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Functional analysis of RALF peptides in soybean immunity and disease resistance

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Abstract

Plant cell-surface-localized pattern-recognition receptors (PRRs) recognize conserved microbial patterns to activate pattern-triggered immunity (PTI), which confers mild and broad-spectrum resistance to most microbes. Rapid alkalization factor (RALF) peptides are a family of secreted peptides that have been reported to regulate many biological processes, including plant immunity. However, little is known for the roles of RALFs in soybean immunity and disease resistance. In total, 24 RALFs have been identified in the soybean *Glycine max*, and we showed that these *GmRALFs* are either transcriptionally induced or suppressed in response to pathogen infection. We characterized the roles of *GmRALFs* in plant immunity by transiently expressing them in *Nicotiana benthamiana*. We examined the effect of *GmRALFs* on PTI responses, including the ROS burst induced by bacterial flg22 or fungal chitin, and oomycete INF1-induced cell death. We further examined the disease resistance to pathogenic bacteria, fungi, and oomycete in plants. We identified several *GmRALFs* that positively or negatively regulate plant immunity. Of these *GmRALFs*, *GmRALF1* and its homolog in *N. benthamiana* exhibited strong immune suppression in all PTI and disease resistance assays. Finally, we obtained *Gmralf1* mutant lines using CRISPR-Cas9 approach. These mutants exhibited significantly enhanced PTI and resistance to *Phytophthora sojae* and *Pseudomonas syringae* infection. In conclusion, our study revealed the common and specific roles of *GmRALFs* in PTI responses and resistance to various pathogens, and identified *GmRALF1* as a candidate susceptibility gene with potential applications for improving soybean disease resistance.

Keywords *Glycine max*, RALF, Pattern-triggered immunity, Disease resistance

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Background

Soybean is an important oilseed crop and source of high-quality plant proteins. The global population increase has led to an increasing demand for soybean production. Worldwide occurring diseases, such as soybean rust, root rot, and leaf spot, have caused serious loss of soybean yield and quality (Lin et al. 2022). Plants primarily rely on their innate immune system to resist invasion of pathogenic microorganisms. Therefore, advancing our understanding of soybean immunity is prerequisite for improving soybean production.

Plants rely mainly on two types of immune receptors to perceive pathogens: the cell-surface-localized pattern recognition receptors (PRRs) and intracellular nucleotide-binding and leucine-rich repeat receptors (NLRs)



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(Yu et al. 2017). PRRs can perceive certain conserved molecular features of microbes, such as bacterial flagellin (or epitope flg22) and fungal cell-wall-derived chitin. These molecules are termed pathogenic/microbe-associated molecular patterns (PAMPs/MAMPs) (DeFalco and Zipfel. 2021). PRRs consist of receptor-like kinases (RLKs) and receptor-like proteins (RLPs) (Tang et al. 2017). Recognition of PAMPs by PRRs requires the involvement of co-receptors or adapters, such as BAK1, CERK1, and SOBIR1 (Chinchilla et al. 2007; Zhang et al. 2013; Cao et al. 2014). Once PRRs and their co-receptors recognize PAMPs, they activate pattern-triggered immunity (PTI) (Yu et al. 2017). For example, Arabidopsis RLK receptor FLS2 recognizes flagellin with the participation of the co-receptor BAK1 (Gomez-Gomez and Bolter 2000; Chinchilla et al. 2007). The RLK proteins LYK4 or LYK5 forms a complex with CERK1 to recognize chitin (Cao et al. 2014). The potato RLP protein ELR interacts with StBAK1 and StSOBIR1 to recognize INF1, a *Phytophthora*-derived PAMP (Du et al. 2015). In addition, PRRs form complexes with many key regulatory components, such as receptor-like cytoplasmic kinases (RLCKs) (Lu et al. 2010; Zhang et al. 2010), malectin-like RLKs (Yeh et al. 2016; Stegmann et al. 2017; Mang et al. 2017), heterotrimeric G proteins (Liang et al. 2016), and NADPH oxidase (Kadota et al. 2015; Li et al. 2014). Upon perception of PAMPs, PRR complexes initiate a series of PTI responses, including ROS bursts, calcium influx, activation of MAPK and CDPK pathways, and defense gene expression (DeFalco and Zipfel. 2021).

Some pathogens suppress plant immunity by secreting virulence effectors into host cells to interfere with the key regulatory components of plant immune signal transduction (Dou and Zhou. 2012). Plant intracellular NLRs perceive the presence of some effectors using multiple strategies, leading to effector-triggered immunity (ETI), which is typically accompanied with hypersensitivity responses (Cui et al. 2015). For example, AvrAc, an effector of *Xanthomonas Campestris*, targets and suppresses plant RLCKs-mediated immunity via its uridylyl transferase activity (Feng et al. 2012). The NLR receptor ZAR1 perceives AvrAc-mediated UMP modification of the RLCK member PBL2, thereby activating ETI (Wang et al. 2015). Although PTI and ETI are mediated by different immune receptors, they share many downstream regulatory components and signaling pathways (Ngou et al. 2021; Yuan et al. 2021). Compared to ETI, PTI provided a milder and broad-spectrum resistance to most microbes, and holds great application prospects to improve broad-spectrum disease resistance in plants.

Rapid alkalization factors (RALFs) are a class of small, secreted peptides that are widely distributed in higher plants. This class was first discovered to possess

the capacity to increase the pH of the extracellular environment in tobacco (Pearce et al. 2001). The matured RALF peptides are cleaved at the second amino acid, usually with a length of ~50 amino acids (Pearce et al. 2010). RALFs usually contain four conserved cysteine residues and a conserved YISY motif folded through two disulfide bonds (Pearce et al. 2010; Campbell and Turner 2017). RALFs usually contain three conserved motifs, namely motifs 1–3. Motif 1 possesses a conserved YISY sequence and is supposed to function in binding with the receptors. Motif 2 contained the conserved RGC(5N) C and RCRR amino acids, which is required for forming disulfide bonds to maintain protein conformation and activity. Motif 3 contains the RRXL cleavage site, which is reported to be cleaved by S1P proteases to generate the matured RALFs in Arabidopsis (Srivastava et al. 2009). In total, 41 and 24 RALFs have been identified in Arabidopsis and soybean, respectively (Jia and Li. 2023). RALFs can be recognized by complexes formed by malectin-like RLKs and glycosylphosphatidylinositol-anchored proteins (GPI-APs) (Xiao et al. 2019), which play important regulatory roles in plant growth, development, abiotic stress response, and disease resistance (Tsukamoto et al. 2010; Zhao et al. 2018; Ge et al. 2019; Xiao et al. 2019; Li et al. 2023). For example, the Arabidopsis malectin-like RLK protein FERONIA (FER) recognizes RALF1 to regulate plant cell expansion, thereby affecting the plant root elongation and pollen tube development (Escobar-Restrepo et al. 2007; Haruta et al. 2014). RALF1 also affects the growth of roots, root hairs, and plant flowering by regulating the kinase activity or localization of FER (Du et al. 2016; Wang et al. 2020; Yu et al. 2020; Zhu et al. 2020). The extracellular domain of FER can bind to pectin to inhibit salt stress-induced cell wall loosening, thereby regulating the resistance to salt stress (Feng et al. 2018). RALF22 and RALF23 regulates the endocytosis of FER to modulate FER-mediated salt tolerance response (Zhao et al. 2018). Arabidopsis RALF4 and RALF19 are recognized by complexes consisting of the malectin-like RLK proteins BUPs 1/2 and ANX1/2, which regulate the bursting of pollen tubes during fertilization (Ge et al. 2017).

