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ORIGINAL ARTICLE

The Raf-like kinase Raf36 negatively regulates plant resistance against the oomycete pathogen *Phytophthora parasitica* by targeting MKK2

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Abstract

Oomycetes represent a unique group of plant pathogens that are phylogenetically distant from true fungi and cause significant crop losses and environmental damage. Understanding of the genetic basis of host plant susceptibility facilitates the development of novel disease resistance strategies. In this study, we report the identification of an *Arabidopsis thaliana* T-DNA mutant with enhanced resistance to *Phytophthora parasitica* with an insertion in the Raf-like mitogen-activated protein kinase kinase kinase gene *Raf36*. We generated additional *raf36* mutants by CRISPR/Cas9 technology as well as *Raf36* complementation and overexpression transformants, with consistent results of infection assays showing that *Raf36* mediates *Arabidopsis* susceptibility to *P. parasitica*. Using a virus-induced gene silencing assay, we silenced *Raf36* homologous genes in *Nicotiana benthamiana* and demonstrated by infection assays the conserved immune function of *Raf36*. Mutagenesis analyses indicated that the kinase activity of Raf36 is important for its immune function and interaction with MKK2, a MAPK kinase. By generating and analysing *mkk2* mutants and *MKK2* complementation and overexpression transformants, we found that *MKK2* is a positive immune regulator in the response to *P. parasitica* infection. Furthermore, infection assay on *mkk2 raf36* double mutant plants indicated that *MKK2* is required for the *raf36*-conferred resistance to *P. parasitica*. Taken together, we identified a Raf-like kinase Raf36 as a novel plant susceptibility factor that functions upstream of MKK2 and directly targets it to negatively regulate plant resistance to *P. parasitica*.

KEYWORDS
Arabidopsis thaliana, *MKK2*, *Nicotiana benthamiana*, *Phytophthora parasitica*, plant susceptibility, *Raf36*, Raf-like MAPKKK

Jinfang Li and Fengyan Deng contributed equally to this work.

1 | INTRODUCTION

Oomycetes are phylogenetically distant from true fungi and include *Phytophthora* plant pathogens that severely threaten agricultural and forestry production (Kamoun et al., 2015). To gain disease resistance, plants have developed two approaches: mobilizing resistance (R) proteins and suppressing susceptibility factors (van Schie & Takken, 2014). Characterizing the genetic basis of plant susceptibility to oomycete pathogens is a promising approach to develop novel disease resistance strategies, and significant progress has been achieved in recent years (Boevink et al., 2016; He et al., 2020; van Schie & Takken, 2014). *Phytophthora parasitica*, which causes destructive diseases in plants and has a broad range of hosts from crops to trees, has emerged as a model oomycete pathogen for such studies (Kamoun et al., 2015; Meng et al., 2014). By employing the compatible *Arabidopsis thaliana* (hereafter *Arabidopsis*)–*P. parasitica* pathosystem, which has been shown to involve the salicylic acid (SA), jasmonic acid (JA), and ethylene (ET) signalling pathways (Attard et al., 2010; Wang et al., 2011), several plant susceptibility factors to *P. parasitica* have been identified recently. For example, the nodulin-related MtN21 family gene *AtRTP1* (*Arabidopsis thaliana Resistance to Phytophthora parasitica 1*) was found to mediate plant susceptibility to *P. parasitica* by regulating reactive oxygen species (ROS) production, cell death progression, and *PR1* expression (Pan et al., 2016). Further investigation showed that *AtRTP1* negatively regulates *P. parasitica* resistance by modulating the unfolded protein response regulators *bZIP60* and *bZIP28* (Qiang et al., 2021). *AtRTP5*, which encodes a WD40-containing protein with unknown function, has been reported to negatively regulate plant resistance by disrupting the SA and JA signalling pathways (Li, Zhao, et al., 2020). The transcription factor *AtERF019* mediates plant susceptibility to *P. parasitica* by suppressing pathogen-associated molecular pattern (PAMP)-triggered immunity (PTI), SA, and JA defence responses (Lu et al., 2020). Interestingly, the *Arabidopsis* VQ motif-containing protein VQ29 has been shown to mediate plant resistance to *P. parasitica* infection independent of known SA, JA, and ET signalling pathways, camalexin biosynthesis, and PTI signalling (Le Berre et al., 2017). This inconsistency can be explained by the sophisticated interaction between *Arabidopsis* and *P. parasitica*. Thus, further studies are warranted to explore the mechanisms of plant defence and plant susceptibility against this pathogen.

Mitogen-activated protein kinase (MAPK) cascades, which often consist of a MAPK kinase kinase (MAPKKK), MAPK kinase (MAPKK), and MAPK, are vital nodes in plant immunity signalling networks and transmit signals from diverse stimuli to regulate downstream defence responses (Bi et al., 2018; Mao et al., 2011; Zhang et al., 2012). Thus, their components are usually targeted and modulated by pathogen and plant factors. For example, the *Phytophthora infestans* RXLR effector PITG20303 targets and stabilizes the potato MAPKK StMKK1 to suppress flg22-triggered PTI and plant resistance (Du et al., 2021). The geminivirus-encoded βC1 protein simultaneously targets both MKK2 and MPK4 to counter host defence and promote infection (Hu et al., 2019). *Arabidopsis* PP2C-type phosphatase AP2C1 can inactivate the stress-responsive MPK4 and MPK6 to modulate plant resistance against *Botrytis cinerea* (Schweighofer et al., 2007). *Arabidopsis* dual-specificity phosphatase MKP1 is a negative regulator of MPK6-mediated PTI responses (Anderson et al., 2011).

Plant MAPKKKs consist of three families: the MEKK family, the Raf-like family, and the ZIK family (Ichimura et al., 2002; Jonak et al., 2002). The MEKK kinases usually function upstream and activate the MAPKK-MAPK cascades (Thulasi Devendrakumar et al., 2018), but the Raf-like kinases interact with different kinds of substrates and participate in diverse life activities (Fàbregas et al., 2020; Hayashi et al., 2020; Wang et al., 2018). Raf-like kinases also play roles in plant–pathogen interactions. For example, AtMKD1 activates the MKK1/5-MPK3/6 cascade to positively regulate resistance to bacterial and fungal pathogens (Asano et al., 2020). AtEDR1 interacts with MKK4/5 to negatively regulate plant resistance to bacterial, fungal, and oomycete pathogens (Zhao et al., 2014). Rice EDR1 interacts with OsMPKK10.2 and perturbs the OsMPKK10.2-OsMPK6 cascade-mediated resistance to bacterial infection (Ma et al., 2021). The Raf-like kinase OsILA1 phosphorylates OsMAPKK4 and suppresses OsMAPKK4-OsMPK6 cascade-mediated resistance to rice bacterial blight (Chen, Wang, Yang, et al., 2021).

Potato Raf-like MAPKKK StVIK is targeted by *P. infestans* RXLR effector Pi17316 to promote late blight disease (Murphy et al., 2018). However, whether other Raf-like kinases are involved in plant–*Phytophthora* interaction and their mechanisms remains largely unknown. Here, we report the identification and characterization of a T-DNA insertion mutant named 105-3 that is resistant to *P. parasitica*. We found that the T-DNA was inserted in a Raf-like kinase gene, *Raf36*, a novel susceptibility factor, by using the established model *Arabidopsis*–*P. parasitica* compatible interaction (Meng et al., 2015; Wang et al., 2011) and a forward genetics approach. Our analyses showed that Raf36 functions upstream of MKK2, a MAPK kinase, by direct targeting, to negatively regulate plant resistance to *P. parasitica*.

