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R2R3-MYB Transcription Factor MdMYB73 Confers Increased Resistance to the Fungal Pathogen Botryosphaeria Dothidea via the Salicylic acid Pathway in Apple

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Abstract

Background: The MYB transcription factor (TF) family is involved in many biological processes. However, the molecular mechanism of the MYB family in the resistance to apple ring rot remains poorly understood.

Results: Here, the R2R3-MYB subfamily member *MdMYB73* was cloned from "*Royal Gala*," and its function was characterized by a positive regulator in controlling defense against *Botryosphaeria dothidea.* qRT-PCR and GUS staining analysis demonstrated that the expression of *MdMYB73* was notably induced in apple fruits and transgenic calli after inoculation with *B. dothidea.* Overexpression of *MdMYB73* enhanced resistance, while suppressing *MdMYB73* expression weakened resistance to *B. dothidea* in apple calli and fruits. The increased resistance to *B. dothidea* was also observed in *MdMYB73*-expressing *Arabidopsis thaliana.* Interestingly, we found that MdMYB73 improved resistance against *B. dothidea*, possibly by the salicylic acid (SA) pathway, since SA content and expression of SA synthesis- and signaling-associated genes were higher in *MdMYB73* overexpression plant materials compared with wild-type controls after inoculation. In addition, we discovered that MdMYB73 interacts with the *B. dothidea* positive regulator MdWRKY31 and together enhance the resistance to *B. dothidea* in apple.

Conclusions: Overall, these findings shed light on the mechanism by which MdMYB73 enhances the resistance to *B. dothidea*, possibly by regulating the SA pathway.

Background

Diseases caused by pathogenic microorganisms largely limit the productivity and quality of crops. As sessile organisms, plants have evolved a variety of effective defense mechanisms in response to the adverse effects of pathogenic attack. When pathogenic microorganisms invade plants, some conservative pathogen- or microbe-associated molecular patterns on the surface are recognized by plant pattern recognition receptors, which initiate the first layer of plant immune responses, which is called pathogen-associated molecular pattern-triggered immunity (PTI) [1–2]. Some pathogenic microorganisms secrete many effector proteins into plant cell spaces or plant cells, which interfere with PTI, thereby helping pathogenic microorganisms to successfully cause disease. Some effector proteins can be recognized by the resistance proteins and activate another level of immune response, named effector-triggered immunity (ETI). ETI is often accompanied by a hypersensitive response at the infection point and accumulation of salicylic acid (SA), which further induces nearby plant cells to develop system acquired resistance (SAR), which is a non-specialized, systematic, and long-lasting defense mechanism [2].

The transcriptional regulation of defense genes is a vital step in the activation of the plant immune response [3]. In this process, transcription factors (TFs) form a complex regulatory network to precisely regulate plant resistance to pathogens. The MYB gene family is the largest family of TFs and is widely

present in animals and plants. These genes are characterized by a MYB domain consisting of approximately 52 amino acids inserted into the main groove of DNA using acid residues in a helix-to-helix conformation [4]. In plants, the MYB gene family is divided based on the number of adjacent incompletely repeated regions they contain into different subfamilies R1-MYB, R2R3-MYB, R1R2R3-MYB and 4R-MYB [5]. So far, a large variety of functions of the MYB genes have been identified, including the control of multifarious processes such as development, differentiation, metabolism, defense, and responses to biotic and abiotic stresses [6]. MYB TFs play a key regulatory role in plant responses to biotic stress. R2R3-MYB subfamily members play significant roles in the regulation of pathogen resistance in different plants. In wheat, overexpression of *TaRIM1*, *TiMYB2R-1*, and *TaPIMP1* improves resistance to *Rhizoctonia cerealis*, *Gaeumannomyces graminis* and *Bipolaris sorokiniana*, respectively [7–9]. The *PacMYBA* gene in sweet cherry positively regulates resistance to *Pst* DC3000 [10]. Overexpression of *SlMYB49* in tomato enhances resistance to *Phytophthora infestans* [11]. Heterologous expression of the tomato (*Solanum pimpinellifolium*) gene *SpMYB* in tobacco increases resistance to the necrotrophic pathogen *Fusarium oxysporum* and *Botrytis cinerea* [12]. In addition, previous studies on disease resistance of the model plant *Arabidopsis* are more in-depth, and a variety of R2R3-MYB TF genes have been identified, including *AtMYB15*, *AtMYB30*, *AtMYB44*, *AtMYB46*, *AtMYB73* and *AtBOS1/AtMYB108* [5–6, 13]. The R2R3-MYB genes are very important in plant immunity and thus should be further studied.

The salicylic acid (SA) signaling pathway mainly regulates the response to biotrophic/hemibiotrophic pathogens and viruses, whereas the jasmonic acid (JA) signal pathway protects plants against necrotrophic pathogens and insects [14]. SA, as an immune signal that rapidly accumulates after infection by pathogens, can induce programmed cell death (PCD) in plants and induce SAR in plants, allowing plants to develop broad-spectrum resistance to multiple pathogens that subsequently invade [15]. The SA pathway involves an increase in defense signals, which is often consistent with (i) the accumulation of reactive oxygen species (ROS), (ii) higher SA content, and (iii) increased transcription of SA synthesis-related (*PAL*, *EDS1*, *PAD4*, etc.) and signaling-related (*NPR1*, *PR1*, *PR5*, etc.) genes [16]. Exogenous SA can activate the expression of the *PR* gene to promote PCD, thereby developing resistance to pathogens in plants [17]. An increase in the content of endogenous SA could improve the resistance of citrus to citrus canker and extend the storage period by stimulating the plant immune response [18]. However, plant mutants with reduced SA synthesis and accumulation fail to trigger plant defense responses and show susceptibility to pathogens [19]. These reports suggest that the hormone SA is very important in the plant immune response.

Apples are loved by consumers all over the world because of its rich nutrition and unique taste. Apple ring rot is one of the most harmful fungal diseases, and seriously damages branches and fruits in most apple growing areas of China. A previous study has shown that the causal agent of apple ring rot is *Botryosphaeria dothidea* [20]. The ultrastructural characteristics of apple fruit tissues infected with *B. dothidea* indicate that the fungus is a typical hemibiotrophic plant pathogen that parasitizes dying or dead tissues to obtain nutrition [21]. It not only directly reduces apple yield, but also seriously affects apple fruit quality. On the one hand, *B. dothidea* rots apple fruit by secreting toxins and enzymes when it invaded [22]. On the other hand, controlling apple ring rot is difficult because *B. dothidea* is a pathogen

that can latently infect the host during the growth stage, causing fruit rot during maturation or storage [23]. So cultivators had to use more pesticides in order to prevent and control *B. dothidea* during the apple production process. All these will lead to the decline of apple quality and threaten food safety. It is necessary to conduct in-depth studies to improve resistance in apples. Interestingly, *MdPR4*, whose expression is regulated by the SA and JA signaling pathways, enhances resistance to *B. dothidea* by directly inhibiting hyphal growth [24]. *Streptomyces rochei* A-1 treatment stimulates a series of defense responses, including activity of peroxidase and expression of *PAL*, to induce resistance against *B. dothidea* in apple fruits [25]. *MdSYP121* participates in balancing penetration resistance and regulating SA-mediated defense and oxidation-reduction processes in the defense response against *B. dothidea* [26]. However, the reports are largely limited due to the complexity of the molecular regulatory mechanisms against *B. dothidea* in apple.

The functions of many R2R3-MYB genes in apple have been identified, and recent research suggested they mainly function in flower development [27], malic acid accumulation and vacuolar acidification [28–29], anthocyanin synthesis and accumulation [30], and the abiotic stress response [31]. In addition, *MdMYB73* has been identified to play an important role in fruit quality regulation [28] and the abiotic stress response [32]. Overexpression of its homologous gene *AtMYB73* in *Arabidopsis thaliana* improved the resistance to *Bipolaris oryzae* [33]. However, whether MdMYB73 contributes to the increase of resistance to *B. dothidea* in apple and the underlying mechanisms remain largely unknown. Here, the R2R3-MYB subfamily member *MdMYB73*, which encodes a TF, was cloned from "*Royal Gala*," and it was characterized to function as a positive regulator in controlling the defense against *B. dothidea.* In addition, we showed that MdMYB73 interacts with the WRKY TF MdWRKY31 and together enhance the resistance to *B. dothidea* in apple. Finally, the potential application of *MdMYB73* in increasing resistance to apple ring rot is discussed.

Results

MdMYB73expression is induced by the fungal pathogenB. dothideain apple

To characterize the function of MdMYB73 in the resistance to apple ring rot, apple fruits were inoculated with *B. dothidea*. qRT-PCR results showed that *MdMYB73* expression was significantly induced by *B. dothidea* (Fig. 1a). To further verify the response of *MdMYB73* to *B. dothidea*, the fusion vector pMdMYB73::GUS, containing the GUS encoding gene driven by the *MdMYB73* promoter, was stably introduced into apple calli. GUS staining assays showed that GUS activity in calli inoculated with *B. dothidea* was much higher than that in uninoculated controls (Fig. 1b, c). These results suggest that *MdMYB73* expression is induced by the fungal pathogen *B. dothidea*, and responds to its infection in apple.

MdMYB73is a positive regulator of the defense response to the fungal pathogenB. dothideain apple calli

To confirm its function in the resistance to *B. dothidea*, overexpression and suppression vectors of *MdMYB73* were transformed into apple "*Orin*" calli. qRT-PCR results showed that these vectors were successfully introduced into apple calli (Fig. 2a). Subsequently, wild-type calli (WT), overexpression calli (OVX), and antisense transgenic calli (ANTI) were inoculated with *B. dothidea* for 4 days. As a result, we found that the plaque areas in *MdMYB73*-OVX and *MdMYB73*-ANTI calli were smaller and larger than in WT controls, respectively (Fig. 2b, c).

ROS exerts immune and signaling transduction effects in plant immune responses [34]. After inoculation, we measured the H_2O_2 and oxygen free radical (OFR) content. The content of H_2O_2 and OFR in *MdMYB73*-OVX calli was significantly higher than in WT controls, and in *MdMYB73*-ANTI calli it was the lowest (Fig. 2d, e). These results suggest that *MdMYB73* is a positive regulator of the defense response to *B. dothidea* in apple calli.

The increased resistance ofMdMYB73toB. dothideain apple calli is closely related to SA content and SA synthesis- and signaling-associated gene expression

The plant hormone SA plays a pivotal role in disease resistance signaling. SA content of WT, *MdMYB73*-OVX, and *MdMYB73*-ANTI apple calli was detected after inoculation with *B. dothidea* in order to study the disease resistance mechanism of *MdMYB73.* The content of SA in *MdMYB73*-ANTI, WT, and *MdMYB73*-OVX calli was increased sequentially (Fig. 3a). At the same time, the expression levels of SA synthesis-associated genes (*PAL*, *EDS1*, and *PAD4*) and SA signaling-associated genes (*PR1*, *PR5*, and *NPR1*) were measured after inoculation of apple calli with *B. dothidea*. The expression levels of SA-associated genes in *MdMYB73*-OVX calli were all the highest in *MdMYB73*-ANTI calli and the lowest in *MdMYB73*-ANTI calli (Fig. 3b–g). These results indicate that the *MdMYB73*-conveyed resistance to *B. dothidea* is closely related to the increase in SA signaling in apple calli.

MdMYB73expression in apple fruits increases resistance toB. dothidea

To further verify the function of *MdMYB73*, the recombinant vectors *MdMYB73*-IL60 and *MdMYB73*-TRV, which could transiently induce and suppress the expression of *MdMYB73*, respectively, were infiltrated into "*Fuji*" apple fruits. The empty vectors IL60 and TRV were used as negative controls. *MdMYB73*-IL60 injection significantly increased *MdMYB73* expression and *MdMYB73*-TRV injection significantly decreased *MdMYB73* expression after infiltration for 2 days (Fig. 4b). Subsequently, these fruits were

inoculated with *B. dothidea.* After 4 days, it was found that the plaque areas in *MdMYB73*-IL60-injected fruits were smaller than in IL60 controls, and the plaque areas in *MdMYB73*-TRV-injected fruits were larger than in TRV controls (Fig. 4a, c). Next, we measured the content of H_2O_2 and OFR, and found that it was significantly higher in *MdMYB73*-IL60-injected fruits and significantly lower in *MdMYB73*-TRV-injected fruits relative to the controls (Fig. 4d, e). In addition, after inoculation with *MdMYB73* transgenic *Arabidopsis* for 4 days, we also found that the plaque areas of the overexpression strains (OE-1, 2, and 3) were smaller (Fig. S1a) and the content of H_2O_2 and OFR was higher in *Arabidopsis* overexpression strains than in wild-type Col controls (Fig. S1b–c). More callose deposition in ectopic expression lines was observed than in wild-type Col controls (Fig. S1d). These results further support that *MdMYB73* is a positive regulator of the defense response to the fungal pathogen *B. dothidea* in apple fruits, just as in apple calli (Fig. 2b–e).

