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A pair of soybean malectin-like domain-containing receptor-like kinases jointly regulate pattern-triggered immunity by forming hetero-oligomers

Qian Zhang[1], Dongmei Wang[2], Zhuoyuan He[3], Yazhou Bao[4], Xiaodan Wang[1], Guangyuan Xu[1], Jun Yang[1], Daolong Dou[1,4*], Xianzhong Feng[2*] and Xiangxiu Liang[1,3*]

Abstract

Plant cells perceive pathogen invasion by recognizing microbial patterns using plasma-membrane-localized pattern-recognition receptors (PRRs) to initiate pattern-triggered immunity (PTI), which confers a moderate immunity to most microbes. For instance, the PRR FLS2 (FLAGELLIN SENSING 2) recognizes bacterial flagellin in the presence of the co-receptor BAK1 and activates a series of PTI responses, such as reactive oxygen species (ROS) burst and mitogen-activated protein kinase (MAPK) activation. We previously showed that soybean malectin/malectin-like domain-containing receptor-like kinase (MRLK) protein GmLMM1 negatively regulates PTI by suppressing FLS2-BAK1 interaction. GmLMM1 replicates in tandem with five other GmMRLKs on chromosome 13. Here, we show that GmMRLK32, the closest homolog to GmLMM1 among the tandem genes of GmLMM1, negatively regulates PTI and disease resistance against bacterial and oomycete pathogens. The *Gmmrlk32* mutant showed enhanced flg22-induced ROS burst and MAPK activation. We revealed that GmMRLK32 interacts with GmFLS2 and GmBAK1, and suppresses flg22-induced GmFLS2-GmBAK1 dimerization in a manner similar to that of GmLMM1. We further showed that GmMRLK32 specifically interacts with GmLMM1 to regulate PTI. In *Nicotiana benthamiana* plants, co-expression of GmMRLK32 and GmLMM1 showed a stronger PTI inhibitory effect on PTI activation than expression of GmMRLK32 or GmLMM1 alone. We uncovered a novel mechanism by which GmMRLK32 and GmLMM1 coordinately regulate PTI by forming hetero-oligomer.

Keywords Soybean, Malectin/malectin-like domain-containing receptor-like kinases, Pattern-recognition receptors (PRRs), Pattern-triggered immunity (PTI)

*Correspondence:
Daolong Dou
ddou@njau.edu.cn
Xianzhong Feng
fengxianzhong@iga.ac.cn
Xiangxiu Liang
liangxiangxiu@scau.edu.cn
Full list of author information is available at the end of the article

Background

Soybeans are important oilseed crops and are a source of plant proteins worldwide. However, soybean production is threatened by many plant diseases, such as bacterial blight, soybean rust, and *Phytophthora* root rot, which cause notable losses on soybean yield and quality (Zorzatto et al. 2015; Whitham et al. 2016). Plants primarily rely on their innate immune system to resist invasion of pathogenic microorganisms. Therefore, it is important to investigate the plant immune system to help improve disease resistance in soybeans. Plant cells have evolved a multilayer immune system mainly consisting of pattern-triggered immunity (PTI) and effector-triggered immunity (ETI) (Jones and Dangl, 2006; Couto and Zipfel, 2016). Plant cell surface-localized pattern-recognition receptors (PRRs) recognize the conserved molecular features of microbes or host cells, and these molecular features are called as pathogen/microbe- or damage-associated molecular patterns (PAMPs/MAMPs or DAMPs), leading to PTI activation (DeFalco and Zipfel, 2021). Certain pathogens can evade PTI by secreting diverse virulence effectors into host cells (Wang et al. 2022). Plant intracellular nucleotide-binding, leucine-rich repeat receptors (NLRs) recognize these effectors and activate stronger ETI to defend against phytopathogens (Jones et al. 2016). Although PTI and ETI are mediated by different immune receptors, they share similar signaling machinery and ETI activates immunity by augmenting PTI (Yuan et al. 2021; Ngou et al. 2021).

PTI plays a key role in plant basal resistance and confers broad-spectrum and mild resistance to most phytopathogens, often with a lower yield penalty (Couto and Zipfel, 2016; Boutrot et al., 2017; Yu et al. 2017). PRRs mainly consist of receptor-like proteins (RLPs) and receptor-like kinases (RLKs) (Tang et al. 2017). RLKs possess an extracellar ligand-binding domain, a transmembrane domain, and an intracellular kinase domain. They can be divided into multiple subfamilies based on the diversity of their extracellular domains, such as the leucine-rich repeat (LRR), lysine motif (LysM), lectin domain, and malectin-like domain. RLPs are structurally similar to that of RLKs, but lack the intracellular kinase domains (Liang et al., 2018; de Azevedo Manhaes et al., 2021; Ngou et al. 2022). PRRs usually recognize PAMPs in the presence of co-receptors to activate the PTI response. For example, the Arabidopsis LRR-RLK receptor FLS2 recognizes bacterial flagellin (or the epitope flg22) in the presence of the co-receptor BAK1 (Chinchilla et al. 2006; Sun et al. 2013). The LRR-RLP receptor RXEG1 from *Nicotiana benthamiana* forms a complex with NbBAK1 and NbSOBIR1 to recognize *Phytophthora sojae*-derived XEG1 (Wang et al. 2018). BAK1 frequently acts as a co-receptor for LRR-type RLKs and RLPs (Ma et al. 2016). Arabidopsis LYK4/5-CERK1 complex recognizes fungal cell wall-derived chitin (Miya et al. 2007; Cao et al. 2014).

Arabidopsis FLS2 and its homologs are the most extensively studied PRRs in plants, which typically serve as paradigms for studying PTI in plants. Upon perception of flg22, FLS2 rapidly associates with BAK1 to form a heterodimer, which subsequently activates several key regulatory components, such as receptor-like cytoplasmic kinases (RLCKs), NADPH oxidase RbohD, calcium-dependent protein kinases (CDPKs), and heterotrimeric G proteins. These regulatory components interact with many PRRs and are the central components of the PRR complexes (Couto and Zipfel, 2016; Yu et al. 2017). Moreover, flg22 induces a series of PTI responses, including transient production of reactive oxygen species (ROS), calcium influx, activation of mitogen-activated protein kinase (MAPK) and calcium-dependent protein kinase (CDPK) cascades, and transcriptional reprogramming (Zhou and Zhang, 2020).

PAMP-induced interaction between PRRs and their co-receptors is a key step to initial plant immunity. This interaction is tightly controlled by multiple regulatory mechanisms to ensure appropriate activation of immunity (DeFalco and Zifel, 2021). For example, BIR2 can suppress the FLS2-BAK1 interaction by competing with FLS2 for binding to BAK1, thereby ensuring moderate immune activation (Halter et al. 2014). NIK1 negatively regulates PTI by suppressing FLS2-BAK1 interaction (Li et al. 2019). The G-type LecRLKs SBP1 and SBP2 specifically regulate the interaction of BAK1 and RLP immune receptors (Bao et al. 2023). NbERK1 positively regulates PTI activation by modulating chitin-triggered NbCERK1-NbLYK4 association (Pi et al. 2023). Several malectin-like domain-containing RLKs (MRLKs) have been reported to regulate the interactions between PRRs and co-receptors (Franck et al. 2018; Dievart et al. 2020; Ortiz-Morea et al. 2022). The extracellular domain of MRLKs contains two tandem malectin-like domains responsible for binding to ligands, such as rapid alkalinization factors (RALFs) (Franck et al. 2018). In Arabidopsis, FER positively regulates immunity by promoting the FLS2-BAK1 interaction (Stegmann et al. 2017), whereas ANX1 and ANX2 attenuate immunity by suppressing flg22-induced FLS2-BAK1 dimerization (Mang et al. 2017). We screened a lesion mimic mutant of GmLMM1 previously and identified GmLMM1 as one of the six tandemly repeated *MRLK* genes that share high similarity with FER and ANX1. We showed that GmLMM1 negatively regulates PTI and disease resistance by inhibiting flg22-induced GmFLS2-GmBAK1 association (Wang et al. 2020). However, whether and how other tandemly repeated GmMRLKs are involved in plant immunity remain unclear.

