MdWRKY46-Enhanced Apple Resistance to *Botryosphaeria dothidea* by Activating the Expression of *MdPBS3.1* in the Salicylic Acid Signaling Pathway

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Salicylic acid (SA) is closely related to disease resistance of plants. WRKY transcription factors have been linked to the growth and development of plants, especially under stress conditions. However, the regulatory mechanism of WRKY proteins involved in SA production and disease resistance in apple is not clear. In this study, *MdPBS3.1* responded to *Botryosphaeria dothidea* and enhanced resistance to *B. dothidea*. Electrophoretic mobility shift assays, yeast one-hybrid assays, and chromatin immunoprecipitation and quantitative PCR demonstrated that MdWRKY46 can directly bind to a W-box motif in the promoter of *MdPBS3.1*. Glucuronidase transactivation and luciferase analysis further showed that MdWRKY46 can activate the expression of *MdPBS3.1*. Finally, *B. dothidea* inoculation in transgenic apple calli and fruits revealed that MdWRKY46 improved resistance to *B. dothidea* by the transcriptional activation of *MdPBS3.1*. Viral vector-based transformation assays indicated that MdWRKY46 elevates SA content and transcription of SA-related genes, including *MdPR1*, *MdPR5*, and *MdNPR1* in an *MdPBS3.1*-dependent way. These findings provide new insights into how MdWRKY46 regulates plant resistance to *B. dothidea* through the SA signaling pathway.

Keywords: apple, *Botryosphaeria dothidea*, *MdPBS3.1*, MdWRKY46, salicylic acid

Salicylic acid (SA), a phenolic derivative, plays important roles in a broad spectrum of pathogenic bacteria (Gu et al. 2017; Lu et al. 2016). Upon infection with a pathogen, plants increase SA production, which affects the redox state of the cell (Chen et al. 1993; Kuźniak et al. 2015; Noctor et al. 2002; Vanacker et al. 2000; Yu et al. 2018). SA can negatively influence the activation of catalase and ascorbate peroxidase or serve as a substrate for ascorbate peroxidase, which may give rise to accumulation of H₂O₂, SA-free radicals, and other reactive oxygen species (ROS) (Chen et al. 1993; Durner and Klessig 1995; Herrera-Vásquez et al. 2015; Kvaratskhelia et al. 1997; Xu et al. 2017). Except for its role in triggering redox changes, SA also plays a vital function in maintaining the cellular redox state, probably by inducing the expression of genes involved in the synthesis of antioxidants (Mou et al. 2003). In spite of the critical role of SA in triggering the plant defense response, redundant free SA may be phytotoxic (Kenton et al. 2000; Lee et al. 1995). To maintain normal plant development and growth, plants regulate free SA transformed to SA-O-β-glucoside (SAG) as the dominant form of SA (Enyedi and Raskin 1993; Jayakannan et al. 2015; Malamy et al. 1992).

The accumulation of SA in plants leads to the expression of pathogenesis-related (PR) proteins and, thus, improves the resistance of plants (Fu and Dong 2013; Klessig et al. 2018; Nawrath and Métraux 1999; Yalpani et al. 1991). When attacked by a pathogen, the accumulation of SA in the *Arabidopsis* SA induction-deficient 1 (*sid1*) mutant is blocked (Nawrath and Métraux 1999). Furthermore, *sid1* mutants are more susceptible to infection by *Pseudomonas syringae* and *Peronospora parasitica*, and the expression of *PR1* is significantly reduced in *sid* mutants after invasion by these pathogens (Nawrath and Métraux 1999). SID2 (ICS1) also is vital for local and systemic resistance and is responsible for the majority of SA biosynthesis after pathogen infection (Abreu and Munné-Bosch 2009; Wildermuth et al. 2001). *Nonexpresser of genes PR1 (NPR1)* in *Arabidopsis* plays an important role in SA-mediated disease resistance and is a regulator in systemic-acquired resistance (SAR) (Li et al. 2018). In the absence of *NPR1*, *Arabidopsis* cannot respond to SAR-induced treatment, and the expression of *PR* genes is reduced, making it more susceptible to infection by pathogens (Cao et al. 1997; Mou et al. 2003; Wang et al. 2018). As a result

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Sequence data can be found in the GenBank/EMBL data libraries under the following accession numbers: *MdPBS3.1* (MD14G1050700); *MdPBS3.2* (MD15G1003500); *MdWRKY46* (MD07G1146900); and *MdACTIN* (EB136338).

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of SA accumulation, the redox changes induced during SAR lead to the conformational switch of NPR1, thereby activating *PR* genes (Mou et al. 2003). A defect in *Arabidopsis thaliana phytoalexin deficient 4 (PAD4)* affects the expression of the *PR1* gene and the response to *Pseudomonas syringae* pv. *maculicola* ES4326. SA is indispensable for the expression of the *PR1* gene. When *pad4* is inoculated with *P. syringae* pv. *maculicola* ES4326, SA accumulation and *PR1* gene expression is reduced, which causes defects in the defense response (Zhou et al. 1998). The *enhanced disease susceptibility 1 (EDS1)/PAD4* complex is capable of inducing expression of SA response genes and resistance to pathogens (Cui et al. 2016). *P. syringae* pv. *tomato* DC3000 treatment can induce the expression of SA-related genes (*PR1*, *PR2*, and *PR5*) and bring about an increase in production of SA (Jia et al. 2018).