The increased resistance ofMdMYB73toB. dothideain apple fruits is associated with SA content and SA synthesis- and signaling-associated gene expression

After the apple fruits were infiltrated with *MdMYB73*-IL60, IL60, *MdMYB73*-TRV, or TRV for 2 days and then inoculated with *B. dothidea* for 4 days, the SA content and the relative expression levels of SA synthesis- and signaling-associated genes in these apple fruits were measured. The results demonstrate that SA content in *MdMYB73*-TRV-injected fruits was much lower than in the TRV control; however, SA content was higher in *MdMYB73*-IL60-injected fruits relative to the IL60 control (Fig. 5a). Simultaneously, the relative expression levels of SA synthesis-associated genes (*EDS1*, *PAD4*, and *PAL*) and SA signaling-associated genes (*PR1*, *PR5*, and *NPR1*) were measured. The expression levels of these genes were all the highest in *MdMYB73*-IL60-injected and the lowest in *MdMYB73*-TRV-injected fruits (Fig. 5b–g). After inoculating *Arabidopsis* with *B. dothidea* for 4 days, we obtained similar results, i.e., SA content and the expression of SA-related genes were significantly upregulated in transgenic *Arabidopsis* overexpressing *MdMYB73* compared with wild-type Col controls (Fig. S1e). These results further suggest that the increased resistance of *MdMYB73* to *B. dothidea* in apple fruits is associated with SA content and with SA synthesis- and signaling-associated gene expression, just as in apple calli (Fig. 3b–g).

MdMYB73 interacts with theB. dothideapositive regulator MdWRKY31

To further clarify the mechanism underlying the resistance conveyed by *MdMYB73* to *B. dothidea*, a yeast two-hybrid screen of an apple cDNA library was performed using MdMYB73 protein as bait. Among the positive clones identified and sequenced, the WRKY TF MdWRKY31 was found. It has been reported that *MdWRKY31* can positively regulate the resistance to *B. dothidea* in apples [35]. Subsequently, the yeast two hybrid assays were used for confirming the interaction between MdMYB73 and MdWRKY31. As a

result, we found MdMYB73 interacted with MdMdWRKY31, but other negative controls were not (Fig. 6a). For further verification, a pull-down assay showed that GST-tagged MdMYB73 and His-tagged MdWRKY31 physically interacted *in vitro* (Fig. 6b). In the bimolecular fluorescence complementation assay, a yellow fluorescent signal appeared in the combination of MdMYB73-YFP^N and MdWRKY31-YFP^C, but no yellow fluorescent signal was observed in the control combinations (Fig. 6c), confirming that MdMYB73 and MdWRKY31 interact with each other. These results indicate that MdMYB73 interacts with the *B. dothidea* positive regulator MdWRKY31.

MdMYB73 and MdWRKY31 together enhance the resistance toB. dothidea

For further evaluate how MdMYB73 and MdWRKY31 regulate plant pathogen defense against *B. dothidea*. The two viral recombinant vectors *MdMYB73*-TRV and *MdWRKY31*-TRV, were used for fruit infiltration into "*Red delicious*", and the empty vector TRV was used as a control. The results showed that the gene expression levels of *MdMYB73* and *MdWRKY31* were strongly decreased in relative to those of TRV control after being infiltrated with *MdMYB73*-TRV and *MdWRKY31*-TRV for 2 days, respectively (Fig. 7b).

Subsequently, the apples were inoculated with *B. dothidea*. After 4 days, our results showed that the fungal plaque areas of *MdMYB73*-TRV + *MdWRKY31*-TRV-injected fruits was the biggest and TRV controls was the smallest in all injected fruits (Fig. 7a, c). Next, we measured the content of H_2O_2 and OFR. The results that it was the lowest in *MdMYB73*-TRV *+ MdWRKY31*-TRV-injected fruits and the highest in TRV control (Fig. 7d, e). These results indicate that the interaction of MdMYB73 and MdWRKY31 together enhance the resistance to *B. dothidea*.

In addition, we also monitored SA content and the relative expression levels of SA synthesis- and signaling-associated genes after the apple fruits were infiltrated with *MdMYB73*-TRV + *MdWRKY31*-TRV, *MdMYB73*-TRV, *MdWRKY31*-TRV or TRV for 2 days and then inoculated with *B. dothidea* for 4 days. As a result, SA content and the transcription levels of SA synthesis- and signaling-associated genes were the lowest in *MdMYB73*-TRV + *MdWRKY31*-TRV-injected fruits and were the highest in TRV control (Fig. 7f–h). These fully indicate that MdMYB73 and MdWRKY31 together enhance the resistance to *B. dothidea* by SA pathway.

We also analyzed the promoters of *MdMYB73* and *MdWRKY31* (Fig. S2). The results showed that the promoter of *MdMYB73* contained a W-box motif which might be bound by MdWRKY31 protein [35]. And the promoter of *MdWRKY31* existed a potential MYB-binding *cis*-element that has been reported to be bound by MdMYB73 protein [28]. These results suggest that MdMYB73 and MdWRKY31 may have a direct regulatory relationship to jointly enhance resistance against *B. dothidea*.

Taken together, these results support that the interaction of MdMYB73 and MdWRKY31 could improve resistance against *B. dothidea* by SA pathway in apples.

Discussion

The quality and safety of agricultural products is a major social issue related to human health and life safety. Diseases and pesticide pollution seriously affect the quality of apples. Apple ring rot, which is caused by *B. dothidea*, a hemibiotrophic pathogen, severely restricts the development of the apple industry [20–21, 23]. Studies of the defense mechanism of plants against pathogenic microorganisms can be of great significance to the healthy development of modern agriculture. In *Arabidopsis*, *AtMYB44, AtMYB73*, and *AtMYB96* enhance the resistance to fungal pathogens [6, 33, 36]. In this study, we found that the MYB TF *MdMYB73* enhances the resistance to *B. dothidea* by the SA signaling pathway in apple and *Arabidopsis*. Furthermore, MdMYB73 interacts with MdWRKY31 and together enhance resistance against *B. dothidea* in apples. These findings provide evidence that MdMYB73 plays a key role in the regulation of plant pathogen resistance.

The production of ROS is a sign of successful recognition of infection and activation of plant immunity. ROS could help to establish a physical barrier at the site of interaction of many pathogens via the oxidative crosslinking of precursor cells during the local synthesis of lignin and suberin polymers, mediate the generation of phytoalexins and secondary metabolites leading to inhibition of pathogen growth, and even directly kill pathogens [37]. In addition, ROS have a signaling function that mediates the activation of defense genes and the establishment of additional defenses [38]. In this study, in apple calli and fruits and *Arabidopsis* overexpressing *MdMYB73*, after inoculation, ROS content was significantly higher than in the control, and an increased resistance to *B. dothidea* was observed (Fig. 2d, e; Fig. 4d, e; Fig. 7d, e; Fig. S1b, c). This was consistent with the notion that increased ROS levels are often closely related to an increase in plant resistance.

SA accumulation is a key factor in multiple modes of disease resistance [16]. EDS1, PAD4, and PAL are involved in SA synthesis and play an important role in the plant immune response [39]. In this study, the expression of SA synthesis-related genes (*EDS1*, *PAD4*, and *PAL*) was significantly increased in the *MdMYB73* overexpression lines and was significantly lower in the *MdMYB73* suppression expression lines after inoculation with *B. dothidea* (Fig. 3b–d; Fig. 5b–d; Fig. 7g; Fig. S1e). This was consistent with the changing trend of SA content (Fig. 3a; Fig. 5a; Fig. 7f; Fig. S1e). Therefore, we speculate that the increased SA content is caused by the stimulatory effects of MdMYB73 on the expression of SA synthesis-related genes (*EDS1*, *PAD4*, and *PAL*) after inoculation with *B. dothidea*. In tobacco and *Arabidopsis*, the reduction of endogenous SA accumulation leads to (i) an inability to induce SAR and (ii) decreased resistance to pathogenic microorganisms, whereas normal resistance can be restored by the addition of exogenous SA functional analogs [16]. Similarly, after inoculation with *B. dothidea*, SA content in all *MdMYB73* suppression expression lines was significantly reduced, leading to a decreased resistance to *B. dothidea*. Opposite results were obtained in the plant materials overexpressing *MdMYB73* (Fig. 3a; Fig. 5a; Fig. 7f; Fig. S1e). Thus, we suspect that the increased resistance to *B. dothidea* is

mediated by SA. To further analyze the SA response, we monitored the expression levels of SA signaling-related genes (*PR1*, *PR5*, and *NPR1*). The expression of pathogen-related proteins (PRs), which indicates the activity of SA signaling, can be induced by pathogens [40]. PR1 and PR5 play an important role in SAR against pathogenic microorganisms [41]. After infection with *B. dothidea*, we monitored the mRNA expression levels of *PR1* and *PR5*, and found that the expression of both *PR1* and *PR5* in *MdMYB73* overexpression and lines was higher and lower compared with the control lines, respectively (Fig. 3e, f; Fig. 5e, f; Fig. 7h; Fig. S1e). *NPR1*, a downstream gene of the SA signaling pathway, encodes a crucial transcriptional coactivator of a series of defense-related genes [42]. In this study, the expression level of *NPR1* was consistent with the trend of *PR1* and *PR5* after inoculation with *B. dothidea* (Fig. 3g; Fig. 5g; Fig. 7h; Fig. S1e). On the other hand, SA could promote callose deposition on the cell wall to form a physical defense against pathogens [43], and also enhance ROS accumulation which in turn activates the synthesis of SA in a self-amplifying loop [44]. We measured callose deposits in *Arabidopsis* leaves and found that SA promotes callose deposition (Fig. S1d). The trend of ROS levels was consistent with SA levels in all plant materials examined (Fig. 2d, e; Fig. 4d, e; Fig. 7d, e; Fig. S1b, c). Therefore, we speculate that SA improves resistance to *B. dothidea* by increasing PR1, PR5, and NPR1 expression, callose deposition, and ROS levels.

The WRKY family is one of the largest transcription families in higher plants and plays an important role in biotic stress. Most WRKY genes respond to pathogen attack and to the endogenous signaling molecule SA [45]. Previous research has shown that WRKY15, 31, and 46 could strengthen the resistance of apple against *B. dothidea* through the SA signaling pathway involving different molecular mechanisms [35, 46–47]. Interestingly, here we found that MdMYB73 interacts with MdWRKY31, as verified by multiple methods (Fig. 6). And MdMYB73 and MdWRKY31 can together promote the resistance against *B. dothidea* in apple fruits (Fig. 7). In addition, we analyzed the promoters of *MdWRKY31* and *MdMYB73* (Fig. S2). The results showed that the promoter of *MdWRKY31* contains a MYB-binding *cis*-element, which has been reported to interact with MdMYB73 [28]. The promoter of *MdMYB73* contains a W-box motif which has been reported to interact with MdWRKY31 [35]. In *Arabidopsis*, AtMYB44 regulates antagonistic interaction by activating SA-mediated defenses and inhibiting JA-mediated defenses through directly binding to the promoter of *AtWRKY70* [48]. MYB response elements have been identified in the promoter regions of WRKY genes, and there may be a regulatory relationship between the two families [49]. Based on these results, we speculate that there might be a direct regulatory relationship between MdWRKY31 and MdMYB73, affecting the expression of SA synthesis- and signaling-associated genes. Zhao et al. reported that MdWRKY31 could inhibit the expression of *hypersensitive-induced reaction 4* (*MdHIR4*), enhancing the resistance against *B. dothidea* through the SA signaling pathway [35]. In addition, *MdHIR4* could reduce the resistance to *B. dothidea* by changing the expression of SA-related genes and also participate in the responses to other biotic stresses through the JA signaling pathway by directly interacting with JAZ proteins [50]. These results provide a new perspective that MdMYB73 might be involved in the crosstalk between the SA and JA signaling pathways, regulating plant resistance responses. In addition, in apple fruits, low soluble sugar levels, low pH values, and high concentrations of antibacterial substances (acids, phenols, etc.) could slow down the growth of fungi

[51]. The hyphae of apple ring rot grow best in a medium of pH 7–8 [52]. MdMYB73 could promote malate accumulation and vacuolar acidification, leading to lower pH in apples [28], which might also be one of the mechanisms underlying the increased resistance to *B. dothidea*. These observations further clarify the functional mechanisms by which *MdMYB73* improves resistance to *B. dothidea*.

Conclusions

Based on the results of this study, we present a model explaining the functional mechanisms by which *MdMYB73* strengthens resistance against *B. dothidea* in apples (Fig. 8). In this model, *MdMYB73* expression is significantly induced by *B. dothidea*, promoting the expression of SA synthesis-associated genes (*EDS1*, *PAD4*, and *PAL*) and increasing SA content. Subsequently, the higher SA levels trigger (i) the expression of the SA signaling-related genes (*PR1*, *PR5*, and *NPR1*), (ii) ROS accumulation, and (iii) callose deposition, strengthening the resistance against *B. dothidea.* MdMYB73 interacts with MdWRKY31 and together enhance the resistance to *B. dothidea*, but the specific mechanisms require further research. A previous study showed that MdWRKY31 could inhibit *MdHIR4* expression, improving the resistance against *B. dothidea* by the SA signaling pathway [35]. However, overexpression of *MdMYB73* leads to lower pH in apples [28, 53], which might be another mechanism by which *MdMYB73* enhances the defense abilities against *B. dothidea.*

In short, this study showed that overexpression of *MdMYB73* allows apples and *Arabidopsis* to acquire *B. dothidea* fungal resistance phenotypes. And the interaction of MdMYB73 and MdWRKY31 together enhance resistance to *B. dothidea*. These results indicate that the *MdMYB73* gene has a great potential in breeding crop plants with boosted immunity to apple ring rot. Moreover, identification of novel TF genes and elucidation of their roles in the regulation of the expression of important genes will improve our understanding of signaling pathways. Meanwhile, measuring the expression levels of the known functional genes can provide references for molecular breeding and the development of plant vaccines. These are of great significance to ensure the sustainable development of agriculture and human health.