GmMRLK32 is the closet homolog of GmLMM1 and in tandem with GmLMM1 on chromosome. Here, we observed that both the *Gmlmm1* and *Gmmrlk32* mutants showed enhanced PTI and disease resistance against bacterial and oomycete pathogens. We further showed that GmLMM1 and GmMRLK32 negatively regulates PTI by suppressing flg22-indcued GmFLS2-GmBAK1 interaction. Moreover, we showed that GmMRLK32 specifically interacted with GmLMM1 and may regulate PTI by forming a hetero-oligomer. Our study discovered a novel mechanism by which MRLK proteins regulate plant PTI.

Results

Ectopic expression of GmMRLK32 in *N. benthamiana* and Arabidopsis suppresses PTI

We previously identified *GmLMM1* as an MRLK gene that negatively regulates PTI and cell death. *GmLMM1* is tandemly repeated with five *GmMRLK* genes on chromosome 13 in the plant of soybean (Wang et al. 2020). Among the GmMRLKs, GmMRLK32 and GmLMM1 showed the highest sequence similarity (Fig. 1a and Additional file 1: Figure S1). To evaluate the role of GmMRLK32 in plant immunity, we examined the flg22-induced ROS burst and MAPK activation in *N. benthamiana* plants by transiently expressing GmMRLKs. GmMRLK32 and GmLMM1 strongly suppressed the flg22-induced ROS burst in *N. benthamiana* (Fig. 1b). As a control, overexpression of GmMRLK36 did not affect flg22-induced ROS production (Fig. 1b). However, chitin-induced ROS production was only slightly suppressed by expression of GmMRLK32 or GmLMM1 (Additional file 2: Figure S2a). Similarly, MAPK activation was significantly suppressed by expression of GmMRLK32 or GmLMM1 in *N. benthamiana*, but not GmMRLK36 (Fig. 1c). We further observed that expression of GmMRLK32 and GmLMM1 suppressed *P. sojae*-derived molecular pattern XEG1-induced cell death (Additional file 2: Figure S2b). Next, we constructed stable *GmMRLK32-HA* and *GmLMM1-HA* transgenic Arabidopsis plants and examined flg22-induced ROS burst and MAPK activation. All GmMRLK32 (GmMRLK32-L1 and L2) and GmLMM1 (GmLMM1-L1 and L2) transgenic plants exhibited significantly reduced ROS production and MAPK activation following flg22 treatment (Fig. 1d, e). In contrast, chitin-induced ROS production was only slightly enhanced by expressing GmMRLK32 in Arabidopsis (Additional file 2: Figure S2c), suggesting that GmMRLK32 specifically regulates immunity mediated by LRR-type PRRs. Immunoblotting assays showed that GmMRLK32-HA and GmLMM1-HA were expressed in Arabidopsis transgenic lines at similar levels (Additional file 2: Figure S2d).

We previously reported that the kinase activity of GmLMM1 is not required for its function in regulating PTI (Wang et al. 2020). Bioinformatic analysis revealed that Lysine 562 was the ATP-binding site of GmMRLK32, which is essential for its kinase activity. Therefore, we constructed GmMRLK32^{K562E}, which carries a mutation by substituting Lysine 562 with Glutamic acid. We then transiently expressed GmMRLK32-HA and GmMRLK32^{K562E}-HA in *N. benthamiana* and examined flg22-induced ROS burst. We observed that GmMRLK32 ^{K562E} suppressed flg22-induced ROS production to a level similar to that of GmMRLK32 (Fig. 1f). Consistent with this notion, the K562E mutation did not affect the suppression effect of GmMRLK32 on XEG1-induced cell death (Additional file 2: Figure S2b). Taken together, these results indicate that GmMRLK32 suppresses plant PTI in a kinase activity-independent manner.

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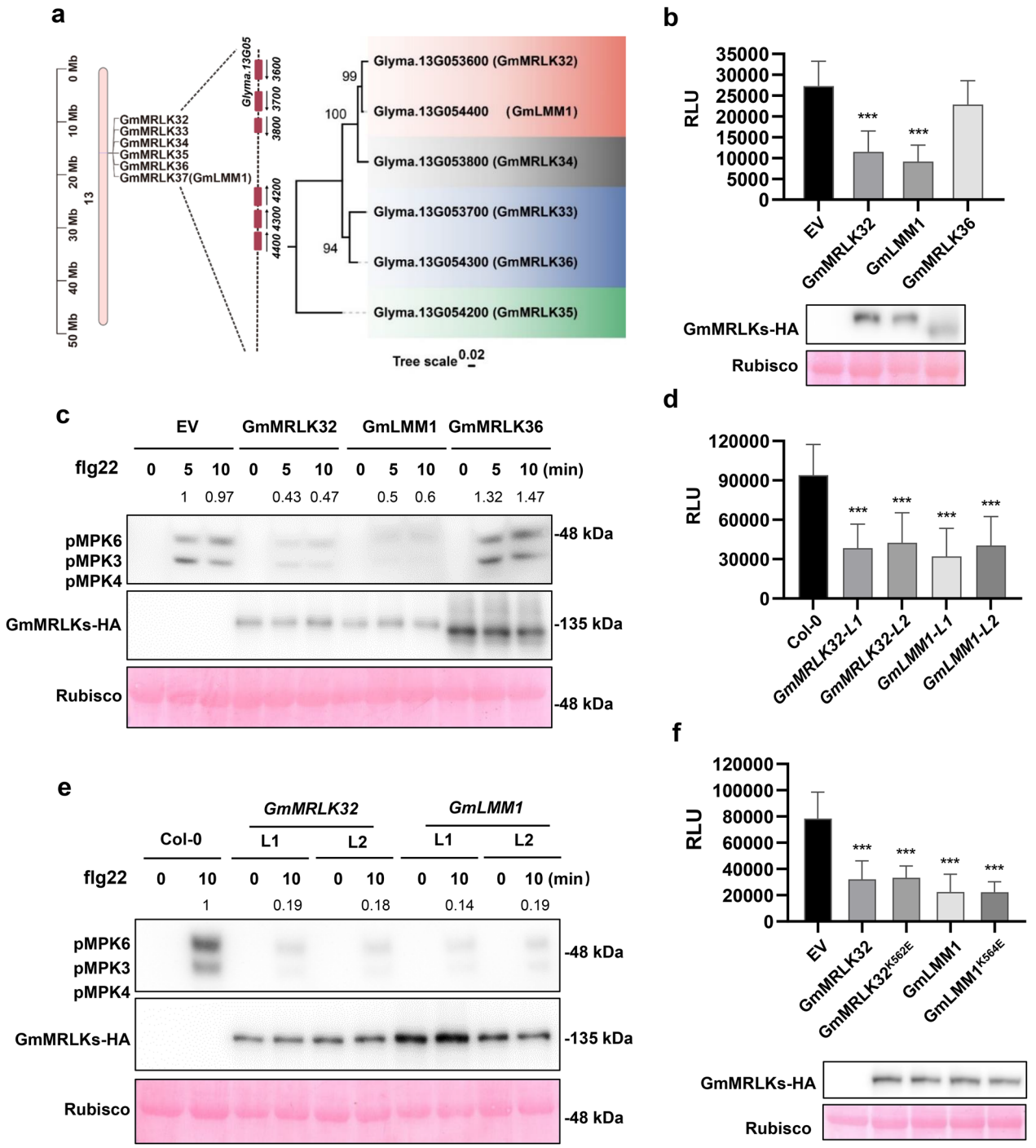
Fig. 1 Overexpression of GmMRLK32 suppresses PTI responses in *N. benthamiana* and Arabidopsis**. a** Phylogenetic analysis of GmMRLK32, GmLMM1, and some GmMRLKs tandem repeats on chromosome 13. **b** GmMRLK32 suppresses flg22-induced ROS in *N. benthamiana*. *Agrobacterium* strains carrying empty vector (EV), GmMRLK32, GmLMM1, or GmMRLK36 were infiltrated into *N. benthamiana* for 2 days, and subjected to flg22-induced ROS examination and the relative luminescence unit (RLU) value was recorded. GmMRLK36 was used as a negative control. (Mean ± SD, n ≥ 6, Student's *t*-test; *** $p < 0.001$). **c** GmMRLK32 suppresses flg22-induced MAPK in *N. benthamiana*. *Agrobacterium* strains carrying empty vector (EV), GmMRLK32, GmLMM1, or GmMRLK36 were infiltrated into *N. benthamiana* for 2 days, and subjected to flg22-induced MAPK examination by anti-pERK immunoblots. Quantitative analysis of MAPK intensity was performed using imageJ and numbers on top of the gel blots indicate ratio of MAPK/Rubisco. **d** GmMRLK32 suppresses flg22-induced ROS production in Arabidopsis. *GmMRLK32-HA* was introduced into Arabidopsis by *Agrobacterium*-mediated stable transformation and independent stable transgenic lines were subjected to flg22-induced ROS examination. (Mean ± SD, n ≥ 6, Student's *t*-test; *** $p < 0.001$). **e** GmMRLK32 suppresses flg22-induced MAPK activation in Arabidopsis. Independent *GmMRLK32-HA* stable transgenic lines were subjected to flg22-induced MAPK examination by anti-pERK immunoblots. Quantitative analysis of MAPK intensity was performed using imageJ and numbers on top of the gel blots indicate ratio of MAPK/Rubisco. **f** GmMRLK32 suppresses flg22-induced ROS production in a kinase-independent manner. The wild-type and kinase dead forms (K562E) of GmMRLK32 were expressed in *N. benthamiana* plants by *Agrobacterium*-mediated transient expression for 2 days, and subjected to flg22-induced ROS examination. (Mean ± SD, n ≥ 6, Student's *t*-test; *** $p < 0.001$). The experiments repeated three times



