INTRODUCTION

In the natural environment, plants encounter various microorganisms, some of which are pathogenic. Due to a lack of specific immune cells, plants have developed a complex immune mechanism composed of microbe-associated molecular pattern (MAMP)/pathogen-associated molecular pattern (PAMP)-triggered immunity (PTI) and effector-triggered immunity (ETI) to resist pathogens (Jones & Dangl, 2006; Macho & Zipfel, 2014). The activation of plant immunity leads to a series of reactions, including an increase in reactive oxygen species (ROS), Ca\(^{2+}\) influx, mitogen-activated protein kinase activation, and regional cell death (Boller & Felix, 2009; Cheng et al., 2019; Spoel & Dong, 2012; Zipfel & Oldroyd, 2017). Numerous proteins, such as the transcription factor BZR1, which is involved in brassinosteroid signalling (Qi et al., 2021), mitogen-activated protein kinase kinases (Rufian et al., 2021), and FLOWERING LOCUS KH domain (FLK) (Fabian et al., 2021), have been reported to regulate plant immune responses. Among these, FLK is an RNA-binding protein (RBP) that regulates plant development (Lim et al., 2004), the ROS-scavenging enzyme catalase, and plant immunity (Fabian et al., 2021). Therefore, we aimed to investigate the role of RBPs in pathogen defence and ROS regulation.

RBPs are pivotal factors manipulating posttranscriptional RNA metabolism during plant growth and stress responses (Lee & Kang, 2016). The K homology (KH) domain-containing proteins in plant defence is still unclear. In this study, we found that a KH domain-containing protein in apple (Malus domestica), HEN4-like (MdKRBP4), is involved in the plant immune response. Silencing of MdKRBP4 compromised reactive oxygen species (ROS) production and enhanced the susceptibility of apple to Valsa mali, whereas transient overexpression of MdKRBP4 stimulated ROS accumulation in apple leaves, indicating that MdKRBP4 is a positive immune regulator. Additionally, MdKRBP4 was proven to interact with the VmEP1 effector secreted by V. mali, which led to decreased accumulation of MdKRBP4. Coexpression of MdKRBP4 with VmEP1 inhibited cell death and ROS production induced by MdKRBP4 in Nicotiana benthamiana. These results indicate that MdKRBP4 functions as a novel positive regulatory factor in plant immunity in M. domestica and is a virulence target of the V. mali effector VmEP1.

KEYWORDS

effector protein, plant immunity, protein stability, RNA-binding protein, Valsa mali
ancient RBPs found in diverse organisms (Nicastro et al., 2015). A few KH domain-containing proteins are involved in RNA metabolism, such as pre-mRNA processing, mRNA stabilization (Rodríguez-Cazorla et al., 2015), and microRNA biogenesis (Karlsson et al., 2015). KH domain-containing proteins, such as SPL11-interacting protein1 (SPIN1) (Vega-Sanchez et al., 2008), HUA ENHANCER4 (HEN4) (Cheng et al., 2003; Ortuno-Miguel et al., 2019), FLK (Lim et al., 2004), PEPPER (PEP) (Ripoll et al., 2006), KHZ1, and KHZ2 (Yan et al., 2017), have been shown to control flowering. Furthermore, some KH domain-containing proteins are crucial for the stress response, such as resistance against Fusarium oxysporum (Thatcher et al., 2015), tomato mosaic virus (Fujisaki & Ishikawa, 2008), and abiotic stress (Chen et al., 2013; Guan et al., 2013). However, the role of KH domain-containing proteins in manipulating development in plants has only been reported Arabidopsis thaliana. Moreover, only a few studies have correlated KH domain-containing proteins with the plant immune response. Only A. thaliana FLK is known to regulate resistance against pathogenic bacteria (Fabian et al., 2021; Lim et al., 2004). Whether other KH domain-containing proteins are involved in regulating host resistance is still unclear. Therefore, it is necessary to identify other KH domain-containing proteins and examine their possible involvement in the plant immune response.

Typically, to counteract the plant immune system and establish a successful infection during colonization, pathogenic microorganisms deploy effectors to avoid host recognition and inhibit the host defense response (Pradhan et al., 2021). These effector molecules manipulate the immune response by targeting key host proteins, such as lectin-like receptor kinase protein (Kanzaki et al., 2008), E3 ubiquitin ligase (Bos et al., 2010; Park et al., 2012), BAK1-associated receptor complexes (Lu et al., 2010), protein kinase (Murphy et al., 2018; Tanaka et al., 2014), exoyct component Sec5 (Du et al., 2015), peroxidase (Hemetsberger et al., 2012; Zhang et al., 2015), protein phosphatase 1 (Boevink et al., 2016), endoplasmic reticulum (ER)-luminal binding immunoglobulin proteins (Jing et al., 2016), cytoplasmic transacetylase (Li et al., 2018), and WRKY transcription factors (Ma et al., 2021), which regulate diverse aspects of plant cell development and metabolism. However, pathogen effectors rarely target KH domain-containing proteins.