Materials And Methods

Plant materials and growth conditions

The apple calli used in this study was induced from the immature embryo of apple cultivar "*Orin*" (*Malus* × *domestica* Borkh.). They were grown on Murashige and Skoog (MS) medium supplemented with 1.5 $mg{\cdot}L^{-1}$ 2, 4-dichlorophenoxyacetic acid (2, 4-D), 0.4 $mg{\cdot}L^{-1}$ 6-benzylaminopurine (6-BA), 30 $g{\cdot}L^{-1}$ sugar and 7.5 $g{\cdot}L^{-1}$ agar at 24°C under dark conditions.

Apple fruits used for the injection of viral vectors were collected from 15-year-old apple trees of the cultivar "*Fuji*" and "*Red delicious*", which were grown in an experimental orchard in Linyi city, Shandong province, China. "*Fuji*" and "*Red delicious*" apple fruits were bagged at 35–40 days after blooming (DAB), harvested at 165 DAB and 130 DAB respectively, and de-bagged before injection.

Seeds of *A. thaliana* ecotype “Columbia” (Col) were sterilized with 75% alcohol and 2% sodium hypochlorite (NaClO) and sown on MS semi-solid medium (8 $g \cdot L^{-1}$ agar and 30 $g \cdot L^{-1}$ sucrose added). They were placed at 4°C for vernalization for 3 days, taken out, and grown in a light growth chamber under a 16 h/8 h light/dark cycle (200 $\mu mol \cdot m^{-2} \cdot s^{-1}$) and at 21°C/18°C (day/night) for 9 days. Then they were cultivated in nutrient soil and grown in an intelligent glasshouse under the same conditions as the incubator.

Nicotiana benthamiana was sown on nutrient soil, and Hoagland nutrient solution was added every week in a greenhouse at 24°C and under a 16 h/8 h light/dark cycle (200 $\mu mol \cdot m^{-2} \cdot s^{-1}$).

B. dothidea grew on potato dextrose agar (PDA) at 28°C without other pathogenic microorganisms in the dark. After 3–4 days, the culture medium was evenly covered with white hyphae of *B. dothidea* in good condition and could be used to inoculate plant materials at this time.

RNA extraction

RNA was extracted from apple calli, apple fruits and *Arabidopsis*, respectively, using RNA Plant Plus Reagent Kit (Qiagen, Shanghai, China). The plant materials were quickly frozen in liquid nitrogen and ground into a powder in a mortar which was treated at 200°C for 4 hours to remove RNase. Then reagents were added in sequence and operations were performed according to instructions of the plant RNA extraction kit. The RNA was stored at -80°C before further use.

MdMYB73 cloning, construction of the expression vectors, and genetic transformation

Primers *MdMYB73*-F: 5’-ATGGAAGCGATGAATATGTGC-3’ and *MdMYB73*-R: 5’-TTAATTTAATCTATGAAGCTC-3’ were designed based on the sequence retrieved from the Apple Genome and Epigenome. RNA extracted from the cultivar “*Royal Gala*” was reverse transcribed into cDNA via the PrimeScript™ RT reagent kit with a gDNA Eraser (Perfect Real Time, TaKaRa, Dalian, China) and used as template for gene amplification. The reaction conditions were as follows: pre-denaturation at 95°C for 5 min; followed by 30 cycles of denaturation at 95°C for 35 s, annealing at 54°C for 35 s, and extension at 72°C for 1 min; and a final extension step at 72°C for 7 min. The products were electrophoresed on a 1.3% agarose gel, the DNA band was cut out, and DNA was recovered. *MdMYB73* was inserted into the pMD19-T cloning vector, which was transformed into competent *E. coli* trans5α cells and sequenced.

The *MdMYB73* DNA fragment was cloned from the cloning vector into the plant expression vector pRI101, downstream of the CaMV 35S promoter, to obtain an overexpression vactor. The sequence of the 5’-terminal part of *MdMYB73* was reverse constructed into pRI101 to obtain a suppression vector. These plasmids were transformed into *Agrobacterium tumefaciens* LBA4404, which were prepared for infection to obtain stable expression plants.

The *MdMYB73* promoter from the cultivar "*Royal Gala*" was inserted into the plasmid pBI121-GUS, which carries the GUS reporter gene. The fusion vector pMdMYB73::GUS was then genetically transformed into calli by the *Agrobacterium*-mediated method. Finally, the transgenic calli were used for GUS staining and the GUS activity assay.

The *MdMYB73* overexpression vector was introduced into *Arabidopsis* by *Agrobacterium*-mediated method, then the overexpressed *Arabidopsis* strains were obtained, which were named as OE-1, OE-2, and OE-3. The *A. tumefaciens* LBA4404 strain carrying the overexpression vector and suppression vector was used to infect the wild-type (WT) calli to obtain overexpression (OVX) and antisense expressing (ANTI) transgenic apple calli. The *Agrobacterium*-mediated genetic transformation of apple calli was performed as described by Hu et al. [54].

Construction of the viral vectors and transient expression in apple fruits

To construct an antisense viral expression vectors, the specific fragments of *MdMYB73* and *MdWRKY31* from the apple fruit cDNA library were amplified and inserted into the antisense TRV vectors under the control of the dual 35S promoter. The resulting vectors (*MdMYB73*-TRV and *MdWRKY31*-TRV) were transformed into *A. tumefaciens* LBA4404, which were injected into apple fruits.

To generate viral overexpression vectors, the full-length cDNA of *MdMYB73* was inserted into the IL60 vector under the control of the 35S promoter. The resulting vector (*MdMYB73*-IL60) was transformed into competent *E. coli* trans5α cells. Large amounts of plasmid were produced for injection.

Fruit injections were performed as previously described by Hu et al. [28].

Quantitative real-time PCR (qRT-PCR) analysis of gene expression

Firstly, the DNA in the total RNA of plants were completely removed by DNase. Secondly, the first strand cDNA was synthesized using the RNA from the previous step by PrimeScript First Strand cDNA Synthesis Kit (Takara, Liaoning, China). Finally, the cDNA was used as templates to conduct real-time quantitative PCR (qRT-PCR) to detect genes expression level via Bio-Rad IQ5 (Hercules, CA, USA). The relative gene expression levels were normalized to the expression of *MdActin* in apple and *AtActin* in *Arabidopsis*. Amplification was carried out using UltraSYBR Mixture (High ROX) (CWBIO, Beijing, China) according to the instructions. The thermal profile for SYBR Green I real-time PCR was 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. The qRT-PCR reaction was repeated at least three biological replicates and the primers used in this study were shown in Table S1.

Pathogen infection assays

Under sterile conditions, MS medium supplemented with 1.5 mg·L^{-1} 2, 4-D, 0.4 mg·L^{-1} 6-BA, 30 g·L^{-1} sugar, and 7.5 g·L^{-1} agar powder was poured into the sterile petri dishes. Wild-type calli and transgenic "*Orin*" calli with the *MdMYB73* gene were subcultured on these medium for 12 days at 24 °C under dark conditions, than were inoculated with 0.5 cm agar disks containing uniform *B. dothidea* hyphae then were cultivated for 5 days on PDA, and cocultured for 4 days in the dark. Apple fruits were inoculated with the same agar disks as the inoculated "*Orin*" calli and cocultured for 4 days in a dark and humid environment at 24°C. Photos of infected calli and apple fruits were taken, and fungal plaque areas were measured and analyzed by ImageJ software. Each test was repeated more than three times.

The leaves of wild-type Col and transgenic *Arabidopsis* with the same growth state and the same leaf order were removed and then inoculated with 0.5 cm agar disks containing uniform *B. dothidea* hyphae. The inoculated leaves were placed in a damp petri dish and cocultivated in the growth chamber of *Arabidopsis* for 4 days. At least 30 leaves were included per treatment.

Determination of SA contents

The method of SA determination was followed as previously described by Han et al. [34]. The experiments were repeated for three times with similar results.

Measurement of ROS

Hydrogen peroxide (H_2O_2) and oxygen free radical (OFR) were measured by using a hydrogen peroxide assay kit (spectrophotometry) (Suzhou Comin Biotechnology Co., Suzhou, China) and the oxygen free radical assay kit (spectrophotometry) (Suzhou Comin Biotechnology Co., Suzhou, China), respectively. The operation methods referred to the kit instructions (http://www.cominbio.com/).

The infected *Arabidopsis* leaves were stained by 3', 3'-diaminobenzidine and nitroblue tetrazolium, the methods were described by Hu et al. [55]. The staining areas were calculated by ImageJ software [34].

Each treatment was repeated for more than three times independently, and similar results were obtained.

Determination of chlorophyll content and callose staining

After inoculating *B. dothidea* on *Arabidopsis* leaves for 4 days, leaves were shredded and mixed, 0.1–0.2 g per serving was weighed, and the materials were put into a test tube with 10 ml absolute ethanol and soaked overnight at room temperature protected from light. When all the leaves turned white, the extracting solution was aspirated and used to determine the chlorophyll content with a

spectrophotometer. Chlorophyll content (mg/g FW) was calculated as follows: chlorophyll content = (6.63 × OD_{665} + 18.08 × OD_{649}) × V_T/W. Each treatment was repeated three times.

Callose deposits in the leaves were visualized by aniline blue staining and callose deposition was statistically analyzed using ImageJ software as previously described by Han et al. [34].

Yeast two-hybrid assay

The full length *MdWRKY31* was inserted into the pGAD424 vector, while the full length *MdMYB73* was constructed into the pGBT9 vector. The pGBT9-MdMYB73 and pGAD424-MdWRKY31 fusion vectors were simultaneously co-transferred into yeast competent cells (Y2H Gold strain). Subsequently, these yeasts plated on the medium lacking Leu and Trp (SD/-Leu-Trp) for the transformation control. Then the colonies were transferred to medium lacking Leu, Trp, His and Ade (SD/-Leu-Trp-His-Ade) for interaction screening.

Pull-down assay

For the GST pull-down assays, *MdMYB73* DNA fragment was constructed into the pGEX-4T-1 vector, and *MdWRKY31*was inserted into pET-32a. All fusion proteins were used to perform the GST pull-down assays as described by Han et al. [56].

Biomolecular fluorescence complementation (BiFC) assay

The yellow fluorescent protein (YFP) gene is divided into two parts, N-terminal region and C-terminal region, which exist on plasmids pSPYNE-*35S* and pSPYCE-*35S*, respectively. The full-length gene *MdMYB73* and *MdWRKY31* were inserted into pSPYNE-*35S* and pSPYCE-*35S*, resulting in fusion vectors MdMYB73-YFP^N and MdWRKY31-YFP^C, respectively. Next, these fusion vectors were transformed into *A. tumefaciens* LBA4404, which was co-injected into tobacco leaves, and the lower epidermis of tobacco was taken after 2–3 days to observe and photograph using a LSM 880 META confocal microscope (Zeiss LSM 510 META, Jena, Germany).

Statistical analysis

All experiments were performed in triplicate. Error bars show the standard deviation of three biological replicates. DPS software was used for data analysis and bars with different letters were significantly different ($P < 0.05$) by Tukey's single factor tests. Data were shown as mean ± standard deviation (SD).

Abbreviations

B.dothidea: *Botryosphaeria dothidea*; Col: Columbia; TF: Transcription factor; qRT-PCR: Quantitative real-time PCR; DAB: Days after blooming; FW: Fresh weight; PDA: Potato dextrose agar; GUS: β-glucuronidase; NaClO: Sodium hypochlorite; SA: Salicylic acid; JA: Jasmonic acid; PTI: Pathogen-associated molecular pattern-triggered immunity; ETI: Effector-triggered immunity; EDS1: Enhanced disease susceptibility 1; PAD4: Phytoalexin deficient; PAL: Phenylalanine ammonialyase; NPR1: Non-expressor of pathogenesis-related genes 1; PR: Pathogenesis-related protein; ROS: Reactive oxygen species; SAR: System acquired resistance; PCD: Programmed cell death; Y2H: Yeast two-hybrid systems; BiFC: Biomolecular fluorescence complementation assay

Declarations

Acknowledgments

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Authors' contributions

Y.-J.H. and D.-G.H. planned and designed the research. K.-D.G., Q.-Y.Z., J.-Q.Y., J.-H.W., F.-J.Z., C.-K.W., Y.-W.Z., C.-H.S. and C.-X.Y. performed experiments, conducted fieldwork, analysed data etc. K.-D.G. and D.-G.H. wrote the manuscript. All authors read and approved the final manuscript.

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Availability of data and materials

All data generated or analysed during this study are included in this published article and its supplementary information files.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare no conflicts of interest.