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GmMRLK32 negatively regulates PTI and disease resistance in soybean

To confirm the role of GmMRLK32 in PTI, we introduced *Gmmrlk32* and *Gmlmm1* soybean mutants, using CRISPR-Cas9 approach in Dongnong 50 (DN50) background. *Gmlmm1* and *Gmmrlk32* each contain a 1 bp and 7 bp deletion in their coding region that causes gene frame shift, respectively (Additional file 3: Figure S3) (Wang et al. 2020). Consistent with our previous report, the lesion mimic spots are observed in the leaves of *Gmlmm1*, but not *Gmmrlk32* (Additional file 3: Figure S3) (Wang et al. 2020). First, we examined flg22-induced

ROS production in the *Gmlmm1* and *Gmmrlk32* mutants. The results showed that both mutants displayed significantly enhanced ROS production upon flg22 treatment (Fig. 2a). We also examined ROS accumulation in leaves treated with *P. sojae* strain P6479, a *Phytophthora* pathogen that can infect soybeans and cause phytophthora root rot (Liu et al. 2014). Compared with DN50, both the mutants showed significantly enhanced ROS accumulation upon *P. sojae* treatment at 16 h (Additional file 4: Figure S4a). We then examined flg22-induced MAPK activation in *Gmlmm1* and *Gmmrlk32* mutants, in which both mutants showed significantly enhanced MAPK activation (Fig. 2b).

To further explore the role of GmMRLK32 in soybean disease resistance, we challenged plants with different pathogens. We infected the *Gmlmm1* and *Gmmrlk32* with *Pseudomonas syringae* pv. *glycinea* (*Psg*), a bacterial pathogen that causes leaf spot on soybean (Ashfield et al. 1995). The mutants displayed greatly enhanced resistance to *Psg* infection (Fig. 2c). Similarly, the *Gmlmm1* and *Gmmrlk32* mutants showed enhanced resistance to *P. sojae* strain P7076 (Fig. 2d and Additional file 4: Figure S4b). Note that *Gmlmm1* displayed a similar level of enhanced PTI as *Gmmrlk32*, but it showed slightly stronger resistance against pathogens than *Gmmrlk32*. We hypothesized that GmLMM1 might

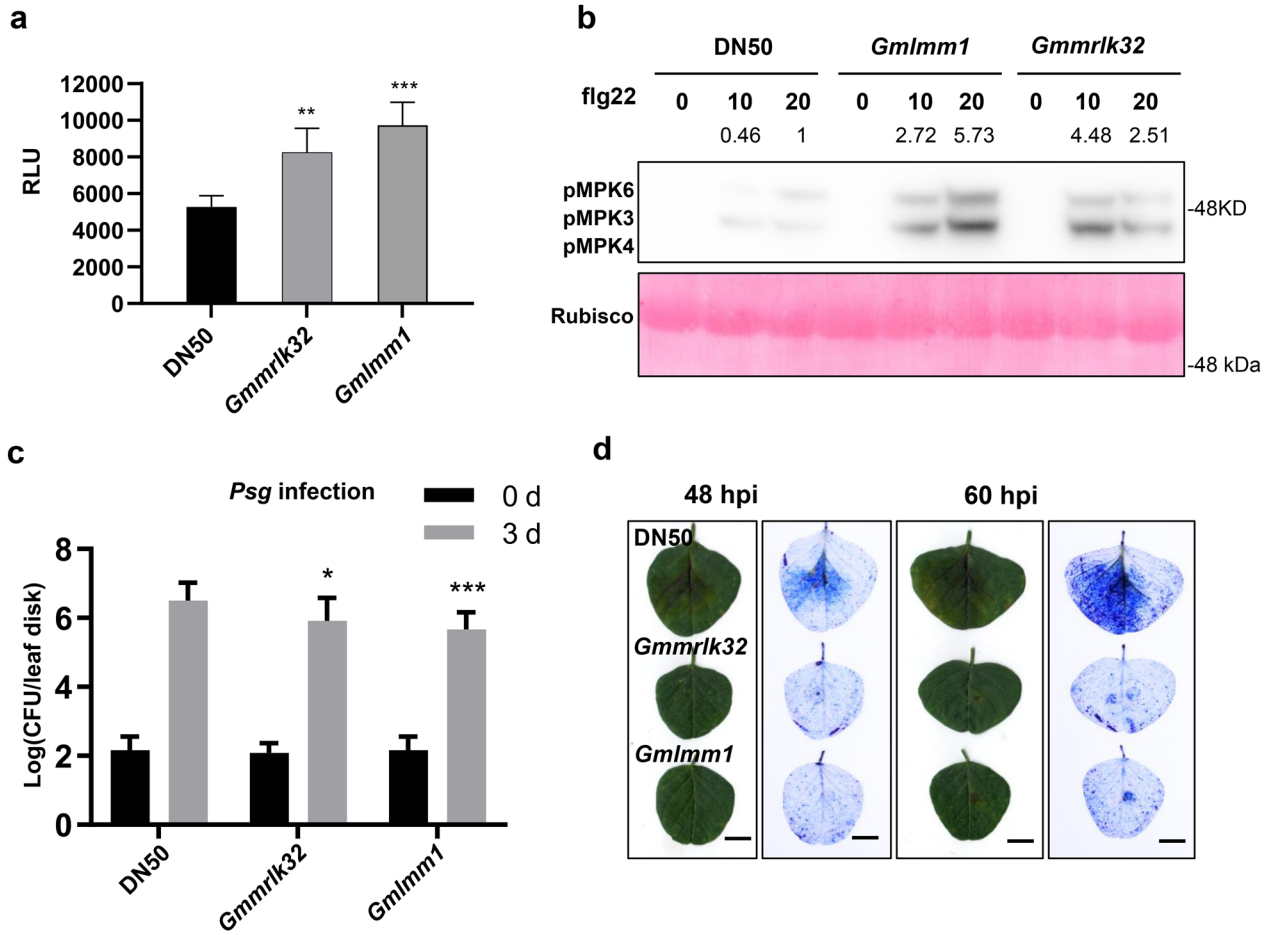

Fig. 2 *Gmmrlk32* mutant display enhanced PTI and disease resistance**. a** The *Gmmrlk32* and *Gmlmm1* mutants showed enhanced flg22-induced ROS. Leaves of the indicated plants at 2 weeks old were subjected to flg22 -induced ROS examination (Mean ± SD, n ≥ 6, Student's *t*-test; ** $p < 0.01$, *** $p < 0.001$). **b** The *Gmmrlk32* and *Gmlmm1* mutants showed enhanced flg22-induced MAPK activation. Leaves of the indicated plants at 2 weeks old were subjected to flg22-induced MAPK examination by anti-pERK immunoblots. Quantitative analysis of MAPK intensity was performed using imageJ and numbers on top of the gel blots indicate ratio of MAPK/Rubisco. **c** The *Gmmrlk32* and *Gmlmm1* mutants exhibited enhanced resistance to *P. syringae* pv. *glycinea* (*Psg*) infection. Leaves of 2-week-old soybean plants were infected with *Psg*, and the bacteria number was calculated at 0 and 3 dpi (Mean ± SD, n ≥ 6, Student's *t*-test; * $p < 0.05$, ***$p < 0.001$). **d** The *Gmmrlk32* and *Gmlmm1* mutants exhibited enhanced resistance to *P. sojae* infection. Leaves of 2-week-old soybean plants were infected with *P. sojae* strain P7076, and the lesions were examined 2 days later via trypan blue staining. Scale bars: 1 cm. The experiment repeated three times

regulate immunity beyond PTI, such as immune-related cell death. Therefore, we conclude that GmMRLK32 and GmLMM1 are negative regulators of PTI and plant disease resistance in soybean.