The ascomycete Valsa mali, which causes apple (Malus domestica) Valsa canker, secretes effector protein 1 (VmEP1) to inhibit BAX-induced programmed cell death (Li et al., 2015) and targets host pathogenesis-related protein 10 (PR10) (Wang et al., 2021). However, the mechanism by which VmEP1 manipulates plant immunity is not known. Previously, we found that VmEP1 targeted MdPR10-mediated resistance against V. mali (Wang et al., 2021). In the present study, VmEP1-GFP was expressed in N. benthamiana leaves and the fusion proteins and associated proteins were purified with anti-GFP magnetic beads. To uncover the virulence mechanism of VmEP1 in V. mali, liquid chromatography–tandem mass spectrometry analysis of the purified VmEP1 and related proteins detected peptides of KH domain-containing proteins (Table 1), with no peptides after purification of green fluorescent protein (GFP) alone. The yeast two-hybrid (Y2H) assay showed that VmEP1 targets a KH domain-containing protein (Figure 1a, Table S1). Therefore, the KH domain-containing protein, named MdKRBP4, was identified as one of the best candidates.

Furthermore, a bimolecular fluorescence complementation (BiFC) assay was performed to verify the association between MdKRBP4 and VmEP1 from different species. The MdKRBP4-nYFP and VmEP1-cYFP constructs were transformed into N. benthamiana leaves using Agrobacterium tumefaciens. The empty vectors (nYFP or cYFP) were used as the negative controls. In contrast to the control, the cells

| Accession | Description |
|-----------|-------------|
| A0A1S3XHV7 | ABC transporter B family member 25-like |
| A0A1U7VME9 | Mitogen-activated protein kinase 12-like |
| A0A1U7YIC1 | LRR receptor-like serine/threonine protein kinase |
| A0A1S4A6X4 | Serine/threonine protein kinase WNK8-like isoform X1 |
| A0A1U7Y2B6 | Cysteine-rich receptor-like protein kinase 42 |
| A0A1S4JR8 | eIF-2α kinase activator GCN1 isoform X2 |
| A0A1U7X8D3 | NAD kinase 2, chloroplastic-like isoform X2 |
| A0A1J6KK09 | E3 ubiquitin protein ligase |
| A0A1S3Z289 | RING-type E3 ubiquitin transferase |
| A0A1U7WC50 | Putative E3 ubiquitin protein ligase |
| A0A1S4BVK9 | E3 ubiquitin protein ligase RNF170-like |
| A0A1U7XI91 | RING-type E3 ubiquitin transferase |
| A0A1S3X1B4 | E3 ubiquitin protein ligase RLIM-like |
| A0A1U7V537 | ABC transporter G family member 14-like |
| A0A1U7V720 | KH domain-containing protein |
| A0A1U7W6H0 | Zinc finger MYM-type protein 1-like |
| A0A1J6L7B6 | Ethylene overproduction protein 1 |

Our data collectively indicate that VmEP1 suppresses plant immunity by promoting MdKRBP4 degradation.

2 | RESULTS

2.1 | MdKRBP4 interacts with VmEP1

In our previous study, the secretory protein VmEP1, with no known function, was shown to suppress plant defence by interrupting MdPR10-mediated resistance against V. mali (Wang et al., 2021). In the present study, VmEP1-GFP was expressed in N. benthamiana leaves and the fusion proteins and associated proteins were purified with anti-GFP magnetic beads. To uncover the virulence mechanism of VmEP1 in V. mali, liquid chromatography–tandem mass spectrometry analysis of the purified VmEP1 and related proteins detected peptides of KH domain-containing proteins (Table 1), with no peptides after purification of green fluorescent protein (GFP) alone. The yeast two-hybrid (Y2H) assay showed that VmEP1 targets a KH domain-containing protein (Figure 1a, Table S1). Therefore, the KH domain-containing protein, named MdKRBP4, was identified as one of the best candidates.

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coexpressing MdKRBP4-nYFP and VmEP1-cYFP displayed a robust yellow fluorescence signal in the nucleus, suggesting an interaction between VmEP1 and MdKRBP4. Meanwhile, no fluorescence was detected in the negative control (Figure 1b). In addition, we tested the interaction between cYFP and nYFP empty vectors; yellow fluorescence was not detected (Figure 1d). Further analysis performed to validate the results of the BiFC assay revealed VmEP1 localization in the nucleus, cytoplasm, and plasma membrane (Figure S1a), and MdKRBP4 localization in the nucleus (Figure S1b). These results suggest that VmEP1 interacts with MdKRBP4 in the nucleus.

Subsequently, VmEP1-HA was coexpressed with MdKRBP4-GFP in N. benthamiana leaves, using GFP as a control, and a coimmunoprecipitation (Co-IP) assay was performed using anti-GFP magnetic beads. Proteins in each sample were detected by western blot with anti-GFP and anti-HA antibodies. The results indicate that all genes were successfully expressed in N. benthamiana leaves (Figure 1c). The immunoblotting assay showed that VmEP1-HA was present in the final GFP-MdKRBP4-precipitated immunocomplex (Figure 1c), indicating that MdKRBP4 interacts with VmEP1. Altogether, these results suggest that MdKRBP4 physically interacts with VmEP1.