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Figures

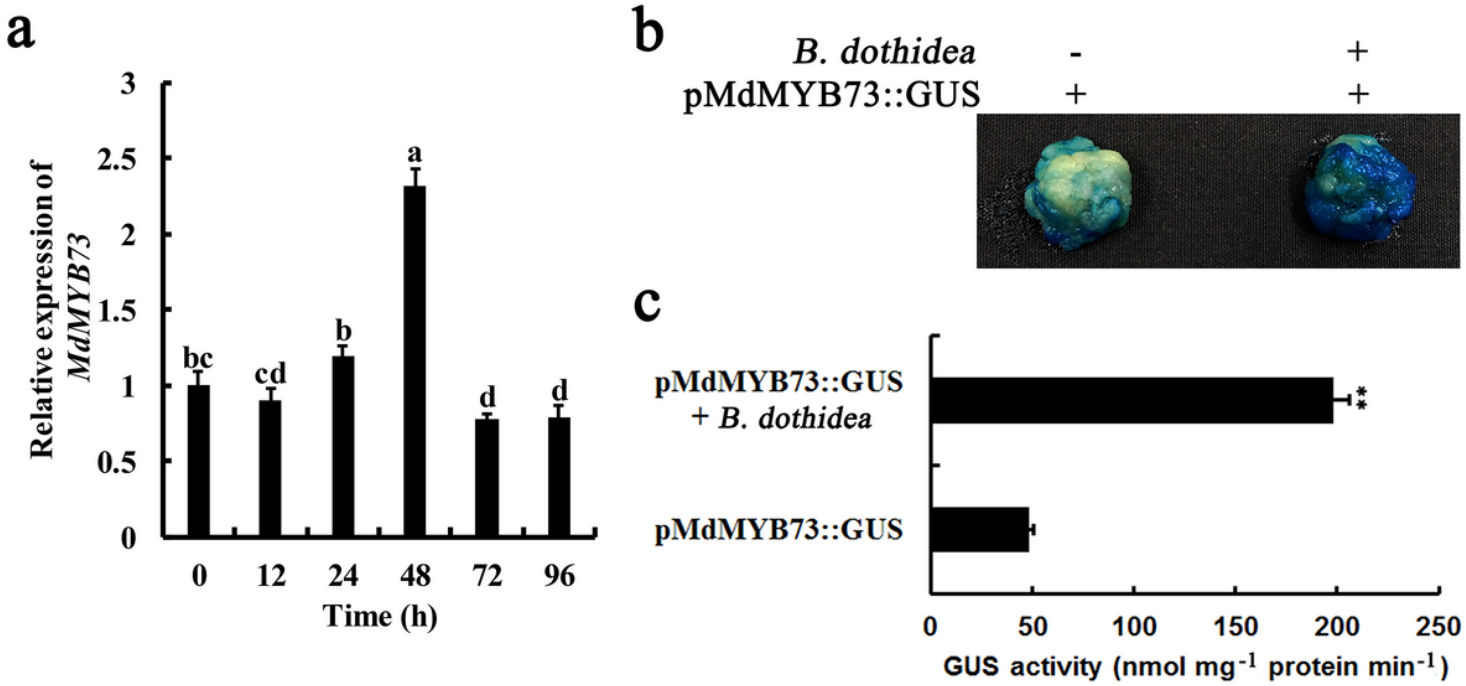
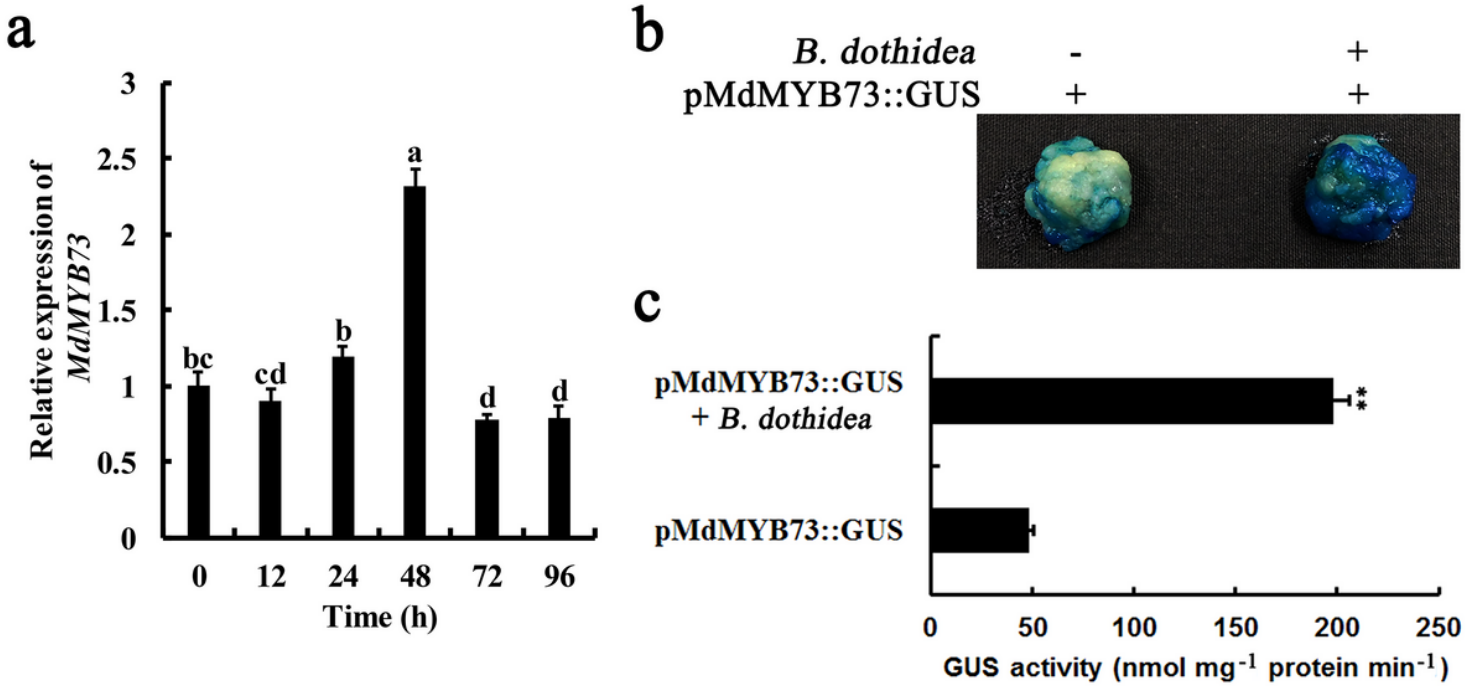


Figure 1

MdMYB73 responds to the induction of B. dothidea in apples. a Relative expression of MdMYB73 in “Fuji” apple fruits infected with B. dothidea after 0, 12, 24, 48, 72, and 96 h. b GUS staining analysis of the transgenic calli of pMdMYB73::GUS after infected and non-infected with B. dothidea. c GUS activity analysis of the transgenic calli of pMdMYB73::GUS after infected and non-infected with B. dothidea. Bars with different letters are significantly different ($P < 0.05$) according to Tukey’s single factor tests. Data are shown as mean ± SD; experiments were replicated at least three times.



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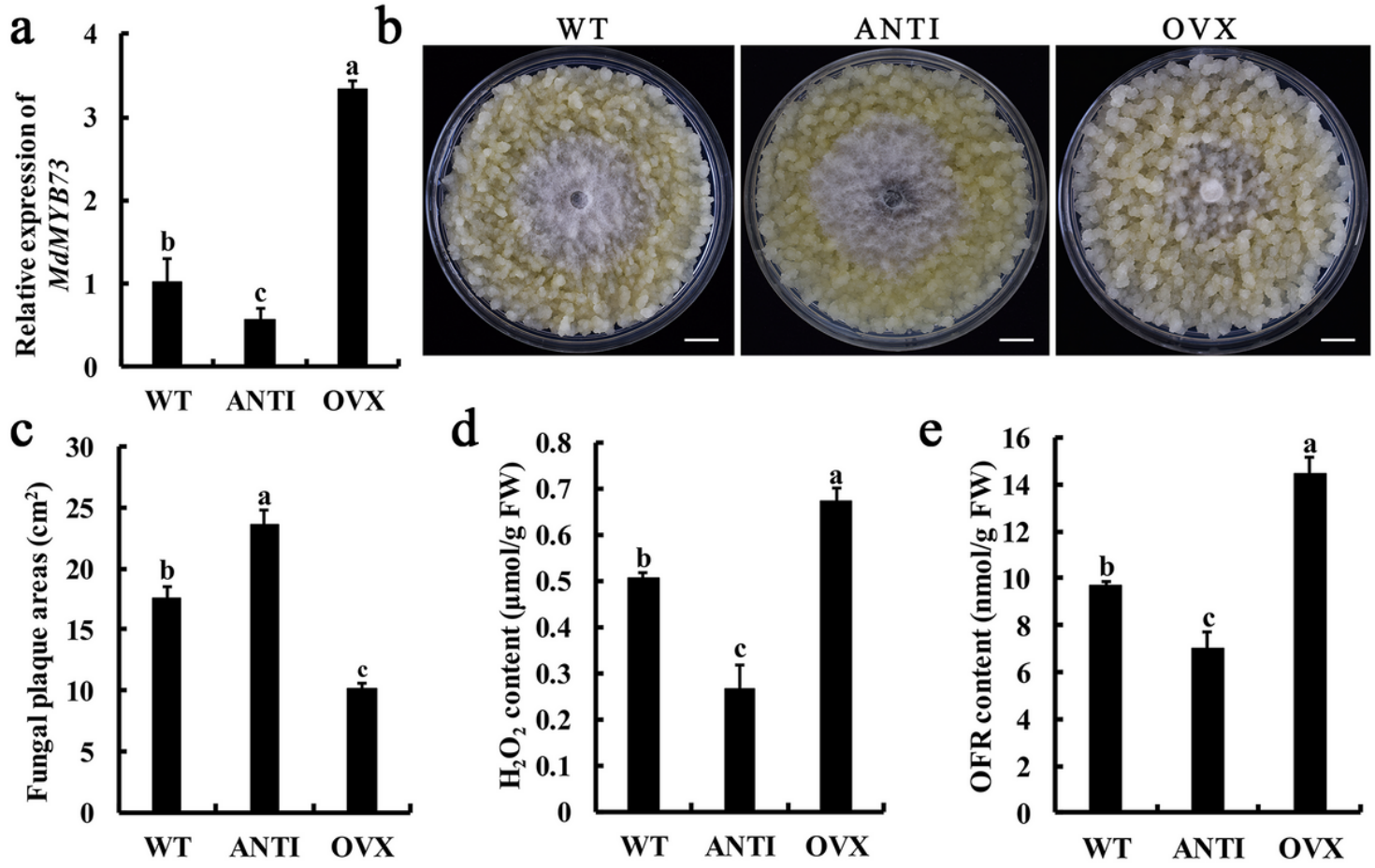
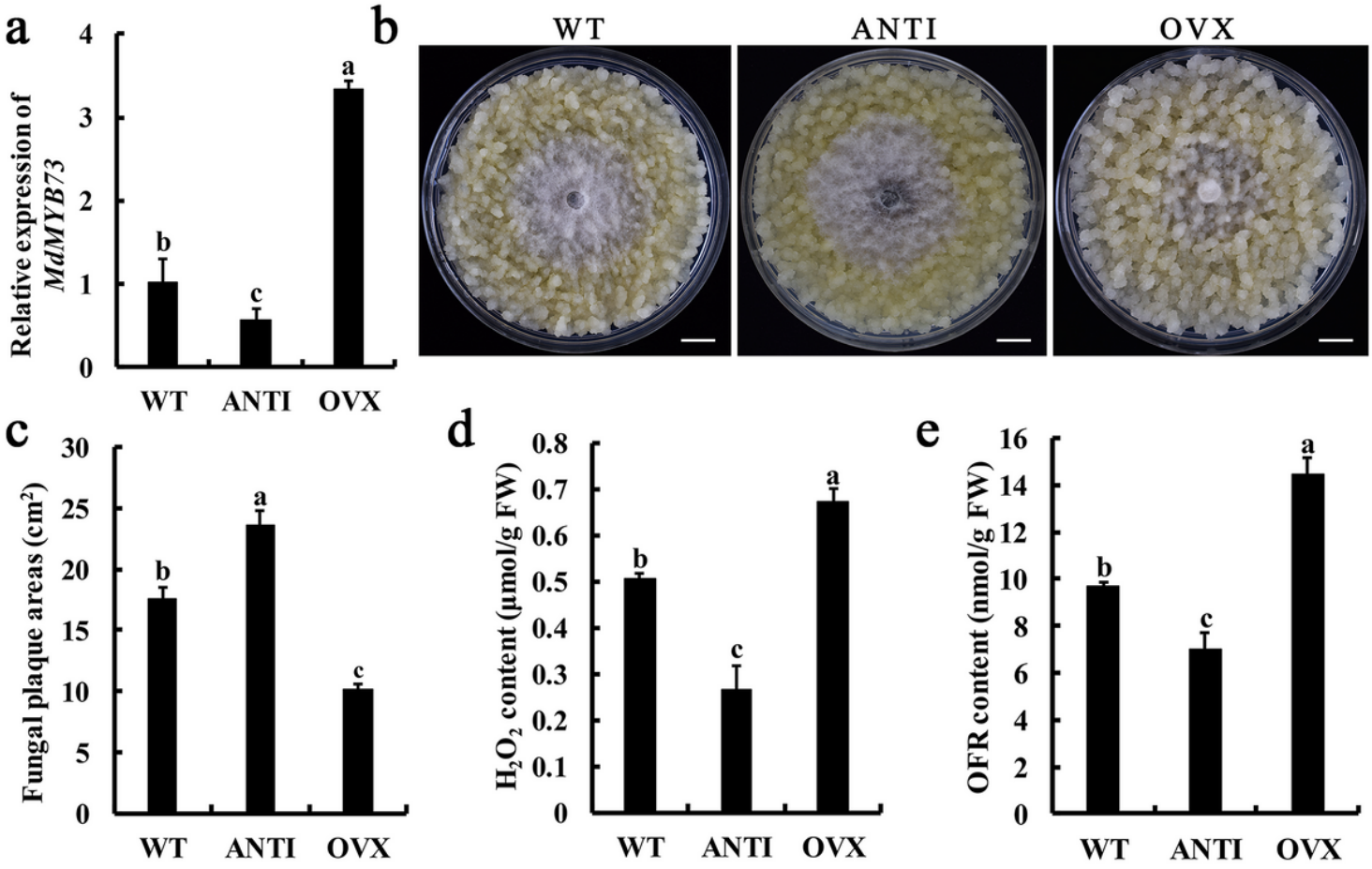


Figure 2

MdMYB73 enhances resistance of apple calli against B. dothidea. a Relative expression in WT, MdMYB73-ANTI, and MdMYB73-OVX apple calli. b The phenotypes after 4 days of inoculation with B. dothidea in WT, MdMYB73-ANTI and MdMYB73-OVX apple calli. Scale bars = 1 cm. c Plaque areas were measured in WT, MdMYB73-ANTI and MdMYB73-OVX apple calli after inoculation with B. dothidea for 4 days. d, e Determination of H2O2 and OFR content in WT, MdMYB73-ANTI and MdMYB73-OVX apple calli after infection with B. dothidea for 4 days. Bars with different letters are significantly different ($P < 0.05$) according to Tukey’s single factor tests. Data are shown as mean ± SD; experiments were replicated at least three times.