2 | RESULTS

2.1 | Identification of *Arabidopsis* mutant 105-3 resistant to *P. parasitica*

To identify plant genes that mediate susceptibility to the oomycete pathogen *P. parasitica*, we screened 6741 T_3 generation *Arabidopsis* T-DNA insertion plants by inoculating detached leaves of the 4-week-old plants with *P. parasitica* zoospores. This led to the identification of the mutant 105-3, which showed restricted water-soaked lesions and less *P. parasitica* biomass compared to the wild-type Col-0 plant at 3 days postinoculation (dpi) (Figure 1a,b). To identify the T-DNA insertion sites in 105-3, we performed thermal asymmetric interlaced (TAIL)-PCR and then subsequent sequence analysis. The results showed a single T-DNA insertion site in mutant 105-3 that occurred immediately downstream of the stop codon of a Raf-like MAPKKK gene named *Raf36* (*AT5G58950*) (Figure 1c). Using a reverse transcription quantitative PCR (RT-qPCR) assay, we found that

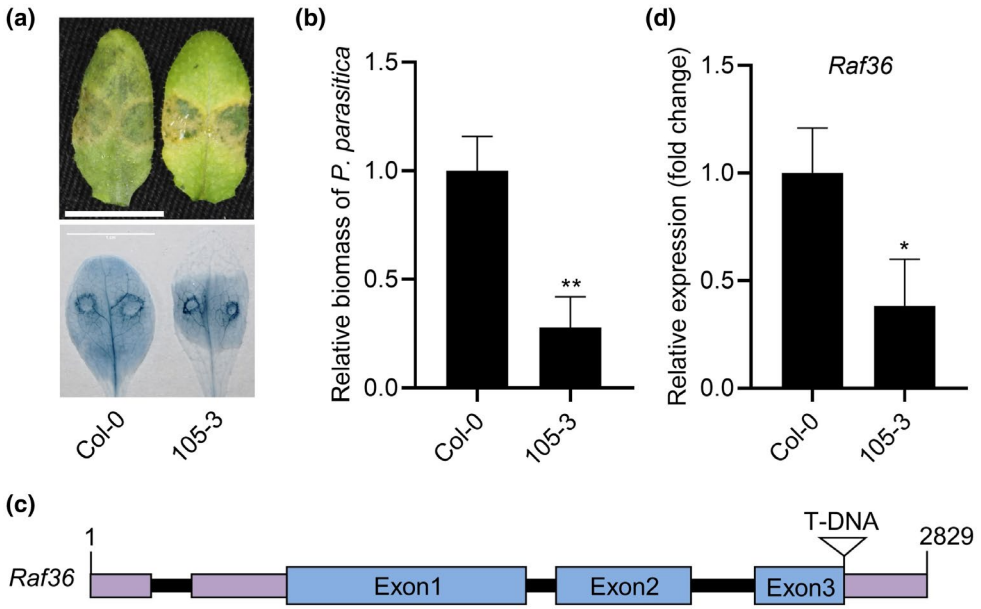


FIGURE 1 *Arabidopsis thaliana* mutant 105-3 showed enhanced resistance to *Phytophthora parasitica*. (a) Disease lesions at 3 days postinoculation (dpi) with *P. parasitica* zoospores before and after trypan blue staining. Scale bars = 1 cm. Detached leaves of at least 20 4-week-old *Arabidopsis* plants were inoculated with 20 μl of *P. parasitica* Pp016 zoospores (100 zoospores/μl). The experiments were performed at least three times and representative photographs are shown. (b) *P. parasitica* biomass at 3 dpi as determined by quantitative PCR (qPCR). *AtUBC9* and *PpWS041* were used as the internal standards for *Arabidopsis* and *P. parasitica*, respectively. Data are presented as the means ± *SD* of three biological replicates. Genomic DNA was extracted from samples containing 10 leaves at 3 dpi per replicate. (c) Gene structure of *Raf36* and the position of the T-DNA insertion. (d) The relative expression level of *Raf36* in 105-3 was determined by reverse transcription qPCR. *AtUBC9* was used as the internal standard for *Arabidopsis*. Data are presented as the means ± *SD* of three biological replicates. Each data point was from three leaves per genotype. (b, d) Asterisks represent a significant difference between mutant plants and wild-type plants as determined by Student's *t* test; $^{*}p < 0.05$, $^{**}p < 0.01$

the transcription of *Raf36* was reduced by more than half in mutant 105-3 compared to the wild-type Col-0 plant (Figure 1d), indicating that the *Raf36* expression was influenced by the inserted T-DNA.

2.2 | *Raf36* mediates *Arabidopsis* susceptibility to *P. parasitica*

To confirm the *raf36*-mediated resistance against *P. parasitica* and further investigate the underlying mechanism, we generated independent *raf36* mutants with nonsense alleles in the first exon of *Raf36* in the Col-0 background using the CRISPR/Cas9 method (Figure 2a). We successfully generated several independent mutants and selected two for further studies (Figure 2a). The predicted protein encoded by *Raf36* was confirmed to be truncated in both the *raf36*-1 and *raf36*-2 mutants because of a 1-nucleotide insertion, which led to a frameshift and premature termination (Figure 2a). We examined the transcript level of *Raf36* in the *raf36*-1 and *raf36*-2 mutants by RT-qPCR and confirmed that *Raf36* expression in both *raf36*-1 and *raf36*-2 mutants was significantly lower compared with that in Col-0 plants (Figure S1a). When detached leaves were inoculated with *P. parasitica* zoospores, both *raf36*-1 and *raf36*-2 mutants showed remarkably smaller lesions compared to Col-0 at 3 dpi (Figure 2b). In addition, a qPCR assay showed that the relative biomass of *P. parasitica* in the leaves of both *raf36*-1 and *raf36*-2 mutants was significantly lower compared with that in Col-0 leaves (Figure 2c). These results suggest that loss of *Raf36* confers enhanced resistance against *P. parasitica*.

We next performed genetic complementation experiments by transferring the coding sequence of *Raf36* with its native promoter into the *raf36*-2 mutant. The transcription of *Raf36* in two lines, *Raf36*-C-5 and *Raf36*-C-9, was confirmed by RT-qPCR (Figure S1b). An infection assay showed that the *Raf36*-C-5 and *Raf36*-C-9 lines had similar water-soaked lesions and *P. parasitica* biomass to Col-0 plants (Figure 2d–f), indicating that plant susceptibility was restored by *Raf36* complementation. We also transferred the *Raf36* coding sequence with the CaMV 35S promoter into *raf36*-1 mutant plants to generate *Raf36* overexpression lines. Two overexpression (OE) transformants, *Raf36*-OE-21 and *Raf36*-OE-42, showed approximately 10-fold higher *Raf36* mRNA accumulation compared to Col-0 plants (Figure S1c). After inoculating the *Raf36*-OE-21 and *Raf36*-OE-42 lines with *P. parasitica* zoospores, the water-soaked lesions were significantly larger than those in Col-0 plants (Figure 2g,h). The *P. parasitica* biomass in leaves of the two *Raf36*-OE lines was also higher than that of Col-0 plants (Figure 2i). The results suggest that *Raf36* overexpression increases plant susceptibility to *P. parasitica*. Thus, *Raf36* is confirmed to be a negative regulator of *Arabidopsis* resistance to *P. parasitica*. To determine whether *Raf36* also plays a role in plant resistance to necrotrophic pathogens, we inoculated *raf36*-1 and *Raf36*-OE-21 plants with *B. cinerea*. The mutant plants