RALF peptides play crucial roles in plant immunity and disease resistance. FER can enhance flg22-induced immunity by promoting the interaction of FLS2 and its co-receptor BAK1 (Stegmann et al. 2017). RALF23 binds to FER to inhibit the promoting effect of FER on the FLS2-BAK1 interaction, thereby suppressing flg22-induced immunity (Stegmann et al. 2017). FER forms a complex with LLG proteins, a family of GPI-AP protein, to sense RALF23 (Xiao et al. 2019). Malectin-like RLK proteins ANX1/2 and GmLMM1 from Arabidopsis and soybean, respectively, have been reported to inhibit the

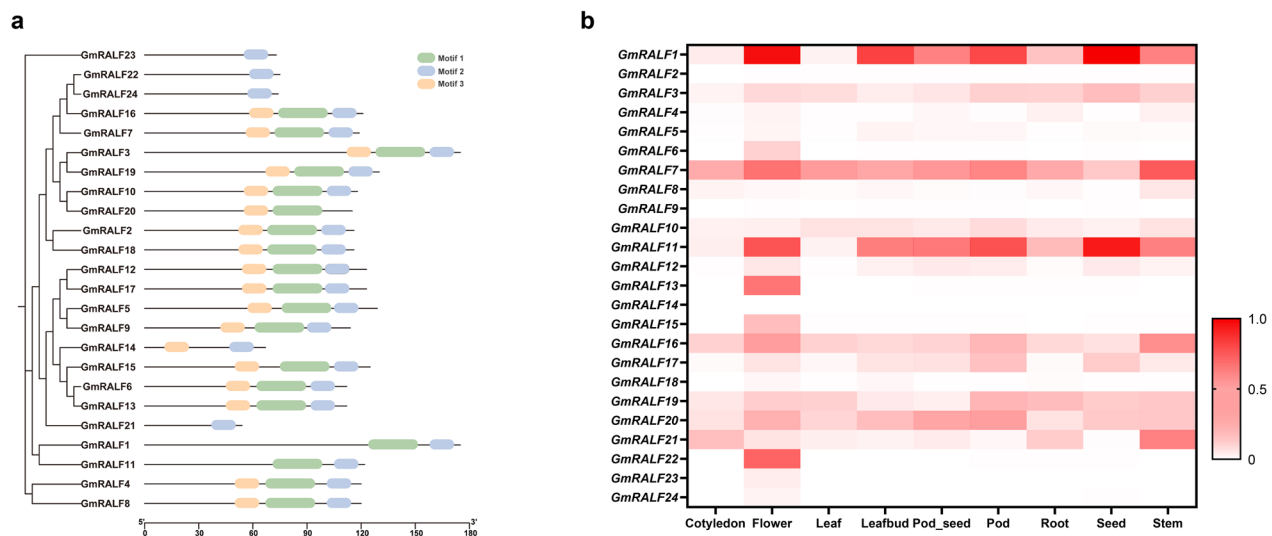


Fig. 1 Analysis of rapid alkalization factor (RALFs) in soybean. **a** Phylogenetic tree and motif analysis of soybean RALFs. The amino acid sequences of GmRALFs were aligned by ClustalW software and the motif structure of the GmRALFs were analysed by Batch SMART software. **b** Analysis of the expression pattern of *GmRALFs* in different tissues. The relative expression level of the *GmRALFs* in different soybean tissues were obtained from SoyMD database (<https://yanglab.hzau.edu.cn/SoyMD>). The heatmap was generated by Graphpad Prism 9.5.0

activation of plant immunity by suppressing the FLS2-BAK1 interaction (Mang et al. 2017; Wang et al. 2020; Zhang et al. 2024). However, immunity-related RALFs recognized by ANX1/2 and GmLMM1 are currently unknown. Some RALFs have been reported to function as danger-associated molecular patterns to induce plant immunity. For example, *Arabidopsis* RALF22 amplifies Pep3-triggered immunity by increasing the expression of PROPEP3 (He et al. 2023). Exogenous application of OsRALF26 in rice significantly inhibits plant root development. OsRALF26 also induces ROS production and the expression of defense genes (Kwon et al. 2024). RALFs play important roles in plant-microbe interactions. For example, nematodes can secrete RALF-like proteins to bind to the extracellular domain of FER to manipulate the FER-mediated signaling pathway and to promote the degradation of MYC2, a key transcription factor in jasmonic acid-mediated immunity (Zhang et al. 2020a; Wu et al. 2021). However, to date, research on RALFs has mainly engaged with *Arabidopsis*. The functions of soybean RALFs are barely studied, particularly their roles in immunity and disease resistance.

In this study, we cloned 24 *GmRALFs* from soybean (*Glycine Max*) and analyzed their functions in plant immunity and disease resistance. We examined the expression patterns of *GmRALFs* in response to various pathogens. Based on transient expression assays in *N. benthamiana*, we analyzed the roles of *GmRALFs* in PTI and disease resistance to bacterial, fungal, and oomycete pathogens. We found that *GmRALFs* are differentially

involved in plant immunity and disease resistance and identified GmRALF1 as a negative regulator of plant immunity. *Gmralf1* mutants exhibit significantly enhanced disease resistance, highlighting *GmRALF1* as a susceptibility gene with potential applications in soybean breeding.

Results

Characterization of GmRALFs in soybean

Based on the structural characteristics of RALFs, we identified 24 *RALFs* in the soybean genome database (*Glycine max_v2.1*) (Fig. 1a and Additional file 1: Table S1), which is consistent with a previous report (Jia and Li. 2023). Protein sequence alignment revealed that the motifs 1–3 are conserved in most GmRALFs, albeit with some variations at individual amino acid positions (Additional file 2: Figure S1). Except for GmRALF20, all other GmRALFs possess a C-terminal motif 2 (Fig. 1a and Additional file 2: Figure S1). While GmRALF1, 11, 21, 22, 23, and 24 lack motif 3, GmRALF21, 22, 23, and 24 only contain motif 2 (Fig. 1a and Additional file 2: Figure S1). Genomic sequence analysis showed that most GmRALFs do not possess introns except for GmRALF9, GmRALF14, and GmRALF20 (Additional file 2: Figure S2a). We then analyzed the chromosomal locations of these *GmRALFs* and found that they were randomly distributed on 15 chromosomes, mostly located in the telomeres (Additional file 2: Figure S2b). To track the evolution and the genetic origin of *RALFs* in soybeans, we conducted an

intraspecific collinearity analysis of *GmRALFs* using the MCSanX tool of TBtools. We found that numerous whole-genome replication events (grey lines) occurred in the *GmRALF* family, with 21 *GmRALFs* forming 28 tandem repeat gene pairs (red lines) in the soybean genome (Additional file 2: Figure S2c). These results indicate that *GmRALFs* have undergone evolutionary events in the soybean genome and that tandem and fragment duplication are the main amplification modes of *GmRALFs*.

Next, we analyzed the expression patterns of *GmRALFs* in different tissues using the SoyMD database (<https://yanglab.hzau.edu.cn/SoyMD>). Among the *GmRALFs*, *GmRALF1*, 7, 11, 13, and 22 exhibited higher expression levels than other *GmRALFs* (Fig. 1b). Most *GmRALFs* were differentially expressed in soybean organs (Fig. 1b), suggesting that they have tissue-specific functions. For example, *GmRALF1* and *GmRALF11* were highly expressed in the flowers, leaf-buds, pods, and seeds (Fig. 1b). *GmRALF22* was specifically highly expressed in flowers (Fig. 1b), suggesting that it regulates the reproductive processes.

Expression patterns of *GmRALFs* in soybean responses to pathogens

To analyze the function of *GmRALFs* in soybean disease resistance, we analyzed the changes in the expression levels of *GmRALFs* in soybean in response to different pathogens. We first examined the expression levels of *GmRALFs* in soybean hypocotyls after oomycete pathogen *Phytophthora sojae* (*P. sojae*) infection. The samples were collected at 0, 24, and 36 h and quantitative PCR (qPCR) analysis showed that the expression levels of 19 *GmRALFs* were significantly increased, whereas the expression of *GmRALF1*, 4, 10, 16, and 20 was significantly decreased (Fig. 2a and Additional file 2: Figure S3). We found that the expression levels of most *GmRALFs* at 36 h of induction were significantly lower than those at 24 h (Fig. 2a and Additional file 2: Figure S3). Next, we examined the expression patterns of *GmRALFs* in soybean leaves after bacterial pathogen *P. syringae* pv. *Glycinea* (*Psg*) infection. Results from qPCR analysis showed that 17 *GmRALFs* were significantly induced by *Psg* treatment for 12 h, and their expression levels mostly returned to near basal levels after *Psg* treatment for 24 h (Fig. 2b and Additional file 2: Figure S4). Seven *GmRALFs*