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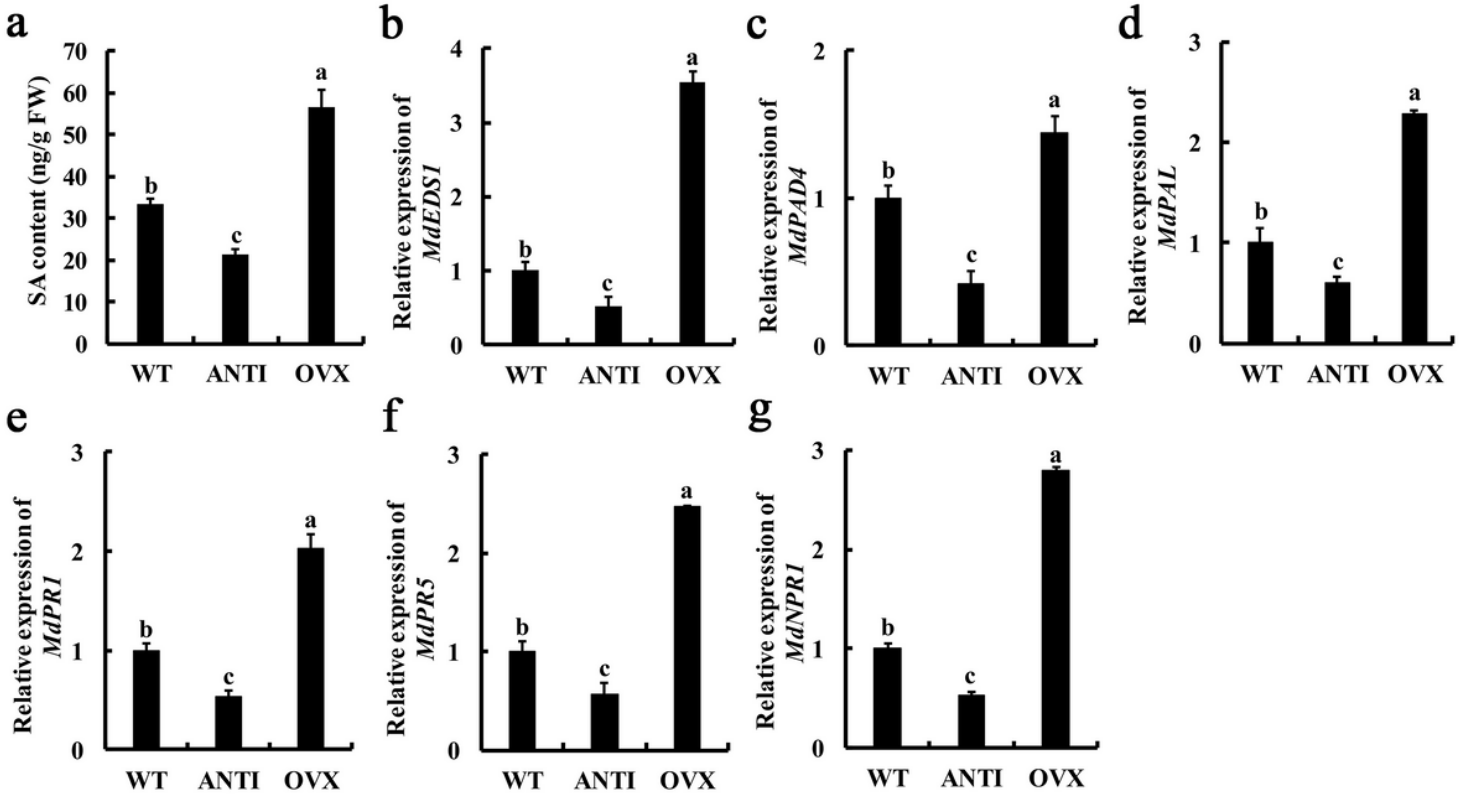


Figure 3

SA content and the relative expression of SA-related genes in apple calli after inoculation with B. dothidea. a SA content of WT, MdMYB73-ANTI and MdMYB73-OVX apple calli after inoculation with B. dothidea. b–d The expression of SA synthesis-related genes (EDS1, PAD4, and PAL) after inoculation with B. dothidea for 4 days in WT, MdMYB73-ANTI and MdMYB73-OVX apple calli. e–g The expression of SA signaling-related genes (PR1, PR5, and NPR1) after inoculation with B. dothidea for 4 days in WT, MdMYB73-ANTI and MdMYB73-OVX apple calli. Bars with different letters are significantly different ($P < 0.05$) according to Tukey's single factor tests. Data are shown as mean ± SD; experiments were replicated at least three times.

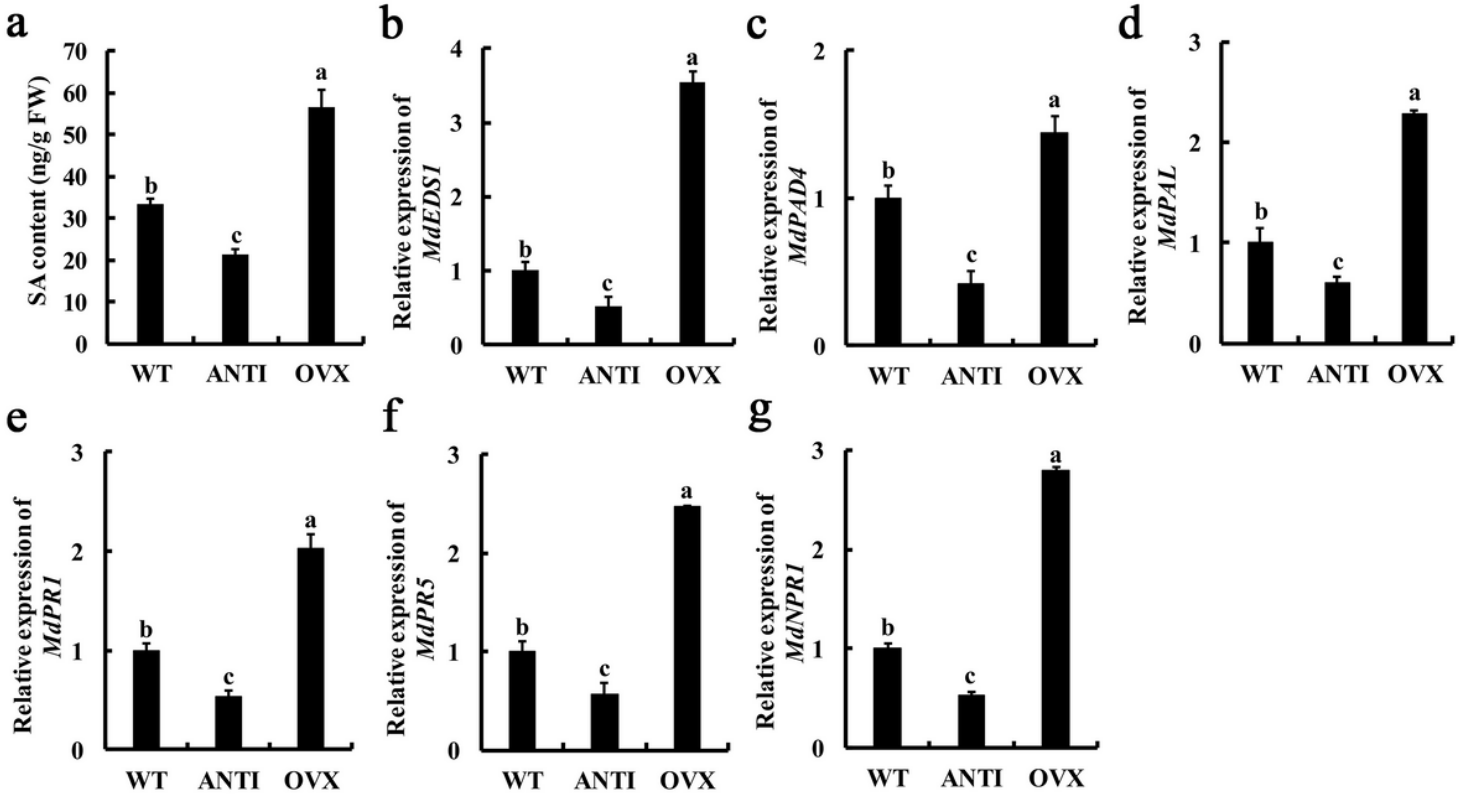


Figure 3

SA content and the relative expression of SA-related genes in apple calli after inoculation with B. dothidea. a SA content of WT, MdMYB73-ANTI and MdMYB73-OVX apple calli after inoculation with B. dothidea. b–d The expression of SA synthesis-related genes (EDS1, PAD4, and PAL) after inoculation with B. dothidea for 4 days in WT, MdMYB73-ANTI and MdMYB73-OVX apple calli. e–g The expression of SA signaling-related genes (PR1, PR5, and NPR1) after inoculation with B. dothidea for 4 days in WT, MdMYB73-ANTI and MdMYB73-OVX apple calli. Bars with different letters are significantly different ($P < 0.05$) according to Tukey's single factor tests. Data are shown as mean ± SD; experiments were replicated at least three times.

