




The MdVQ37-MdWRKY100 complex regulates salicylic acid content and *MdRPM1* expression to modulate resistance to *Glomerella* leaf spot in apples

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Received 26 October 2023;

revised 26 January 2024;

accepted 29 March 2024.

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Summary

Glomerella leaf spot (GLS), caused by the fungus *Colletotrichum fructicola*, is considered one of the most destructive diseases affecting apples. The VQ-WRKY complex plays a crucial role in the response of plants to biotic stresses. However, our understanding of the defensive role of the VQ-WRKY complex on woody plants, particularly apples, under biotic stress, remains limited. In this study, we elucidated the molecular mechanisms underlying the defensive role of the apple MdVQ37-MdWRKY100 module in response to GLS infection. The overexpression of *MdWRKY100* enhanced resistance to *C. fructicola*, whereas *MdWRKY100* RNA interference in apple plants reduced resistance to *C. fructicola* by affecting salicylic acid (SA) content and the expression level of the CC-NBS-LRR resistance gene *MdRPM1*. DAP-seq, Y1H, EMSA, and RT-qPCR assays indicated that *MdWRKY100* inhibited the expression of *MdWRKY17*, a positive regulatory factor gene of SA degradation, upregulated the expression of *MdPAL1*, a key enzyme gene of SA biosynthesis, and promoted *MdRPM1* expression by directly binding to their promoters. Transient overexpression and silencing experiments showed that *MdPAL1* and *MdRPM1* positively regulated GLS resistance in apples. Furthermore, the overexpression of *MdVQ37* increased the susceptibility to *C. fructicola* by reducing the SA content and expression level of *MdRPM1*. Additionally, *MdVQ37* interacted with *MdWRKY100*, which repressed the transcriptional activity of *MdWRKY100*. In summary, these results revealed the molecular mechanism through which the apple MdVQ37-MdWRKY100 module responds to GLS infection by regulating SA content and *MdRPM1* expression, providing novel insights into the involvement of the VQ-WRKY complex in plant pathogen defence responses.

Keywords: Apple, SA, *Glomerella* leaf spot, VQ protein, WRKY.

Introduction

Apple (*Malus × domestica* Borkh.) is a deciduous tree belonging to the Rosaceae family. It is extensively cultivated in temperate regions worldwide and is an economically important fruit tree. Its fruit has a unique flavour and high nutritional value and is cherished by people. However, in natural environments, various pathogens can infect perennial apple trees. *Glomerella* leaf spot (GLS) is a fungal epidemic caused by *Colletotrichum fructicola* that occurs in almost all apple producing areas (Munir *et al.*, 2016; Oo *et al.*, 2018; Velho *et al.*, 2015; Zhang *et al.*, 2008a). GLS infects fruit and leaves, resulting in significantly decline in fruit quality and yield (Munir *et al.*, 2016; Oo *et al.*, 2018; Velho *et al.*, 2015; Zhang *et al.*, 2019a). Chemical treatments are commonly used to control GLS; however, they pose new challenges to food safety, environmental protection, and planting costs (Shan

et al., 2021; Zhang *et al.*, 2019a). Breeding pathogen-resistant varieties and selecting pathogen-resistant apple resources through molecular breeding are the most effective strategies to combat apple fungal diseases (Shan *et al.*, 2021; Zhang *et al.*, 2018, 2019a). Therefore, identifying new candidate genes and elucidating the molecular mechanisms underlying their participation in GLS are crucial for the molecular breeding of disease-resistant varieties.

Several studies have revealed that in effector-triggered immunity (ETI), disease-resistant proteins (R proteins) interact with effector proteins produced by pathogens in a gene-for-gene manner, leading to rapid and robust defence responses in plants (Dodds and Rathjen, 2010; Meng *et al.*, 2018). Most plant R proteins are NBS-LRRs that contain nucleotide binding sites (NB) and leucine-rich repeats (LRRs) (Dangl and Jones, 2001). Based on the structural characteristics of the N-terminus domain, NBS-LRR proteins can be divided into two classes: TIR-NBS-LRR (TNL),

which contains the Toll/interleukin-1 receptor (TIR) domain, and CC-NBS-LRR (CNL), which contains the coiled-coil (CC) domain (Mackey *et al.*, 2002; Meng *et al.*, 2018). Previous studies have shown that the TIR and CC domains of NBS-LRR proteins are involved in downstream signal regulation and the initiation of plant defence responses (Mackey *et al.*, 2002; Meng *et al.*, 2018). Resistance to *Pseudomonas syringae* pv. *maculicola* 1 (*RPM1*) encodes a CC-NBS-LRR protein and was the first disease resistance gene to be identified using molecular markers in the study of natural variations in *Arabidopsis* (Bisgrove *et al.*, 1994; Grant *et al.*, 1995). In apples, the TIR-NBS-LRR class genes, *MdNLR16*, *Md-TN1-GLS*, and *MdTNL1-1*, have been reported to be involved in response to *Alternaria alternata* f. sp. *mali* (ALT1), *Glomerella cingulata*, and GLS infection, respectively (Lv *et al.*, 2022; Meng *et al.*, 2018; Zhang *et al.*, 2019a). In addition, the hairpin RNA *MdhpRNA277* produces *mdm-siR277-1* and *mdm-siR277-2*, which target CC-NBS-LRR genes in response to ALT1 infection (Zhang *et al.*, 2018). However, there are no reports on the involvement of CC-NBS-LRR proteins in GLS tolerance in apples.

Salicylic acid (SA), an endogenous signalling molecule in plants, plays a crucial role in plant–pathogen interactions, and its level is required for PAMP-triggered immunity (PTI), ETI, and systemic acquired resistance (SAR) (Zhang and Li, 2019). SA content in plants is primarily determined by SA homeostasis, including SA biosynthesis (regulated by the isochorismate synthase [ICS] and phenylalanine ammonia lyase [PAL] pathways) and SA metabolism (primarily controlled by Downy Mildew Resistant6 [*DMR6*] and *SAG108*) (Zhang *et al.*, 2017b). In apples, *C. fructicola* effector CfEC12 interacts with *MdNIMIN2*, a NIM1-interacting (NIMIN) protein that putatively modulates NPR1 activity in response to SA signalling (Shang *et al.*, 2024). Furthermore, applying exogenous SA can significantly increase apple resistance to GLS (Zhang *et al.*, 2016). Some studies have shown that *Arabidopsis* WRKY TFs directly bind to the promoter of SA synthesis, metabolism, and signal transduction pathway-related genes, including *ISOCHORISMATE SYNTHASE1* (*ICS1*), *AVRPPHB SUSCEPTIBLE 3* (*PBS3*), *Plant Antitoxin Deficiency 3* (*PAD3*), *ENHANCED DISEASE SUSCEPTIBILITY 1* (*EDS1*) and *pathogenesis-related* (*PR*), to regulate plant response to pathogen infection (Birkenbihl *et al.*, 2012; Shan *et al.*, 2021; van Verk *et al.*, 2011). Similar molecular mechanisms have been observed in apples. For example, *MdWRKY15* and *MdWRKY46* activate the transcription of the SA synthase genes *MdICS1* and *MdPBS3.1* by directly binding to their promoters to enhance SA accumulation and confer resistance to *Botryosphaeria dothidea* (Zhao *et al.*, 2019, 2020). *MdWRKY17* reduces SA accumulation by activating the SA degradation gene *MdDMR6*, thereby increasing the sensitivity to GLS (Shan *et al.*, 2021).

VQ proteins are a class of transcriptional regulatory cofactors containing VQ domains, named after 10 highly conserved amino acid residues (FxxhVQxhTG) in the VQ domain (Jing and Lin, 2015). VQ proteins typically interact with other proteins, such as WRKY, Phytochrome-Interacting Factors1 (PIF1), ABSCISIC ACID-INSENSITIVE5 (ABI5), and MAPK, to perform biological functions. Currently, research on the interaction between VQ proteins and WRKY transcription factors is extensive, as they collaboratively participate in regulating specific biological processes and stress responses. (Hu *et al.*, 2013; Jiang and Yu, 2016; Lei *et al.*, 2018; Ma *et al.*, 2023a, 2023b). The interaction

between *MdVQ10* and *MdWRKY75* in apples regulates wound-triggered leaf senescence (Zhang *et al.*, 2023a). However, the molecular mechanisms through which woody plant VQ-WRKY complexes regulate disease resistance remain unclear.

The aim of this study was to elucidate the molecular mechanisms underlying the defensive role of the apple *MdVQ37-MdWRKY100* module in response to GLS infection caused by the fungus *C. fructicola*. Moreover, we investigated the impact of *MdWRKY100* overexpression and RNA interference on GLS resistance in apple plants, focusing on their influence on SA content and the expression of the CC-NBS-LRR resistance gene *MdRPM1*. The results of the study may elucidate the crucial role of the WRKY-VQ complex in the response of apples to GLS and contribute to improving the molecular breeding of apple varieties with GLS resistance.