**FIGURE 1** VmEP1 interacts with MdKRBP4 in vivo. (a) The interaction of VmEP1 with MdKRBP4 in a GAL4 yeast two-hybrid system. VmEP1 interacts with MdKRBP4, as indicated by yeast two-hybrid assays. Double dropout (DDO) medium is SD–Leu/–Trp medium. Quadruple dropout (QDO) medium/X is SD–Ade/–His/–Leu/–Trp/+/X–α-Gal medium. (b) Bimolecular fluorescence complementation assays showing the interaction of VmEP1 with MdKRBP4 in Nicotiana benthamiana leaves. Agrobacterium tumefaciens containing VmEP1-cYFP/nYFP-MdKRBP4 were coinfiltrated in N. benthamiana leaves. VmEP1-cYFP/nYFP, nYFP-MdKRBP4/cYFP, and nYFP/cYFP were used as negative controls. Bar = 20 μm. (c) Coimmunoprecipitation assays using anti-GFP magnetic beads. Pairs of VmEP1-HA and MdKRBP4-GFP/GFP were coexpressed in N. benthamiana leaves. The immunoprecipitated proteins were resolved by SDS-PAGE and analysed by western blot with the anti-GFP or anti-HA antibody. Molecular mass markers are shown (in kilodaltons). Blots were stained with Coomassie brilliant blue (CBB) to verify equal loading. This assay was repeated three times.
2.2 | Overexpression of MdKRBP4 activates the immune response

Our studies revealed that VmEP1 targets MdKRBP4. Therefore, we speculated that MdKRBP4 is a vital factor regulating the immune response in plants, and we examined the accumulation of ROS using 3,3′-diaminobenzidine (DAB) staining and mRNA accumulation of defence-related genes (PR1, PR2, PR5) using reverse transcription-quantitative PCR (RT-qPCR) (Boutrot & Zipfel, 2017; Heath, 2000; Pontier et al., 1994; Zipfel & Oldroyd, 2017). Compared with controls, apple leaves overexpressing MdKRBP4 showed high production of ROS, indicating a role of MdKRBP4 in inducing ROS accumulation (Figure 2a). Meanwhile, the expression levels of ROS accumulation-regulating genes (Figure 2c) and salicylic acid pathway-related genes, such as MdPR1, MdPR2, and MdPR5 (Figure 2d), were considerably enhanced 3 days post-infiltration. The relative transcript level of MdPR10, a target of VmEP1 (Wang et al., 2021), was also enhanced in apple leaves expressing MdKRBP4 (Figure 2d). These results suggest that the transient expression of MdKRBP4 activates the plant immune response and induces ROS accumulation.

2.3 | MdKRBP4 induces cell death in N. benthamiana leaves

ROS are a critical signal that triggers and activates cell death in plants (Jacobson, 1996; Petrov & Van Breusegem, 2012). Therefore, we investigated whether MdKRBP4 induces cell death in apple leaves. Although RT-qPCR analysis showed an up-regulation of the hypersensitive response-related genes MdHSRP203J (Pontier et al., 1998) and MdHIN1 (Takahashi et al., 2004) (Figure 2e) a cell death phenotype in apple leaves expressing MdKRBP4 was not detected. Interestingly, we found that MdKRBP4 overexpression significantly up-regulated the transcript levels of the hypersensitive response-related genes NbHSRP203J and NbHIN1 (Figure 3a) and induced cell death in N. benthamiana leaves (Figure 3b). Furthermore, staining with trypan blue confirmed that the lesions represented MdKRBP4-induced cell death in N. benthamiana leaves (Figure 3c). Meanwhile, DAB staining showed that the transient expression of MdKRBP4 promoted ROS accumulation in N. benthamiana compared to controls (Figure 3e,f). In addition, the RT-qPCR analysis indicated that MdKRBP4 up-regulated the expression of NbRBOHD and NbCAT2, genes regulating ROS accumulation (Figure 3g). These

