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*-conferrered 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
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 (Boeving 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 MKP6-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 OsSLA1 phosphorylates OsMPKK4 and suppresses OsMPKK4-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$_{2}$ 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...
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 transforming 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 *NbRaf36* 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 cloned into a tobacco rattle virus (TRV)-based virus-induced gene silencing (VIGS) vector to silence all four 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 control plants (Figure 3a). After inoculating the detached leaves with *P. parasitica* (Figure S2), the lesions in *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.,...
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 \( \text{Raf36}^{\Delta105} \), \( \text{Raf36}^{\Delta154} \), \( \text{Raf36}^{\Delta171} \), and \( \text{Raf36}^{\Delta184} \), 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.
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 S4b,c). 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 its native promoter into *mkk2-2* mutants. 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 5f,g). The results indicate that the genetic complementation of MKK2 restores plant resistance against *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*
The 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 5b). 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
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 Hyałoperonospora arabidopsis 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 proRaf36::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 SacI and SpeI. To generate proMKK2::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 SacI and SpeI.

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 Xhol and Xbol or pART27-pro35S-4Myc vector (Fan et al., 2018) digested with Xhol and HindIII 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 KpnI and SalI (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 AarI 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 pGBKTK7 vector (Clontech), and the coding sequence of MKK2 was amplified and recombined into the vector pGADT7 (Clontech). Restriction enzymes EcoRI and BamHI 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
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 replicates were performed with genomic DNA extracted from 4- to 6-week-old N. benthamiana leaves were photographed and stained with trypan blue at 3 dpi. Ment was performed at least three times, and representative samples containing 10 leaves at 3 dpi per replicate. At least 12 replicates were performed with genomic DNA extracted from 4- to 6-week-old N. benthamiana plants after 3 weeks of VIGS were used for RT-qPCR and P. parasitica infection. PCR primers are listed in Table S1.

**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 OD600 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-a-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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**SUPPORTING INFORMATION**

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