Results

MdWRKY100 positively regulates resistance to GLS in apples

Our previous studies have shown that *MdWRKY100* positively regulates resistance to bitter rot and salt stress in apples (Ma *et al.*, 2021; Zhang *et al.*, 2019b). This study revealed that the *MdWRKY100* transcript was significantly upregulated after inoculation with *C. fructicola* for 48 and 72 h (Figure S1). To further investigate the biological role of *MdWRKY100* in GLS tolerance, we used the previously obtained *MdWRKY100* overexpression (OE) line *MdWRKY100-OE 4* as well as the *MdWRKY100* RNA-interference (RNAi) line *MdWRKY100-RNAi 3*. After *C. fructicola* inoculation for 4 or 6 days, the *MdWRKY100-OE 4* seedlings showed highly resistance to *C. fructicola*; however, the *MdWRKY100-RNAi 3* seedlings displayed the opposite phenotype compared to wild-type (WT) seedlings (Figure 1a). Disease index analysis confirmed this phenotypic difference (Figure 1b). Free SA, total SA content, and PAL activity in the *MdWRKY100-OE 4* seedlings were significantly higher than those in the WT seedlings. In contrast, free SA, total SA content, and PAL activity in the *MdWRKY100-RNAi 3* seedlings were significantly lower than those in the WT seedlings (Figure 1c–e). These results suggest that apple *MdWRKY100* enhances resistance against GLS by altering SA content and PAL activity.

MdWRKY100 binds and inhibits the *MdWRKY17* expression, and activates the *MdPAL1* and *MdRPM1* expression

DNA affinity purification sequencing (DAP-seq) was performed to map the genome-wide *MdWRKY100* DNA-binding sites to further explore the molecular mechanism and regulatory pathway through which *MdWRKY100* regulates GLS infection. The in vitro synthesized *MdWRKY100* protein was used to conduct affinity purification of sheared genomic DNA of mature 'Gala' leaves, followed by deep sequencing. In total 8750 highly reliable *MdWRKY100* binding sites were identified; 24.9% *MdWRKY100* binding peaks was distributed in the promoter regions, and 50.2%, 13.1%, and 3.9% were in the intergenic, intronic, and exon regions, respectively (Figure S2). MEME suite analysis showed that the motif sequence with the most significant enrichment of *MdWRKY100* binding sites was 'TTGACT/C', which was a typical W-box element (Figure 2a). By screening the functional annotation of *MdWRKY100* binding

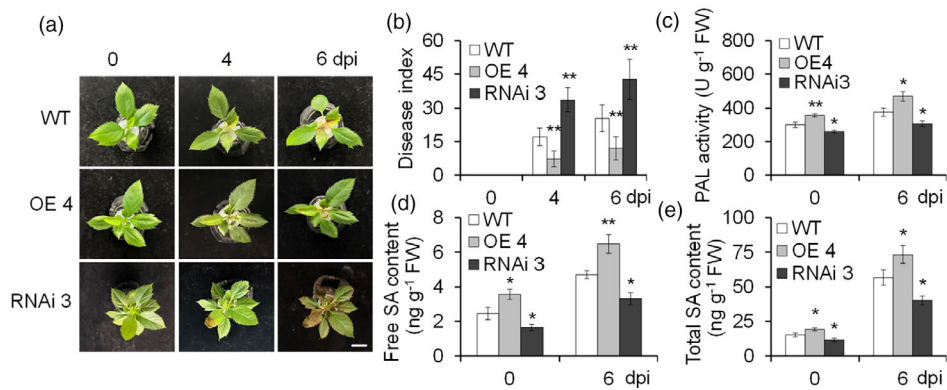


Figure 1 MdWRKY100 positively regulates apple defence against *Colletotrichum fructicola*. Phenotypes (a) and disease index (b) of apple seedlings of WT, MdWRKY100 overexpression line (MdWRKY100-OE 4) and MdWRKY100 RNAi line (MdWRKY100-RNAi 3) after inoculation with *C. fructicola* for 4 and 6 days. Scale bar: 1 cm. Error bars represent SE ($n = 18$). The experiments were repeated thrice with similar results. ** in each panel indicates significantly different values relative to WT at $P < 0.01$, using Student's *t*-test. The PAL activity (c) and the contents of endogenous free SA (d) and total SA (e) in apple seedlings of WT, MdWRKY100-OE 4, and MdWRKY100-RNAi 3 after inoculation with *C. fructicola* for 6 days. Error bars represent SD based on 3 independent replicates. ** and * in each panel indicate significantly different values relative to WT at $P < 0.01$ and $P < 0.05$, using Student's *t*-test, respectively.

sites, we found that a previously reported susceptibility transcription factor during *C. fructicola* infection, *MdWRKY17*, a key enzyme gene of SA synthesis, *MdPAL1* (Figure S3), and CC-NBS-LRR resistance genes (Figure S4) may be the target genes of MdWRKY100 (Table S1). RT-qPCR results showed that the expression levels of *MdPAL1* and *MdRPM1* were upregulated by *C. fructicola* infection (Figure 2b), whereas those of *MdCNL1-MdCNL3* were unaffected by *C. fructicola* infection, indicating that *MdPAL1* and *MdRPM1* may play important roles in the apple's response to *C. fructicola* infection. To verify the reliability of the DAP-seq results, yeast one-hybrid (Y1H) and electrophoretic mobility shift assay (EMSA) assays were carried out. In Y1H assays, co-transformed yeast cells containing pGADT7 empty vector and pAbAi-MdWRKY17-*Pro*, pAbAi-MdPAL1-*Pro*, or pAbAi-MdRPM1-*Pro* could not grow on -Leu medium with 200 ng/mL AbA; however, co-transformed yeast cells containing pGADT7-MdWRKY100 and pAbAi-MdWRKY17-*Pro*, pAbAi-MdPAL1-*Pro* or pAbAi-MdRPM1-*Pro* could grow on -Leu medium with 200 ng/mL AbA (Figure 2c). In the EMSA assays, MdWRKY100 was bound to *Pro-MdWRKY17* (Figure S5), *Pro-MdPAL1* (Figure S6), and *Pro-MdRPM1* (Figure S7) probes (Figure 2d). When the W-box (TTGAC) in the *MdWRKY17*, *MdPAL1*, and *MdRPM1* promoters was mutated to TTTT, these bindings disappeared, indicating that the binding was specific. These results indicate that MdWRKY100 directly binds to the *MdWRKY17*, *MdPAL1*, and *MdRPM1* promoters. Furthermore, we examined *MdWRKY17*, *MdPAL1*, and *MdRPM1* expression levels in the WT and *MdWRKY100* transgenic apple lines. The relative expression levels of *MdRPM1* and *MdPAL1* in MdWRKY100-OE 4 seedlings were significantly higher than those in WT seedlings. The relative expression level of *MdWRKY17* in MdWRKY100-OE 4 seedlings was significantly lower than that in WT seedlings (Figure 2e). The relative expression levels of *MdWRKY17*, *MdPAL1*, and *MdRPM1* in the MdWRKY100-RNAi 3 seedlings displayed opposite trends. These results suggest that MdWRKY100 directly binds to the promoters of *MdWRKY17*, *MdPAL1*, and *MdRPM1* to inhibit *MdWRKY17* expression and activate *MdPAL1* and *MdRPM1* expression.

MdPAL1 and MdRPM1 positively regulate resistance to GLS in apples

To further investigate whether *MdPAL1* is involved in resistance to GLS, we conducted transient overexpression and silencing *MdPAL1* in the leaves of tissue-cultured 'Gala' apple plantlets (Figure 3a). The transcription abundance of *MdPAL1* and PAL activity increased in *MdPAL1*-OE leaves and decreased in *MdPAL1*-RNAi leaves 2 days after agroinfiltration compared with their controls (Figure 3b,c). We inoculated these leaves with *C. fructicola* for 5 days. We found that the overexpression of *MdPAL1* significantly reduced the lesion area by increasing the free SA and total SA contents. In contrast, silencing *MdPAL1* significantly increased the lesion area by reducing free SA and total SA contents compared with their controls (Figure 3d-f). These results indicate that *MdPAL1* positively regulates apple resistance to GLS by increasing SA content.

We also investigated whether *MdRPM1* participates in GLS resistance. Transient overexpression and silencing of *MdRPM1* in the leaves of 'Gala' tissue-cultured apple plantlets were performed (Figure 3g). The transcription abundance of *MdRPM1* increased in *MdRPM1*-OE leaves and decreased in *MdRPM1*-RNAi leaves at 2 days after agroinfiltration compared with that in the controls (Figure 3h). Similarly, we inoculated these leaves with *C. fructicola* for 5 days and found that the overexpression of *MdRPM1* significantly reduced the lesion area. In contrast, silencing of *MdRPM1* significantly increased the lesion area compared to that of the controls (Figure 3i). Furthermore, the inoculation of *C. fructicola* in *MdRPM1*-OE or *MdRPM1*-RNAi leaves for 5 days altered endogenous free SA and total SA contents (Figure 3j,k). These results indicate that *MdRPM1* positively regulates apple resistance to GLS.