![Figure 2](image-url)
Overexpression of MdKRBP4 induces cell death and the accumulation of reactive oxygen species (ROS) in *Nicotiana benthamiana* leaves. (a) Relative transcript levels of hypersensitive response-related genes in *N. benthamiana* leaves expressing GFP or MdKRBP4 were analysed by reverse transcription-quantitative PCR (RT-qPCR) with NbActin. *N. benthamiana* leaves expressing MdKRBP4 or GFP control for 3 days were used for RNA extraction. Relative transcript levels of genes were normalized to NbActin and calibrated to the levels of the empty vector (EV) control (set as 1) (mean ± standard deviation; *n* = 3; **p < 0.01, Student's *t* test). Similar results were obtained from three individual experiments. (b) Photographs were taken at 5 days postinoculation under UV light. *N. benthamiana* leaves were transiently expressing EV (left) and MdKRBP4 (right). (c) Trypan blue staining showing the cell death symptoms of the EV (left) and MdKRBP4 (right) in leaves of *N. benthamiana*. (d) An anti-GFP antibody was used to analyse expression of the marked constructs. (e) 3,3′-diaminobenzidine (DAB) staining shows ROS accumulation in *N. benthamiana* leaves expressing MdKRBP4 (right). (f) Quantification of ROS accumulation in *N. benthamiana* leaves, as determined by ImageJ software. Error bars indicate ± standard deviation, *n* > 10; ***p < 0.001, Student’s *t* test. (g) The mRNA level of ROS accumulation-regulated key genes in *N. benthamiana*, as revealed by RT-qPCR 3 days postinfiltration (dpi) of overexpression construct OE-MdKRBP4. The transcript levels were analysed by RT-qPCR. Relative expression levels were normalized to NbActin and calibrated to the levels of the EV control (set as 1). Error bars indicate ± standard deviation, *n* = 3; ****p < 0.0001, Student’s *t* test. These experiments were repeated three times with similar results. (h) Analysis of KH domain-containing proteins in *Malus domestica*. The sequences were obtained from an *M. domestica* genome database by bioinformatics analysis. The tree was constructed with the maximum-likelihood method. Coloured bars represent different subgroups. (i) Cell death was quantified by measuring electrolyte leakage (mean ± standard deviation; *n* = 4; **p < 0.01, Student’s *t* test).
results suggest that MdKRBP4 induces ROS accumulation, resulting in cell death in *N. benthamiana*.

KH domain-containing proteins possess a conserved VIGXXGXXI motif (Burd & Dreyfuss, 1994). We obtained VIGXXGXXI-containing proteins from the apple genome database (ASM211411 v1). A total of 39 VIGXXGXXI-containing proteins including MdKRBP4 (XP_028954217.1) were identified (Figure 3h). Phylogenetic tree analysis divided these proteins into six subgroups (Figure 3h). We randomly selected and cloned genes from each subgroup. Transient expression of XP_028954217.1 (MdKRBP4), XP_008371618.2, XP_008375627.2, XP_008350638.2, XP_008386871.1, XP_008393654.2, and XP_008384622.2 from each subgroup showed that only the genes from the HEN4 subgroup caused cell death in *N. benthamiana* (Figure 3i). In addition, *MdKRBP4* overexpression resulted in the highest electrolyte leakage, indicating maximum cell death (Figure 3i). These results imply that *MdKRBP4* is essential for plant immunity.

### 2.4 | MdKRBP4 positively regulates apple resistance to *V. mali*

Subsequently, we constructed RNA interference vectors and transferred them to apple plants via *Agrobacterium*-mediated transformation to investigate whether *MdKRBP4* positively regulates immunity. After RT-qPCR evaluation, we obtained two silencing lines (SL5 and SL6) in which *MdKRBP4* expression was less than 35% of that in wild-type (WT) plants (Figure 4c). Then, we infected WT, SL5, and SL6 plants with *V. mali*. The SL5 and SL6 plants were more susceptible than the WT (Figure 4a,b). In addition, ROS accumulation in SL5 and SL6 apple leaves inoculated with *V. mali* was obviously less than that in inoculated WT apple leaves (Figure 4d,e), indicating that silencing of *MdKRBP4* weakened ROS generation. We also found that silencing of *MdKRBP4* down-regulated the expression of *MdPR1*, *MdPR2*, and *MdPR5* (Figure 4f). These results suggest that *MdKRBP4* positively regulates apple resistance to *V. mali*.

### 2.5 | VmEP1 inhibits plant immunity induced by MdKRBP4

We further tested whether VmEP1 affects MdKRBP4-induced cell death and ROS accumulation. The result showed that VmEP1 attenuated MdKRBP4-induced cell death in *N. benthamiana* (Figure 5a). Moreover, MdKRBP4-triggered electrolyte leakage in *N. benthamiana* was significantly attenuated in the presence of VmEP1 compared with controls (Figure 5b). The analysis of actin quantity showed that the quantity of actin in *N. benthamiana* leaves expressing *MdKRBP4* was lower than that in *N. benthamiana* leaves expressing *MdKRBP4/VmEP1* (Figure 5c). We then conducted DAB staining to investigate the variations in ROS accumulation induced by MdKRBP4 in the presence of VmEP1. ROS accumulation in leaves coexpressing *MdKRBP4/VmEP1* was lower than that in leaves expressing *MdKRBP4* alone (Figure 5d,e). Previously, Yin demonstrated that MdSRLK3, a G-type lectin S-receptor-like protein kinase from apple, induces cell death in *N. benthamiana* (Yin, 2018). Therefore, we used the coexpression of *MdSRLK3* and VmEP1 as a control. The results showed that MdSRLK3-induced cell death was not affected by VmEP1 (Figure 5a), indicating a specific effect of VmEP1 on MdKRBP4. Collectively, these results suggest that VmEP1 inhibits host immunity by targeting MdKRBP4 and attenuating the immune response triggered by MdKRBP4.