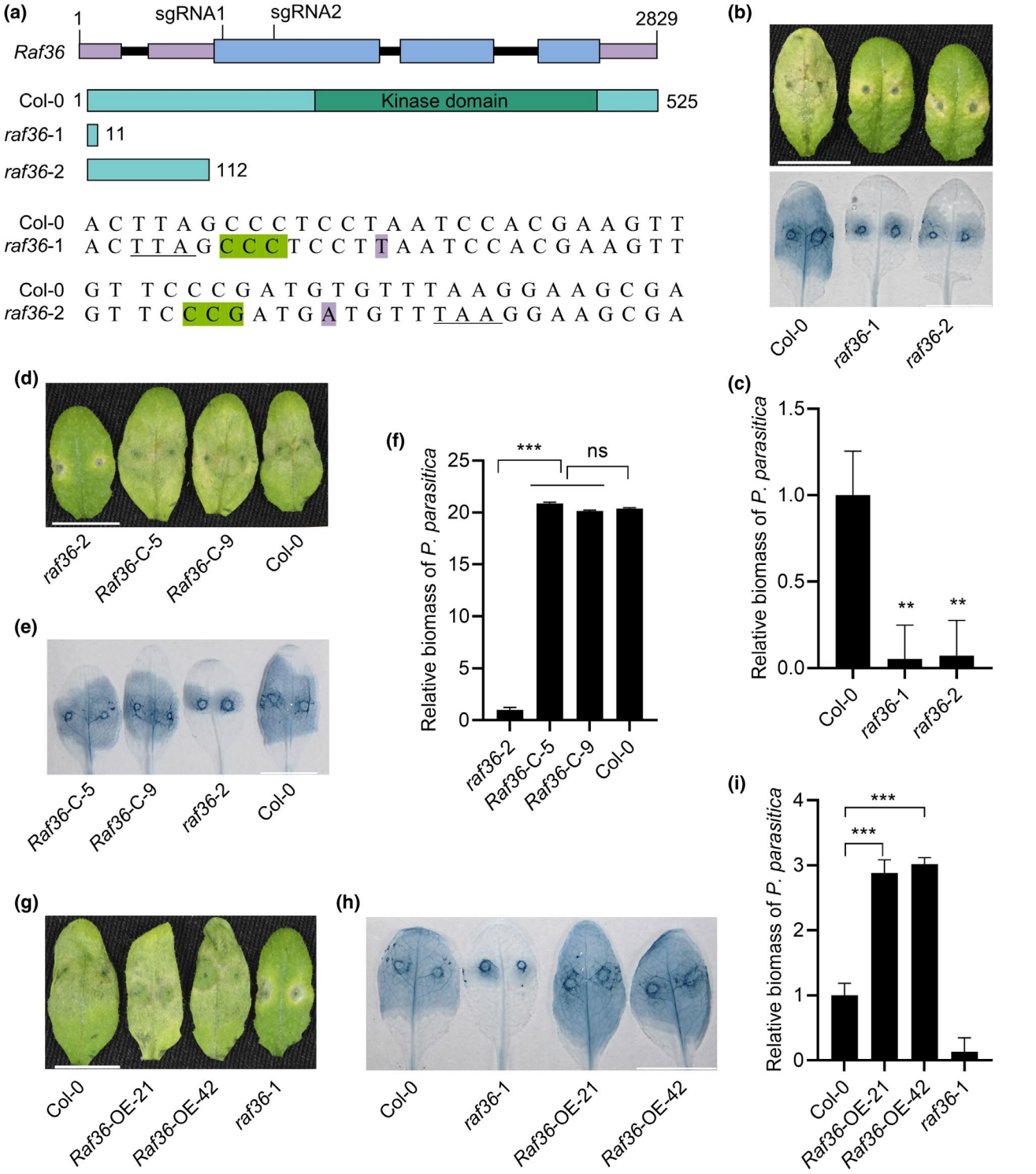


FIGURE 2 *Raf36* is required for *Arabidopsis thaliana* susceptibility to *Phytophthora parasitica*. (a) Sequences of mutant alleles of *Raf36* in *raf36* homozygous plants. The target sites of sgRNAs in *Raf36* genomic DNA and the Raf36 truncated proteins are shown. The PAM sequences of the *Raf36* sgRNAs are highlighted in light green. The bases inserted by nonhomologous end joining are highlighted in purple. The underlined bases indicate the stop codon introduced by a frameshift. (b, d, e, g, h) Disease lesions on leaves of *raf36* mutants (b), *Raf36* complementation (C) lines (d, e), and Raf36 overexpression (OE) lines (g, h) at 3 days postinoculation (dpi) with *P. parasitica* before and after trypan blue staining. Scale bars = 1 cm. Detached leaves of at least 20 4-week-old *Arabidopsis* plants were inoculated with 20 μl of *P. parasitica* Pp016 zoospores (100 zoospores/μl). The experiments were performed at least three times and representative photographs are shown. (c, f, i) *P. parasitica* biomass in leaves of *raf36* mutants (c), *Raf36*-C lines (f), and *Raf36*-OE lines (i) at 3 dpi as determined by quantitative PCR. *AtUBC9* and *PpWS041* were used as the internal standards for *Arabidopsis* and *P. parasitica*, respectively. Data are presented as the means ± *SD* of three biological replicates. Genomic DNA was extracted from samples containing 10 leaves at 3 dpi per replicate. Asterisks represent a significant difference between mutant plants and wild-type plants as determined by Student's *t* test; $^{**}p < 0.01$, $^{***}p < 0.001$, ns $p > 0.05$

showed indistinguishable disease lesions compared with Col-0 plants, indicating that *Raf36* is not related to plant resistance to *B. cinerea* (Figure S2).

2.3 | Silencing of *NbRaf36s* by virus-induced gene silencing enhanced plant resistance to *P. parasitica*

Raf36 is conserved in the representative hosts of *P. parasitica* such as *N. benthamiana* (Figure S3). To demonstrate whether the *AtRaf36*-mediated plant susceptibility is also conserved in *N. benthamiana*, the fragment of 369–613 bp in *NbRaf36*-3 cDNA was selected and cloned into a tobacco rattle virus (TRV)-based virus-induced gene silencing (VIGS) vector to silence all four *Raf36* homologous genes, *NbRaf36-1*, *NbRaf36-2*, *NbRaf36-3*, and *NbRaf36-4*. The transcriptions of four *NbRaf36* genes in *TRV2-NbRaf36-3* plants were reduced to 20% or lower compared to that in the *TRV2-GFP* control plants (Figure 3a). After inoculating the detached leaves with *P. parasitica*, the lesions in *TRV2-NbRaf36-3* plants were smaller than that in *TRV2-GFP* plants (Figure 3b,c), indicating that *NbRaf36* genes negatively regulate *N. benthamiana* resistance to *P. parasitica*.

2.4 | The kinase activity of Raf36 is vital for its interaction with MKK2 and the susceptibility function

To understand the mechanisms underlying *AtRaf36*-mediated plant susceptibility, we attempted to identify the candidate interacting proteins. AtMKK2, a MAPK kinase, has been reported to interact with Raf36 by yeast two-hybrid (Y2H) assay and in vitro pull-down assay (Himbert, 2009; Li, 2016). We used a co-immunoprecipitation (co-IP) assay and a luciferase complementation imaging (LCI) assay to investigate whether AtRaf36 interacts with AtMKK2 in planta. For the co-IP assay, we co-expressed *Flag-AtRaf36* with *AtMKK1*- or *AtMKK2-Myc* in leaves of *N. benthamiana*. AtMKK1 was considered because of its high sequence similarity to AtMKK2. Total proteins were extracted from the leaves and then immunoprecipitated with anti-Flag magnetic beads, and the immunoprecipitated proteins were detected with an anti-Myc antibody. The results showed that AtRaf36 could immunoprecipitate AtMKK2 but not AtMKK1, indicating that AtRaf36 interacts with AtMKK2 in *N. benthamiana* (Figure 4a).

For the LCI assay, we fused *AtRaf36* and *AtMPK6* to the N-terminus of luciferase protein (*NLuc*) and *AtMKK2* and *AtMKK1* to the C-terminus of luciferase (*CLuc*); the *CLuc-AtMKK2* and *AtMPK6-NLuc* pair was used as the positive control (Cao et al., 2014). After co-expression of these construct pairs in *N. benthamiana* leaves, fluorescence signals were detected for both the *AtRaf36-NLuc* and *CLuc-AtMKK2* pair and the positive control, but not for the *AtRaf36-NLuc* and *CLuc-AtMKK1* pair, supporting the notion that AtRaf36 interacts with AtMKK2 in *N. benthamiana* (Figure 4b). Collectively, these results suggest that AtRaf36 interacts with AtMKK2 in planta.