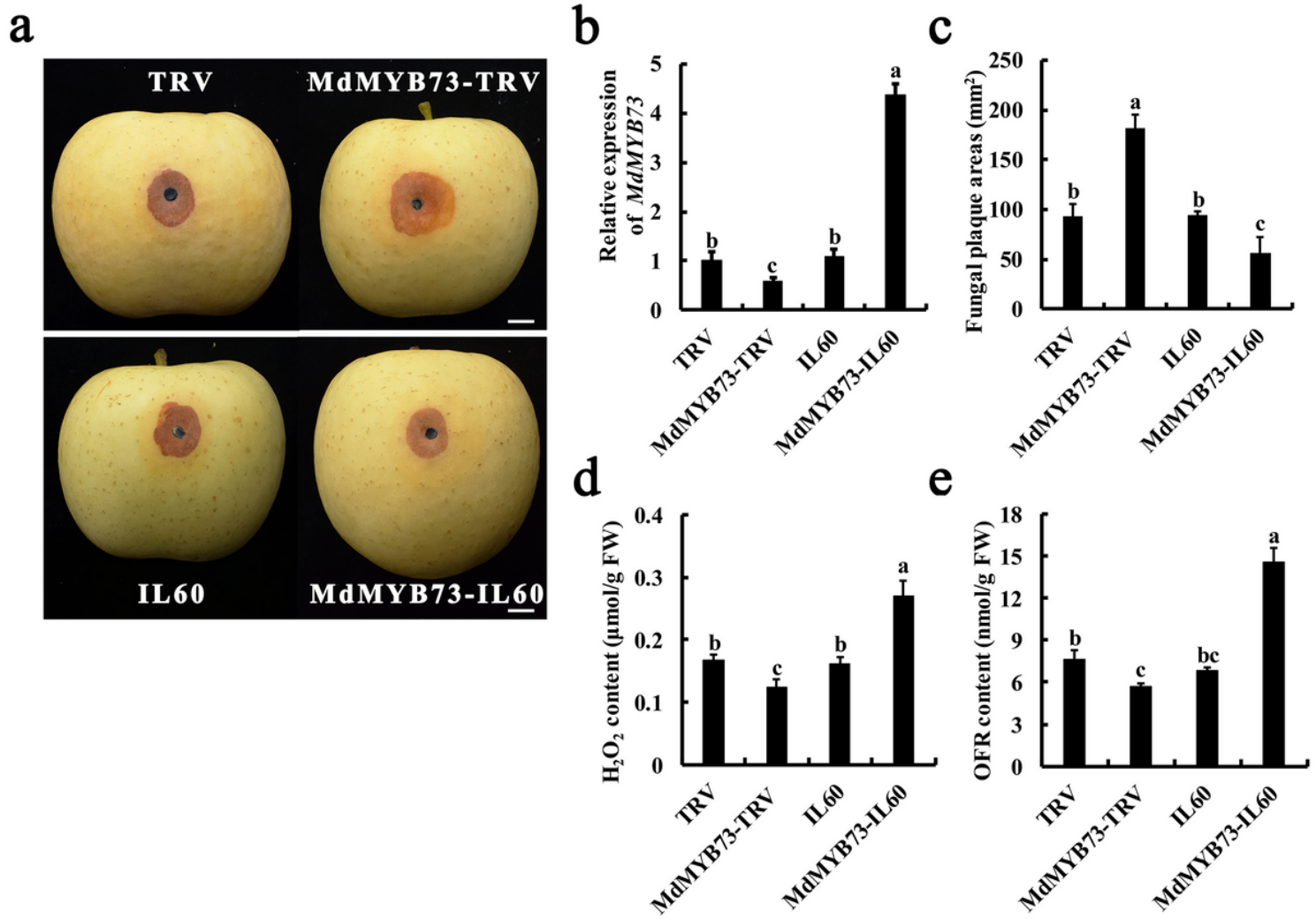
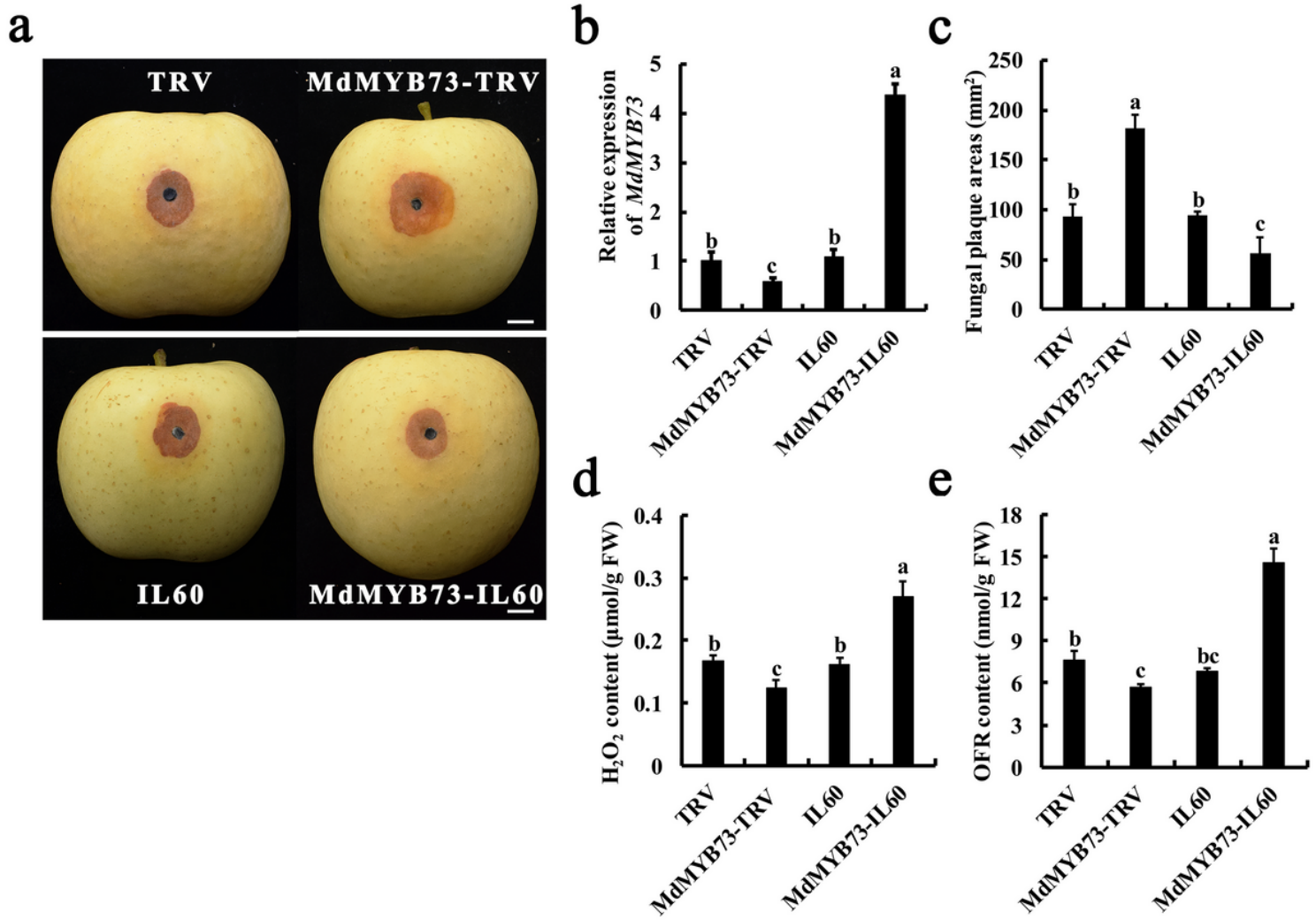


Figure 4

MdMYB73 improves resistance of apple fruits against B. dothidea. a The phenotypes of TRV-, MdMYB73-TRV-, IL60- and MdMYB73-IL60-injected fruits after infection with B. dothidea for 4 days. Scale bars = 1 cm. b The expression of MdMYB73 in TRV-, MdMYB73-TRV-, IL60- and MdMYB73-IL60-injected “Fuji” apple fruits after infiltration for 2 days. c Plaque areas of apple fruits were measured after infection with B. dothidea for 4 days. d, e Determination of H2O2 and OFR content in TRV-, MdMYB73-TRV-, IL60- and MdMYB73-IL60-injected fruits after infection with B. dothidea for 4 days. Bars with different letters are significantly different ($P < 0.05$) according to Tukey’s single factor tests. Data are shown as mean ± SD; experiments were replicated at least three times.



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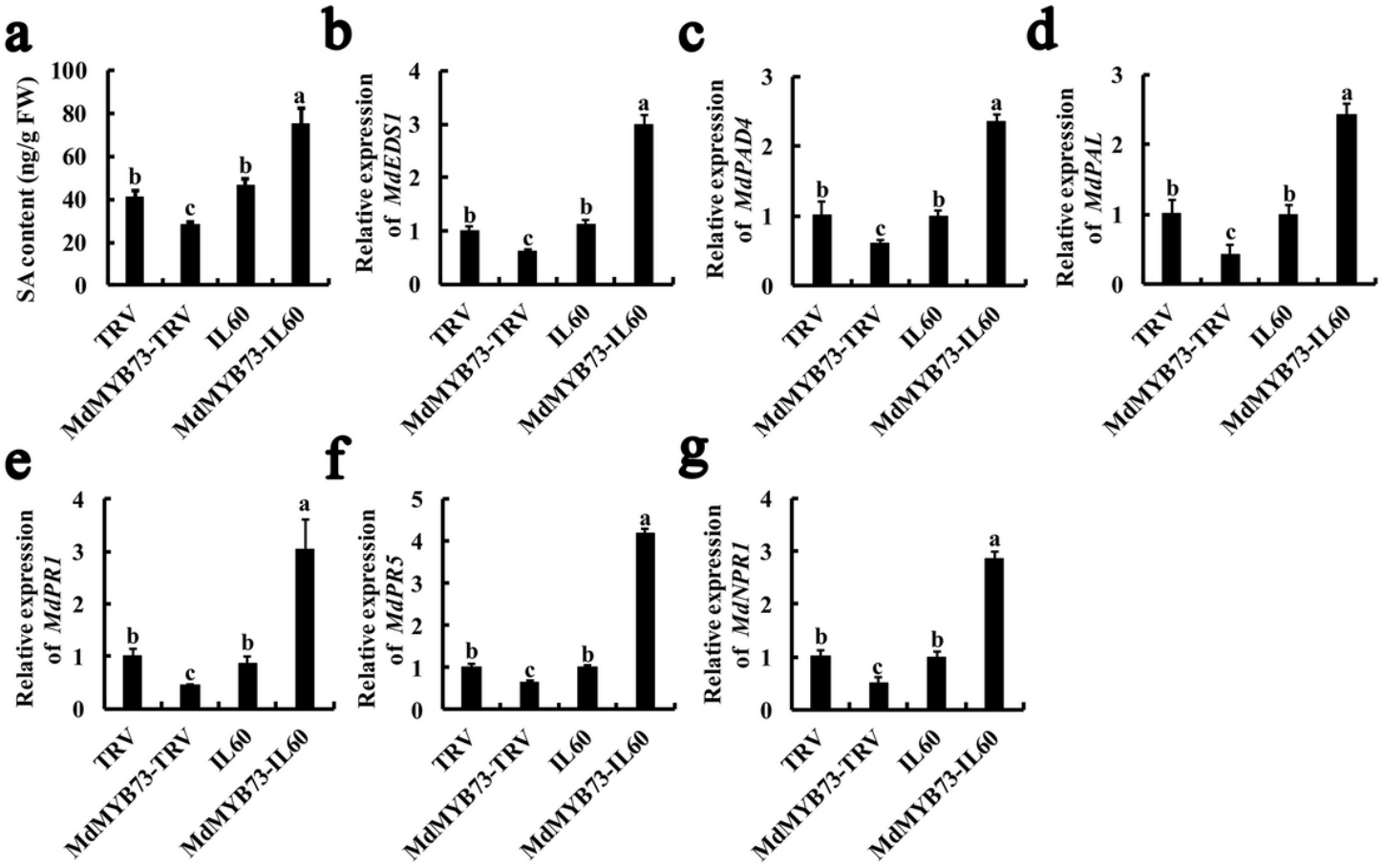
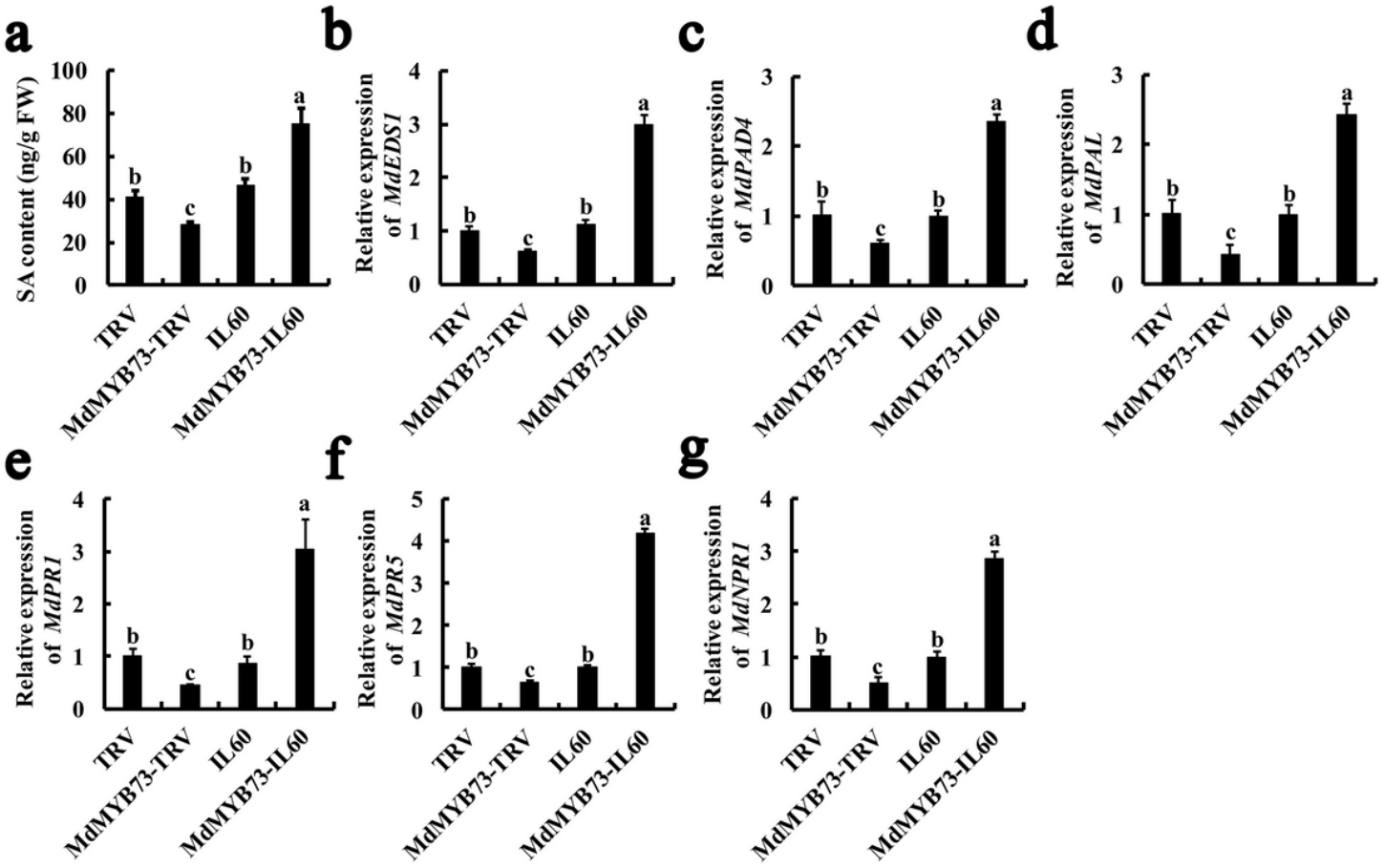


Figure 5

SA content and the relative expression of SA-related genes in apple fruits after infection with B. dothidea for 4 days. a After infection with B. dothidea, SA content was measured in TRV-, MdMYB73-TRV-, IL60- and MdMYB73-IL60-injected fruits. b–d After infection with B. dothidea, the relative expression of SA synthesis-associated genes (EDS1, PAD4, and PAL) was measured in TRV-, MdMYB73-TRV-, IL60- and MdMYB73-IL60-injected fruits. e–g After infection with B. dothidea, the relative expression of SA signaling-associated genes (PR1, PR5, and NPR1) was measured in TRV-, MdMYB73-TRV-, IL60- and MdMYB73-IL60-injected fruits. Bars with different letters are significantly different ($P < 0.05$) according to Tukey's single factor tests. Data are shown as mean ± SD; experiments were replicated at least three times.