MdWRKY100 physically interacts with MdVQ37

In our previous studies, we obtained two *MdVQ37*-overexpressing transgenic lines and found that the overexpression of *MdVQ37* reduced drought, salt, and heat tolerance (Dong et al., 2021, 2022a, 2022b). Previous studies have suggested that VQ proteins interact with group I and II WRKY transcription factors to regulate

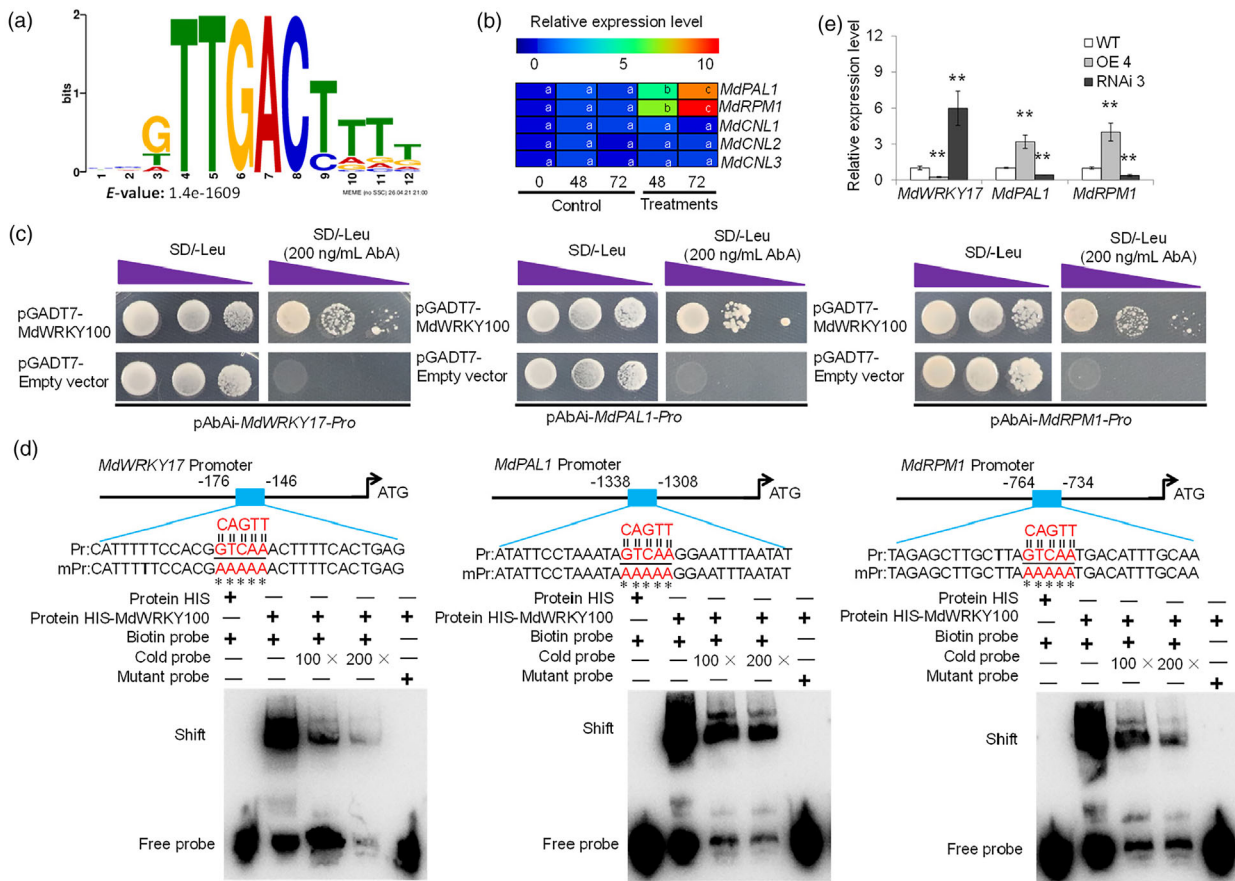


Figure 2 MdWRKY100 binds to the *MdWRKY17*, *MdPAL1* and, *MdrRPM1* promoters. (a) Identified DNA-binding motif of the MdWRKY100 protein by DAP-seq. The e-value of motif 'GTTGACTTTT' is 1.4e-1609. Recombinant MdWRKY100 proteins were used to perform DAP-seq. (b) Relative expression levels of *MdPAL1*, *MdrRPM1*, and *MdCNL1/2/3* after *Colletotrichum fructicola* infection. After qRT-PCR data were re-analysed, relative expression was calculated with respect to control samples (i.e., Control 0). Heat maps were generated using TIGR MeV v4.8.1 software. The bar at the top of the heat map presents relative expression values. Different letters in each panel indicate values are significantly different at $P < 0.05$, based on one-way ANOVA and Duncan's test. (c) Y1H assay of MdWRKY100 binding to the *MdWRKY17*, *MdPAL1*, and *MdrRPM1* promoter fragments. The prey vector containing MdWRKY100 and the bait vectors pAbAi-MdWRKY17-Pro, pAbAi-MdPAL1-Pro, and pAbAi-MdrRPM1-Pro were introduced into yeast strain Y1H, and the interaction between bait and prey enhanced Aureobasidin A (AbA) resistance. Yeast cells were grown on SD-Leu media with 200 ng/mL AbA. The bait vector pAbAi-MdWRKY17-Pro plus pGADT7 empty vector, the bait vector pAbAi-MdPAL1-Pro plus pGADT7 empty vector, and the bait vector pAbAi-MdrRPM1-Pro plus pGADT7 empty vector were also transformed into Y1H as negative controls. (d) EMSA confirming the in vitro binding of MdWRKY100 to the TTAGC motif of the *MdWRKY17*, *MdPAL1*, and *MdrRPM1* promoters. The labelled probe (Pr) containing the TTAGC motif was synthesized based on the sequence of the *MdWRKY17*, *MdPAL1*, and *MdrRPM1* promoters. In the mPr probe, TTAGC sequences are mutated to AAAAA. W-box sequences are underlined. *Mutated sites. HIS alone was used as a negative control of the binding. (e) Expression patterns of *MdWRKY17*, *MdrRPM1*, and *MdPAL1* in *MdWRKY100* transgenic lines. Error bars represent SD based on three biological replicates. ** in each panel indicates significantly different values relative to WT at $P < 0.01$, using Student's *t*-test.

plant defence responses (Jing and Lin, 2015). Given that MdWRKY100 is a group I WRKY protein (Zhang *et al.*, 2019b), we speculated that MdVQ37 may interact with MdWRKY100. Therefore, yeast two-hybrid (Y2H) assay was used to detect the interaction between MdWRKY100 and MdVQ37. The full-length MdWRKY100 cDNA with a deleted activation domain was fused to the pGBT9 bait vector (MdWRKY100 Δ 1–227). As shown in Figure 4a, MdWRKY100 Δ 1–227 protein interacted with MdVQ37 protein. To determine which regions of MdWRKY100 and MdVQ37 proteins are required for their interactions, we separated MdWRKY100 into its N-WRKY (location 236–289), C-WRKY (location 409–463), and C-terminus domains (location 478–571) and divided MdVQ37 into its N-terminus (location 1–31), VQ motif (location 32–41), and C-terminus domain (location 91–181). The

Y2H assay indicated that the deletion of the C-terminus of MdVQ37 or the N-WRKY and C-terminus domains of MdWRKY100 did not affect these interactions. However, deleting the N-terminus and VQ motif of MdVQ37 or the C-WRKY domain of MdWRKY100 eliminated these physical interactions. These results demonstrate that the C-WRKY domain of MdWRKY100 and the N-terminus and VQ motif domains of MdVQ37 are critical for physical interactions in yeast.

We performed bimolecular fluorescence complementation (BiFC), split-LUC, and pull-down assays to verify the interaction between MdWRKY100 and MdVQ37 *in vivo* and *in vitro*. In the BiFC assays, when MdWRKY100-N was co-infiltrated with MdVQ37-C in tobacco leaves, a YFP signal was detected in the nucleus; however, YFP-C + MdWRKY100-N, MdVQ37-C + YFP-N, and