GmMRLK32 is involved in GmFLS2 immune receptor complex

GmLMM1 regulates PTI by coupling with the PRR complexes. It interacts with GmFLS2, GmBAK1, and other key components of the PRR complex, such as heterotrimeric G proteins and RLCKs (Wang et al. 2020). Therefore, we examined the interactions between GmMRLK32 and the PRR complex. GmMRLK32 interacted with GmFLS2 (Glyma.08g083300) and NbFLS2, as per luciferase complementation image (LCI) and co-immunoprecipitation (co-IP) assay results, in *N. benthamiana* (Fig. 3a, b). LCI and co-IP assays further showed that GmMRLK32 are in a complex with GmBAK1a (Glyma.15G051600), GmBAK1b (Glyma.08G180800), and NbBAK1 (Fig. 3c, d). Immunoblotting assays showed that the indicated proteins were expressed in *N. benthamiana* in the LCI assays (Additional file 5: Figure S5a, b). LCI assays showed that GmMRLK32 also interacts with soybean heterotrimeric G protein subunits GmGα and GmGβ (Glyma.17g226700, Glyma.06G05700, Glyma.11G118500, and Glyma.04G013100) and RLCKs (Glyma.14G068700 and Glyma.02G247600) (Additional file 6: Figure S6a–c), the known key components in PRR complexes (Couto and Zipfel, 2016). This result further confirmed that GmMRLK32 is coupled to PRR complexes. We then examined the effect of flg22 treatment on GmMRLK32-GmFLS2 interaction. Co-IP assays showed that flg22 treatment significantly enhanced GmMRLK32-GmFLS2 and GmLMM1-GmFLS2 interactions (Fig. 3e). However, flg22 treatment did not affect the GmMRLK32-GmBAK1a or GmLMM1-GmBAK1a interactions (Fig. 3f), suggesting that GmMRLK32-GmFLS2 and GmLMM1-GmFLS2 interactions are dynamically regulated by flg22. Together, we showed that GmMRLK32 and GmLMM1 are components of plant PRR complexes.

GmMRLK32 negatively regulates GmFLS2-GmBAK1 interaction

We have previously shown that GmLMM1 regulates PTI by modulating the GmFLS2-GmBAK1 interaction (Wang et al. 2020). Therefore, we evaluated the effect of GmMRLK32 on flg22-induced GmFLS2-GmBAK1 dimerization. Consistent with our previous findings, our co-IP assays showed that GmLMM1 strongly suppressed flg22-induced GmFLS2-GmBAK1 interaction. GmMRLK32 significantly suppressed GmFLS2-GmBAK1a interactions at a level comparable to that of GmLMM1 (Fig. 4a). Similarly, GmMRLK32 suppressed GmFLS2-GmBAK1b interaction (Fig. 4b). Consistent with this notion, we observed that GmMRLK32 and GmLMM1 suppressed GmFLS2-GmBAK1a interaction in the LCI assays (Fig. 4c). We further showed that both GmMRLK32^{K562E} and GmMRLK32 inhibited the flg22-induced GmFLS2-GmBAK1a interaction (Fig. 4d), which supports the notion that the kinase activity of GmMRLK32 is not required for its function in regulating PTI. Taken together, the results indicated that GmMRLK32 and GmLMM1 regulate PTI by suppressing the interaction of PRRs and their co-receptors.

GmMRLK32 forms hetero-oligomers with GmLMM1

The aforementioned results indicated that GmMRLK32 and GmLMM1 regulate PTI in a similar manner. Considering the high protein sequence similarity between GmLMM1 and GmMRLK32, it is confusing that the *Gmlmm1* and *Gmmrlk32* mutants showed similar levels of flg22-induced ROS burst and MAPK activation (Fig. 2a, b). We hypothesized that GmMRLK32 and GmLMM1 regulates PTI synergistically. Therefore, we performed LCI and co-IP assays to examine

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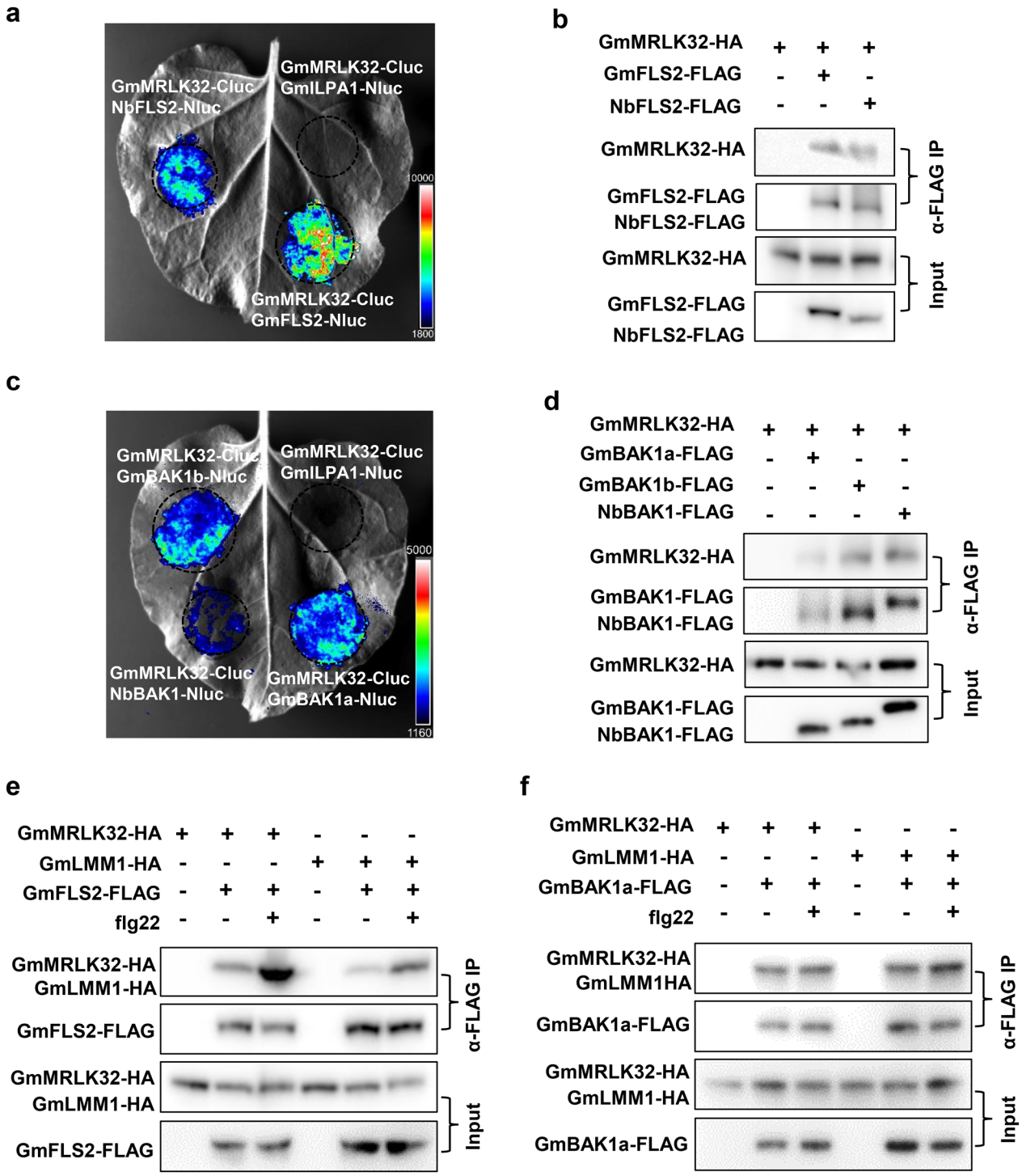
Fig. 3 GmMRLK32 interacts with GmFLS2 and GmBAK1. **a** GmMRLK32 interacts with GmFLS2 and NbFLS2 in *N. benthamiana* by luciferase complementation image (LCI) assays. The indicated constructs were expressed in *N. benthamiana* by *Agrobacterium*-mediated transient expression assay, and LCI assays were performed to examine the protein interactions. GmILPA1 was used as a negative control. The protein expression levels are shown in Additional file 5: Figure S5a. **b** GmMRLK32 interacts with GmFLS2 and NbFLS2 in *N. benthamiana*, as determined via co-IP assays. The indicated constructs were expressed in *N. benthamiana* by *Agrobacterium*-mediated transient expression assay, and protein interactions were examined by co-IP assay. **c** GmMRLK32 interacts with GmBAK1 and NbBAK1 in *N. benthamiana* by LCI assay. The indicated constructs were expressed in *N. benthamiana* by *Agrobacterium*-mediated transient expression assay, and LCI assays were performed to examine the protein interactions. The protein expression levels are shown in Additional file 5: Figure S5b. **d** GmMRLK32 interacts with GmBAK1 and NbBAK1 in *N. benthamiana* by co-IP assays. The indicated constructs were expressed in *N. benthamiana* by *Agrobacterium*-mediated transient expression assay, and protein interactions were examined by co-IP assay. **e** The GmMLRK32-GmFLS2 interaction was dynamically regulated by flg22. The indicated constructs were expressed in *N. benthamiana* by *Agrobacterium*-mediated transient expression assay, treated with 1 μM flg22, and protein interactions were examined by co-IP assay. **f** The GmMRLK32-GmBAK1a interaction was not affected by flg22 treatment. The indicated constructs were expressed in *N. benthamiana* by *Agrobacterium*-mediated transient expression assay, treated with 1 μM flg22, and protein interactions were examined by co-IP assay. The experiments were performed three times with similar results