Like other members of the Raf-like MAPKKK family, the *Arabidopsis* Raf36 protein consists of a putative N-terminal regulatory region and a kinase domain-containing C-terminal region (Figure 4c). The regions required for interactions between Raf-like kinases and MAPKKs are not typical (Asano et al., 2020; Ma et al.,

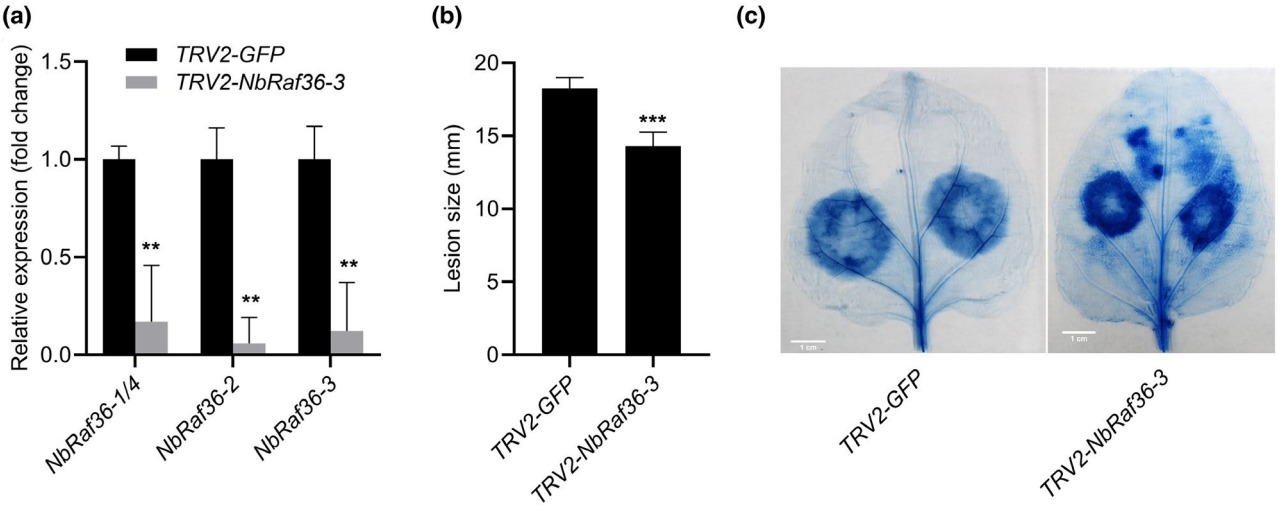
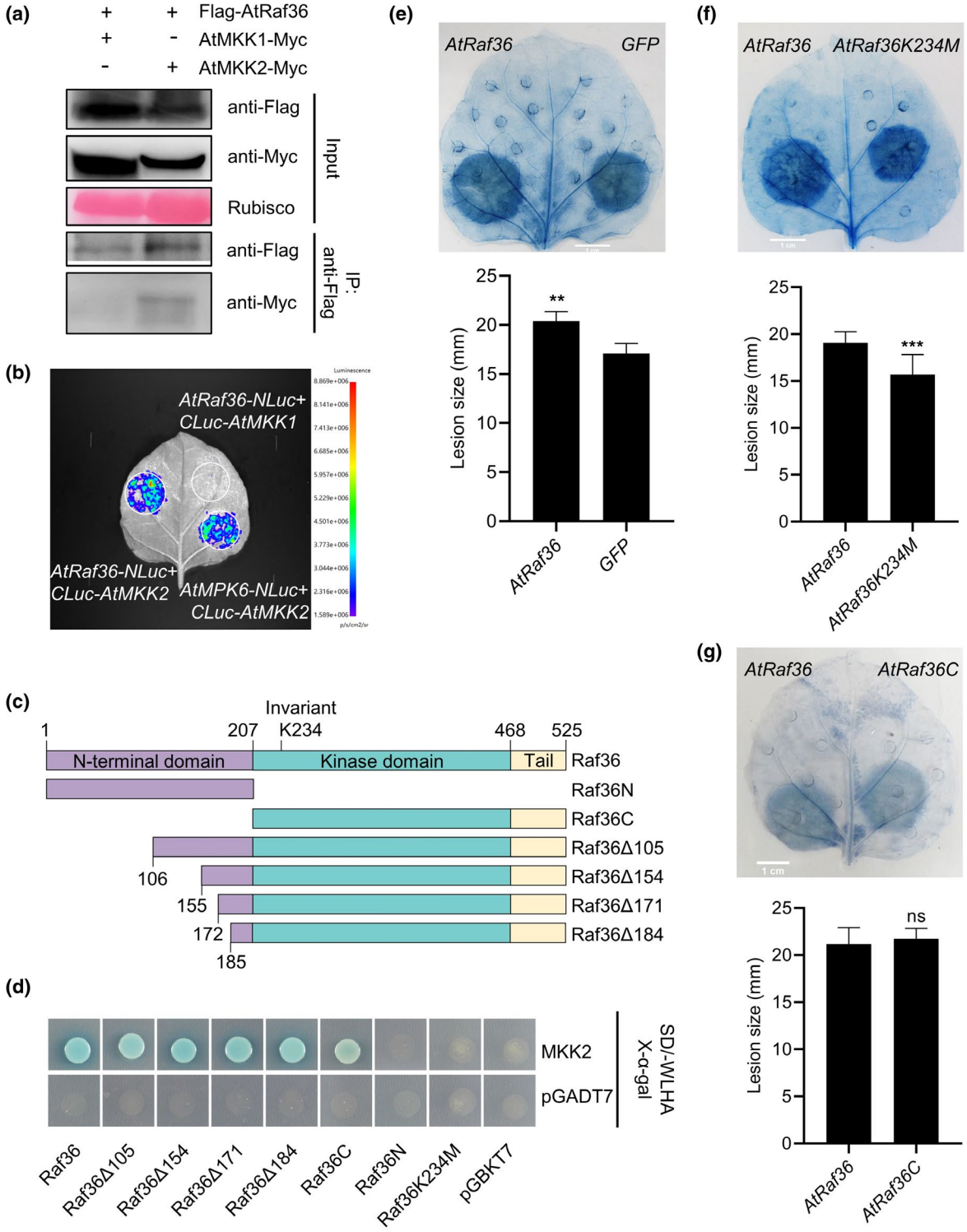


FIGURE 3 Silencing of *NbRaf36* genes by virus-induced gene silencing (VIGS) enhanced *Nicotiana benthamiana* resistance to *Phytophthora parasitica*. (a) The transcriptions of *NbRaf36* genes in *TRV2-NbRaf36-3* plants and the *TRV2-GFP* plants. Transcriptions of *NbRaf36-1* and *NbRaf36-4* were determined by one primer pair. *NbβActin* was used as the internal standard. Data are presented as means ± *SD* of three biological replicates. Each data point was from three leaves per genotype. (b, c) *TRV2-NbRaf36-3* plants show smaller lesions than *TRV2-GFP* plants. Detached leaves of *N. benthamiana* plants were inoculated with 20 μl of *P. parasitica* Pp016 zoospores (25 zoospores/μl). The lesions in leaves were measured and analysed (b) at 2 days postinoculation and then stained with trypan blue (c). Scale bars = 1 cm. Data are presented as mean lesion diameter ± *SD* from at least 12 plants per genotype. Asterisks represent a significant difference between *TRV2-NbRaf36-3* plants and the *TRV2-GFP* plants as determined by Student's *t* test; $^{**}p < 0.01$, $^{***}p < 0.001$. The experiments were performed at least three times with similar results and representative photographs are shown



2021; Zhao et al., 2014). To determine which regions of the Raf36 protein are essential for its interaction with MKK2, we tested the interactions of the N-terminal domain (Raf36N) and the C-terminal domain (Raf36C) with MKK2 in a Y2H system (Figure 4c,d). The results showed that the yeast co-transformed with BD-Raf36N and AD-MKK2 could not grow on quadruple dropout (QDO) selective medium (Figure 4d), whereas yeast co-transformed with BD-Raf36C and AD-MKK2 could, although the growth was weaker than that of yeast co-transformed with MKK2 and the full-length Raf36 (Figure 4d). To determine which N-terminal sequences of Raf36 contribute to the interaction with MKK2, we generated a series of N-terminal truncation mutants, namely Raf36Δ105, Raf36Δ154, Raf36Δ171, and Raf36Δ184, and then evaluated the interactions of these truncated Raf36 proteins with MKK2 in yeast cells (Figure 4c). The growth intensity of the co-transformed yeast remained unaltered, even when the first 184 amino acids of Raf36 were truncated