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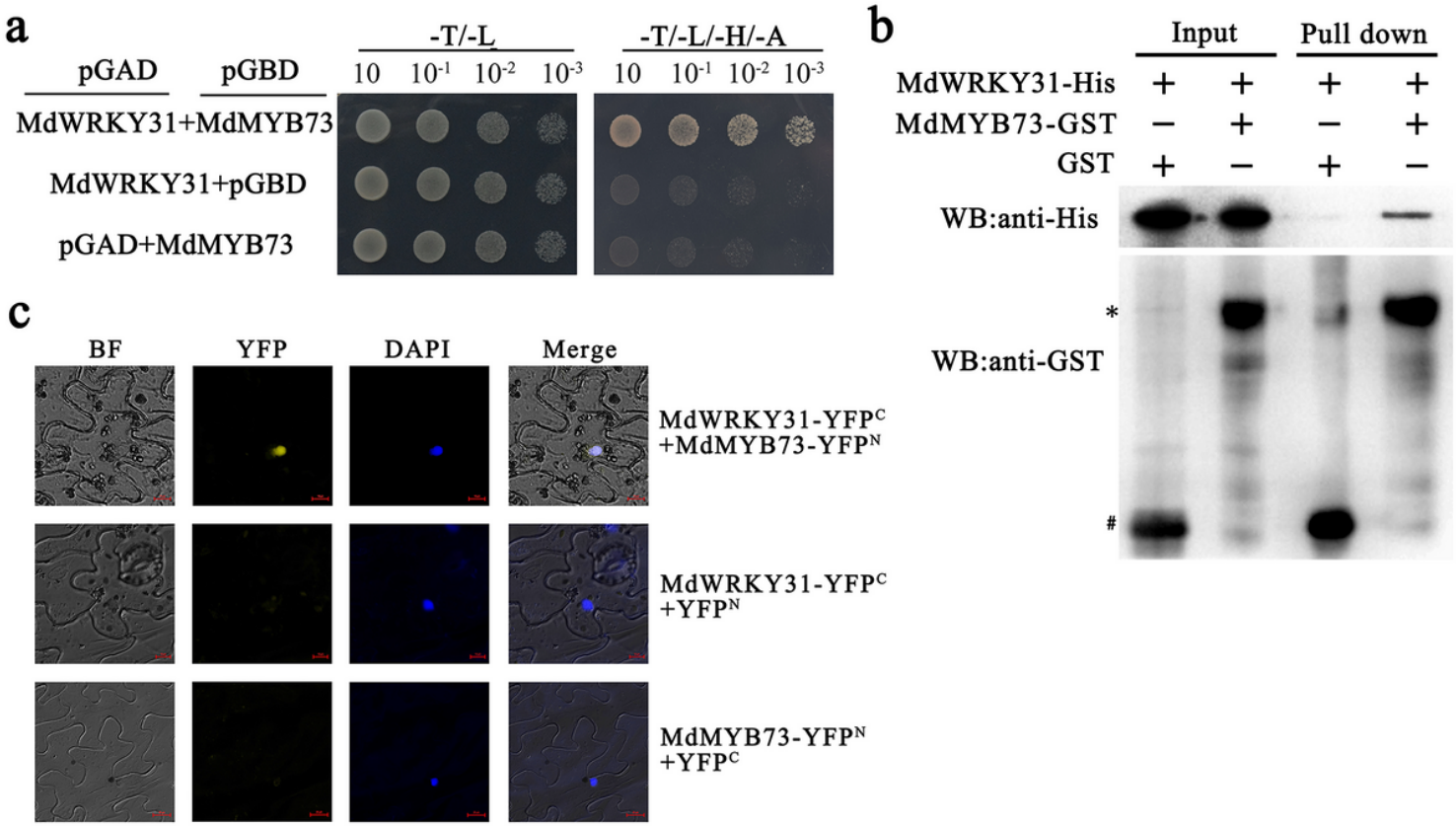
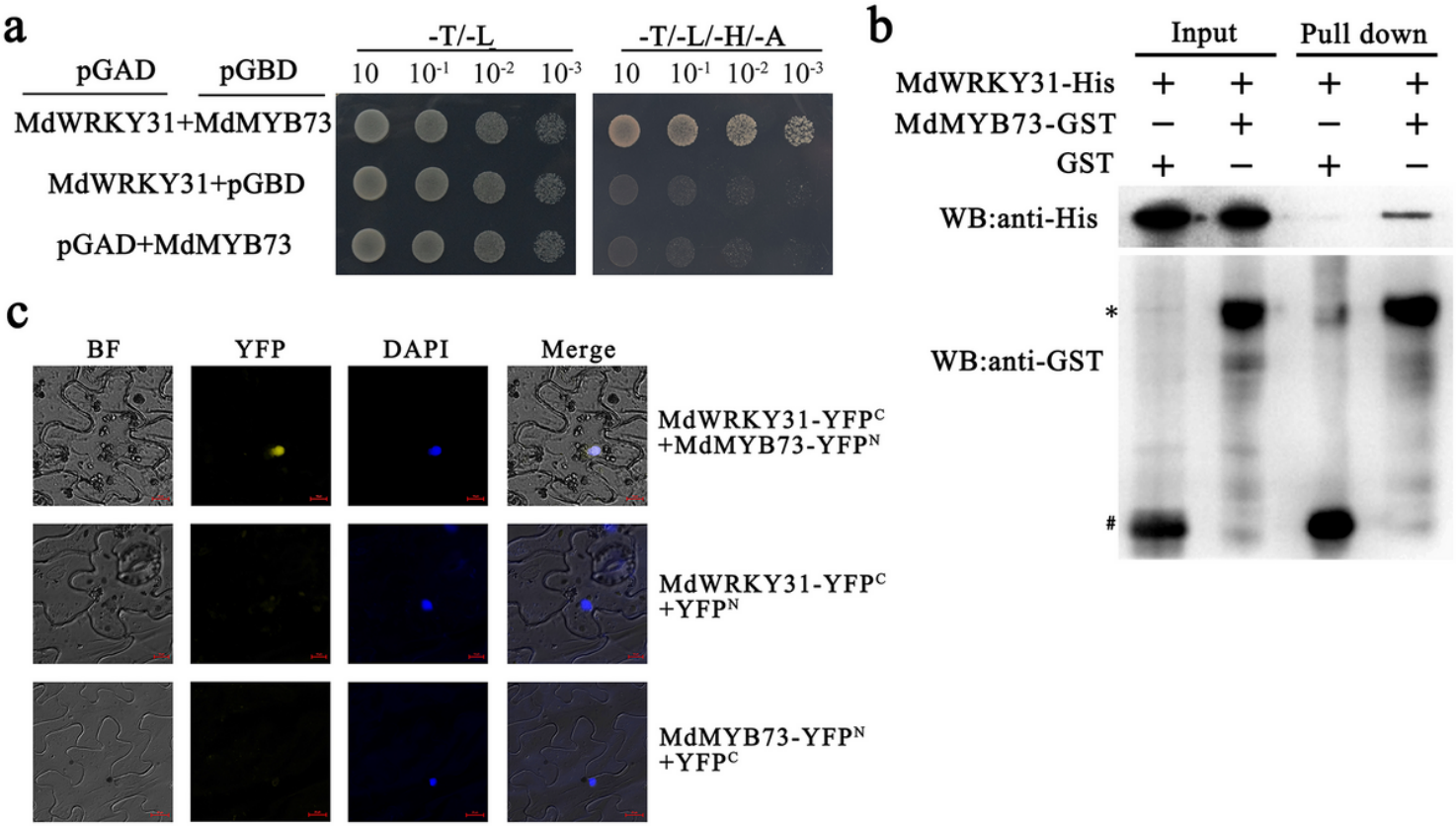


Figure 6

MdMYB73 interacts with MdWRKY31. a The interaction of MdMYB73 and MdWRKY31 was verified via yeast two-hybrid assays. b In vitro pull-down assays were performed to verify the interaction between MdMYB73 and MdWRKY31. Pound sign represents GST protein. Asterisk represents MdMYB73-GST protein. c MdMYB73 was confirmed to interact with MdWRKY31 in the bimolecular fluorescence complementary assay. Scale bars = 10 μm.



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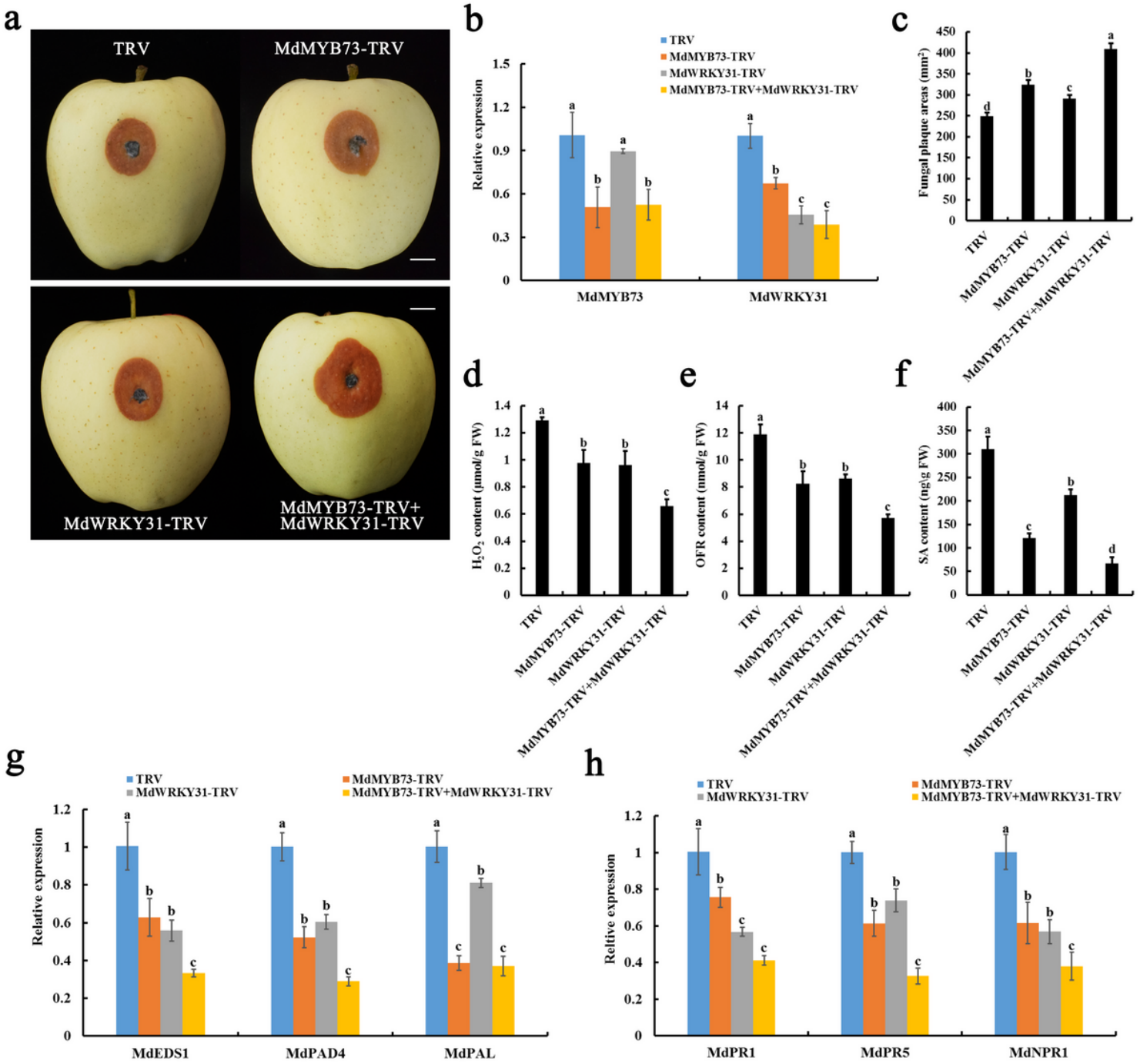
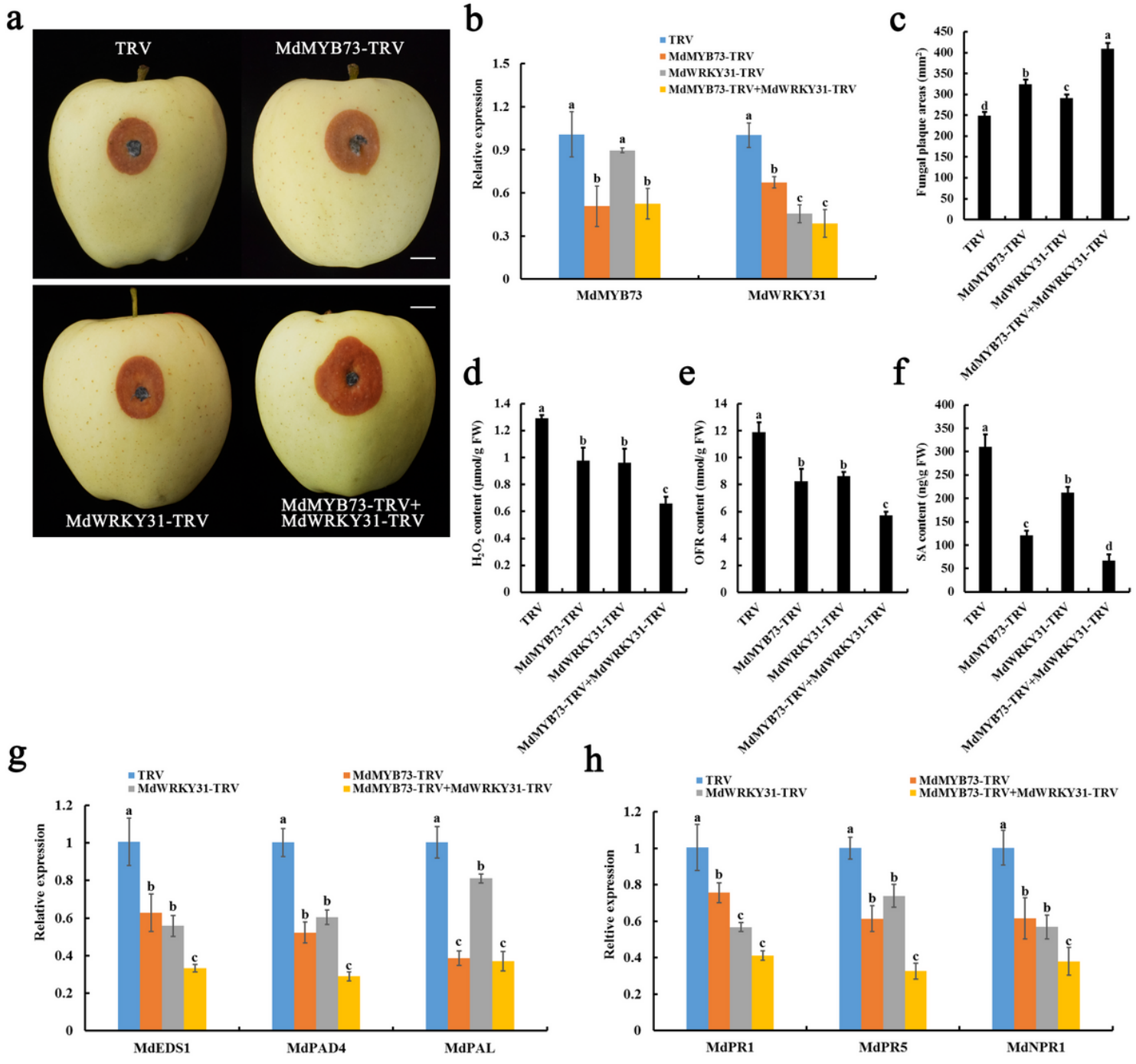