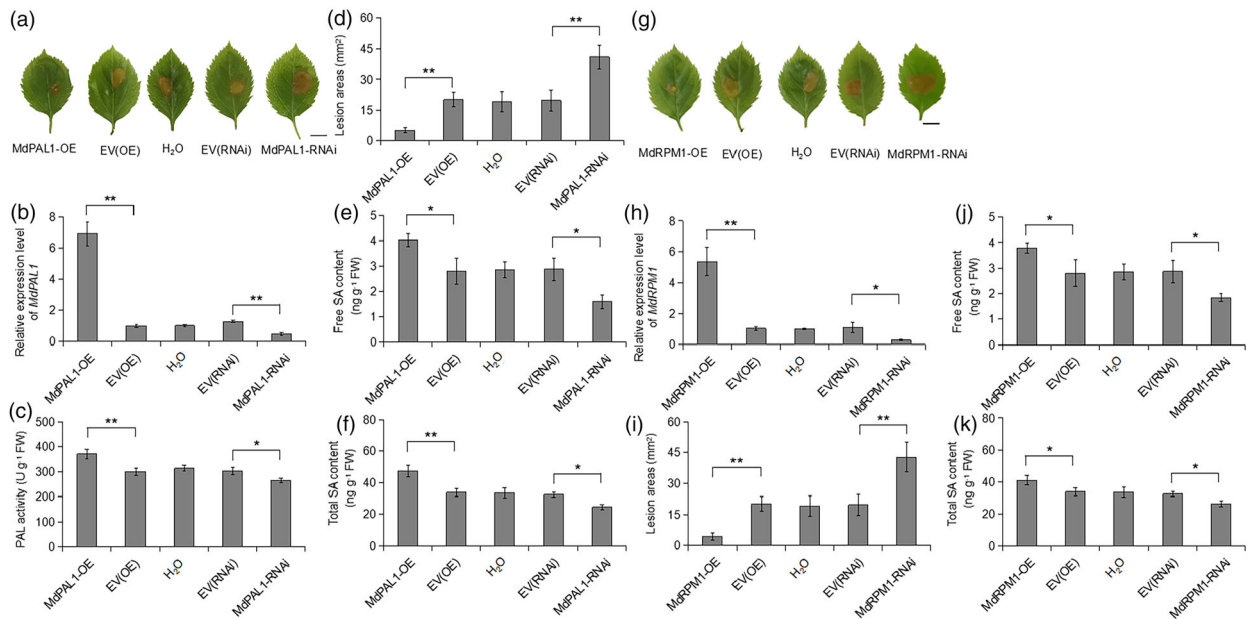


Figure 3 Transient overexpression and silencing of *MdPAL1* and *MdRPM1* in apple leaves (a) Disease symptoms on infiltrated leaves in which *MdPAL1* was overexpressed or silenced at 48 h after inoculation with *C. fructicola* for 5 days. H₂O; pK7GWIWG2D empty vector: EV(RNAi); pK7GWIWG2D-*MdPAL1* fusion vector: *MdPAL1*-RNAi; pCambia2300 empty vector: EV(OE); pCambia2300-*MdPAL1* fusion vector: *MdPAL1*-OE. Scale bar: 1 cm. (b) Relative expression levels of *MdPAL1* in infiltrated leaves in which *MdPAL1* was overexpressed or silenced at 48 h. Error bars represent SD based on three biological replicates. Statistics by Student's *t*-test, ****P* < 0.01. (c) PAL activities in infiltrated leaves in which *MdPAL1* was overexpressed or silenced at 48 h after inoculation with *C. fructicola* for 5 days. Error bars represent SD based on three biological replicates. Statistics by Student's *t*-test, **P* < 0.05 and ***P* < 0.01. (d) and (i) Lesion areas in infiltrated leaves after inoculation with *C. fructicola* for 5 days. Error bars represent SE (*n* = 18). The experiments were repeated thrice with similar results. Statistics by Student's *t*-test, ***P* < 0.01. The contents of endogenous free SA (e) and total SA (f) in infiltrated leaves in which *MdPAL1* was overexpressed or silenced at 48 h after inoculation with *C. fructicola* for 5 days. Error bars represent SD based on three biological replicates. Statistics by Student's *t*-test, **P* < 0.05 and ***P* < 0.01. (g) Disease symptoms on infiltrated leaves in which *MdRPM1* was overexpressed or silenced at 48 h after inoculation with *C. fructicola* for 5 days. Scale bar: 1 cm. (h) Relative expression levels of *MdRPM1* in infiltrated leaves in which *MdRPM1* was overexpressed or silenced at 48 h. Error bars represent SD based on 3 biological replicates. Statistics by Student's *t*-test, **P* < 0.05 and ***P* < 0.01. The contents of endogenous free SA (j) and total SA (k) in infiltrated leaves in which *MdRPM1* was overexpressed or silenced at 48 h after inoculation with *C. fructicola* for 5 days. Error bars represent SD based on three biological replicates. Statistics by Student's *t*-test, **P* < 0.05 and ***P* < 0.01.

YFP-C + YFP-N showed no fluorescence (Figure 4b). In the split-LUC assays, strong LUC activity was observed when MdWRKY100-cLuc was co-injected with MdVQ37-nLuc or when MdWRKY100-nLuc was co-injected with MdVQ37-cLuc into tobacco leaves (*Nicotiana benthamiana*) (Figure 4c). In pull-down assays, MdWRKY100-HIS was captured by MdVQ37-MBP but not by MBP (Figure 4d). These observations suggest that MdWRKY100 physically interacts with MdVQ37 *in vivo* and *in vitro*.

MdVQ37 negatively regulates resistance to GLS in apples

The RT-qPCR results showed that *MdVQ37* expression was induced after inoculation with *C. fructicola* for 48 and 72 h (Figure S8a). Sub-cellular localization results indicated that MdVQ37 was localized in the nucleus (Figure S8b). To investigate the biological role of MdVQ37 in GLS tolerance, two *MdVQ37*-overexpressing transgenic lines were used. Upon *C. fructicola* inoculation for 5 days, transgenic apple lines were found hypersensitive to *C. fructicola* compared to WT plants (Figure 5a). Compared to that in WT plants, the lesion area in *MdVQ37*-overexpressing transgenic lines was significantly increased, whereas PAL activity, free SA content, and total SA content were significantly decreased (Figure 5b–e). These results

suggest that apple MdVQ37 negatively regulates resistance to GLS by altering SA content and PAL activity.

MdVQ37 represses the transcriptional activity of MdWRKY100

Having demonstrated the MdWRKY100-MdVQ37 interaction (Figure 4), further investigations were conducted to understand whether MdVQ37 could affect the transcriptional function of MdWRKY100 using a transient expression system. To test this possibility, the *MdWRKY17*, *MdRPM1*, and *MdPAL1* promoters were inserted into the pGreenII 0800-LUC vector and co-transformed into tobacco leaves with pGreenII 62-SK-MdWRKY100 or a combination of pGreenII 62-SK-MdWRKY100/pGreenII 62-SK-MdVQ37 (Figure 6a). We found that MdWRKY100 induced the accumulation of *MdRPM1* and *MdPAL1* transcripts, with increased transactivation by almost 2.8-fold and 2.2-fold, respectively; however, MdWRKY100 inhibited the accumulation of *MdWRKY17* transcripts (Figure 6b). Notably, the combination of MdVQ37 and MdWRKY100 downregulated the transcript levels of *MdRPM1* and *MdPAL1* and upregulated the transcript levels of *MdWRKY17* *in vivo* (Figure 6b). Furthermore, we examined the expression