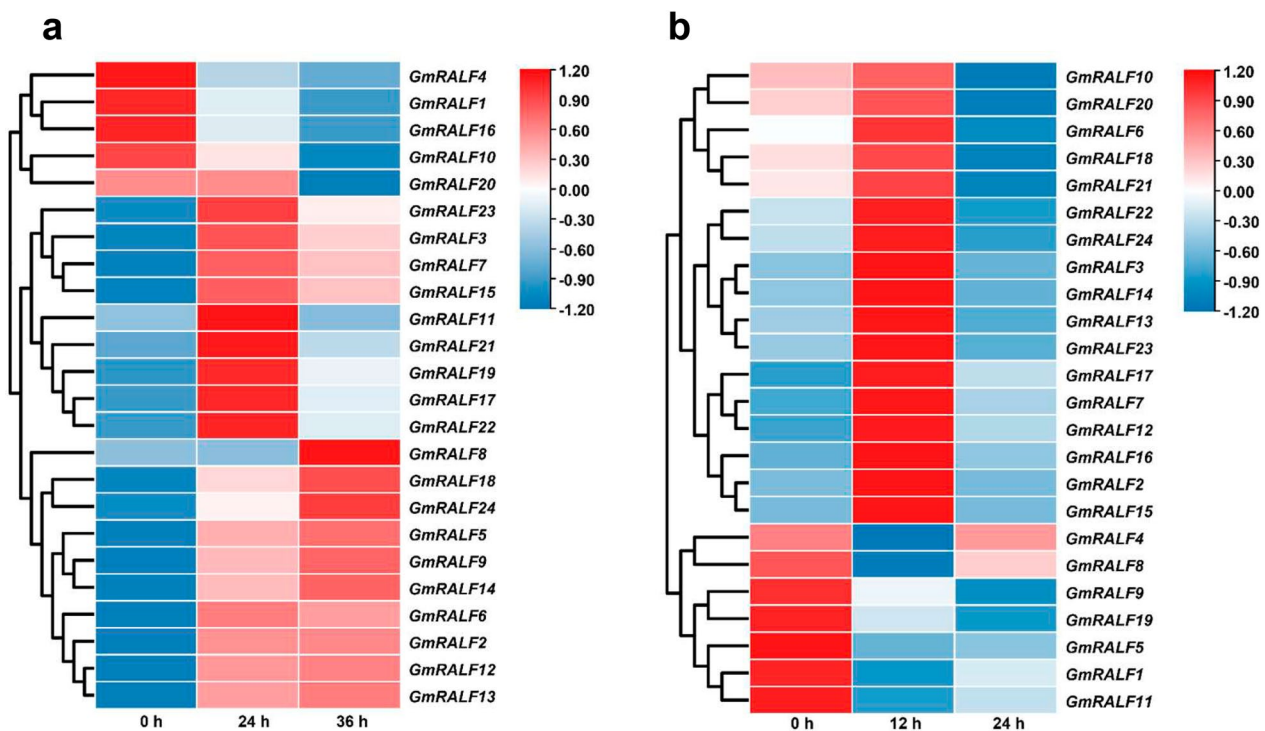


Fig. 2 Transcriptional expression levels of *GmRALFs* upon *Pseudomonas syringae* pv. *glycinea* and *Phytophthora sojae* treatment. **a** Transcriptional levels of *GmRALFs* in response to *Phytophthora sojae* (*P. sojae*) for 0, 24, and 36 h. The hypocotyls of etiolated soybean seedlings at 4-days-old were treated with *P. sojae* for indicated time, total RNA was extracted, and the relative expression level of *GmRALFs* were analysed by qPCR. The results were shown in Additional file 2: Figure S3 and summarized with heatmap in (a). **b** Transcriptional levels of *GmRALFs* in response to *Pseudomonas syringae* pv. *Glycinea* (*Psg*) for 0, 12, and 24 h. The leaves of soybean plants at 2-week-old were treated with *Psg* for indicated time. Total RNA was extracted and the relative expression levels of *GmRALFs* were analysed by qPCR. The results were shown in Additional file 2: Figure S4 and summarized by heatmap in (b)

(*GmRALF1*, 4, 5, 8, 9, 11, and 19) showed significantly reduced expression levels after 12 and 24 h of *Psg* treatment (Fig. 2b and Additional file 2: Figure S4). We found that *GmRALF1* and *GmRALF4* were significantly down-regulated upon infection with both pathogens (Fig. 2), suggesting that these genes may negatively regulate soybean immunity. Taken together, these results indicate that *GmRALFs* transcriptionally respond to pathogen infection.

Identification of *GmRALFs* involved in PTI

To analyze the role of *GmRALFs* in PTI, we amplified the coding sequences of *GmRALFs* and cloned them into the pCambia1300-35S-HA-RBS vector. We then expressed these *GmRALFs* using *Agrobacterium*-mediated transient expression assay in *N. benthamiana* to analyze their effects on PTI. Anti-HA immunoblotting analysis

showed that all the *GmRALFs* were typically expressed in *N. benthamiana* (Additional file 2: Figure S5). We selected the bacterial flagellin epitope flg22, fungal-derived chitin, and oomycete-derived INF1 to activate plant PTI. Flg22 and chitin induce ROS burst, and INF1 induces strong cell death in *N. benthamiana*. We transiently expressed these *GmRALFs* in *N. benthamiana* for 24 h and analyzed flg22- and chitin-induced ROS production. The results showed that most *GmRALFs* significantly promoted flg22-induced ROS burst (Fig. 3a, b and Additional file 2: Figure S6a). *GmRALF5*, 8, 10, and 24 significantly enhanced chitin-induced ROS burst (Fig. 3c and Additional file 2: Figure S6b), whereas *GmRALF1* and *GmRALF18* significantly reduced flg22- and chitin-induced ROS burst (Fig. 3a–c and Additional file 2: Figure S6). We then analyzed the effect of *GmRALFs* on INF1-induced cell death. We transiently expressed