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GmMRLK32-GmLMM1 interactions. LCI assays showed that GmLMM1 strongly interacted with GmMRLK32 in *N. benthamiana* (Fig. 5a). Immunoblotting assays showed that GmLMM1 and GmMRLK32 were expressed in *N. benthamiana* in the LCI assays (Additional file 5: Figure S5c). We then confirmed GmMRLK32-GmLMM1 interaction using co-IP assays (Fig. 5b and Additional file 7: Figure S7); We observed that GmMRLK32 was dissociated from GmLMM1 upon flg22 treatment in co-IP assays (Fig. 5b and

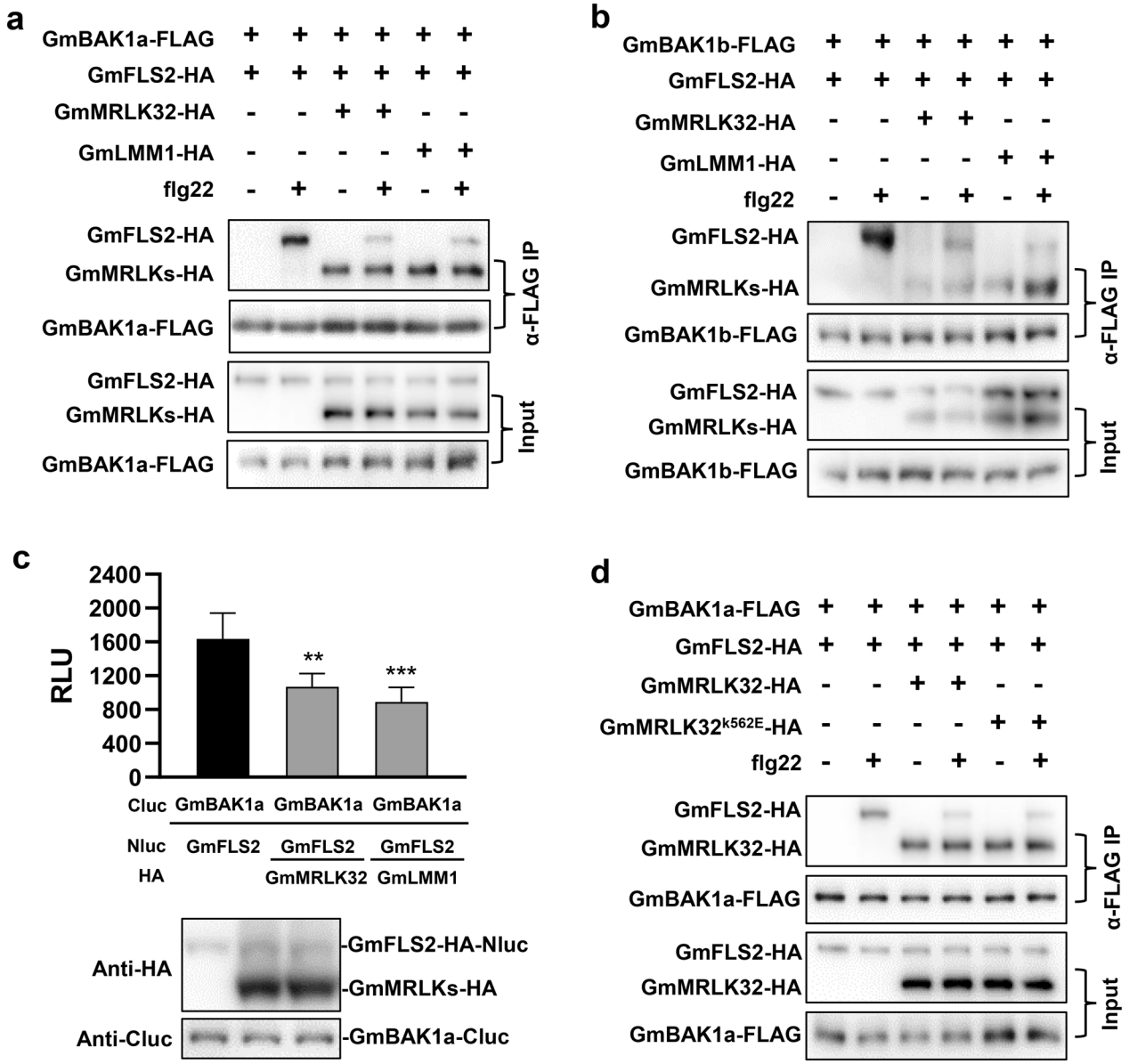

Fig. 4 GmMRLK32 suppresses flg22-iduced GmFLS2-GmBAK1 interactions**. a** GmMRLK32 and GmLMM1 suppress flg22-induced GmFLS2-GmBAK1a interaction. GmFLS2 and GmBAK1a were co-expressed in *N. benthamiana* along with GmLMM1 or GmMRLK32, treated with or without flg22, and the GmFLS2-GmBAK1 interactions were examined by co-IP assays. **b** GmMRLK32 suppresses flg22-induced GmFLS2-GmBAK1b interaction. GmFLS2 and GmBAK1b were co-expressed in *N. benthamiana* along with GmMRLK32, treated with or without flg22, and subjected to co-IP assays. **c** GmMRLK32 inhibits GmFLS2-GmBAK1a interaction by LCI assays. The indicated constructs were transiently expressed in *N. benthamiana* and LCI assays were performed to examine the protein interactions. The protein expression levels were shown by immunoblotting. (Mean ± SD, n ≥ 8, Student's *t*-test; ** $p < 0.01$, *** $p < 0.001$). **d** GmMRLK32 suppresses flg22-induced GmFLS2-GmBAK1a interaction in a kinase-independent manner. GmFLS2 and GmBAK1a were co-expressed in *N. benthamiana* along with GmMRLK32 or GmMRLK32^{K562E}, treated with or without flg22, and the GmFLS2-GmBAK1a interactions were examined by co-IP assays

Additional file 7: Figure S7), suggesting that this interaction is dynamically regulated by flg22. To determine the specificity of GmLMM1-GmMRLK32 interaction, we analyzed the interaction between GmLMM1-HA and GmLMM1-FLAG. Although we detected the formation of the GmLMM1 homodimer, this interaction was not affected by flg22 treatment (Fig. 5c). Together, we hypothesized that GmMRLK32 regulates PTI by forming a complex with GmLMM1.