### 2.6 | MdKRBP4 is a virulence target of VmEP1

Furthermore, we verified whether MdKRBP4 is a virulence target of VmEP1. A VmEP1 deletion mutant (*ΔVmEP1*) (Li et al., 2015) and WT *V. mali* were inoculated on WT apple leaves and SL5 leaves. The average lesion diameter of WT *V. mali* was smaller than that of *ΔVmEP1* in WT apple leaves (Figure 6a,b). The average lesion diameter of WT *V. mali* was also smaller than that of *ΔVmEP1* in SL5 leaves (Figure 6c,d). However, the lesion growth rate on SL5 leaves was slower compared to the lesion growth rate on WT leaves (Figure 6e), indicating that MdKRBP4 is required for the virulence function of VmEP1. These results suggest that MdKRBP4 is essential for the virulence of VmEP1.

### 2.7 | VmEP1 promotes MdKRBP4 degradation and inhibits MdPR10 expression

Immunoprecipitation (IP) previously revealed that the MdKRBP4 protein is easily degraded (Dharma et al., 2005). The peptide-aldehyde proteasome inhibitor MG132 suppressed MdKRBP4 degradation (Figure 7a), indicating that MdKRBP4 is degraded by the 26S proteasome pathway in plants (Dreher & Callis, 2007). Initial experiments proved that VmEP1 mitigated ROS production and cell death induced by MdKRBP4 (Figure 3); therefore, we tested whether VmEP1 decreases the MdKRBP4 protein level. We measured the MdKRBP4 protein level after coexpression of *MdKRBP4* with VmEP1 in *N. benthamiana* leaves. Agrobacteria containing either *MdKRBP4-GFP* or GFP were coinfiltrated with VmEP1-HA, and leaves were collected at 48 h postinoculation (hpi). As exhibited in Figure 7b, lower fluorescence of MdKRBP4-GFP was detected in the presence of VmEP1-HA than that of GFP in the presence of VmEP1-HA (Figure 7b, left), suggesting that the transient expression of VmEP1 decreases MdKRBP4 protein accumulation. Interestingly, MG132 treatment blocked the degradation of MdKRBP4 by VmEP1 (Figure 7b, right). However, the interaction between VmEP1 and MdPR10, a target protein of VmEP1 (Wang et al., 2021), did not result in degradation of MdPR10 (Figure S2), indicating that VmEP1 specifically promotes MdKRBP4 degradation. These results indicate that the interaction between VmEP1 and MdKRBP4 reduces MdKRBP4 accumulation by promoting its degradation.

Previous studies have shown that VmEP1 interacts with MdPR10, an immune-related protein (Wang et al., 2021). In the present study,
FIGURE 4  Silencing of MdKRBP4 enhances sensitivity of apple to Valsa mali. (a) Silencing of MdKRBP4 in apple leaves promotes V. mali infection. Wild-type (WT) apple leaves and MdKRBP4-silenced apple leaves (SL5, SL6) were inoculated with V. mali. Representative disease symptoms were photographed at 24 h postinoculation (hpi). (b) Average lesion diameters were measured at 24 hpi. Bars indicate ± standard deviation, n > 15; ***p < 0.001, ****p < 0.0001, Student’s t test. These experiments were repeated three times with similar results. (c) Silencing efficiency of MdKRBP4 in apple leaves was determined by reverse transcription-quantitative PCR (RT-qPCR) (mean ± standard deviation; n = 4; **p < 0.01, ***p < 0.001, Student’s t test). These experiments were repeated three times with similar results. (d) 3,3′-diaminobenzidine (DAB) staining shows reactive oxygen species (ROS) accumulation in apple leaves incubated with V. mali. Bar = 100 μm. (e) Quantification of ROS accumulation in apple leaves, as determined by ImageJ software. Means and standard deviation were calculated from three independent experiments. Bars indicate ± standard deviation; n > 15; ****p < 0.0001, Student’s t test. (f) Relative transcript levels of MdPR1, MdPR2, and MdPR5 in SL5 were analysed by RT-qPCR with MdEF-1α. Relative transcript levels of genes were normalized to MdEF-1α and calibrated to the levels of the empty vector (EV) control (set as 1) (mean ± standard deviation; n = 4; ****p < 0.0001; Student’s t test). Similar results were obtained from three individual experiments.
we found that VmEP1 targets MdKRBP4 and regulates the plant immune response. Overexpression of MdKRBP4 increased the relative expression level of MdPR10 (Figure 2d). Coincidentally, the relative expression level of MdPR10 was reduced in SL5, indicating that MdKRBP4 indirectly regulates MdPR10 expression (Figure 7c). Our results show that MdKRBP4, as a positive regulator of plant immunity, enhances apple resistance to V. mali by up-regulating MdPR10 expression and ROS accumulation. VmEP1 secreted by V. mali interacts with MdKRBP4 in the nucleus, reducing the accumulation of MdKRBP4. The decrease in MdKRBP4 indirectly down-regulates...
DISCUSSION