FIGURE 4 Mutagenesis analysis of AtRaf36 to identify protein regions important for its interaction with AtMKK2 and its role in immunity. (a) AtRaf36 interacts with AtMKK2 in a co-immunoprecipitation (co-IP) assay. Total proteins were extracted from *Nicotiana benthamiana* leaves expressing *pro35S::Flag-AtRaf36* with *pro35S::AtMKK1-Myc* or *pro35S::AtMKK2-Myc*. Anti-Flag magnetic beads were used for immunoprecipitation and the precipitated proteins were analysed by immunoblotting using an anti-Myc antibody. The assay was performed at least three times and representative photographs are shown. (b) AtRaf36 interacts with AtMKK2 in *N. benthamiana* in a firefly luciferase complementation (LCI) assay. The AtMKK2-AtMPK6 pair was used as the positive control. The fluorescence signal in leaves infiltrated with the indicated construct pairs was captured by a CCD camera at 2.5 days after injection. The assay was performed at least three times and representative photographs are shown. (c) Schematic representation of full-length Raf36 and truncated forms: Raf36Δ105 (amino acids Δ1–105 of Raf36), Raf36Δ154, Raf36Δ171, Raf36Δ184, Raf36C, and Raf36N. (d) The interaction between Raf36, the truncated mutants, or the kinase-dead mutant ($Raf36^{K234M}$) and MKK2 in a yeast two-hybrid system. Yeast cells containing the indicated plasmids were spotted onto SD/−Trp/−Leu/−His/−Ade (SD/−WLHA)/X-α-gal medium. Photographs were taken after 3 days of incubation. The assay was performed at least three times and representative photographs are shown. (e, f) The kinase-dead mutant of AtRaf36 does not confer susceptibility to *Phytophthora parasitica*. (g) The AtRaf36 C-terminus is enough for susceptibility to *P. parasitica*. Scale bars = 1 cm. Each construct was agoinfiltrated in one panel of the leaves for 2 days, followed by infection assay. The lesions in leaves were examined at 2 days postinoculation with *P. parasitica* Pp016 zoospores (25 zoospores/μl) and then stained with trypan blue. Data are presented as mean lesion diameter ± *SD* from at least 12 leaves. Asterisks represent a significant difference between the mutants and the wild-type Raf36 as determined by Student's *t* test; $^{**}p < 0.01$, $^{***}p < 0.001$, ns $p > 0.05$. (e–g) The experiments were performed at least three times with similar results and representative photographs are shown

(Figure 4d). These results indicate that the entire C-terminal domain of Raf36 and the N-terminal 185–206 amino acids of Raf36 are important for its interaction with MKK2. Previous studies showed that Raf36 is a true kinase (Himbert, 2009; Kamiyama et al., 2021); we further tested the ability of the Raf36 kinase-deficient mutant $Raf36^{K234M}$ to interact with MKK2 in yeast. In contrast to yeast cells co-transformed with wild-type BD-Raf36 and AD-MKK2, those transformed with BD-$Raf36^{K234M}$ and AD-MKK2 could not grow on QDO medium (Figure 4d). This indicates that the kinase activity of the Raf36 protein is crucial for the interaction with MKK2.

We also evaluated the role of kinase activity in *Arabidopsis Raf36*-mediated susceptibility to *P. parasitica* in *N. benthamiana*. We transiently expressed *Arabidopsis Raf36*, $Raf36^{K234M}$, *Raf36C*, and control *GFP* in *N. benthamiana* leaves and subsequently inoculated them with *P. parasitica*. We found that *AtRaf36* overexpression caused larger disease lesions than the control, whereas $AtRaf36^{K234M}$ expression led to smaller lesions than the *AtRaf36* overexpression plants (Figure 4e,f). This indicates that the K234M mutation altered *AtRaf36*-mediated susceptibility to *P. parasitica*. Moreover, when overexpressing *AtRaf36C* in *N. benthamiana* leaves and subsequently inoculating them with *P. parasitica*, the disease lesions were not affected (Figure 4g). This indicates that the kinase activity of AtRaf36 is important for its role in plant susceptibility. It is also suggested that AtRaf36 may regulate its substrates involved in plant–*P. parasitica* interaction by phosphorylation.

2.5 | *MKK2* positively regulates *Arabidopsis* resistance to *P. parasitica*

To investigate the potential function of *MKK2* in plant immunity to *P. parasitica*, we generated *MKK2* knockout mutant lines, complementation lines, and overexpression lines for further analyses. We first generated *mkk2* mutants with nonsense mutations in exons in the Col-0 background using CRISPR/Cas9 (Figure 5a). The predicted MKK2 proteins in two independent knockout mutants, *mkk2*-1 and *mkk2*-2, were truncated because of a 1-nucleotide insertion that caused premature termination (Figure 5a). We examined the *MKK2* expression level in the two knockout mutants by RT-qPCR and found that it was significantly lower compared with that in Col-0 plants (Figure S4a). When challenged with *P. parasitica*, the *mkk2*-1 and *mkk2*-2 mutants displayed larger water-soaked lesions and increased *P. parasitica* colonization than Col-0 plants (Figure 5b,c), suggesting that the knockout of *MKK2* disrupts plant resistance to *P. parasitica*. We then transferred the *MKK2* coding sequence with its native promoter into *mkk2*-2 mutant plants. Two complementation lines, *MKK2*-C-11 and *MKK2*-C-28, showed a similar expression level of *MKK2* to Col-0 plants (Figure S4b). They also exhibited similar disease lesions and *P. parasitica* colonization to those of Col-0 plants but distinct from those of *mkk2*-2 plants (Figure 5d,e). The results indicate that the genetic complementation of *MKK2* restores plant resistance against *P. parasitica*.

To investigate the effects of *MKK2* overexpression on plant resistance, we transferred the *MKK2* coding sequence with the CaMV 35S promoter into Col-0 plants. Two individual lines, *MKK2*-OE-5 and *MKK2*-OE-9, with an approximately 80-fold increase in *MKK2* expression than Col-0 plants were further analysed (Figure S4c). The two lines exhibited smaller lesions and significantly less *P. parasitica* colonization than Col-0 plants (Figure 5f,g), indicating that overexpression of *MKK2* increased plant resistance to *P. parasitica*. Taken together, these results suggest that *MKK2* positively regulates plant resistance to *P. parasitica*.

2.6 | *MKK2* is required for *raf36*-mediated resistance to *P. parasitica*

These findings prompted us to further investigate whether *MKK2* might be involved in regulating the *raf36*-mediated immune signalling pathway. Toward this end, we generated an *mkk2 raf36* double knockout mutant using CRISPR/Cas9 technology. The single guide RNA (sgRNA) targeting *MKK2* (*MKK2*-sgRNA2) (Figure 5a) was used to knock out *MKK2* in *raf36*-1 background, and the *mkk2*-2 *raf36*-1