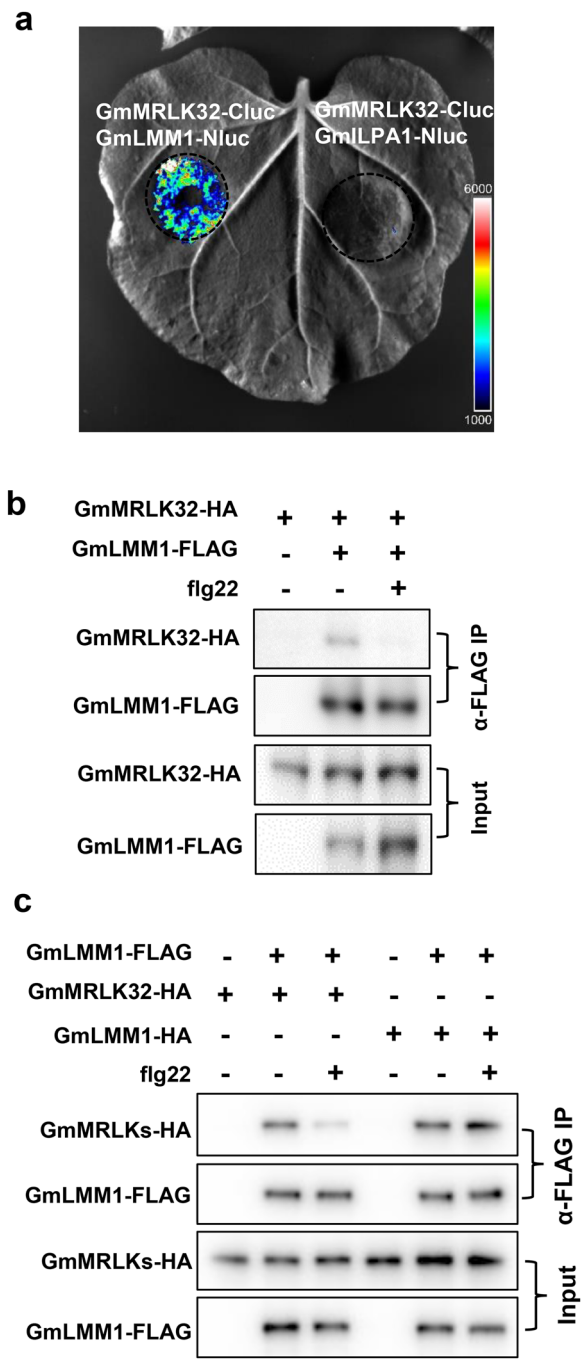

Fig. 5 GmMRLK32 specifically interacts with GmLMM1. **a** GmMRLK32 interacts with GmLMM1 in *N. benthamiana* by LCI assay. The indicated constructs were expressed in *N. benthamiana* by *Agrobacterium*-mediated transient expression assay, and LCI assays were performed to examine the protein interactions. The protein expression levels were examined by immunoblotting as shown in Additional file 5: Figure S5c. **b** GmMRLK32 dynamically interacts with GmLMM1 in *N. benthamiana* by co-IP assay. GmMRLK32-HA and GmLMM1-FLAG were expressed in *N. benthamiana* by *Agrobacterium*-mediated transient expression assay, treated with or without flg22, and protein interactions were examined by co-IP assay. **c** flg22 specifically regulates GmMRLK32-GmLMM1 interaction. The indicated constructs were expressed in *N. benthamiana* by *Agrobacterium*-mediated transient expression assay, treated with or without flg22, and protein interactions were examined by co-IP assay

GmMRLK32 and GmLMM1 may coordinately regulate PTI

To verify the hypothesis that these two MRLKs jointly regulate PTI, we examined flg22-induced ROS burst and MAPK activation in *N. benthamiana* plants that were infiltrated with *Agrobacterium* carrying GmMRLK32-HA and empty vectors (EV), GmLMM1-HA and EV, and GmMRLK32-HA and GmLMM1-HA (Fig. 6a). When proteins were expressed at similar levels for the three groups, we found that co-expression of GmMRLK32 and GmLMM1 exhibited the strongest inhibitory effect on flg22-induced ROS production, as compared with the expression of GmMRLK32 or GmLMM1 alone (Fig. 6a). Notably, GmMRLK32 and GmLMM1 have similar molecular weights and cannot be separated via SDS-PAGE. Similarly, co-expression of GmMRLK32 and GmLMM1 almost completely abolished flg22-induced MAPK activation in *N. benthamiana* plants (Fig. 6b). Next, we inoculated *N. benthamiana* leaves with *P. capsici*, a destructive pathogen that can infect most dicots. Leaves co-expressing GmMRLK32-HA and GmLMM1-HA exhibited the most serious susceptibility to *P. capsici* (Fig. 6c). Co-expression of GmMRLK32-HA and GmLMM1-HA also displayed enhanced suppression effect on XEG1-induced cell death (Fig. 6d). These results are consistent with the notion that GmMRLK32 and GmLMM1 might regulate PTI by forming hetero-oligomers (Fig. 6e).

Discussion

In this study, we identified a pair of closely related soybean MRLKs, namely GmMRLK32 and GmLMM1, which are tandemly repeated on chromosome 13 and negatively regulate plant disease resistance. The MRLK family is a very important class of plant RLK proteins that play crucial regulatory roles in multiple biological processes. Here, we showed that GmMRLK32 and GmLMM1 are involved in PRR complexes and negatively regulate flg22-induced immune responses by suppressing GmFLS2-GmBAK1 interaction. We further for the first time showed that two MRLKs might jointly regulate PTI by forming hetero-oligomers. This study demonstrates a new mechanism for MRLKs to regulate plant PTI and advances our understanding of the regulatory mechanism of MRLKs.

Our previous study identified GmLMM1 as a negative regulator of PTI in a screen of soybean lesion mimicking mutants (Wang et al. 2020). Subsequently, we performed a systematic reverse screening for GmMRLKs that can affect plant PTI and ETI responses based on transient expression assays in *N. benthamiana*. In this screening, we observed that the overexpression of GmMRLK32 strongly suppressed flg22-induced ROS production (Zhang et al. 2022). Overexpression of either GmMRLK32 or GmLMM1 in *N. benthamiana* and Arabidopsis attenuated flg22-induced ROS and MAPK activation. We observed that GmLMM1 and GmMRLK32 were involved in the GmFLS2 receptor complex and negatively regulated GmFLS2-GmBAK1 interaction. GmMRLK32 and GmLMM1 are the closest homologs and are probably generated by gene duplication events (Wang et al. 2020). Therefore, we hypothesized that they may function redundancy in the regulation of PTI.

In contrast to this hypothesis, both *Gmmrlk32* and *Gmlmm1* mutants exhibited similar levels of enhanced PTI. Protein interaction assays showed that GmMRLK32 interacts with GmLMM1. This result prompted us to hypothesize that GmMRLK32 and GmLMM1 coordinately regulate PTI by forming hetero-oligomers. Consistent with this notion, co-overexpression of GmLMM1 and GmMRLK32 resulted in enhanced suppression of PTI, when compared to overexpression of GmLMM1 or GmMRLK32 alone. Notably, the suppression of PTI by transient expression of GmLMM1 or GmMRLK32 in *N. benthamiana* suggests that GmMRLK32/GmLMM1 might forms hetero-oligomers with endogenous NbMRLKs. Unfortunately, we failed to generate *Gmlmm1 Gmmrlk32* double mutant. To further support the notion that GmLMM1 and GmMRLK32 form hetero-oligomers to regulate immunity, the immune phenotype of *Gmmrlk32 Gmlmm1* double mutants should be examined in future studies. To further elucidate the molecular mechanism, the exact number of GmLMM1 and GmMRLK32 proteins in the oligomer also needs to be clarified via biochemical experiments. Several MRLKs from Arabidopsis, such as FER, and ANX, were reported to regulate the FLS2-BAK1 interaction (Stegmann et al. 2017; Mang et al. 2017; Gronnier et al. 2022). It would be interesting to investigate whether these MRLKs adopt similar strategies to regulate PTI activation.