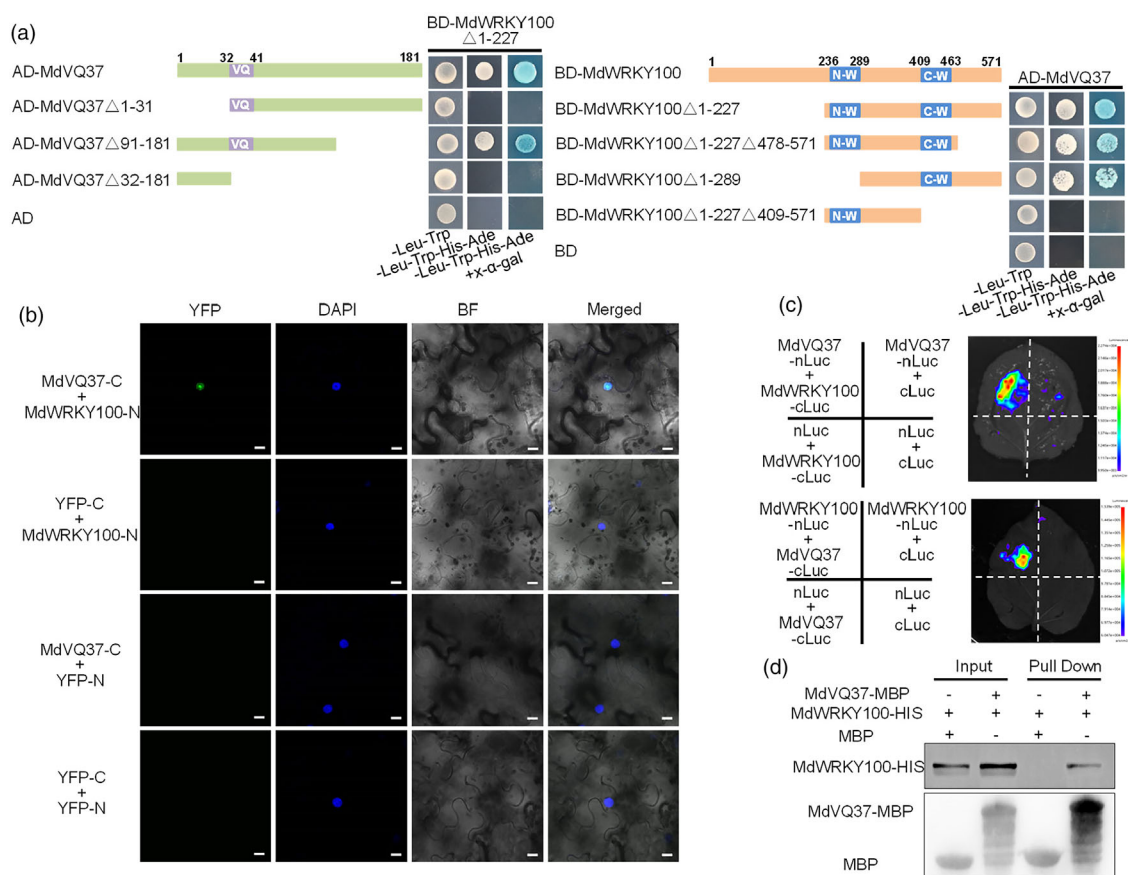


Figure 4 MdVQ37 physically interacts with MdWRKY100 *in vitro* and *in vivo*. (a) According to the domains of MdVQ37 (N-terminus domain, VQ motif, and C-terminus domain) and MdWRKY100 (N-terminus domain, N-WRKY, C-WRKY, and C-terminus domain), truncated MdVQ37 and MdWRKY100 with specific deletions were inserted into the pGAD424 prey vector and pGBT9 bait vector, respectively, and used to assess interactions. pGAD424 fusion prey vectors were co-transformed with pGBT9 fusion bait vectors into yeast cells. Positive interactions were indicated by the ability of cells to grow on synthetic dropout medium with additive x-α-gal but lacking Leu, Trp, His, and Ade. Empty AD prey vector plus BD-MdWRKY100Δ1–227 fusion bait vector and Empty BD bait vector plus AD-MdVQ37 fusion prey vector were used as negative controls. (b) Assay of bimolecular fluorescence complementation (BiFC), showing fluorescence in nuclear compartments of tobacco leaf epidermal cells that resulted from the complementation of the C-terminus part of YFP fused to MdVQ37 (MdVQ37-C) with N-terminus part of YFP fused to MdWRKY100 (MdWRKY100-N). No signal was observed from negative controls. Scale bar: 10 μm. (c) Split-luciferase complementation assay showing the interaction of MdVQ37 and MdWRKY100. The construct combinations of MdVQ37 and MdWRKY100 were co-transformed into *N. benthamiana* leaves. (d) Pull-down assay for detecting the interaction between MdVQ37 and MdWRKY100. MdVQ37-MBP or purified MBP was incubated with MdWRKY100-His protein and purified using an MBP Purification Kit. Resultant protein samples were immunoblotted with anti-MBP or anti-HIS antibodies. The negative control was MBP.

levels of *MdWRKY100*, *MdWRKY17*, *MdRPM1*, and *MdPAL1* in the WT and *MdVQ37* transgenic apple lines. The relative expression levels of *MdWRKY100*, *MdRPM1*, and *MdPAL1* in *MdVQ37* transgenic apple lines were significantly lower than those in the WT plants. The relative expression level of *MdWRKY17* in *MdVQ37* transgenic apple lines was significantly higher than that in the WT plants (Figure 6c). These results indicate that MdVQ37 acts as a negative mediator of MdWRKY100 and represses its transcriptional activity.

Discussion

GLS is one of the most destructive diseases affecting apple production and restricting apple income in China. Multiple studies have shown that the WRKY transcription factor, as an important regulator of host–pathogen interactions, is an important component of the plant defence response signalling network and can

positively or negatively regulate the defence response to multiple plant pathogens (Jiang *et al.*, 2017; Rushton *et al.*, 2010). In *Arabidopsis* and tomato, WRKY transcription factors reportedly interact with VQ proteins to jointly regulate the response to pathogens jointly (Huang *et al.*, 2022; Jing and Lin, 2015). With the advancements in research, it has been proven that the interaction mechanism between WRKY and VQ in *Arabidopsis*, soybean, and apple only exists in some members of the WRKY family in groups I and IIc (Cheng *et al.*, 2012; Dong *et al.*, 2018; Zhou *et al.*, 2016). These studies suggest that the interaction between WRKY and VQ regulates plant response to pathogens, a conserved molecular mechanism. Therefore, exploring the physical interactions between the apple WRKY transcription factor and VQ proteins may provide novel insights into the molecular mechanisms underlying plant defence responses. In apples, MdWRKY100, which encodes a group I WRKY transcription factor, positively regulates the resistance to *Colletotrichum*

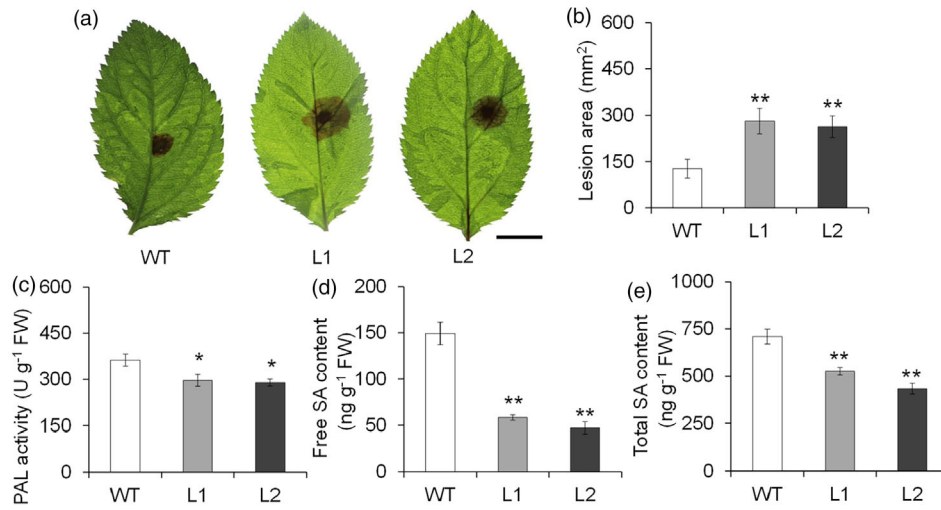


Figure 5 *MdVQ37* overexpression reduces resistance to *Colletotrichum fructicola* infection in transgenic apples. Phenotypes (a) and lesion areas (b) of detached leaves of wild-type (WT) and *MdVQ37* overexpression lines (L1 and L2) after inoculation with *C. fructicola* for 5 days. Scale bar: 1 cm. Error bars represent SE ($n = 18$). The experiments were repeated thrice, and similar results were obtained. ** in each panel indicates significantly different values relative to WT at $P < 0.01$, using Student's *t*-test. The PAL activity (c) and contents of endogenous free SA (d) and total SA (e) in the detached leaves of the WT and *MdVQ37* overexpression lines (L1 and L2) after inoculation with *C. fructicola* for 5 days. Error bars represent the standard deviation (SD) based on three independent replicates. * and ** in each panel indicates significantly different values relative to WT at $P < 0.05$ and $P < 0.01$, respectively, using Student's *t*-test.