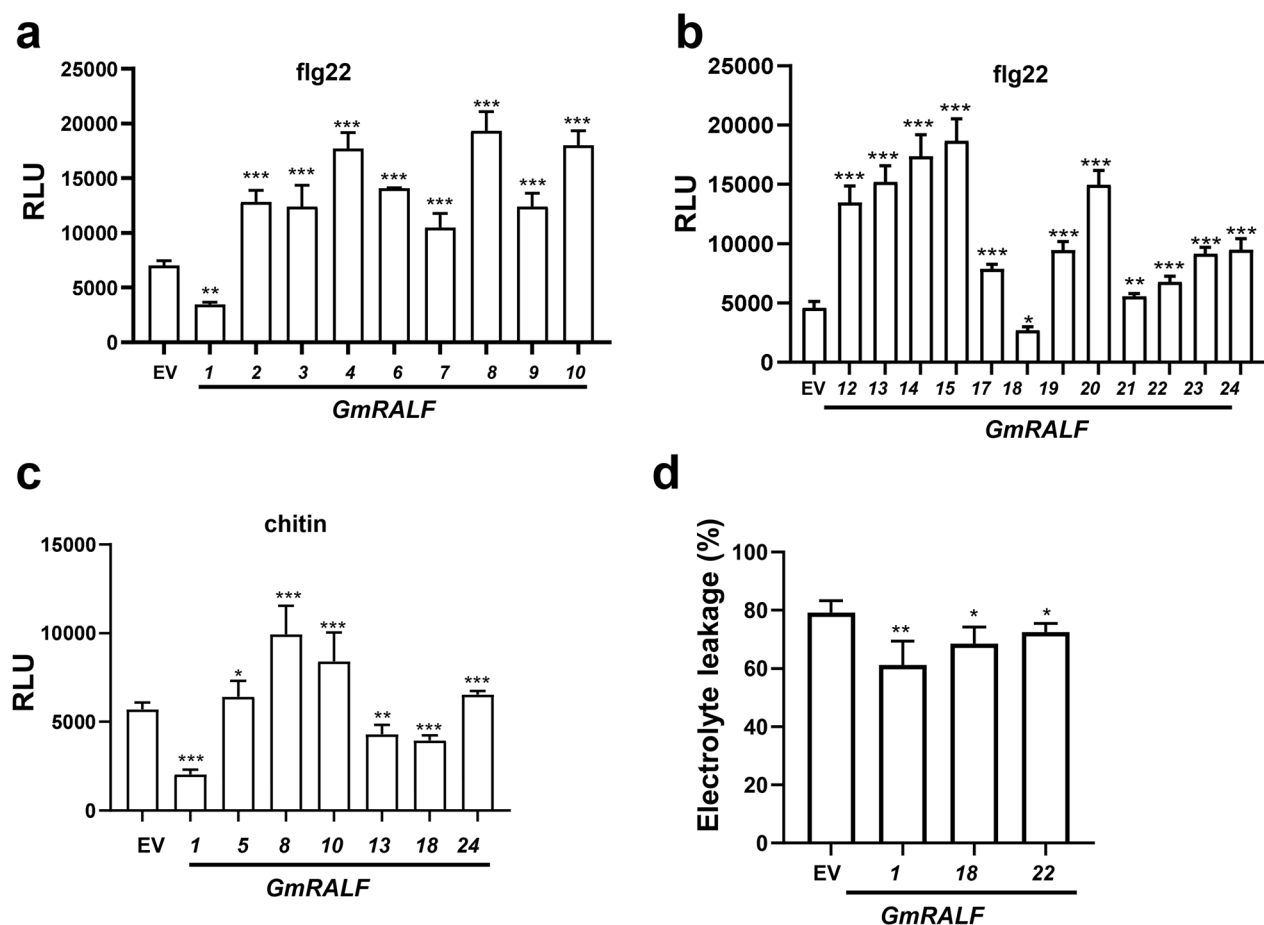


Fig. 3 Analysis of the roles of *GmRALFs* in plant immunity induced by flg22, chitin, and INF1. **a–c** Effect of *GmRALFs* on ROS burst induced by flg22 (**a**, **b**) and chitin (**c**). The indicated *GmRALFs* were cloned into pCambia1300 vector and expressed in *N. benthamiana* by *Agrobacterium*-mediated transient expression assay for 2 days and subjected to flg22- and chitin-induced ROS examination assays (Mean $\pm$ SD, $n \geq 6$, Student's *t*-test; * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$). EV, empty vector. **d** Effect of *GmRALFs* on INF1-induced cell death. The indicated *GmRALFs* were expressed in *N. benthamiana* by *Agrobacterium*-mediated transient expression assay for 1 day, and infiltrated with *Agrobacterium* carrying INF1. The cell death phenotype was examined by measuring the electrolyte leakage 2 days later (Mean $\pm$ SD, $n \geq 8$, Student's *t*-test; * $P < 0.05$, ** $P < 0.01$)

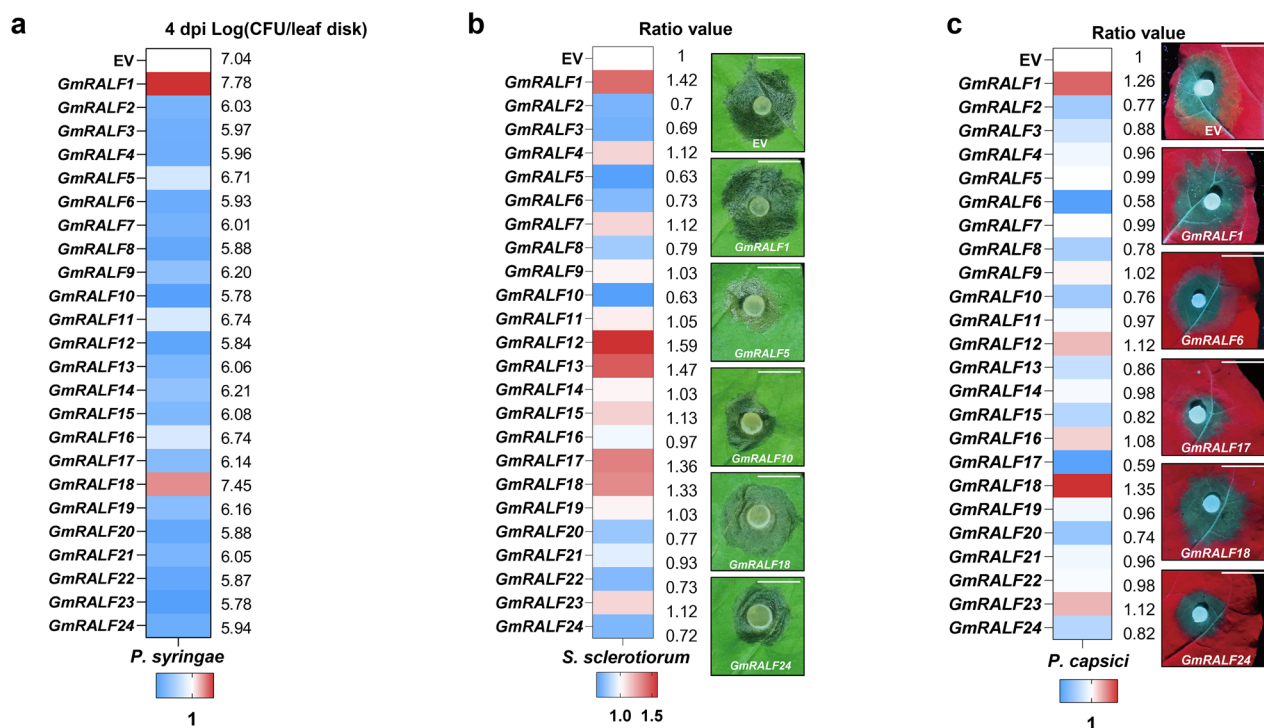


Fig. 4 Analysis of the roles of GmRALFs in plant resistance to bacterial, fungal, and oomycete pathogens. **a** Effect of GmRALFs on plant resistance to *Pseudomonas syringae* pv. tomato DC3000 (*Pst* DC3000) Δ hopQ1. The indicated GmRALFs were transiently expressed in *N. benthamiana* for 1 day, and the leaves were infiltrated with *Pst* DC3000 Δ hopQ1 and the samples were collected at 0 and 4 days after inoculation. The bacterial population was determined, calculated, and shown in in Figure S8 (Mean $\pm$ SD, $n \geq 6$, One-way ANOVA, $P < 0.05$). To summarize, the logarithmic value of bacterial number in leaf disk at day 4 was shown by heatmap in (a). EV, empty vector. **b** Effect of GmRALFs on plant resistance to *Sclerotinia sclerotiorum*. The indicated GmRALFs were transiently expressed in *N. benthamiana* for 1 day, and the leaves were inoculated with *Sclerotinia sclerotiorum* (*S. sclerotiorum*) and disease symptoms were photographed 24 h later. The lesion area was measured, calculated, and shown in Additional file 2: Figure S9 (Mean $\pm$ SD, $n \geq 8$, Student's *t*-test; $*P < 0.05$, $**P < 0.01$, ns, no significance). To summarize, the ratio value of GmRALF/EV was shown with heatmap in Fig. 4b. Representative images of the disease symptoms were displayed on the right panel. Bar, 1 cm. **c** Effect of GmRALFs on plant resistance to *Phytophthora capsici*. The indicated GmRALFs were transiently expressed in *N. benthamiana* for 1 day, and the leaves were inoculated with *Phytophthora capsici* (*P. capsici*) and photographs were taken 24–30 h later under UV light. The lesion areas were measured, calculated, and shown in Figure S10 (Mean $\pm$ SD, $n \geq 8$, Student's *t*-test; $*P < 0.05$, $**P < 0.01$, ns, no significance). The ratio value of GmRALF/EV were shown with heatmap in (c). Representative images of the disease symptoms were displayed on the right panel. Bar, 1 cm

GmRALFs in *N. benthamiana* for 24 h, followed by infiltration of *Agrobacterium* carrying INF1 and the cell death phenotype was visualized 2 days later. We observed that GmRALF1 significantly suppressed INF1-induced cell death, whereas GmRALF22 and GmRALF18 only slightly reduced cell death (Fig. 3d and Additional file 2: Figure S7). In summary, we showed that GmRALFs have both common and specific functions in regulating PTI. Notably, GmRALF1 plays a negative role in immunity induced by different PAMPs (Fig. 3), serving as a potential susceptibility gene.