We previously examined flg22-induced ROS production in *N. benthamiana* plants overexpressing GmMRLKs which are replicated in tandem with GmMRLK32 and GmLMM1. The results showed that GmMRLK34, but not GmMRLK33, GmMRLK35, or GmMRLK36, suppressed flg22-induced ROS production (Zhang et al. 2022), suggesting that these six GmMRLKs are functionally differentiated in regulating plant immunity. The exact role of GmMRLK34 in soybean disease resistance and whether it regulates PTI by forming oligomers require further investigation. We showed that overexpression of GmMRLK32 or GmLMM1 in *N. benthamiana* only slightly increased chitin-induced ROS production, indicating that these two GmMRLKs may preferentially regulate immune responses mediated by PRRs with extracellular LRR motifs. Consistent with this notion, we showed that GmMRLK32 and GmLMM1 also suppress the LRR-RLP receptor RXEG1-mediated cell death phenotype. Therefore, GmMLRK32 and GmLMM1 might also regulate the interaction between RLP receptor and BAK1 co-receptor. We further showed that the kinase activities of GmMRLK32 and GmLMM1 are not required to regulate plant immunity. Therefore, this hetero-oligomer may function as a scaffold to regulate the interactions between PRRs and their co-receptors. Additionally, flg22-induced GmLMM1-GmMRLK32 dissociation indicated that this interaction was dynamically regulated in PRR complexes.

It is worth noting that the lesion mimicking phenotype was not observed in *Gmmrlk32* mutant (Wang et al. 2020), suggesting a divergence between the functions of GmLMM1 and GmMRLK32 in the regulation of autoimmunity-related cell death. GmLMM1 may play a role in plant ETI and ETI-associated HR. Differences in 29 amino acids were observed between the two proteins.

(See figure on next page.)

Fig. 6 GmMRLK32 and GmLMM1 jointly regulate PTI and disease resistance. **a** and **b** Co-expression of GmMRLK32 and GmLMM1 showed stronger suppression effect on PTI than expression of GmMRLK32 or GmLMM1 alone. The indicated constructs were expressed in *N. benthamiana* by *Agrobacterium*-mediated transient expression assays and subjected to flg22-induced ROS and MAPK examination. (Mean ± SD, n ≥ 8, Student's *t*-test; different letters indicate significant difference at $p < 0.01$). Quantitative analysis of MAPK intensity was performed by imageJ and numbers on top of the gel blots indicate ratio of MAPK/Rubisco. **c** *N. benthamiana* plants co-expressing GmMRLK32 and GmLMM1 were more susceptible than plants expressing GmMRLK32 or GmLMM1 alone. The indicated constructs were expressed in *N. benthamiana* by *Agrobacterium*-mediated transient expression assay for one day; The resulted tissue was inoculated with *P. capsici* strain LT263, and photographed under UV light 48 h later. The lesion area was measured. (Mean ± SD, n ≥ 8, Student's *t*-test; different letters indicate significant difference at $p < 0.05$). **d** Co-expression of GmMRLK32 and GmLMM1 showed stronger suppression effect on XEG1-induced cell death, compared to expression of GmMRLK32 or GmLMM1 alone. The indicated constructs were transiently expressed in *N. benthamiana* 1 day before infiltration of XEG1, and cell death phenotypes were examined two days later. **e** A model illustrating that GmMRLK32 and GmLMM1 regulating GmFLS2-GmBAK1 interaction by forming hetero-oligomers

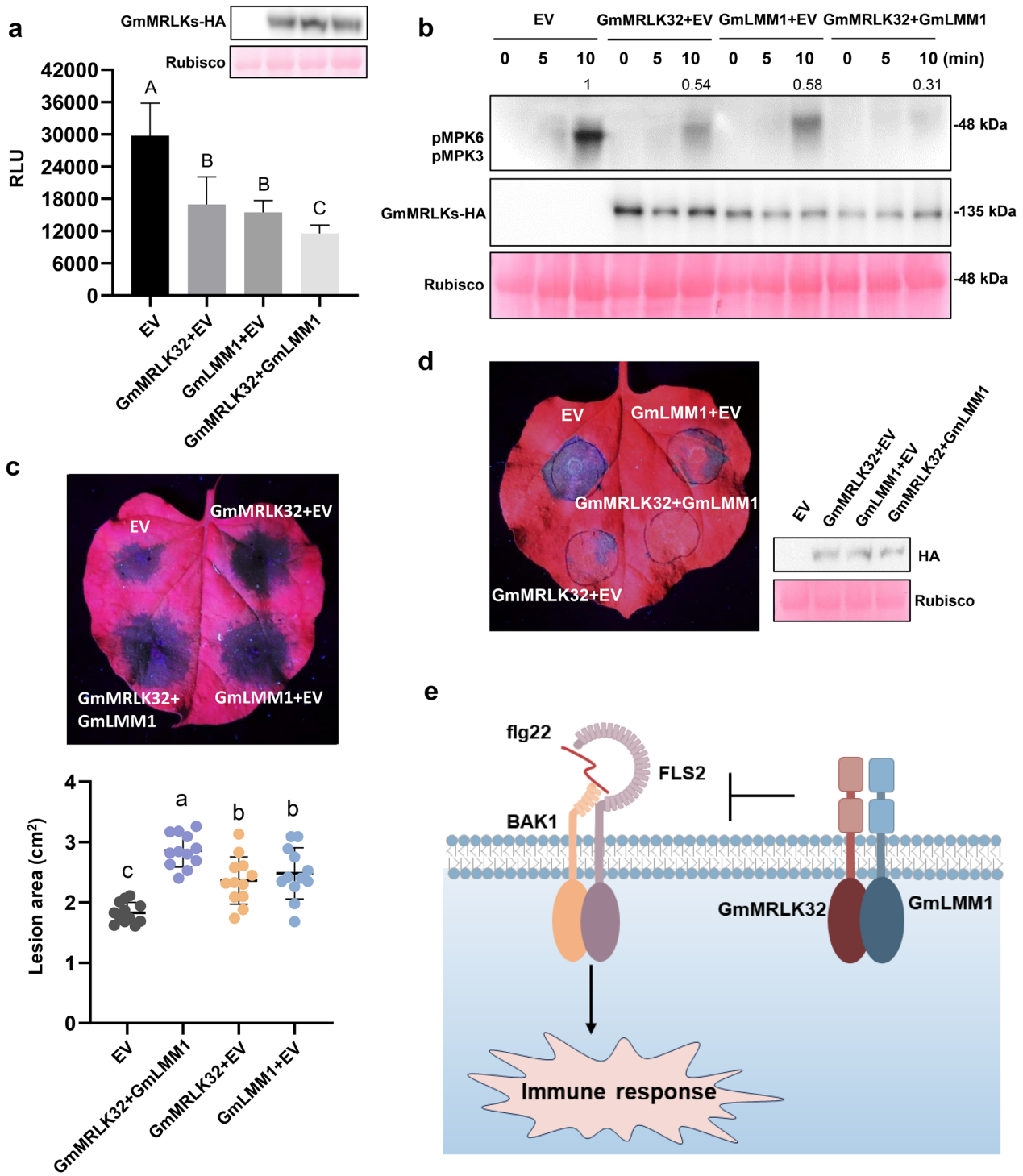

Fig. 6 (See legend on previous page.)

Further studies are required to identify the key amino acids responsible for functional differentiation. We speculated that GmLMM1 might be guarded by certain NLR receptor and the loss of GmLMM1 caused a constitutive activation of ETI, which caused the autoimmunity related lesion mimic phenotype. We also showed that the overexpression of GmMRLK36, another GmMRLK protein that is in tandem with GmLMM1 and GmMRLK32, did not suppress flg22-induced ROS. All six GmMRLK genes in this region may originate from gene duplication (Wang

et al. 2020). The functional differentiation between these six genes deserves further investigation.

MRLKs recognize a family of secreted RALF peptides that regulate multiple biological processes, including plant growth, development, and responses to biotic and abiotic stresses (Franck et al. 2018). For example, FER recognizes RALF1 and RALF23 to regulate plant growth and immunity, respectively (Escobar-Restrepo et al. 2007; Haruta et al. 2014; Stegmann et al. 2017). Whether GmLMM1 and GmMRLK32 possess the capacity for RALF binding and the function of their corresponding RALFs still requires investigation. Another interesting topic to study is whether the GmMRLK32-GmLMM1 oligomer is regulated by RALF peptides.

We previously showed that GmLMM1 also regulates the fertility of sexual organs and embryo development (Wang et al. 2020), suggesting that GmLMM1 is involved in the balance between plant growth, reproduction, and disease resistance. However, it is unclear whether GmLMM1 and GmMRLK32 form oligomers that regulate these processes. The exact role of GmMRLK32 in processes other than disease resistance remains unclear. In summary, we propose a model in which GmMRLK32 and GmLMM1 form hetero-oligomers to regulate PTI and soybean disease resistance (Fig. 6e). This study provides clues for further understanding plant immunity and may help to facilitate the improvement of soybean disease resistance based on modern genetic engineering techniques, such as gene editing.