Research has shown that KH domain-containing proteins such as PEPPER, SPIN1, FLK, and HEN4 regulate the development of *A. thaliana* (Cheng et al., 2003; Lim et al., 2004; Ripoll et al., 2009; Vega-Sanchez et al., 2008). In the present study we chose PEPPER, SPIN1, FLK, and HEN4 from Cruciferae, Gramineae, Musaceae, Celastraceae, Rosaceae, and Malvaceae and constructed a phylogenetic tree using ClustalW. The results show that KH domain-containing proteins regulating plant development can be grouped into four distinct classes (Figure S3). ATHEN4 and MdKRBP4 were within the same subgroup, and multiple sequence alignment of the HEN4 subgroup indicated that they had a KH domain (Figure S4). Subsequently, we identified eight genes (Table S2) as putative apple HEN4-encoding genes via BLASTP search against the apple proteome using the characterized AtHEN4, which delays flowering (Cheng et al., 2003; Ortuno-Miquel et al., 2019). This observation implied that the apple genes might also control flowering. However, no evidence indicated that HEN4 participates in the plant immune response. Remarkably, in the present study we found that silencing of MdKRBP4 increased the susceptibility of apple to *V. mali* (Figure 4), indicating MdKRBP4 positively regulates the plant immune response. Overexpression of

MdPR10 expression and ROS accumulation, reducing apple resistance to *V. mali* (Figure 7d).
MdKRBP4 induced an ROS burst, indicating that MdKRBP4 participates in the plant immune response by regulating ROS levels.

ROS, such as the superoxide anion (O$_2$•$^-$) and hydrogen peroxide (H$_2$O$_2$), play an important role in resisting infection of pathogens during the plant defence response (Shetty et al., 2008). However, pathogens differ in their sensitivities to ROS. For instance, 0.1 mM H$_2$O$_2$ suppresses the infection of Pectobacterium carotovorum and Phytophthora infestans (Wu et al., 1995). ROS are toxic to plants. Studies have revealed that a regulated increase in ROS benefits cell differentiation and proliferation (Schafer & Buettner, 2001), while an excess leads to a series of physiological changes, such as nucleic acid degradation, lipid peroxidation, and enzyme inactivation (Lamb & Dixon, 1997; Trachootham et al., 2009). For example, 6–8 mM H$_2$O$_2$ leads to the death of suspension-cultured soybean
cells (Levine et al., 1994). Interestingly, in the present study we detected cell death (Figure 5a) and abundant accumulation of ROS (Figure 5g) in N. benthamiana leaves overexpressing MdKRBP4. However, we only observed ROS accumulation, but no cell death, in M. domestica leaves (Figure 2a). Previous studies demonstrated that different plants can tolerate different concentrations of H$_2$O$_2$, such as 60μM–7 mM in Arabidopsis and 1–2 mM in maize and rice (Wang et al., 2013). Based on the above studies, we speculate that perhaps N. benthamiana leaves are more sensitive to H$_2$O$_2$ than M. domestica leaves, which leads to cell death in N. benthamiana leaves. Another possibility is that MdKRBP4-induced ROS accumulation in N. benthamiana leaves is greater than in M. domestica leaves, and therefore cell death was not observed in apple leaves expressing MdKRBP4.

Biologic and abiotic stresses lead to ROS accumulation. Rapid production of ROS is a typical characteristic of the hypersensitive response following the recognition of pathogen infection (Lamb & Dixon, 1997; Wojtaszek, 1997). Pathogens adopt measures to inhibit ROS production. For example, the Puccinia striiformis effector Pst18363 can target and stabilize wheat Nudix hydrolase 23 (TaNUDX23) to suppress ROS accumulation and facilitate infection (Yang et al., 2020). In the present study we detected ROS production in MdKRBP4-expressing leaves of apple (Figure 2) and N. benthamiana (Figure 5d). Furthermore, stable silencing of MdKRBP4 decreased V. mali-induced ROS accumulation (Figure 4d). These results indicate that MdKRBP4 modulates ROS production to positively regulate the plant immune response. We also found that the V. mali effector VmEP1 inhibited cell death (Figure 5a) and decreased MdKRBP4-induced ROS accumulation in N. benthamiana (Figure 5c). BIFC and Co-IP assays demonstrated the interaction between VmEP1 and MdKRBP4 (Figure 1). These results indicate that VmEP1 promotes V. mali infection by restraining MdKRBP4-induced ROS accumulation.

Pathogens use effectors as key weapons to attack host plants because they can manipulate plant immunity by disrupting host protein function and promoting infection. For example, Pseudomonas syringae HopZ1a suppresses local and systemic plant immunity by acetylating mitogen-activated protein kinase kinase 7 (MKK7) (Rufian et al., 2021). The Magnaporthe oryzae effector AvrPiz-t suppresses the ubiquitin ligase activity of the rice RING E3 ubiquitin ligase APIP6 to suppress PAMP-triggered immunity (Park et al., 2012). AVR3a of P. infestans restrains host cell death by targeting and stabilizing host U-box E3 ligase CMPG1 during the biotrophic phase of infection (Bos et al., 2010), and Avr1d from Phytophthora sojae enhances the stability of the soybean E3 ubiquitin ligase GmPUiB13 to facilitate infection (Lin et al., 2021). These studies illustrated that effectors control host immunity in various ways, especially manipulating host protein ubiquitination. In the present study we found that the proteasome inhibitor MG132 stabilized MdKRBP4 (Figure 7a), suggesting that the MdKRBP4 protein is degraded via the 26S proteasome pathway. We demonstrated that VmEP1 targets MdKRBP4 (Figure 1), leading to MdKRBP4 degradation (Figure 7a). The ubiquitin E3 ligase PUB17 degrades a KH domain-containing protein, StKH17 (McLellan et al., 2020). These results imply that VmEP1 restrains ROS accumulation and promotes V. mali infection by disrupting MdKRBP4 ubiquitination.