Identification of GmRALFs in resistance to various pathogens

Next, we analyzed the role of GmRALFs in plant resistance to various pathogens. We challenged GmRALF-expressing *N. benthamiana* plants with the bacterial

pathogen *Pseudomonas syringae* pv. tomato DC3000 (*Pst* DC3000) Δ hopQ1, fungal pathogen *Sclerotinia sclerotiorum* (*S. sclerotiorum*), and oomycete pathogen *Phytophthora capsici* (*P. capsici*). Note that HopQ1 is an effector that can be recognized by the NLR receptor NbROQ1 and induces hypersensitive response in *N. benthamiana* (Schultink et al. 2017). Therefore, *Pst* DC3000 Δ hopQ1 is used for bacterial infection assays in *N. benthamiana* plants. We revealed that most GmRALFs enhanced resistance to *Pst* DC3000 Δ hopQ1 in plant, whereas GmRALF1 and GmRALF18 significantly reduced resistance to *Pst* DC3000 Δ hopQ1 (Fig. 4a and Additional file 2: Figure S8). In the *S. sclerotiorum* infection assays, nine GmRALFs (GmRALF2, 3, 5, 6, 8, 10, 20, 22, and 24) promoted plant resistance to fungal infection, whereas five GmRALFs (GmRALF1, 12, 13, 17, and 18) reduced plant resistance to fungal infection (Fig. 4b and

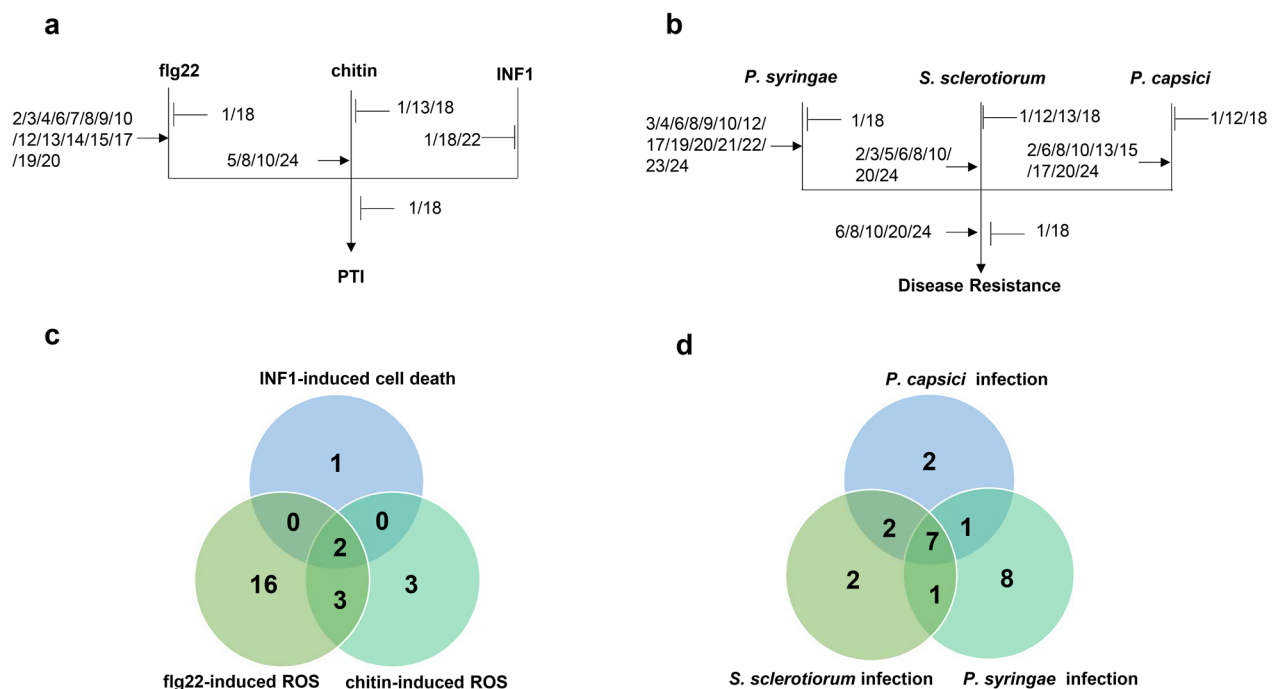


Fig. 5 Summary of the roles of GmRALFs in PTI and disease resistance. **a, b** Classification of GmRALFs based on their positive or negative effects on different PTI responses (**a**) and plant resistance to various pathogens (**b**). T bar and arrows indicate negative and positive effects, respectively. **c** Venn diagram showing the number of GmRALFs that function in immunity induced by flg22, chitin, and INF1. **d** Venn diagram showing the number of GmRALFs that function in plant resistance to *Psg*, *S. sclerotiorum*, and *P. capsici*

Additional file 2: Figure S9). In the *P. capsici* inoculation experiment, six GmRALFs (GmRALF2, 6, 8, 10, 17, and 20) promoted plant resistance to pathogen infection, and four GmRALFs (GmRALF1, 12, 18, and 23) significantly reduced plant resistance to *P. capsici* (Fig. 4c and Additional file 2: Figure S10). In summary, GmRALFs play distinct roles in plant resistance to various pathogens. GmRALF2, 6, 8, 10, and 20 play positive regulatory roles in plant resistance to all three pathogens (Fig. 4). GmRALF1 and GmRALF18 negatively regulate plant resistance to multiple pathogens (Fig. 4), suggesting that they are negative regulators of plant immunity.

Summary of the roles of GmRALFs in plant immunity and disease resistance

Based on the immunity-related experiments performed above, we summarized the results of all the PTI and disease resistance assays, including flg22- and chitin-induced ROS burst, INF1-induced cell death, and resistance to various pathogens. We analyzed the common and specific roles of GmRALFs in PTI and disease resistance. In total, 15 and 4 GmRALFs positively regulate flg22- and chitin-induced ROS production, respectively (Fig. 5a). GmRALF8 and GmRALF10 promoted both flg22- and chitin-induced ROS burst (Fig. 5a). Owing to the strong cell death phenotype

induced by INF1, no GmRALF that promote INF1-induced cell death was identified. Notably, GmRALF1 and GmRALF18 specifically suppressed the immune responses triggered by all three PAMPs (Fig. 5a). While GmRALF13 specifically suppressed chitin-induced ROS burst, GmRALF22 negatively regulated INF1-induced cell death (Fig. 5a). In disease resistance assays, we observed that 14, 8, and 9 GmRALFs enhanced plant resistance to *Pst* DC3000 Δ hopQ1, *S. sclerotiorum*, and *P. capsici*, respectively (Fig. 5b). Among these, GmRALF6, 8, 10, 20, and 24 enhanced plant resistance to all three pathogens (Fig. 5b). Consistent with the results of the PTI assays, GmRALF1 and GmRALF18 negatively regulate plant resistance to all three pathogens (Fig. 5b). Together, the results of the PTI and disease resistance assays exhibited a high degree of consistency.

In total, 21, 7, and 3 GmRALFs were involved in immunity triggered by flg22, chitin, and INF1, respectively (Fig. 5c). GmRALF1 and GmRALF18 regulate immunity induced by all three PAMPs. Additionally, 17, 12, and 12 GmRALFs were involved in plant resistance to *Pst* DC3000 Δ hopQ1, *S. sclerotiorum*, and *P. capsici*, respectively (Fig. 5d). Among these, 7 GmRALFs (GmRALF1, 6, 8, 10, 18, 20, and 24) were involved in plant resistance to all three pathogens. While GmRALF1 and GmRALF18