Figure 7

MdMYB73 and MdWRKY31 together enhance the resistance against B. dothidea in apple fruits. a The phenotypes of MdMYB73-TRV+MdWRKY31-TRV-, MdMYB73-TRV-, MdWRKY31-TRV- and TRV-injected fruits after infection with B. dothidea for 4 days. Scale bars = 1 cm. b The gene expression levels of MdMYB73 and MdWRKY31 in TRV-, MdMYB73-TRV-, MdWRKY31-TRV- and MdMYB73-TRV+MdWRKY31-TRV-injected “Red delicious” apple fruits after infiltration for 2 days. c Plaque areas of apple fruits were monitored after inoculated with B. dothidea for 4 days. d, e Determination of H2O2 and OFR content in MdMYB73-TRV+MdWRKY31-TRV-, MdMYB73-TRV-, MdWRKY31-TRV- and TRV-injected fruits after infection with B. dothidea for 4 days. f SA content was measured in TRV-, MdMYB73-TRV-, MdWRKY31-

TRV- and MdMYB73-TRV+MdWRKY31-TRV-injected fruits after infection with B. dothidea. g The relative expression of SA synthesis-associated genes (EDS1, PAD4, and PAL) was measured in TRV-, MdMYB73-TRV-, MdWRKY31-TRV- and MdMYB73-TRV+MdWRKY31-TRV-injected fruits after infection with B. dothidea. h The relative expression of SA signaling-associated genes (PR1, PR5, and NPR1) was measured in TRV-, MdMYB73-TRV-, MdWRKY31-TRV- and MdMYB73-TRV+MdWRKY31-TRV-injected fruits after infection with B. dothidea. Bars with different letters are significantly different ($P < 0.05$) according to Tukey's single factor tests. Data are shown as mean ± SD; experiments were replicated at least three times.



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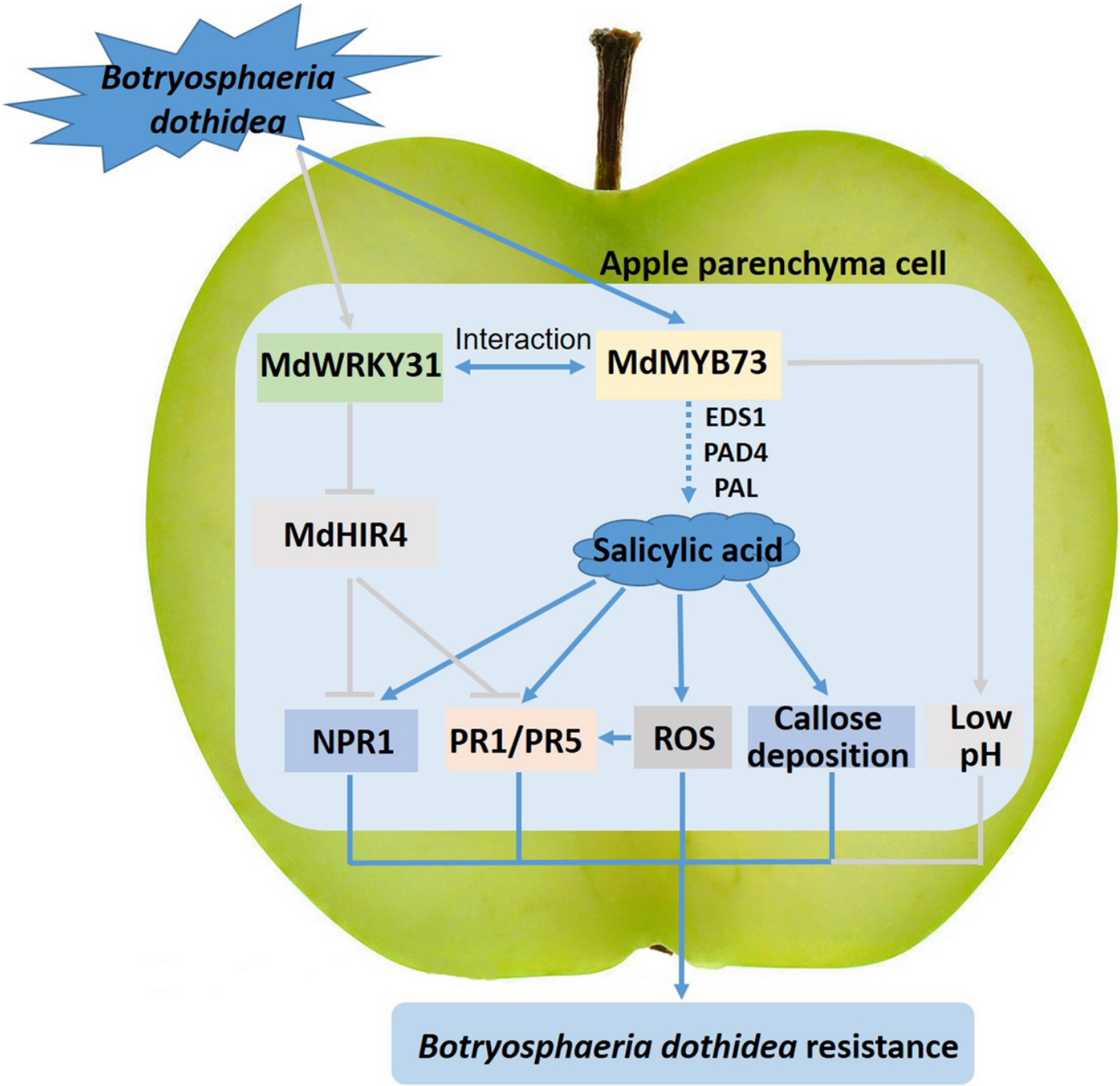

Figure 8

Working model of MdMYB73 in the regulation of resistance against B. dothidea. B. dothidea induces the expression of MdMYB73, which increases the expression of SA synthesis-associated genes (EDS1, PAD4, and PAL), leading to increased SA content. Subsequently, the higher SA levels lead to the expression of SA signaling-associated genes (PR1, PR5, and NPR1), ROS accumulation, and callose deposition, enhancing resistance against B. dothidea. On the other hand, MdMYB73 interacts with MdWRKY31, and could be involved in the MdWRKY31-MdHIR4-mediated pathway in the resistance against B. dothidea. In addition, overexpression of MdMYB73 leads to lower pH in apples, which might be another pathway to

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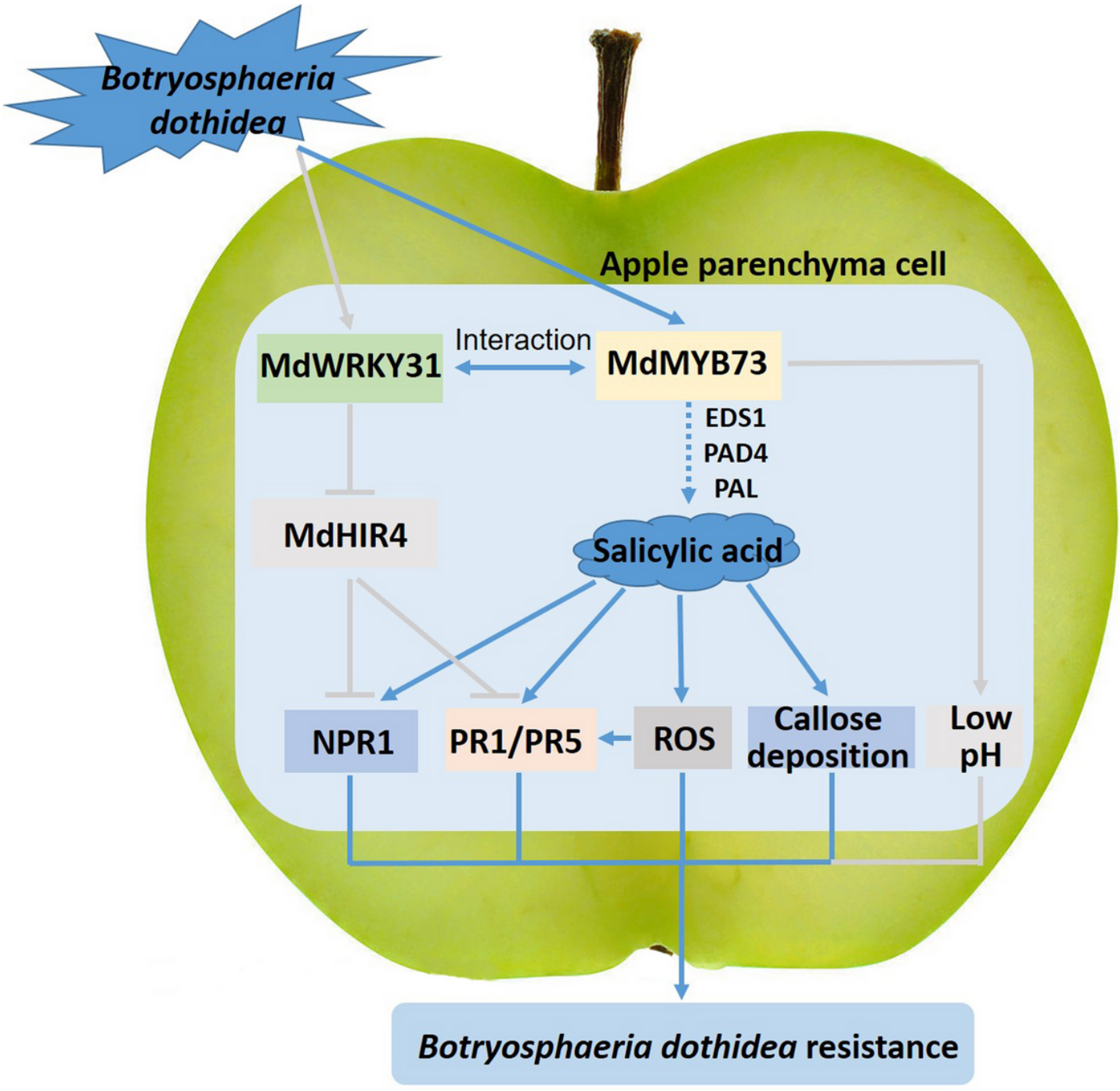

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