Our findings prove that MdKRBP4 has a novel function in plant immunity. We found that the VmEP1 effector promotes the degradation of the positive immune regulator MdKRBP4 to compromise plant defence. These findings provide a basis for detailed research on apple resistance. However, additional studies are necessary to elucidate the MdKRBP4-mediated immune regulatory mechanism.

4 | EXPERIMENTAL PROCEDURES

4.1 | Plant and microbe materials

The tissue-cultured plantlets of M. domestica ‘Gala’ and MdKRBP4-silenced transgenic plants were initially grown for 4 weeks on Murashige and Skoog (MS) agar supplemented with 0.3 mg/L 6-benzylaminopurine and 0.2 mg/L indole-3-acetic acid. They were cultured at 25°C under a 14/10-h light/dark photoperiod with a light intensity of 60μmol·m$^{-2}$·s$^{-1}$. N. benthamiana plantlets were cultured in an incubator under a 16/8-h light/dark photoperiod at 25°C/22°C and used for Agrobacterium infiltration experiments.

V. mali was acquired from the State Key Laboratory of Crop Stress Biology for Arid Areas, Northwest A&F University, Shaanxi, Yangling, China and cultured on potato dextrose agar at 25°C.

4.2 | Transient expression of MdKRBP4 in N. benthamiana and M. domestica

The transient expression constructs were generated with the plant expression vector pCAMBIA1302 using standard protocols. The vectors were then transformed into A. tumefaciens GV3101 (pSoup-P19) by electroporation. The Agrobacterium strains containing constructs were cultured in yeast extract peptone broth in a shaker at 200rpm at 28°C for 18h. The bacteria were harvested in infiltration buffer (10mM MgCl$_2$, 10mM MES, and 200μM acetosyringone, pH 5.7) and incubated for 3 h without light at room temperature before use. Five-week-old N. benthamiana leaves were infused with the binary constructs using a 1-ml syringe without a needle.

The infiltration of apple leaves was carried out as previously described (Wang et al., 2021). Briefly, Agrobacterium suspensions were used to prepare 50ml Agrobacterium containing the pCAMBIA1302 constructs, which was then vacuum-infiltrated into apple seedling leaves under 100kPa for 10 min. The treated seedlings were cultured on MS medium for 2 days and then used for experiments.

4.3 | Y2H assay

MdKRBP4 and VmEP1 were cloned into the binary vectors pGADT7 and pGBD7, respectively. Primers used in this study are summarized in Table S3. The polyethylene glycol-mediated conversion method
stated in the Yeast Protocols Handbook (Clontech) was used to transform binary vectors into *Saccharomyces cerevisiae* AH109. pGaDT7-MdKRBP4 and pGBDT7-VmEP1 were cotransformed into yeast cells, and then transformants were selected on synthetic dropout (SD) medium without tryptophan (Trp) and leucine (Leu). Then, single clones were transferred onto SD medium lacking adenine (Adε), histidine (His), Leu, and Trp, containing X-α-Gal for selection of interaction.

### 4.4 | BiFC assay

*MdKRBP4* and *VmEP1* were cloned into the binary vectors cYFP and nYFP, respectively. Table S3 contains the information of primers applied in the present study. The obtained vectors were transformed into *A. tumefaciens* GV3101 and coexpressed in *N. benthamiana* leaves. A confocal laser scanning microscope (FV3000; Olympus) was used to detect the results at about 48 hpi.

### 4.5 | Co-IP assay

Co-IP assays were executed to verify the protein interactions in vivo. To construct the *MdKRBP4*-GFP vector, *MdKRBP4* was cloned into pCAMBIA1302 via homologous recombination, resulting in pCAMBIA1302-MdKRBP4, in which the expression of the VmEP1-GFP fusion is driven by a CaMV 35S promoter. VmEP1 was fused into the vector PICH86988 with an HA tag at its C-terminus. Table S3 contains the information of primers applied in the present study. All plasmids were transformed into *A. tumefaciens* GV3101 and expressed in *N. benthamiana* leaves by coinfiltration. After infiltration with *A. tumefaciens* with either VmEP1-GFP or GFP, *N. benthamiana* leaf tissues were collected at about 36 hpi and frozen in liquid nitrogen. The proteins were extracted in native lysis buffer containing 1% protease inhibitor cocktail and 1 mM phenylmethylsulfonyl fluoride (Solarbio). The protein extracts were cleaned by two rounds of centrifugation and tissue debris was discarded. Next, the total proteins were incubated with 20 μl anti-GFP magnetic beads (Epizyme) for 3 h at 4°C. Then, the magnetic beads were washed three times with TBST buffer (50 mM Tris–HCl, pH 7.5, 8 g/L NaCl, 0.2 g/L polysorbate 20) to elute the proteins, which were boiled in 1x SDS-PAGE loading buffer for 10 min. The total proteins were separated by 10% SDS-PAGE. The gel sections were excised and then digested with trypsin. Finally, mass spectrometry was conducted to identify interacting proteins as previously described (Hu et al., 2014).