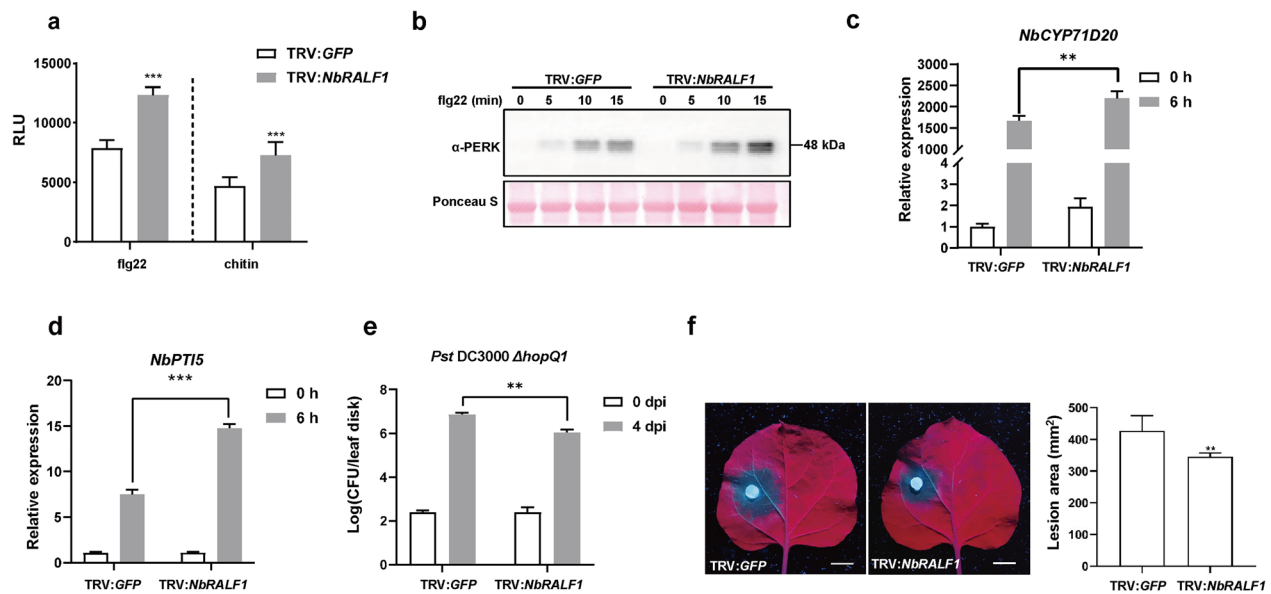


Fig. 6 Silencing of *NbRALF1* led to enhanced PTI and disease resistance. **a** Flg22- and chitin-induced ROS is enhanced in *NbRALF1*-silenced plants. The *NbRALF1*-silenced plants were subjected to flg22- and chitin-induced ROS examination assays, and the RLU values were recorded and calculated (mean $\pm$ SD, $n \geq 6$, Student's *t*-test, *** $P < 0.001$). **b** Flg22-induced MAPK activation is enhanced in *NbRALF1*-silenced plants. The TRV:GFP and TRV:*NbRALF1* plants were subjected to flg22-induced MAPK examination by anti-pERK immunoblots. **c, d** Flg22-induced defense gene expression is enhanced in *NbRALF1*-silenced plants. The TRV:GFP and TRV:*NbRALF1* plants were treated with flg22 (1 μ M) for 0 and 6 h. Total RNA was extracted and the expression of *NbCYP71D20* (**c**) and *NbPTI5* (**d**) were examined by qPCR analysis (mean $\pm$ SD, Student's *t*-test, ** $P < 0.01$, *** $P < 0.001$). **e** *NbRALF1*-silenced plants showed reduced susceptibility to *Pst* DC3000 Δ hopQ1. The indicated plants were infiltrated with *Pst* DC3000 Δ hopQ1 and the bacterial number was determined at 0 and 4 days after inoculation (mean $\pm$ SD, $n \geq 8$, Student's *t*-test, *** $P < 0.001$). **f** *NbRALF1*-silenced plants showed reduced susceptibility to *P. capsici*. The indicated plants were inoculated with *P. capsici* strain LT263 and photographed under UV light 36–48 h later. The lesion area was measured and calculated (mean $\pm$ SD, $n \geq 8$, Student's *t*-test, ** $P < 0.01$)

play negative roles in plant disease resistance, the other five *GmRALF*s positively regulate plant disease resistance (Fig. 5d).

NbRALF1 negatively regulates PTI and disease resistance

Our results revealed that *GmRALF1* and *GmRALF18* are negative regulators of immunity and candidate susceptibility genes. *GmRALF1* exhibited stronger immune suppression ability than *GmRALF18*. To further analyze the roles of *GmRALF1* in plant immunity, we identified a homologous gene of *GmRALF1* in *N. benthamiana* and named it as *NbRALF1* (NbD036943.1). We transiently overexpressed *NbRALF1* in *N. benthamiana* to analyze its effects on PTI and disease resistance (Additional file 2: Figure S11a). We found that the ROS burst induced by flg22 and chitin was significantly reduced in *NbRALF1* expressing leaves (Additional file 2: Figure S12a). Next, we noticed that the expression of *NbRALF1* also significantly reduced plant resistance to *Pst* DC3000 Δ hopQ1, *S. sclerotiorum*, and *P. capsici* (Additional file 2: Figure S12b–d). We silenced *NbRALF1* using a virus-induced gene silencing (VIGS) approach (Additional file 2: Figure S11b). The results showed that silencing of *NbRALF1* significantly enhanced flg22- and chitin-induced ROS

production (Fig. 6a). Flg22-induced MAPK activation was also greatly enhanced in *NbRALF1*-silenced plants (Fig. 6b). We subsequently observed that silencing of *NbRALF1* led to significantly enhanced flg22-induced expression of the defense marker genes *NbCYP71D20* and *NbPTI5* (Fig. 6c,d). Further analysis showed that *NbRALF1*-silenced plants exhibited significantly enhanced resistance to *Pst* DC3000 Δ hopQ1 and *P. capsici* (Fig. 6e,f).

GmRALF1 negatively regulates PTI and disease resistance

To determine whether *GmRALF1* is a negative regulator of plant immunity, we designed a gRNA targeting *GmRALF1* and obtained two *Gmralf1* mutant lines (*Gmralf1*-L1 and *Gmralf1*-L2) using CRISPR-Cas9 approach. *Gmralf1*-L1 is a biallelic mutant, with a 1-bp insertion in one allele and a 1-bp deletion in the other allele. *Gmralf1*-L2 carries a 1 bp deletion at position 365 bp of the coding sequence. (Fig. 7a). We then analyzed the flg22-induced ROS burst and defense gene expression in *Gmralf1* mutants. We showed that flg22 induces a stronger ROS production in *Gmralf1* mutants than in Williams 82 (WT) plants (Fig. 7b). Similarly, the *Gmralf1* mutants exhibited higher levels

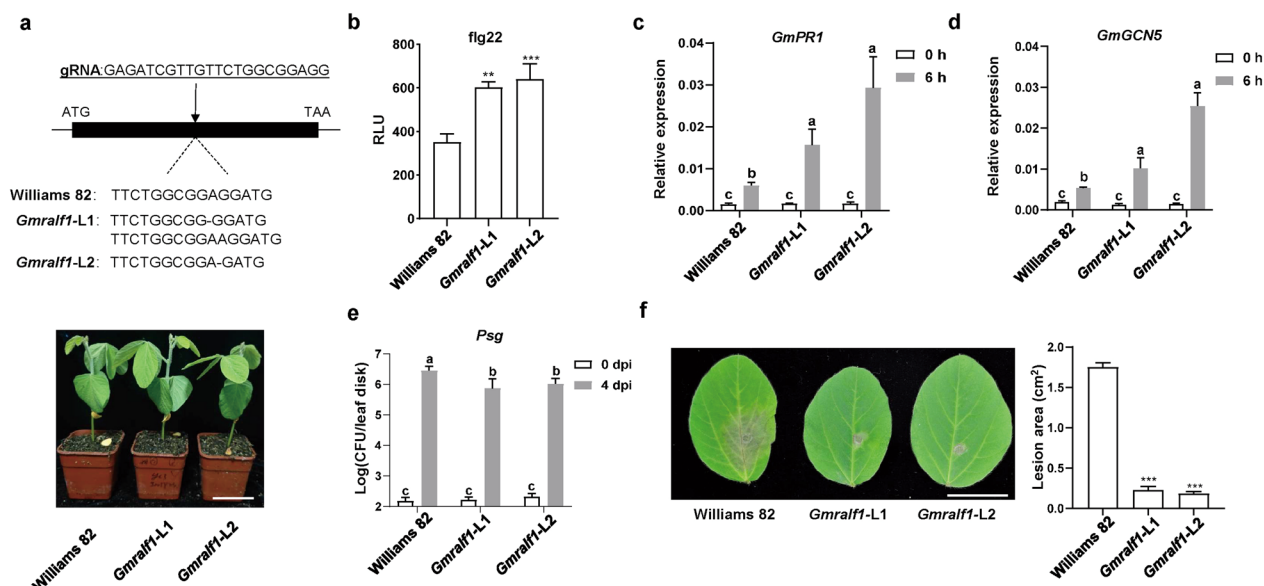


Fig. 7 The *Gmralf1* mutants exhibited enhanced PTI and disease resistance. **a** Construction of *Gmralf1* mutant plants. A gRNA targeting *GmRALF1* was designed and two mutant lines were obtained by CRISPR-Cas9 approach in the Williams 82 background. *Gmralf1*-L1 is a biallelic mutant that carries a 1-bp insertion in one allele and 1-bp deletion in the other allele. *Gmralf1*-L2 carries a 1-bp deletion in the coding region. The lower panel shows the morphology of 3-week-old *Gmralf1* mutant plants. Bar, 5 cm. **b** Flg22-induced ROS is enhanced in *Gmralf1* mutants. Leaves of the indicated soybean plants at 2 weeks old were subjected to flg22-induced ROS assays (Mean $\pm$ SD, $n \geq 6$, Student's *t*-test, ** $P < 0.01$, *** $P < 0.001$). **c,d** Flg22-induced defense gene expression is enhanced in *Gmralf1* mutants. Leaves of the indicated plants were infiltrated with flg22 for 0 and 6 h. Total RNA was extracted and the expression of *GmPR1* (**c**) and *GmGCN5* (**d**) was examined by qPCR analysis (Mean $\pm$ SD, One-way ANOVA, different letters indicate $P < 0.05$). **e** *Gmralf1* mutants exhibited enhanced resistance to *Psg*. The indicated plants were infiltrated with *Psg* and the bacterial number was determined at 0 and 4 days after inoculation (Mean $\pm$ SD, $n \geq 8$, One-way ANOVA, different letters indicate $P < 0.05$). **f** *Gmralf1* mutants exhibited enhanced resistance to *P. sojae*. The indicated plants were inoculated with *P. sojae* strain P7076, and disease symptom was photographed 2 days later. The lesion area was measured and calculated (mean $\pm$ SD, $n \geq 8$, Student's *t*-test, *** $P < 0.001$)

of flg22-induced expression of defense genes *GmPR1* and *GmGCN5* than the WT plants (Fig. 7c,d). We then challenged the WT and *Gmralf1* mutants with *Psg*, and the results showed that *Gmralf1* mutants exhibited significantly enhanced resistance to *Psg* (Fig. 7e). Similarly, we observed that mutation of *GmRALF1* greatly enhanced disease resistance to *P. sojae* infection in soybean (Fig. 7f).