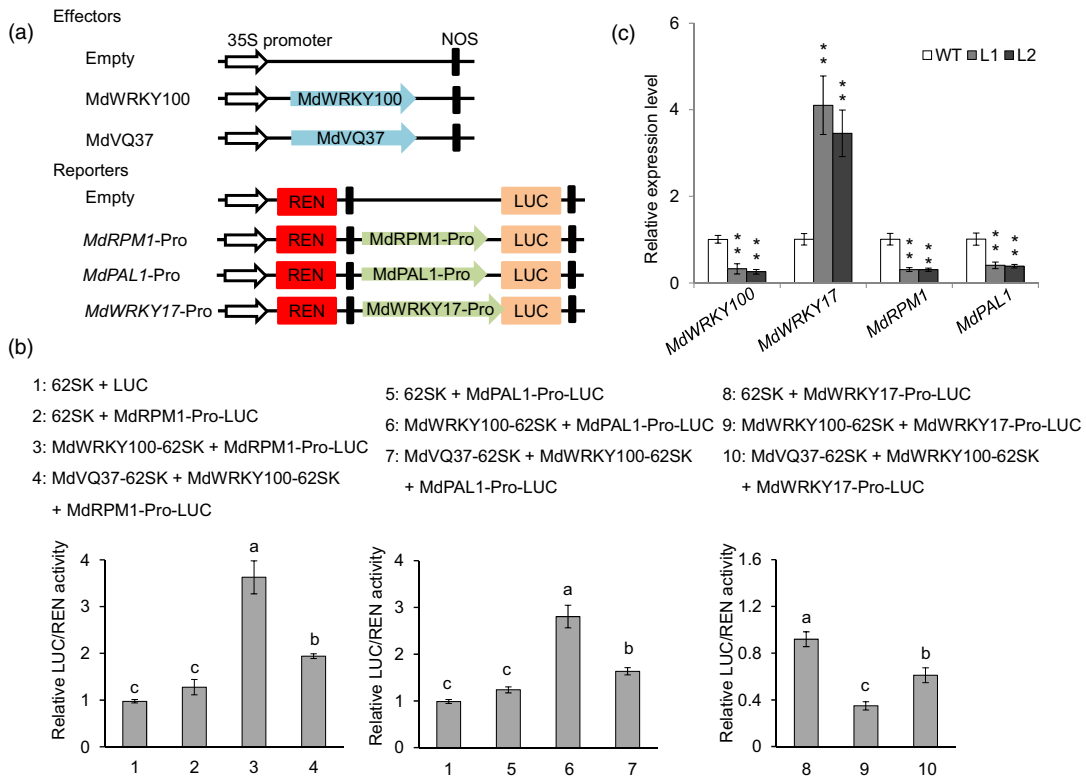


Figure 6 *MdVQ37* inhibits the transcriptional activity of *MdWRKY100*. (a) Schematic showing the constructs used in the transient expression assays. The *MdVQ37* and *MdWRKY100* genes were cloned into the pGreenII 62-SK vector to generate the effector constructs. The reporters were generated by recombining the promoter fragments of *MdRPM1*, *MdPAL1*, and *MdWRKY17* into the pGreenII 0800-LUC vector. (b) Dual-luciferase reporter assay results. Different combinations of reporter and effector plasmids were co-infiltrated into the leaves of *N. benthamiana*. LUC/REN activity detection to *MdVQ37* and *MdWRKY100* co-transformation to repress the transcriptional activity of *MdWRKY100*. Empty vector was used as the reference. Error bars represent SD based on three biological replicates. Different letters indicate significant differences between different combinations, according to one-way ANOVA and Duncan's test ($P < 0.05$). (c) Expression patterns of *MdWRKY100*, *MdWRKY17*, *MdRPM1*, and *MdPAL1* in *MdVQ37* overexpression lines. Error bars represent SD based on three biological replicates. * and ** in each panel indicates significantly different values relative to WT at $P < 0.05$ and $P < 0.01$, respectively, using Student's *t*-test.

gloeosporioides infection (Zhang *et al.*, 2019b). Our previous study demonstrated that apple VQ proteins interact with group I WRKY transcription factors. Based on our acquisition of *MdVQ37* overexpression apple transgenic plants, we speculated that the MdWRKY100 transcription factor may interact with MdVQ37. This speculation was confirmed through yeast two-hybrid experiments, and the results showed that the C-terminus WRKY domain of MdWRKY100 and the VQ motif of MdVQ37 were crucial for the interaction between MdWRKY100 and MdVQ37 (Figure 4a). Furthermore, we demonstrated through BiFC, LCI, and pull-down experiments that MdWRKY100 and MdVQ37 interact both in vivo and in vitro (Figure 4b–d). To characterize the role of the MdWRKY100-MdVQ37 complex in the defence response of apples against *C. fructicola*, we investigated the performance of overexpression and silencing of *MdWRKY100*, as well as the overexpression of *MdVQ37* in response to *C. fructicola* infection. Phenotypic analysis showed that the *MdWRKY100* overexpression lines showed enhanced resistance to *C. fructicola*, whereas the *MdWRKY100* RNAi and *MdVQ37* overexpression lines showed weakened resistance to *C. fructicola* by increasing SA content (Figures 1 and 5). These results demonstrate the regulatory role of the MdWRKY100-MdVQ37 complex in the response of apples to *C. fructicola* infection by affecting the biosynthesis or degradation of SA.

WRKY transcription factor can specifically bind to the cis-acting element W-box on the target gene promoter to activate or inhibit the expression of the target gene and participate in regulating the response of plants to biotic stress (Jiang *et al.*, 2017; Rushton *et al.*, 2010). For example, AtWRKY57 competes with AtWRKY33 to regulate the expression of the downstream target genes *JASMONATE ZIM DOMAIN 1* (*JAZ1*) and *JAZ5*, thereby affecting the JA-mediated defence signalling pathway involved in the regulation of *B. cinerea* resistance (Jiang and Yu, 2016). OsWRKY51 binds to the W-box element of the *OsPR10a* promoter and functions as a positive regulator of the defence response against *Xanthomonas oryzae pv. oryzae* (Hwang *et al.*, 2016). Apple MdWRKY79 can bind to and activate the NLR resistance gene, *MdNLR16*, thereby enhancing resistance to *Alternaria alternata* (Meng *et al.*, 2018). In this study, we identified MdWRKY100 as a positive regulator of GLS infection. To further explore the molecular mechanism through which MdWRKY100 regulates GLS infection, we conducted DAP-seq sequencing and found that *MdWRKY17*, *MdPAL1*, and *MdRPM1* were potential target genes of MdWRKY100 (Figure 2a). Furthermore, we demonstrated through Y1H, EMSA, transient expression, and RT-qPCR experiments that MdWRKY100 could bind to the W-box motif of these target gene promoters, thereby inhibiting the expression of *MdWRKY17* and promoting the expression of *MdPAL1* and *MdRPM1* (Figures 2b–e and 6a,b). This result suggests that MdWRKY100 is involved in the response to GLS infection by regulating these three target genes.

Plant SA homeostasis is dependent on SA biosynthesis and metabolism. Under biotic stress, both an increase in SA biosynthesis and a decrease in metabolic levels can enhance plant resistance to biotic stress. During the SA biosynthesis process, PAL is an important enzyme for SA biosynthesis in *Arabidopsis*, tobacco, pepper, soybean, and wheat (Kim and Hwang, 2014; Mauch-Mani and Slusarenko, 1996; Shadle *et al.*, 2003; Shine *et al.*, 2016). For example, the overexpression of pepper *CaPAL1* increases PAL activity and SA content to enhance resistance to microbial pathogen infection in transgenic *Arabidopsis* plants (Kim and Hwang, 2014). The overexpression of

GmPAL2.1 remarkably increased SA content to improve resistance to *Phytophthora sojae* infection in soybean plants (Zhang *et al.*, 2017a). Silencing of wheat *AevPAL1* reduces nematode resistance in roots by decreasing the accumulation of SA (Zhang *et al.*, 2021). The rice TPR-domain RNA-binding protein, BSR-K1, regulates *OspAL1* mRNA levels to confer broad-spectrum disease resistance (Zhou *et al.*, 2018). However, the role of PAL in GLS tolerance remains unclear. In this study, we discovered that MdPAL1 positively contributes to GLS resistance by increasing SA accumulation (Figure 3a–f), indicating that MdPAL1 plays an essential role in GLS infection in apples. Mutations in the tomato SA degradation gene *SIDMR6* confer broad-spectrum resistance to fungi, bacteria, and oomycetes (Thomazella *et al.*, 2021). The CRISPR/Cas9-mediated simultaneous mutation of three *salicylic acid 5-hydroxylase* (*OsS5H/OsDMR6*) confers resistance to rice blast and bacterial blight diseases (Liu *et al.*, 2023). Wheat-susceptible gene TaWRKY76 directly activates *TaDMR6* expression to inhibit SA accumulation and reduce aphid resistance (Zhang *et al.*, 2023b). The apple susceptibility gene MdWRKY17 directly activates the *MdDMR6* expression to promote SA degradation and weaken resistance to GLS (Shan *et al.*, 2021). The present study demonstrated that MdWRKY100 inhibited the expression of *MdWRKY17* and reduced the degradation of SA; however, MdWRKY100 promoted the expression of *MdPAL1*, increased the accumulation of SA, and comprehensively improved the resistance to GLS in apples (Figures 1–3a–f and 6a,b).

CC-NBS-LRR proteins have been proven to play crucial roles in plant responses to pathogen infection in multiple plant species (Du *et al.*, 2021; Grant *et al.*, 1995; Wang *et al.*, 2020, 2021). For example, transgenic rice lines overexpressing *OsRLR1* (*RPM1 like resistance gene 1*) enhanced resistance to rice blast fungi and *Xanthomonas oryzae* through direct interaction with OsWRKY19, which facilitated the function of OsWRKY19 in the activation of the *OsPR10* gene (Du *et al.*, 2021). The dominant rice resistance gene, *OsRSR1* (disease resistance gene *RPM1*), was correlated with sheath blight resistance in a genome-wide association study, and OsRSR1 was validated through overexpression and knock-down assays (Wang *et al.*, 2021). Wheat TaRPM1 positively contributes to *Puccinia striiformis* f. sp. *tritici* resistance in high-temperature wheat seedlings through the BSMV virus-induced gene silencing (VIGS) system (Wang *et al.*, 2020). In addition, silencing of a CC-NBS-LRR gene, *GbRVd*, in cotton through VIGS substantially downregulated the SA content, resulting in increased susceptibility to *Verticillium dahlia* (Yang *et al.*, 2016). However, the role of apple CC-NBS-LRR protein, MdRPM1, in GLS tolerance remains unclear. The present study demonstrated that MdRPM1 positively regulates resistance to GLS in apples by affecting SA content through overexpression and RNAi assays, indicating that MdWRKY100 increases resistance to GLS by increasing SA content and upregulating *MdRPM1* expression (Figures 1–3g,h and 6a,b).

