f34c90af-9a2a-4279-a6d2-09cbdcb323a2 (Kourelis et al. 2019). Gene sequence of *NbERK1* (Niben101Scf05948g04005.1) can be found in the Sol Genomics Network database.

Declarations

Ethics approval and consent to participate
Not applicable.

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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