We further analyzed the growth phenotype and yield-related traits of *Gmralf1* mutant. The *Gmralf1*-L1 and *Gmralf1*-L2 mutant lines at 2-month-old were slightly smaller than the WT plants (Additional file 2: Figure S13a). Consistent with this, the *Gmralf1* mutant lines exhibited reduced stem length compared to that of WT (Additional file 2: Figure S13b). However, the *Gmralf1* mutants showed similar levels of branch number and hundred-grain weight (Additional file 2: Figure S13c,d). Taking together, *GmRALF1* negatively regulates soybean disease resistance and possesses the application potential to be engineered for soybean breeding.

Discussion

Over the past decade, RALF peptides and their corresponding receptors, malectin-like RLKs, have been extensively studied in Arabidopsis. However, the functions of soybean RALFs, especially their role in plant immunity, have not been well studied. In this study, we systematically characterized their common and specific roles in plant PTI and disease resistance using transient expression assays in *N. benthamiana* plants. We also identified *GmRALF1* as a candidate susceptibility gene.

We found that the transcriptional levels of all soybean *RALFs* were altered upon *Psg* and *P. sojae* infection, with more than 2/3 of them showing significantly increased expression, whereas the other 1/3 of them showed certain degree of reduced expression. This finding suggests that *GmRALFs* play different roles in plant immunity. PTI confers mild resistance to most microbes and is important for improving broad-spectrum plant resistance. Therefore, we analyzed the effects of *GmRALFs* on plant immunity by transiently expressing them in *N. benthamiana* and examined the immune responses

induced by three different microbial-derived PAMPs: flg22, chitin, and INF1. We found that GmRALFs have both specific and common functions in the regulation of immune responses induced by different PAMPs. Notably, GmRALF1 and GmRALF18 inhibited immune responses induced by all three PAMPs. However, due to the strong cell death induced by INF1, we did not identify GmRALFs that suppress INF-induced cell death.

We further investigated the functional similarities and differences between RALFs in soybean and Arabidopsis. Abarca et al. (2021) found that more than 50% of RALFs in Arabidopsis can promote the activation of plant immunity (Abarca et al. 2021). We identified 21 GmRALFs that are involved in plant immunity, with majority of them exerting a positive effect. Among them, GmRALF1 and GmRALF18 were found to be the negative regulators of plant immunity. It is noteworthy that Arabidopsis and soybean possess 37 and 24 RALFs, respectively, yet the number of leucine-rich repeat (LRR) proteins in soybean is substantially higher than that in Arabidopsis (Zhang et al. 2022), hinting a possibly functional divergence between them. Arabidopsis RALF23 was reported to be a negative regulator of immunity (Xiao et al. 2019), while its homologous *GmRALF10* and *GmRALF20* in soybean are positive regulators of immunity. We showed that *GmRALF1* inhibits plant immunity, but its orthologues in Arabidopsis, *RALF34*, has not been reported to be involved in plant immunity. *RALF34* is involved in the bursting of pollen tubes during reproduction (Ge et al. 2017). We noticed that GmRALF1 was highly expressed in flower organs, suggesting that GmRALF1 may also participate in the regulation of soybean reproduction.

In Arabidopsis, leucine-rich repeat (LRR) proteins recognize RALFs to regulate plant immunity (Stegmann et al. 2017). For instance, RALF23 is recognized by FER, which interacts with the immune receptor FLS2 and co-receptor BAK1 to regulate the flg22-induced FLS2-BAK1 interaction, thereby modulating the activation of plant immunity (Xiao et al. 2019). Whether GmRALFs are also present in the PRR complexes and adopt a similar mechanism to regulate plant immunity remains unclear. Our previous studies showed that the soybean leucine-rich repeat (LRR) proteins GmLMM1 and GmMRLK32 regulate FLS2-BAK1 interaction (Wang et al. 2020; Zhang et al. 2024). However, GmRALFs that bind GmLMM1 or GmMRLK32 have not been identified. To date, the receptors for all GmRALFs still need to be identified and investigated.

We further analyzed plant resistance to bacterial, fungal, and oomycete pathogen infections in GmRALF-expressing *N. benthamiana* plants. The results of the pathogen infection experiments were consistent with those of the PTI assays. We identified multiple GmRALFs that promoted or suppressed plant resistance to various

pathogens. Among these, GmRALF2, 6, 8, 10, and 20 positively regulated plant resistance to all three types of pathogen. GmRALF1 and GmRALF18 significantly suppressed plant resistance to multiple pathogens. GmRALF1 exhibited a stronger inhibitory effect on PTI and plant disease resistance than GmRALF18 did. We found that the GmRALF1 homolog in *N. benthamiana* also inhibited plant immunity. Notably, we observed that *GmRALF18* is transcriptionally suppressed by pathogen infection, which is inconsistent with its negative role in plant immunity. We concluded that GmRALF18 may function to prevent immune overactivation through a feedback regulatory mechanism.

To clarify the role of GmRALF1 in plant immunity, we generated the knockout mutants of *GmRALF1*. In the *Gmralf1* mutant, flg22-induced ROS production and defense gene expression were significantly enhanced. *Gmralf1* mutants also exhibited enhanced resistance to *Psg* and *P. sojae* infections. These results confirmed that *GmRALF1* is a susceptibility gene. Notably, silencing of *NbRALF1*, the homolog of *GmRALF1* in *N. benthamiana*, also resulted in enhanced immunity and disease resistance, suggesting that *GmRALF1* might be a widespread susceptibility gene in plants. Therefore, *GmRALF1* may be used to improve soybean resistance through approaches such as gene editing.

In this study, we mainly used the *Agrobacterium*-mediated transient expression system in tobacco to search for RALFs involved in disease resistance in soybean. However, it should be noted that there may be some bias in the results in analyzing the function of soybean RALFs by heterologous systems. Therefore, future research needs to obtain *Gmralf* mutants and explore their specific functions in soybean immunity. So far, there are no relevant reports on the identification of the receptors for GmRALF peptides. Future research needs to search for leucine-rich repeat (LRR) proteins that can recognize GmRALFs. In addition, given that RALFs play a crucial role in plant growth, development, reproduction, and response to abiotic and biotic stresses (Zhang et al. 2020b; Liu et al. 2024), future research should focus on the function analysis of RALFs in multiple physiological processes, and explore the application potentials of RALFs in improving crop yield and stress resistance.

Conclusions

In summary, we identified 24 GmRALFs in *Glycine max* and revealed their common and specific roles in plant immunity and disease resistance. We cloned the *GmRALFs* and transiently expressed them in *N. benthamiana* to analyze their effects on different PTI responses and disease resistance against bacterial, fungal, and oomycete pathogens. We showed that most identified

GmRALFs positively regulate plant immunity. Notably, the expression of *GmRALF1* and *GmRALF18* significantly suppressed plant immunity and disease resistance. Furthermore, we generated the *Gmralf1* mutant lines and showed that the mutants exhibited significantly enhanced immunity and disease resistance, suggesting that GmRALF1 could be used to improve soybean disease resistance.

Methods

Plant materials and growth conditions

Soybean cultivars Williams 82 were used as wild type and *Gmralf1* mutants were generated in the background of Williams 82. Soybean plants used for ROS burst and pathogen infection assays were grown in a growth chamber at 28°C and 50% humidity with a 14 h/10 h photoperiod. *N. benthamiana* plants used for transient expression assays were grown at 23°C and 70% relative humidity with a 12 h/12 h photoperiod.