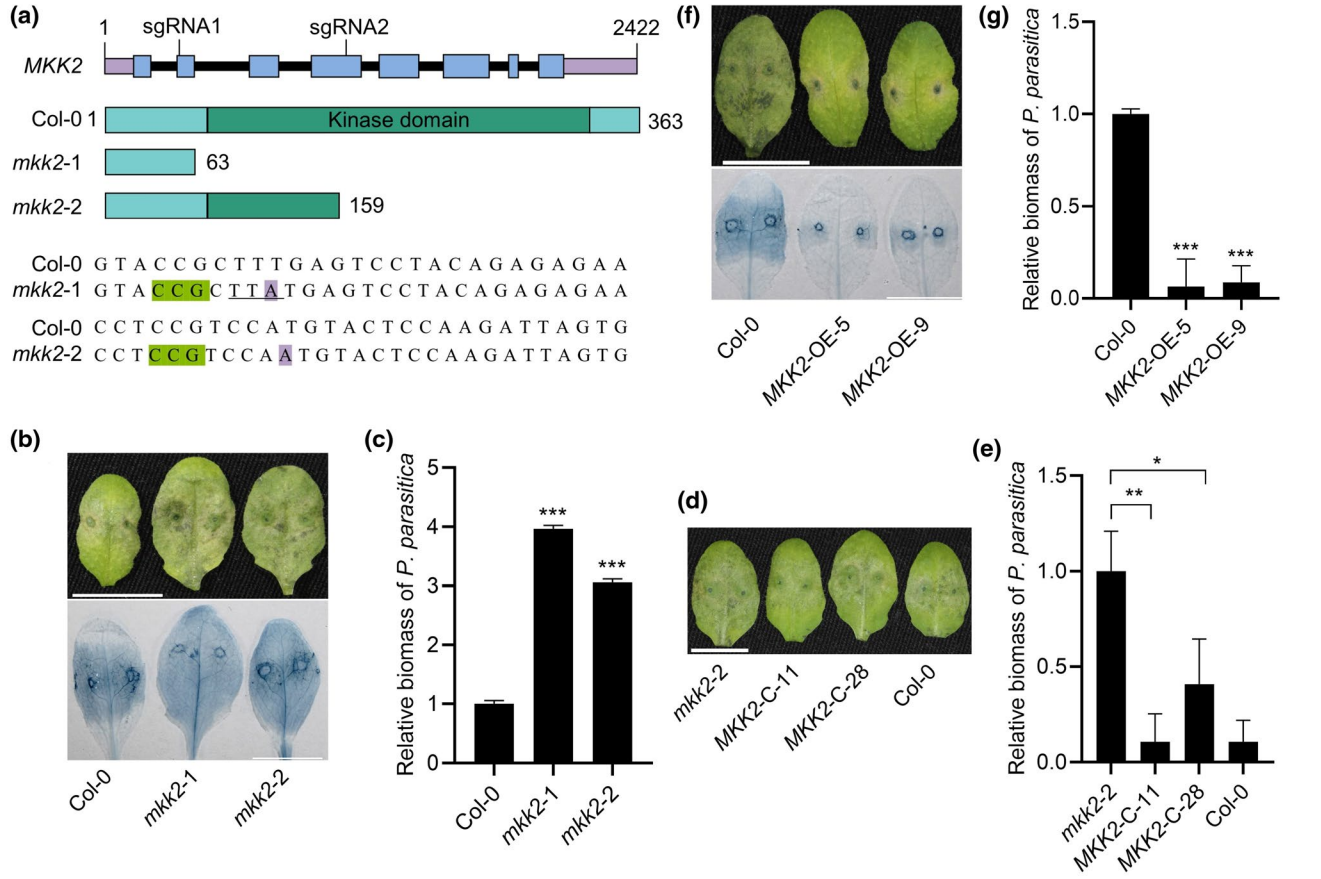


FIGURE 5 *MKK2* is required for *Arabidopsis thaliana* resistance to *Phytophthora parasitica*. (a) Sequences of mutant *MKK2* alleles in *mkk2* homozygous plants. The sgRNA target sites in *MKK2* genomic DNA and the truncated MKK2 proteins are shown. The PAM sequences of *MKK2* sgRNAs are highlighted in light green. The bases inserted by nonhomologous end joining are highlighted in purple. The underlined bases indicate the stop codon introduced by a frameshift. (b, d, f) Disease lesions on leaves of *mkk2* mutants (b), *MKK2* complementation (C) lines (d), and *MKK2* overexpression (OE) lines (f) at 3 days postinoculation (dpi) with *P. parasitica* before and after trypan blue staining. Scale bars = 1 cm. Detached leaves of at least 20 4-week-old *Arabidopsis* plants were inoculated with 20 μl of *P. parasitica* Pp016 zoospores (100 zoospores/μl). The experiments were performed at least three times and representative photographs are shown. (c, e, g) *P. parasitica* biomass in leaves of *mkk2* mutants (c), *MKK2*-C lines (e), and *MKK2*-OE lines (g) at 3 days postinoculation (dpi) as determined by quantitative PCR. *AtUBC9* and *PpWS041* were used as the internal standards for *Arabidopsis* and *P. parasitica*, respectively. Data are presented as means ± *SD* of three biological replicates. Genomic DNA was extracted from samples with 10 leaves at 3 dpi per replicate. Asterisks represent a significant difference between mutant plants and wild-type plants as determined by Student's *t* test; $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$

mutant that exhibited the same *mkk2* mutation as the *mkk2*-2 mutant was chosen for further analysis (Figure 5a). Following *P. parasitica* infection, we analysed the disease lesions and quantified *P. parasitica* colonization on leaves of *mkk2*-2 *raf36*-1 mutant plants. In comparison with the *raf36*-1 mutant, *mkk2*-2 *raf36*-1 mutant plants showed significantly more susceptibility, which resembled the phenotype of *mkk2*-2 mutant plants (Figure 6). These results imply that *raf36*-mediated plant resistance might be counteracted by the *mkk2* mutation, suggesting that *MKK2* functions downstream of *Raf36* in *raf36*-mediated resistance to *P. parasitica*.

3 | DISCUSSION

During compatible plant–pathogen interactions, pathogens deliver virulence factors and recruit diverse plant susceptibility factors to enable successful infection. In turn, plants mobilize resistance (R) proteins and suppress susceptibility factors to resist the infection (van Schie & Takken, 2014). NOD-like receptor (NLR)-type R protein-mediated dominant resistance is usually specific and easy to overcome, whereas pattern recognition receptor-mediated dominant resistance and the susceptibility factor-mediated recessive resistance are probably more broad-spectrum and durable (Kou & Wang, 2010; Li, Deng, et al., 2020; van Schie & Takken, 2014). Here we identified an *Arabidopsis* T-DNA mutant for enhanced resistance to *P. parasitica* with an insertion in *Raf36*, a Raf-like MAPKKK gene. We demonstrate that Raf36 is a novel plant susceptibility factor that functions upstream of MKK2 by direct targeting to negatively regulate plant resistance to *P. parasitica*.

There are approximately 80 MAPKKKs in *Arabidopsis*, 48 of which belong to the Raf-like family (Ichimura et al., 2002; Jonak et al., 2002). Raf-like MAPKKKs often play important roles of plants in the responses

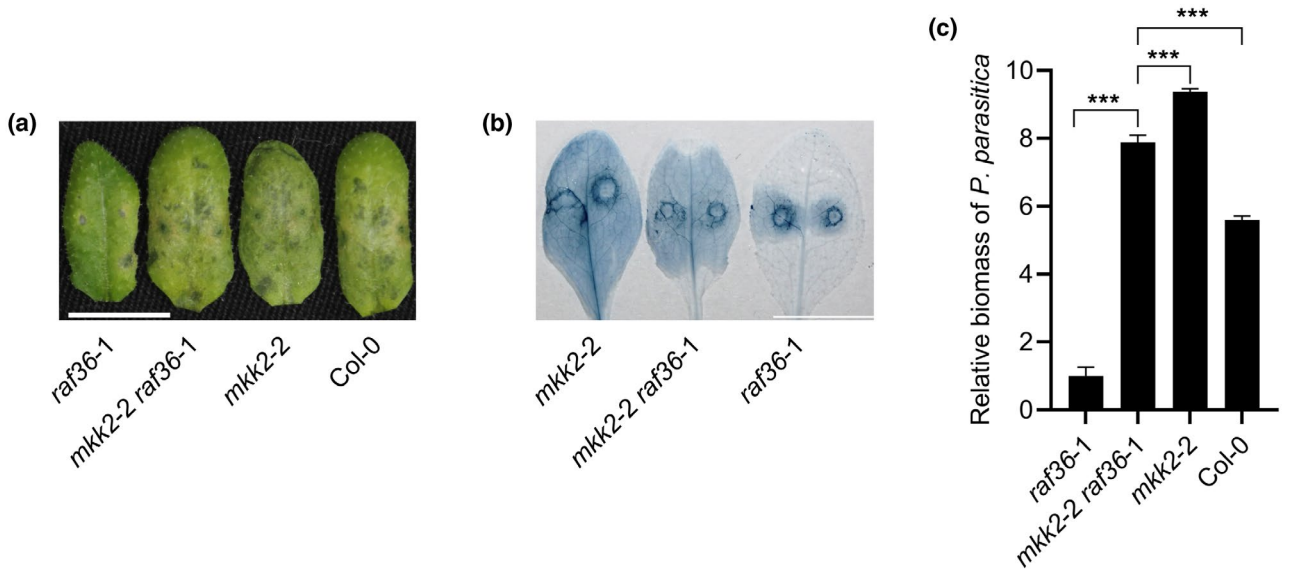


FIGURE 6 Mutation of *MKK2* suppresses *raf36*-mediated resistance to *Phytophthora parasitica*. (a, b) Disease lesions on leaves of *raf36*-1, *mkk2*-2, and *mkk2*-2 *raf36*-1 mutants at 3 days postinoculation (dpi) before (a) and after (b) trypan blue staining. Scale bars = 1 cm. Detached leaves of at least 20 4-week-old *Arabidopsis thaliana* plants were inoculated with 20 μl of *P. parasitica* Pp016 zoospores (100 zoospores/μl). The experiment was performed at least three times and a representative photograph is shown. (c) *P. parasitica* biomass in leaves of *raf36*-1, *mkk2*-2, and *mkk2*-2 *raf36*-1 mutants at 3 dpi as determined by quantitative PCR. *AtUBC9* and *PpWS041* were used as the internal standards for *Arabidopsis* and *P. parasitica*, respectively. Data are presented as means ± *SD* of three biological replicates. Genomic DNA was extracted from samples with 10 leaves at 3 dpi per replicate. Asterisks represent a significant difference between mutant plants and wild-type plants as determined by Student's *t* test; $^{***}p < 0.001$