Transcriptional regulators typically inhibit or promote the transcriptional activity of WRKY transcription factors to regulate the transcript levels of their target genes (Jing and Lin, 2015; Li *et al.*, 2014; Pan *et al.*, 2018; Wang *et al.*, 2022). For example, *Arabidopsis* VQ18 and VQ26 physically interact with ABI5 and inhibit its transcriptional activity, thereby negatively modulating the ABA response during seed germination (Pan *et al.*, 2018). VQ29 interacted with PIF1, and the interaction decreased the stability of protein PIF1 and repressed *XYLOGLUCAN ENDO-TRANSGLYCOSYLASE7* expression to mediate the inhibition of

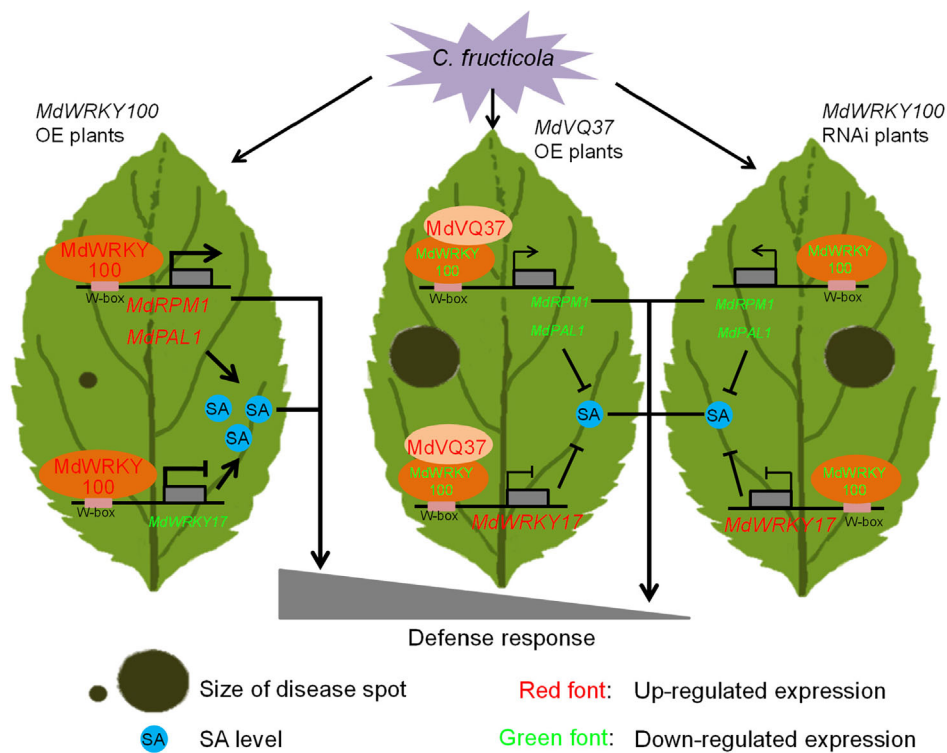


Figure 7 Model for MdWRKY100-MdVQ37 module-mediated GLS resistance in apples. On the one hand, the overexpression of *MdWRKY100* improves *C. fructicola* tolerance, on the one hand by activating *MdRPM1* expression, and on the other hand, the activation of *MdPAL1* expression reduces *MdWRKY17* expression, thereby increasing SA accumulation. However, the overexpression of *MdVQ37* or RNAi silencing of *MdWRKY100* enhances *C. fructicola* susceptibility to inhibit the transcriptional activity of *MdWRKY100*, along with decreases in *MdRPM1* expression and SA accumulation.

hypocotyl elongation during early seedling development in *Arabidopsis* (Li et al., 2014). SIVQ7 acts as a positive interacting partner of the SIWRKY37 transcription factor and increases the transcript levels of *SIWRKY53* and *SISGR1* to regulate dark-induced leaf senescence in tomatoes (Wang et al., 2022). In the present study, MdVQ37 acted as a negative interacting partner of MdWRKY100 and repressed its transcriptional activity of MdWRKY100 to negatively regulate GLS tolerance in apples (Figures 4–6).

In summary, we proposed a functional model of the MdWRKY100-MdVQ37 complex in apples, which responds to GLS infection via two pathways (Figure 7). The overexpression of *MdWRKY100* in transgenic apple plants activated the expression of the target gene *MdRPM1* to improve GLS tolerance. In contrast, *MdWRKY100* upregulated *MdPAL1* expression and downregulated *MdWRKY17* expression, leading to SA accumulation that induces resistance to GLS infection. In contrast, the overexpression of *MdVQ37* in transgenic apple plants inhibited the transcriptional activity of MdWRKY100 through the interaction between its VQ motif and the C-terminus WRKY domain of MdWRKY100, which reduced the expression of *MdRPM1* and SA accumulation, thereby increasing the sensitivity to GLS infection.

Materials and methods

Plant materials and stress treatments

Apples (*Malus domestica* Borkh.) variety ‘Gala’ (GL-3) was used for genetic transformation and expression analysis (Dai et al.,

2013). The overexpression of *MdVQ37* in transgenic apple lines (Dong et al., 2021) and overexpression of RNAi *MdWRKY100* in transgenic apple lines (Zhang et al., 2019b) were performed in our previous studies. For the expression analysis of *MdVQ37*, *MdWRKY100*, *MdRPM1*, and *MdPAL1* under *C. fructicola* infection, *C. fructicola* was cultured on potato dextrose agar medium at 25 °C for 7 d, and the fungal spores were collected and suspended in sterile water until the spore concentration was 10^6 cfu mL⁻¹ for fungal inoculation. The detached, fully expanded leaves of 60-day-old GL-3 plants were sprayed with spore suspension, incubated in a plastic box containing moist filter paper, and covered in a plastic box with vinyl film to maintain humidity at 25 °C. At 0, 48, and 72 h of treatment, detached leaves were collected for gene expression analysis.

To phenotypically assess *MdVQ37* transgenic apple plants and transiently overexpressed or RNA-interfered *MdRPM1* and *MdPAL1*, the detached leaves from overexpression *MdVQ37* transgenic apple lines and WT plants, as well as those from *in vitro*-cultured GL-3 and control seedlings with overexpression or RNA interference of *MdRPM1* and *MdPAL1* at 48 h were inoculated with a 10 µL spore suspension at the center, following the methodology described by Shan et al. (2021). After 5 days of treatment, the detached leaves were collected for phenotypic comparison and SA content analysis.

To phenotype *MdWRKY100* transgenic apple plants, transgenic apple seedlings with overexpression or RNA interference of *MdWRKY100* were sprayed with a spore suspension, incubated in Petri dishes containing moist filter paper at 25 °C, and then

sampled at 0, 4, and 6 days after infection for detecting disease index and SA content.

Gene cloning and expression analysis

To clone *MdVQ37*, *MdWRKY100*, *MdRPM1*, and *MdPAL1*, their complete open reading frames (ORFs) were obtained from the fully expanded leaves of 'Royal Gala' apples via RT-PCR using the specific primers listed in Table S1. The qRT-PCR experiments were conducted according to the manufacturer's instructions using a QuantStudio 5 instrument (Applied Biosystems, Foster City, CA, USA) in conjunction with TransStart Top Green qRT-PCR SuperMix (Transgen Biotech, Beijing, China), as previously described (Dong *et al.*, 2018). The obtained data were calculated and analysed by the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001), and the quantification results were normalized by *MdMDH* (Duan *et al.*, 2023). Three biological replicates were used in each experiment.

Vector construction and genetic transformation

ORFs without the stop codon of *MdRPM1* or *MdPAL1* were ligated into the overexpression (OE) vector pCambia2300. Specific 300 bp fragments of *MdRPM1* and *MdPAL1* were introduced into the RNAi vector pK7GWIWG2D. Overexpression or RNA interference of *MdRPM1* and *MdPAL1* was performed by transiently transforming the leaves of *in vitro*-cultured GL-3 seedlings using *Agrobacterium* strain EHA105 carrying pCambia2300-MdRPM1, pCambia2300-MdPAL1, pK7GWIWG2D-MdRPM1, or pK7GWIWG2D-MdPAL1, as described by Zhang *et al.* (2018) and Shan *et al.* (2021). The expression levels of *MdRPM1* and *MdPAL1* and PAL activity were determined 2 days after transient transformation, and GLS tolerance was recorded 5 days after infection, as described above. The experiment was independently repeated thrice.

Sub-cellular localization assays

The CDS without the stop codons of *MdVQ37* was cloned into the pCambia2300-GFP vector and transformed into *A. tumefaciens* strain 'GV3101' by heat shock. Sub-cellular localization assays were carried out according to the method previously reported (Duan *et al.*, 2023).

Yeast two-hybrid assays

Y2H assays were performed following the manufacturer's instructions (Clontech Laboratories, Mountain View, CA, USA). The full-length MdWRKY100 protein showed strong self-activation, and truncated fragments of MdWRKY100 were used to test these interactions. The truncated fragments of MdWRKY100 were introduced into the pGBT9 bait vector. The full-length cDNA and truncated fragments of MdVQ37 were cloned into a pGAD424 prey vector. Different combinations of these constructs were transformed into the yeast strain 'Y2H Gold' using the lithium acetate method. The transformed yeast cells were cultured on yeast synthetic dropout medium lacking Trp and Leu (SD/-Trp-Leu) and SD/-Trp-Leu-His-Ade to observe yeast growth at 30 °C for 3 days. An assay of β -galactosidase activity was performed using X- α -gal. Empty pGBT9 and pGAD424 vectors were used as negative controls (Dong *et al.*, 2020).