Bioinformatic analysis

The RALF protein sequences of Arabidopsis and soybean were obtained from TAIR and SoyBase databases. For phylogenetic analysis, the sequences were aligned by ClustalW and the phylogenetic tree was constructed using Neighbor-Joining (NJ) method. The conserved motifs of GmRALFs were determined using MEME database (<http://alternate.meme-suite.org/tools/meme>). The phylogenetic tree and conserved motifs were visualized and integrated into graphics by TBtools (Chen et al. 2020). Chromosomal locations of GmRALFs were obtained from Phytozome database (Glycine max_v2.1) and Gene location visualize of TBtools was used for visual analysis (Schmutz et al. 2010). Intraspecific collinearity analysis was analyzed using the MCSanX of TBtools.

Plasmid constructions and *Agrobacterium*-mediated transient expression assay

The coding sequences of the indicated *GmRALFs* were amplified and cloned into pCambia1300-35S-HA-RBS vector. To generate the *Gmralf1* knockout lines, a guide-RNA targeting *GmRALF1* was designed and ligated into the VK005-04-soU6-2-GmUbi3 vector. (Du et al. 2016). The primers used in this study were listed in Additional file 1: Table S1.

Agrobacterium strain GV3101 carrying the indicated constructs were grown overnight in liquid LB medium. The bacteria were collected by centrifugation and resuspended in the infiltration buffer (10 MES pH 5.7, 10 mmol/L MgCl₂, 200 μM acetosyringone) to an OD₆₀₀ value of 1.0. The bacteria were infiltrated into *N. benthamiana* leaves using a needleless syringe.

Pathogen infection assays

To perform the *P. sojae* infection assays in soybean, leaves of 2-week-old soybean plants were infected with *P. sojae* isolate P7076 at 25°C in dark. The disease symptom was photographed 60–72 h later. To perform *P. syringae* pv. *glycinea* (Psg) infection assays in soybean, leaves of 2-week-old soybean plants were infiltrated with bacteria at a concentration of 5×10⁵ CFU/mL. Leaf disks were taken and the bacterial number was determined 4 days later (Wang et al. 2020).

To perform the *Pst* DC3000 Δ hopQ infection assays in *N. benthamiana*, four-week-old *N. benthamiana* plants were infiltrated with bacteria at a concentration of 1×10⁵ CFU/mL. Leaf disks were taken and the bacterial number was determined 3 days later (Li et al. 2022). To perform the *P. capsici* infection assays in *N. benthamiana*, detached leaves of 4-week-old *N. benthamiana* plants were inoculated with *P. capsici* strain LT263 at 25°C in a high-humidity plastic box for 36–48 h. The lesion area was photographed under UV light and calculated by Image J software (Liang et al. 2021). To perform the *S. sclerotiorum* inoculation assay in *N. benthamiana*, the *S. sclerotiorum* strain was cultured on potato dextrose agar (PDA) medium at 25°C for 2–3 days. Detached leaves of 4-week-old *N. benthamiana* plants were inoculated with fresh mycelial plugs for 1 day. The disease symptom was photographed and lesion areas were calculated by Image J software (Chen et al. 2024).

Reactive oxygen species burst assay

To perform flg22- or chitin-induced ROS production assays, leaf disks from 4-week-old *N. benthamiana* or 2–3-week-old soybean plants were incubated with 200 μL ddH₂O in a 96-well plate for 8–12 h. The water was then replaced with luminescence detection buffer (10 mg/mL horseradish peroxidase and 20 mM luminol) containing 1 μM flg22 or 200 μg/mL chitin. Relative luminescence units (RLU) were recorded using a luminometer (TECAN).

INF1-induced cell death assay

To analyze the effect of GmRALFs on INF1-induced cell death, The GmRALFs were transiently expressed in *N. benthamiana* for 1 day and the same area was infiltrated with *Agrobacterium* carrying INF1 for 2 days. The cell death phenotype was visualized and photographed under UV light. To examine the cell death by electrolyte leakage assay, leaf disks were taken and floated on water for 3 h at 23°C. The electrical conductivity was measured with conductivity meter (METTLER TOLEDO) and denoted as 'value A'. Samples were then boiled for 20–30 min and cooled to room temperature for a second electrical

conductivity measurement, and denoted as ‘value B’. The ion leakage was calculated as $(\text{value A}/\text{value B}) \times 100\%$.

RNA isolation and qRT-PCR assay

The plant samples were ground into powder in liquid nitrogen and total RNA was isolated using TRIzol reagent (Invitrogen) according to the manufacturer’s instructions. The first-strand cDNA was synthesized using Maxima First Strand cDNA Synthesis Kit (Thermo) and qRT-PCR was performed using specific primers and SYBR Premix Ex Taq Kit (Takara). The primers used for qRT-PCR were listed in Additional file 1: Table S1.

Generation of soybean CRISPR lines

The indicated construct was introduced into *Agrobacterium tumefaciens* strain EHA105 by electroschock transformation. Cotyledonary explants of a soybean cultivar Williams 82 were transformed with the EHA105 strain carrying the indicated knockout constructs. *GmRALF1* was amplified from the plants obtained via tissue culture, followed by sequencing to ascertain potential gene editing.

Virus-induced gene silencing assay

To perform the virus-induced gene silencing in *N. benthamiana*, *A. tumefaciens* strains carrying TRV2 or TRV1 vector were resuspended in an infiltration solution (10 mmol/L MES pH5.7, 10 mmol/L MgCl_2) and adjusted to a final OD_{600} value of 1.0. Equal amount of TRV1 and TRV2 was mixed and infiltrated into primary leaves during the four-leaf stage. TRV2:*GFP* was used as negative control and gene silencing efficiency was determined by qPCR analysis.

Abbreviations

CDPK	Calcium dependent protein kinase
ETI	Effector-triggered immunity
GPI-Aps	Glycosylphosphatidylinositol-anchored protein
MAPK	Mitogen-activated protein kinase
NLR	Nucleotide-binding domain and leucine-rich repeat
PAMP	Pathogen-associated molecular patterns
PRR	Pattern recognition receptor
PTI	Pattern-triggered immunity
RALF	Rapid alkalization factor
RLCK	Receptor-like cytoplasmic kinases
RLK	Receptor-like kinase
RLP	Receptor-like protein

Supplementary Information

The online version contains supplementary material available at <https://doi.org/10.1186/s42483-025-00361-1>.

Additional file 1: Table S1. Coding sequences of all the *GmRALFs*.
Table S2. Primers used in this study.

Additional file 2: Figure S1. Protein sequence alignment of *GmRALFs*.
Figure S2. Chromosome structure and intraspecific collinearity analysis of *GmRALFs*. **Figure S3.** Expression of *GmRALFs* in response to *Phytophthora sojae* (*P. sojae*) treatment. **Figure S4.** Expression of *GmRALFs* in response

to *Pseudomonas syringae* pv. *glyciniae* (*Psg*). **Figure S5.** Examination of the protein expression of *GmRALFs* in *N. benthamiana*. **Figure S6.** Analysis of the roles of *GmRALFs* in flg22- and chitin-induced ROS burst. **Figure S7.** Analysis of the roles of *GmRALFs* in INF1-induced cell death. **Figure S8.** Analysis of the roles of *GmRALFs* in plant resistance to bacterial pathogen *Pseudomonas syringae* pv. *tomato* DC3000 ΔhopQ1 (*Pst* DC3000 ΔhopQ1). **Figure S9.** Analysis of the roles of *GmRALFs* in plant resistance to fungal pathogen *Sclerotinia sclerotiorum* (*S. sclerotiorum*). **Figure S10.** Analysis of the roles of *GmRALFs* in plant resistance to oomycete pathogen *Phytophthora capsici* (*P. capsici*). **Figure S11.** Examination of the protein expression of NbRALF1-HA and silencing efficiency of NbRALF1 in *N. benthamiana* plants. **Figure S12.** Transient expression of NbRALF1 in *N. benthamiana* plants leads to reduced PTI and disease resistance. **Figure S13.** Analysis of the growth phenotype and yield-related traits of *Gmralf1*.

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Author contributions

RC, XL, and DD coordinated the research and wrote the paper. WW and RC performed the majority of the experiments. ZH, GX, and JY contribute to pathogen infection assays and plasmid constructions. DW and XF contributed to generation of soybean knockout mutants.

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Availability of data and materials

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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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