to diverse activities (Fàbregas et al., 2020; Hayashi et al., 2020; Wang et al., 2018). Some Raf-like MAPKKKs are negative regulators of plant resistance to diverse pathogens (Chen, Wang, Yang, et al., 2021; Ma et al., 2021; Murphy et al., 2018; Zhao et al., 2014). Our results demonstrate that the *Arabidopsis* group C5 Raf-like kinase Raf36 is a negative regulator in plant resistance to the hemibiotrophic oomycete pathogen *P. parasitica* (Figures 1 and 2), providing another example of Raf-like MAPKKKs as negative regulators against biotic stresses. One previous study showed that *Raf36* is required for resistance to the necrotrophic fungus *Alternaria brassicicola* (Himbert, 2009), but our results show that *Raf36* did not alter resistance to the necrotrophic fungus *B. cinerea* (Figure S2). Although plants usually share camalexin-based resistance to necrotrophs (Kristin & Tesfaye, 2010), there are differential resistance mechanisms against *A. brassicicola* and *B. cinerea* infection (Ono et al., 2020; van Wees et al., 2003; Zhu et al., 2014). We suggest that *Raf36* may mediate responses to these two necrotrophic pathogens and *P. parasitica* through different mechanisms. Besides being a susceptibility factor for plant disease, *Raf36* was recently identified as a negative regulator of the abiotic stress-associated abscisic acid (ABA) response (Kamiyama et al., 2021), suggesting the important roles of *Raf36* in both biotic and abiotic stresses.

N. benthamiana is one of the hosts of *P. parasitica*. *Raf36* homologous genes-silenced *N. benthamiana* plants showed enhanced resistance (Figure 3), indicating a conserved function of *Raf36* as a plant susceptibility factor to *P. parasitica*. *Raf36* homologs also exist in other hosts of *P. parasitica* (Figure S3), such as the solanaceous plants potato, tomato, and pepper; further studies in them will expand our knowledge on *Raf36*-mediated susceptibility.

Previous studies showed that Raf36 interacts with the MAPK kinase MKK2 in yeast and in vitro (Himbert, 2009; Li, 2016). Our results demonstrate that they also interacted with each other in planta (Figure 4a,b). The kinase domain of Raf36 was required for Raf36–MKK2 interaction while its N-terminal domain was not necessary for it but contributed (Figure 4d). The regions of Raf-like kinases required for their interaction with MAPKKs are not typical. For example, the N-terminal domain and the kinase domain of the Raf-like MAPKKK MKD1 are required for interactions with its substrates MKK1 and MKK5 (Asano et al., 2020). However, in the interaction between EDR1 and MKK4/5, the N-terminal region of EDR1 is the only domain required (Zhao et al., 2014) and in the interaction between OsEDR1 and OsMPKK10.2, the kinase domain of OsEDR1 is the only domain required (Ma et al., 2021). As plant Raf-like kinases can regulate the activity or protein level of their substrate MAPKKs to affect their function (Asano et al., 2020; Ma et al., 2021; Zhao et al., 2014), it is necessary to check the relationship between Raf36–MKK2 interaction and their regulation further.

MKK2 is considered to be an important component of the MEKK1-MKK1/2-MPK4 cascade, which is associated with abiotic and biotic stress responses in plants (Thulasi Devendrakumar et al., 2018; Zhao et al., 2017). This cascade positively regulates basal resistance to *Pseudomonas syringae* DC3000 and *Hyaloperonospora arabidopsidis* Noco2 (Zhang et al., 2012). Our genetic analysis showed that MKK2 positively regulates *Arabidopsis* resistance to *P. parasitica* (Figure 5), indicating the typical role of MKK2 as a positive regulator in *Arabidopsis* basal resistance. Although MKK1 is highly homologous to MKK2 in *Arabidopsis*, it did not interact with

Raf36 (Figure 4a,b) (Himbert, 2009; Li, 2016), indicating a potential function division between MKK1 and MKK2. Though MKK1 and MKK2 play redundant roles in the MEKK1-MKK1/2-MPK4 cascade, evidence suggests that they can work independently in other cascades. The *Arabidopsis* MKD1-MKK1/5 pathway plays a positive role in resistance to the necrotrophic pathogen *Fusarium sporotrichioides* (Asano et al., 2020), whereas the MEKK1-MKK2-MPK4/6 cascade positively regulates salt tolerance (Teige et al., 2004). MKK2 homologs also play different roles in plant immunity to different pathogens. For example, our previous work showed that *StMKK1*, the gene orthologous to *MKK1/2* in potato, is a negative regulator of plant resistance to the hemibiotrophic pathogen *P. infestans* and the necrotrophic pathogen *Ralstonia solanacearum*, but it positively regulates resistance to the necrotrophic pathogen *B. cinerea* (Chen, Wang, Cai, et al., 2021). Moreover, the cotton *GhMKK1* and maize *ZmMKK1* show opposite functions in *N. benthamiana* responses against *R. solanacearum* (Cai et al., 2014; Lu et al., 2013). These findings suggest divergence of orthologous *MKK2* gene functions even in response to the same pathogen. Further investigations will be needed to illustrate the specific mechanisms of *MKK2* and its orthologs in plant–pathogen interactions.

As Raf36 and MKK2 physically interact with each other and play opposite roles on *P. parasitica* infection, we hypothesize that they may relate to one signalling pathway. Further analysis showed that the *mkk2 raf36* double mutant disrupted *raf36*-mediated resistance (Figure 6), suggesting that Raf36 genetically works upstream of MKK2. It is reported that Raf-like kinases regulate the function of MAPKKs in different ways. *Arabidopsis* Raf-like kinase EDR1 interacts with MKK4/5 and decreases the MKK4/5 protein levels (Zhao et al., 2014). The rice homolog of EDR1 interacts with OsMPKK10.2 and negatively regulates its activity by an unclear mechanism (Ma et al., 2021). Another Raf-like kinase, OsILA1, phosphorylates the T34 site in the N-terminal domain of OsMAPKK4 to negatively regulate its activity (Chen, Wang, Yang, et al., 2021). Consistent with the report that Raf36 can phosphorylate MKK2 in vitro (Himbert, 2009), our results showed that the kinase activity of Raf36 is required for plant susceptibility to *P. parasitica* and its interaction with MKK2 (Figure 4c–g). Further investigation of the relationship between Raf36 and the MKK2-containing MAPK cascade will be useful to understand the mechanism of Raf36-mediated susceptibility in the plant–*P. parasitica* interaction.

4 | EXPERIMENTAL PROCEDURES

4.1 | Construction of plasmids

To generate pro*Raf36*::*Raf36-Flag* transgenic plants, a 1853-bp fragment upstream of the start codon was PCR amplified from Col-0 genomic DNA, fused to the *Raf36* coding sequence with a C-terminal *Flag*, and cloned into pART27 digested with *Sac*I and *Spe*I. To generate pro*MKK2*::*MKK2-Flag* transgenic plants, a genomic fragment spanning 1881 bp upstream of the start codon and the entire coding region of *MKK2* with a C-terminal *Flag* was fused and cloned into vector pART27 (Gleave, 1992) digested with *Sac*I and *Spe*I.

To generate constructs for co-IP assays, the corresponding cDNA fragments of *Raf36*, *MKK1*, and *MKK2* were amplified from Col-0 total RNA by RT-PCR. The coding sequences were amplified by FastPfu DNA polymerase (Transgene) and cloned into the pART27-pro35S-3Flag vector (Zhang et al., 2020) digested with *Xho*I and *Xba*I or pART27-pro35S-4Myc vector (Fan et al., 2018) digested with *Xho*I and *Hin*dIII using a ClonExpress II One Step Cloning Kit (Vazyme Biotech) or T4 DNA ligase (Thermo Scientific).