Bimolecular fluorescence complementation assays

The BiFC assays were performed as described by Li *et al.* (2012). Briefly, the coding sequences of MdWRKY100 and MdVQ37 were

amplified by PCR and inserted into pSPYNE-35S and pSPYCE-35S, respectively. The obtained fusion vectors, empty pSPYNE-35S vector, and empty pSPYCE-35S vector were transformed into *A. tumefaciens* strain 'GV3101' and co-injected into tobacco leaves in different combinations. After 48 h of infection, yellow fluorescence in the infected leaves was observed using a confocal laser scanning microscope (LSM510 META; Zeiss, Oberkochen, Germany). 4',6-diamidino-2-phenylindole (DAPI) was used to stain cell nuclei.

Split-luciferase complementation assay

Split-luciferase completion assays were performed as described by Jing *et al.* (2022). Briefly, the coding sequences of MdWRKY100 and MdVQ37 were amplified by PCR and inserted into the pCAMBIA1300-nLuc or pCAMBIA1300-cLuc vectors. The obtained fusion vectors, empty pCAMBIA1300-nLuc vector, and empty pCAMBIA-cLuc vector were transformed into *A. tumefaciens* strain 'EHA105' and co-injected into tobacco leaves in different combinations. After 3 days of infection, 1 mM fluorescein (Promega) was sprayed onto the leaves, and the fluorescence signal in the infected leaves was observed using a CCD (Lumazone Pylon 2048 B).

Pull-down assay

MdWRKY100-HIS and MdVQ37-MBP fusion proteins were produced in *Escherichia coli* strain BL21 and purified using HIS and MBP purification columns (Beyotime, Shanghai, China). Protein mixtures were captured using the MBP Purification Kit (Thermo Fisher Scientific). Eluted proteins were detected using anti-HIS and anti-MBP antibodies.

DAP-seq sampling and data analysis

DNA purification and sequencing were performed by Bluescape Scientific Co., Ltd. (Hebei, China). The plant genomic DNA extraction kit (Tiangen Biotechnology) was used to extract genomic DNA from mature leaves of 'Gala' (Dong *et al.*, 2023). Clean DNA beads (Bluescape Scientific Co., Ltd., Hebei, China) were used to purify the gDNA, and a NEXTFLEX Rapid DNA Seq Kit (PerkinElmer, Inc., Austin, TX, USA) was used to construct an affinity-purified DNA library. The MdWRKY100 coding sequence was cloned into the pFN19K HaloTag T7 SP6 Flexi vector and expressed using a TNT SP6 Coupled Wheat Germ Extraction System (Promega, Madison, WI, USA). The expressed Halo-MdWRKY100 fusion protein was purified and captured using Magne Halo-tag beads (Promega, Madison, WI, USA). The MdWRKY100-bound beads were incubated with adapter-ligated gDNA libraries, and the technology was repeated twice. A negative control lacking MdWRKY100-bound beads was incubated with adapter-ligated gDNA libraries. The eluted DNA was sequenced in Illumina NavoSeq6000. The Bowtie2 software aligned the DAP-seq reads with the apple GDDH13 v1.1 genome (Langmead and Salzberg, 2012). The MACS2 callpeak and IDR software were used to merge the peaks ($P < 0.05$) and score the reliability of the repeated peaks (Zhang *et al.*, 2008b). MEME-CHIP and Homer software were used to analyse the conserved motifs in the peak areas (Machanic and Bailey, 2011) and annotate the bound peaks (Heinz *et al.*, 2010), respectively.

Yeast one hybrid assays

Y1H assay was performed according to the manufacturer's instructions (Clontech Laboratories, Mountain View, CA, USA). The coding sequence of MdWRKY100 was amplified using PCR

and inserted into the pGADT7 vector. The promoters of *MdWRKY17* and *MdRPM1* were inserted into the pAbAi vector. The recombinant plasmids were transformed into the yeast strain Y1H. Transformed yeast cells were cultured in yeast synthetic dropout medium lacking Leu (SD/-Leu) and 200 ng/mL AbA to confirm positive interactions. Empty pGADT7 vector served as a negative control.

Electrophoretic mobility shift assay

MdWRKY100-HIS was obtained as described for the pull-down assay. EMSAs were performed using the LightShift Chemiluminescent EMSA kit (Thermo Scientific, Waltham, MA, USA) according to the manufacturer's instructions. Unlabeled probes were used as competitors, and HIS was used as a negative control, and the biotin-labelled DNA probes are listed in Table S2.

Dual-luciferase transient expression assay

The dual-luciferase transient expression assay assays were performed as described by Yang *et al.* (2023). The promoters of *MdWRKY17* and *MdRPM1* were individually inserted into the pGreenII 0800-LUC vector, and the ORF sequences of *MdVQ37* and *MdWRKY100* were cloned into the pGreenII 62-SK vector. The empty pGreenII 62-SK vector was used as a negative control. These vectors were transformed into *Agrobacterium* strain *GV3101* and co-injected into tobacco leaves in different combinations. After 2 days of incubation, LUC and Renilla luciferase (REN) activities were measured using a dual-luciferase assay kit (Yeasen, Shanghai, China). Transcriptional activation of the target promoters was calculated as the ratio of LUC to REN. At least six transient assays were performed for each combination.

Measurement of SA contents

SA content was determined using 0.1 g leaf samples taken from WT and *MdVQ37* overexpression apple plants after inoculation with *C. fructicola* for 5 days, leaf tissue collected from WT and *MdWRKY100* transgenic apple seedlings after inoculation with *C. fructicola* for 6 days, and infiltrated leaves from MdPAL1-OE, EV(OE), H₂O, MdPAL1-RNAi, and EV(RNAi) after inoculation with *C. fructicola* for 5 days. SA was extracted, purified, and analysed using liquid chromatography-mass spectrometry according to a previously described method (Dong *et al.*, 2021).

PAL activity assay

PAL activity was measured using a PAL activity kit (cas: PAL-2-Y) (Comin, Suzhou, China) according to the manufacturer's instructions. Briefly, 0.1 g of apple leaves was suspended in a 1 mL extraction buffer and centrifuged at 10000g for 10 min. The supernatant was collected as a crude extract for PAL activity assay.

Statistical analyses

All data were analysed using IBM SPSS (version 20) statistical software (IBM, Chicago, IL, USA). One-way analysis of variance (ANOVA) was used to compare statistically significant differences between the control plants and transgenic lines using Duncan's test ($P < 0.05$) or Student's *t*-test ($P < 0.05$, $P < 0.01$).

Accession numbers

Sequence data from this article can be found in the APPLE GENOME DATABASE (<https://iris.angers.inra.fr/gddh13/>) as follows: MdWR

KY100 (MDP0000514115), MdVQ37 (MDP0000248043), MdWRKY17 (MD12G1181000), MdPAL1 (MD07G1172700), MdRPM1 (MD02G1199100), MdCNL1 (MD07G1056600), MdCNL2 (MD11G1069800), and MdCNL3 (MD08G1239100).

Acknowledgements

This work was supported by grants from the Natural Science Foundation of Hebei Province (C2022204086 and C2023204128), the National Natural Science Foundation of China (31401852, 32072524 and 32272655), the Hebei Agriculture Research System (HBCT2024150206), and the Key Research and Development Program of Xingtai City (2022zz064).

Conflict of interest

The authors declare that they have no competing interests.

Author contributions

F.M., Y.M. Y.T., X.Z., and Q.D. conceived and designed the experiments. Q.D., D.D., F.W., K.Y., Y.S., Y.W., and Z.J. performed the experiments. Q.D., D. W., C.X., P.J., H.L., S.G., G.Q., and K.M. analysed the data. Y.M. and Q.D. wrote the manuscript. Q.D., D.W., K.M., and Y.T. revised the manuscript. All authors read and approved the final version of the manuscript.

Conflict of interest

The authors declare no competing financial interests.

Data availability statement

The data that supports the findings of this study are available in the supplementary material of this article.

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Supporting information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Figure S1 Relative expression level of *MdWRKY100* after *C. fructicola* infection.

Figure S2 Genome-wide analysis of *MdWRKY100*-binding sites.

Figure S3 The phylogenetic tree of apple and *Arabidopsis* phenylalanine ammonia-lyase proteins.

Figure S4 The phylogenetic tree of MdRPM1, MdCNL1, MdCNL2, MdCNL3, and *Arabidopsis* NBS-LRR proteins.

Figure S5 The promoter sequence of *MdWRKY17* gene.

Figure S6 The promoter sequence of *MdPAL1* gene.

Figure S7 The promoter sequence of *MdRPM1* gene.

Figure S8 Expression pattern and sub-cellular localization of MdVQ37.

Table S1 MdWRKY100 DAP-seq peaks located upstream of the transcription start site (TSS) of *MdWRKY17*, *MdPAL1* and CC-NBS-LRR resistance genes.

Table S2 A list of primers used in this study.