### 4.7 | Construction of transgenic apple

RNA interference vectors for apple were generated using the plant expression vector pK7GWIWG2D. The specific PCR fragments of *MdKRBP4* were inserted into pK7GWIWG2D(I). Table S3 contains the information of primers used in this study. The total proteins of leaves expressing VmEP1 were extracted and purified in native lysis buffer. The total protein mixture was centrifuged at 14,000 × g for 10 min and tissue debris was discarded. Next, 30 μl anti-GFP magnetic beads (Shanghai Epizyme Biomedical Technology) was mixed into the supernatant and samples were incubated for 3 h at 4°C on a rotator. The anti-GFP magnetic beads were washed about five times using TBST and the beads were boiled with 1x SDS-PAGE loading buffer for 10 min. The total proteins were separated by 10% SDS-PAGE. The gel was then stained using a kit from Thermo (MAN0011539) following the manufacturer’s instructions. After staining with silver stain and subsequent destaining, gel sections were excised and then digested with trypsin. Finally, mass spectrometry was conducted to identify interacting proteins as previously described (Hu et al., 2014).

### 4.8 | Pathogen inoculation

*V. mali* (03–8) was cultured on potato dextrose agar for 2 days at 28°C. Plugs (3 mm diameter) of *V. mali* were incubated onto transgenic apple leaves for about 24 h at 28°C using the stab inoculation method. Resulting lesions were photographed and the size was evaluated by ImageJ. Each of the experiments was done on at least 30 apple seedling leaves, and for all inoculation assays three biological repeats were carried out.

### 4.9 | Isolation of RNA and RT-qPCR

RNA was isolated from samples (50 μg) using the EasyPure Plant RNA kit (Transgen) and processed with DNase I. RNA samples (2 μg) were used for the RT-qPCR analyses. The RT-qPCR efficiency was determined using a standard curve method. The relative expression of the target genes was calculated using the 2^(-ΔΔCt) method.
reverse-transcribed using high-capacity cDNA reverse transcription kits (Applied Biosystems) and subjected to qPCR using gene-specific primers (available upon request). cDNA samples were amplified using 2xRealStar Green Power mixture (GenStar) and a Roche LightCycler 96 SW1.1 real-time PCR system (Roche). The elongation factor 1α (EF-1α) gene of *M. domestica* and *Actin* of *N. benthamiana* were used for normalization, and gene expression was calculated by the comparative $C_t$ method. Primers used in this study are summarized in Table S3. All experiments were repeated independently three times.

4.10 | DAB staining

$H_2O_2$ accumulation in plant tissue was examined by staining with DAB as described previously (Xiao et al., 2003). Leaf pieces were infiltrated in DAB solution (Sigma) (1 mg/ml, pH 3.8) and shaken at room temperature for 12 h in the light. The leaf pieces were then destained with 95% ethanol. The cleared leaves were transferred to 50% glycerol. A microscope (Olympus) was used to take photographs. The accumulation of ROS was evaluated by ImageJ.

4.11 | Trypan blue staining

*N. benthamiana* leaves expressing MdKRBP4 or empty vector were stained by boiling for 10 min in lactophenol–trypan blue solution (10 ml lactic acid, 10 ml glycerol, 10 g phenol, 10 mg trypan blue, 10 ml distilled water). Then, they were decoloured with gentle shaking in a chloral hydrate solution (2.5 g/ml) for 12 h. Samples were photographed under natural light.

4.12 | Electrolyte leakage

Cell death was quantified by determining electrolyte leakage using a previously described method (Ma et al., 2021; Nayyar & Chander, 2004). Samples from *N. benthamiana* (diameter 1 cm) expressing MdKRBP4 were immersed in nanopure water (5 ml) for 3 h at room temperature to determine the electrical conductivity ($E_1$). A conductivity meter (Five Easy Plus Conductivity) was used to measure the conductivity. Then the samples were boiled for 10 min and the second electrical conductivity ($E_2$) was measured after the fluid recovered to ambient temperature. Electrolyte leakage was calculated as follows: electrolyte leakage (%) = ($E_1$/$E_2$) x 100. This assay was repeated three times.

4.13 | Bioinformatics analysis

Multiple sequence alignment of HEN4 was performed with Clustal Omega (https://www.ebi.ac.uk/Tools/msa/). Phylogenetic trees were established using MEGA 7 software with the neighbour-joining method (Kumar et al., 2016).

**AUTHOR CONTRIBUTIONS**

W.W. and L.H. conceived the research. W.W. mainly performed the experiments. W.S., W.G., and L.L. assisted with the rest of the experiments. J.N. and L.X. participated in the preparation of the manuscript. L.H. revised the manuscript.

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**CONFLICT OF INTEREST**

The authors of this paper declare no competing interests exist.

**DATA AVAILABILITY STATEMENT**

The data used in this study are available from the corresponding author.

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**SUPPORTING INFORMATION**

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