For constructs used in the LCI assay, the coding sequences of *Raf36*, *MKK1*, *MKK2*, and *MPK6* were amplified and cloned into pCAMBIA1300-CLuc or pCAMBIA1300-NLuc vector digested with *Kpn*I and *Sal*I (Zhou et al., 2018).

To generate the *raf36* or *mkk2* single mutants, two 20-bp sequences targeting *Raf36* (*Raf36*-sgRNA1 and *Raf36*-sgRNA2) or *MKK2* (*MKK2*-sgRNA1 and *MKK2*-sgRNA2) were designed by the online tool CRISPR-P 2.0 (http://crispr.hzau.edu.cn/CRISPR2/) and cloned into the *Aar*I site of the pKI1.1R binary vector (Tsutsui & Higashiyama, 2016).

For the Y2H assay, the coding sequences of full-length and truncated *Raf36* were amplified and recombined into the pGBKT7 vector (Clontech), and the coding sequence of *MKK2* was amplified and recombined into the vector pGADT7 (Clontech). Restriction enzymes *Eco*RI and *Bam*HI were used to digest the two vectors.

sgRNAs and PCR primers are listed in Table S1.

4.2 | Plant materials and growth conditions

The T-DNA mutants were kindly provided by Professor Jianru Zuo (Zhang et al., 2005). The *raf36*-1, *raf36*-2, *mkk2*-1, and *mkk2*-2 mutants were generated by a CRISPR/Cas9 method described previously (Tsutsui & Higashiyama, 2016) in the *Arabidopsis* ecotype Col-0 background. The *mkk2*-2 *raf36*-1 double mutant was generated by knocking out *MKK2* in the *raf36*-1 mutant background. *proRaf36::Raf36-Flag* was introduced into *raf36*-2 or *proMKK2::MKK2-Flag* was introduced into *mkk2*-2 to generate *Raf36* or *MKK2* complementation lines, respectively, using *Agrobacterium tumefaciens*-mediated transformation (Zhang et al., 2006). *pro35S::Raf36* was introduced into *raf36*-1 mutant plants to generate *Raf36* overexpression lines. *pro35S::MKK2* was introduced into Col-0 plants to generate *MKK2* overexpression lines using standard protocols. These lines were confirmed by allele-specific genotyping and the presence of transgenic antibiotic resistance. *Arabidopsis* and *N. benthamiana* plants were grown at 23°C with an 11/13 h day/night photoperiod for 4 weeks before use.

4.3 | Pathogen culture conditions and infection assays

P. parasitica culturing, zoospore production, and assays using detached leaves of *Arabidopsis* have been described previously

(Wang et al., 2011). Detached leaves of at least 20 4-week-old *Arabidopsis* plants per genotype were inoculated with 20 μl of *P. parasitica* Pp016 zoospores (100 zoospores/μl). Each experiment was performed at least three times, and representative leaves were photographed and stained with trypan blue at 3 dpi. For the quantification of *P. parasitica* biomass, three biological replicates were performed with genomic DNA extracted from samples containing 10 leaves at 3 dpi per replicate. At least 12 detached leaves of *N. benthamiana* plants per construct were inoculated with 20 μl of *P. parasitica* Pp016 zoospores (25 zoospores/μl). Lesion diameters were measured at 2 dpi. The experiments were performed at least three times with similar results. *B. cinerea* Bc001 was isolated from tomato and cultured on potato dextrose agar (PDA) in a growth chamber at 20°C for 10–12 days. Spores were harvested in potato dextrose broth (PDB, 24 g/L) and subsequently vortexed to release the spores, which were filtered through four layers of gauze to remove hyphae. Spore density was adjusted to 5×10^5 spores/ml with PDB. Plants were infected by dropping 5 μl of the spore suspension on fully expanded leaves. At least 20 different individual *Arabidopsis* plants were used for *B. cinerea* infection. Lesion diameters were measured at 2 dpi. The experiments were performed at least three times with similar results.

4.4 | DNA and RNA isolation and PCR assay

Genomic DNA was extracted using the cetyltrimethylammonium bromide (CTAB) method (Clarke, 2009) and used as a template in qPCR experiments to quantify the pathogen biomass.

Total RNA was extracted using TRIzol (Invitrogen). One microgram of total RNA was reverse transcribed with the PrimeScript RT reagent kit with gDNA eraser (Perfect Real Time) (Takara). qPCR was performed in a LightCycler 480 real-time PCR system (Roche) using an UltraSYBR Mixture kit (CWBIO), following the manufacturer's protocol. The relative expression level of each gene was determined using the $2^{-\Delta\Delta Ct}$ method (Livak & Schmittgen, 2001) and normalized to *Arabidopsis AtUBC9* or *N. benthamiana NbβActin*. RT-qPCR primers are listed in Table S1.

TAIL-PCR (Liu et al., 1995) was used to obtain the flanking sequence of T-DNA in 105-3 mutant as described previously (Zhang et al., 2005). Primers are listed in Table S1.

4.5 | Transient expression in *N. benthamiana*

A. tumefaciens GV3101 transformed with each construct was grown in Luria-Bertani broth with appropriate antibiotics at 28°C overnight. Cells were resuspended in infiltration buffer (10 mM 2-(*N*-morpholino)-ethanesulfonic acid, 10 mM $MgCl_2$, 200 μM acetosyringone) at $OD_{600\ nm}$ = 0.2–0.5 and incubated at room temperature for 1 h before infiltration. Appropriate assays were done after transient expression in *N. benthamiana* for 2 days.

4.6 | VIGS in *N. benthamiana*

The method was performed as previously described (Senthil-Kumar & Mysore, 2014). The fragment of 369–613 bp in *NbRaf36*-3 (*Niben101Scf05713g04009.1*) cDNA was used to silence all four *NbRaf36* genes. *A. tumefaciens* GV3101 harbouring each construct was adjusted to a final concentration of $OD_{600\ nm}$ = 0.2. At least 12 plants were used for *NbRaf36* or *GFP* silencing. Three-week-old *N. benthamiana* plants were used for VIGS, plants after 3 weeks of VIGS were used for RT-qPCR and *P. parasitica* infection. PCR primers are listed in Table S1.

4.7 | Immunoblotting and co-IP assay

Protein extraction and immunoblotting were described previously (Fan et al., 2018). For the co-IP assay, 1 ml of protein extract was incubated with anti-Flag magnetic beads (Bimake) following the standard protocol and the precipitated proteins were analysed by immunoblotting using an anti-Myc antibody. Antibodies used for immunoblotting were as follows: mouse anti-DDDDK-Tag mAb (ABclonal), mouse anti-Myc-Tag mAb (ABclonal), and horseradish peroxidase goat anti-mouse immunoglobulin G (IgG) (L) antibody (ABclonal).

4.8 | LCI assay

The assay was performed as previously described (Zhou et al., 2018). Leaves of 4-week-old *N. benthamiana* plants were infiltrated with *A. tumefaciens* GV3101 cells containing the indicated plasmids, and leaves were excised 2.5 days after inoculation. After being sprayed evenly with 1 mM luciferin (Promega), leaves were placed in darkness for 10 min before detection. A low-light cooled CCD imaging apparatus (PlantView100; BTL) was used to capture luciferase images. Each interaction pair was tested on leaves from at least eight different plants grown in different pots at the same time.

4.9 | Y2H assay

For Y2H assays, constructs were co-transformed into yeast strain AH109 (Clontech) using the Matchmaker Two-Hybrid System 3 protocol (Clontech). The transformants were selected on synthetic dropout (SD/−Trp−Leu−His−Ade) agar plates containing adenine and histidine (SD/−Trp−Leu) for 2 days. At least four colonies of each transformant were dissolved in 10 μl of double-deionized water and dropped on SD/−Trp−Leu−His−Ade medium, respectively, with X-α-gal for 2–4 days.

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CONFLICT OF INTEREST

The authors declare no competing interests.

AUTHOR CONTRIBUTIONS

J.L. and W.S. designed the research. J.L., F.D., Y. M., and H.W. performed the experiments. J.L., X.Q., Y. M., and W.S. wrote the manuscript. All authors discussed and interpreted the results.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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