Methods

Plant materials

The soybean cultivar Dongnong 50 (DN50) was used as the wild type in this study. *Gmmrlk32* and *Gmlmm1* were generated using the CRISPR-Cas9 approach as previously described (Wang et al. 2020). *N. benthamiana* plants were grown in a growth chamber at 25°C and 60% relative humidity with a photoperiod of 16 h of light /8 h of darkness. Wild-type Arabidopsis (Col-0) plants were grown at 23°C and 70% relative humidity with a 12 h/12 h photoperiod.

Plasmid construction

For the transient expression assay in *N. benthamiana* and the generation of stable transgenic Arabidopsis lines, the coding sequences of the indicated genes were amplified and cloned into pCAMBIA1300-35S-HA-RBS or pCAMBIA1300-35S-FLAG-RBS vectors. To perform the luciferase complementation image (LCI) assay, the coding sequences of the corresponding genes were amplified and inserted into the pCAMBIA1300-35S-Cluc-RBS or pCAMBIA1300-35S-HA-Nluc-RBS vectors. Point mutations in *GmMRLK32* and *GmLMM1* were generated via site-directed mutagenesis.

Agrobacterium-mediated transient expression in *N. benthamiana*

The indicated pCAMBIA1300 constructs were introduced into *Agrobacterium* strain GV3101 via electroshock transformation. *Agrobacterium* strains carrying the indicated constructs were grown overnight in liquid Luria-Bertani (LB) medium, collected by centrifugation, and washed twice with sterile water. The bacteria were resuspended in the infiltration solution (10 mmol/L $MgCl_2$, 10 MES pH 5.7, 200 μM acetosyringone) to a OD_{600} value of 0.6, and then were infiltrated into *N. benthamiana* leaves using a needleless syringe.

Pattern-induced ROS burst

Microbial pattern-induced ROS production assay was performed as previously described (Wang et al. 2020). Leaf disks from 2-week-old soybean, 4-week-old *N. benthamiana*, or 4-week-old Arabidopsis plants were incubated overnight with 200 μL of sterile water in a 96-well plate. The samples were treated with luminescence detection buffer (20 mM luminol [Sigma-Aldrich] and 10 mg/mL horseradish peroxidase [Sigma-Aldrich]) with the addition of 1 μM flg22 (Sangon) or 200 μg/mL chitin (Sigma-Aldrich). Relative luminescence units (RLU) were recorded using a luminometer (TECAN).

Pattern-induced MAPK activation

Examination of microbial patterns-induced MAPK activation in leaves of soybean and *N. benthamiana* referred to the previously described method (Li et al. 2022). Leaf discs were taken and placed in a 96-well plate containing 150 μL sterile water overnight. The samples were treated with 1 μM flg22 for the indicated time. For examination of MAPK activation in Arabidopsis, 10-day-old Arabidopsis seedlings in ½MS medium were sprayed with 1 μM flg22 for the indicated time. Total protein was extracted with protein extraction buffer (50 mM HEPES pH 7.5, 150 mM KCl, 5 mM EDTA, 0.5% Triton X-100, 1 mM DTT, proteinase inhibitor cocktail [Roche]), and MAPK activation was examined using anti-pERK immunoblots (Sigma-Aldrich).

Pathogen infection

To perform *P. syringae* pv. *glycinea* (*Psg*) infection assays, leaves from the indicated soybean plants at

2-week-old were infiltrated with bacteria at a concentration of 5×10^5 CFU/mL with a needleless syringe. Leaf disks were collected and the bacterial count was determined at 2 h and 3 days later (Wang et al. 2020).

To perform the *P. sojae* infection assays, leaves of the indicated soybean plants at 2-week-old were infected with *P. sojae* isolate P7076 for the indicated time at 25°C in dark. The lesion area was examined using trypan blue staining (Song et al., 2015).

To perform the *P. capsici* infection assays, the detached leaves from *N. benthamiana* plants at 4-week-old were infected with *P. capsici* isolate LT263 and inoculated under high humidity at 25°C in dark. The lesion area was photographed under UV light 36–48 h later (Liang et al. 2021).

Trypan blue and DAB staining

To perform the trypan blue staining assays, soybean leaves were boiled for 10 min in trypan blue solution (0.25 mg/mL), incubated at 25°C for 12 h, and de-stained in chloral hydrate (2.5 mg/mL) for 48 h. The disease phenotype was visualized by capturing photographs using a camera.

To perform the DAB staining assays, leaves were submerged in DAB solution (1 mg/mL, pH 3.8) at 25°C in dark for 8 h, transferred to 95% ethanol, boiled for 10 min, and incubated in 95% ethanol to remove the chlorophyll completely. ROS accumulation was visualized under a microscope or using a camera (Wang et al. 2020).

Luciferase complementation image (LCI)

The LCI assay was performed strictly following a previously described protocol (Zhou et al. 2018). *Agrobacterium* strains carrying the indicated Cluc and HA-Nluc constructs were co-infiltrated into the leaves of *N. benthamiana* for 2 d. Leaf disks were taken and incubated with 1 mM luciferin (Biovision) in a 96-well plate for 10–20 min, and RLU was measured using a microplate luminometer (Tecan F200). The protein expression levels of the indicated constructs were examined using anti-Cluc and anti-HA antibodies (Sigma-Aldrich).

Co-immunoprecipitation (co-IP)

The indicated pCAMBIA1300-35S-HA-RBS or pCAMBIA1300-35S-FLAG-RBS constructs were co-expressed in *N. benthamiana* leaves for 2 days using *Agrobacterium*-mediated transient expression assays. The leaves were ground in liquid nitrogen, extracted with IP buffer (50 mM HEPES [pH 7.5], 150 mM KCl, 1 mM EDTA, 0.3%Triton X-100, 1 mM DTT, proteinase inhibitor cocktail [Roche]), centrifuged at 27,000 *g* for 10 min, and the supernatant was collected. For anti-FLAG immunoprecipitation, total protein was incubated with 20 μL ANTI-FLAG® M2 agarose (Sigma) for 2 h, washed with IP buffer four times, and eluted with 60 μL 3×FLAG peptide (Sigma) for 45 min. Then, the beads were washed four times with IP buffer, and boiled at 100℃ for five minutes. Protein interactions were examined using anti-HA and anti-FLAG antibodies (Sigma-Aldrich).

Abbreviations

MAPK	Mitogen activated protein kinase
MRLK	Malectin/malectin-like domain-containing receptor-like kinases
PRR	Pattern-recognition receptor
PTI	Pattern-triggered immunity
RLK	Receptor-like kinase
RLP	Receptor-like protein
ROS	Reavtive oxygen species

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s42483-024-00232-1.

Additional file1: **Figure S1.** Sequence alignment of GmMRLK32 and GmLMM1.

Additional file2: **Figure S2.** Effect of GmMRLK32 on chitin-induced ROS burst and XEG1-induced cell death.

Additional file3: **Figure S3.** Schematic representation of *Gmmrlk32* and *Gmlmm1* mutants.

Additional file4: **Figure S4.** ROS accumulation and disease resistance of *Gmmlrk32* and *Gmlmm1* mutants upon *P. sojae* treatment.

Additional file5: **Figure S5.** Examination of protein expression levels in LCI assays.

Additional file6: **Figure S6.** GmMRLK32 interacts with soybean RLCKs and G proteins in *N. benthamiana*.

Additional file7: **Figure S7.** GmMRLK32 interacts with GmLMM1 in *N. benthamiana* by co-IP assay.2.

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Author contributions

DD, XF, and XL coordinated the research and wrote the paper. QZ performed the majority of the experiments. DW, HZ, BY, JY, and XW contribute to pathogen infection assays and plasmid constructions. GX contributed to the protein interaction assays. All authors read and approved the final manuscript.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

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.

Author details
[1]MOA Key Laboratory of Pest Monitoring and Green Management, Department of Plant Pathology, College of Plant Protection, China Agricultural University, Beijing 100193, China. [2]Key Laboratory of Soybean Molecular Design Breeding, Northeast Institute of Geography and Agroecology, The Innovative Academy of Seed Design, Chinese Academy of Sciences, Changchun 130102, China. [3]College of Life Sciences, South China Agricultural University, Guangzhou 510642, China. [4]College of Plant Protection, Nanjing Agricultural University, Nanjing 210095, China.

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