AVR PPHB SUSCEPTIBLE 3 (PBS3), a member of the GH3 acyl adenylase family, functions upstream of SA synthesis, either in a regulatory capacity, which may include an amplification loop, or in SA biosynthesis (Nobuta et al. 2007). The *pbs3* mutant in *Arabidopsis* affects SAG accumulation and SA-dependent gene expression, and the *pbs3* mutant shows a deficiency in resistance upon invasion by pathogens. PBS3 plays an important role in regulating SA-dependent resistance responses (Nobuta et al. 2007). The HopW1-1 of *P. syringae* pv. *maculicola* ES4326 effector in *P. syringae* can target *PBS3* and alter the SA signaling pathway (Lee et al. 2008). *PBS3* acts upstream of the SA signal to regulate pathogen resistance (Wang et al. 2011), but the transcriptional regulation of *PBS3* in plants is not clear.

In a complex environment, plants are subjected to a variety of biological stresses, among which transcription factors play a crucial role in plant immunity induced by biological stresses (Pandey and Somssich 2009; Sarris et al. 2015). In strawberries, for instance, the expression of *FvWRKY42* and *PR1* is induced by SA and increases plant resistance to powdery mildew (Wei et al. 2018). *Arabidopsis* expression of *AtWRKY46* is upregulated by *P. syringae* or SA, and *AtWRKY46* can coordinate with *WRKY70* and *WRKY53* to increase resistance to *P. syringae* (Hu et al. 2012). In addition, several WRKY transcription factors act as negative regulators of biotic resistance. In *Arabidopsis*, analyses of mutants has revealed that the loss of *WRKY11* function increases resistance to *P. syringae* pv. *tomato* strains and that resistance is further enhanced in *wrky11 wrky17* double-mutant plants (Journot-Catalino et al. 2006). *Arabidopsis WRKY18/40* can negatively regulate the expression of *EDS1* and *PAD4* and promote powdery mildew infection (Pandey et al. 2010). In *Capsicum annuum*, *CaWRKY58* negatively regulates plant resistance to *Ralstonia solanacearum*, and plants with decreased *CaWRKY58* expression display increased resistance to *R. solanacearum* (Wang et al. 2013). These findings indicate that WRKY proteins play crucial roles in plant immune responses.

Botryosphaeria dothidea is a model strain of *Botryosphaeria* spp. (Marsberg et al. 2017). On apple, *B. dothidea* mainly harms the branches and fruits. It also adversely affects tree growth, releasing spores that infect the fruit, which results in rot and in the death of branches and young trees. The expression of *MdPR-4* in apples can be induced by SA, which mediates the resistance of apples to *B. dothidea* (Bai et al. 2013). After the inhibition of *syntaxin-related 121 (SYP121)* expression in apples, resistance to *B. dothidea* is improved (He et al. 2018). However, the regulatory mechanism of WRKY transcription factors in relation to *B. dothidea* infection is not well studied.

In this study, we found that *MdPBS3.1* expression can be induced by *B. dothidea*, and resistance to *B. dothidea* is improved by *MdPBS3.1* overexpression. The WRKY transcription factor MdWRKY46 activated *MdPBS3.1* expression by directly binding to the W-box motif in the promoter of *MdPBS3.1*, which positively regulated the resistance to *B. dothidea* through the

SA-mediated pathway. These findings provide important insights into the function and the novel regulatory mechanism of MdWRKY46 in mediating resistance to *B. dothidea* through the SA signaling pathway in apples, which may help to defend apples from infection by *B. dothidea*.

RESULTS

MdPBS3.1 response to *B. dothidea*.

In plants, pathogens are able to induce SA accumulation (Nawrath and Métraux 1999). *PBS3* plays an important role in the production of SA in plants (Nobuta et al. 2007). *B. dothidea* is an important pathogen causing disease of apple branches and fruits, and it is considered a major threat to the fruit-producing industry. To investigate the relationship between *B. dothidea* and *PBS3* in apples, a BLAST search against the apple genome database was conducted using *Arabidopsis AtPBS3* (At5g13320) as the query. As a result, 21 *PBS3*-like genes were found, and a sequence alignment of 21 probable *PBS3* homologs in apple with *Arabidopsis PBS3* was made (Supplementary Fig. S1). Two proteins, MdPBS3.1 (MDP0000257967) and MdPBS3.2 (MDP0000716496), were selected according to their high protein similarity to *AtPBS3*. On the basis of a new apple genome database, MdPBS3.1 (MDP0000257967) is MdPBS3.1 (MD14G1050700), and MdPBS3.2 (MDP0000716496) is MdPBS3.1 (MD15G1003500).

The expression levels of *MdPBS3.1* and *MdPBS3.2* were examined after infection of 'Fuji' apples with *B. dothidea*. Compared with the control, the *MdPBS3.1* expression level was significantly increased 1 and 2 days after inoculation with *B. dothidea*. Although the *MdPBS3.2* expression level also increased 1 and 2 days after inoculation with *B. dothidea*, the extent of the increase was not comparable to that of *MdPBS3.1* (Fig. 1A); therefore, the decision was made to focus on *MdPBS3.1* in this study.

To investigate the function of *MdPBS3.1*, the National Center for Biotechnology Information (NCBI) conserved-domains online software was used to analyze the conserved domain of the MdPBS3.1 protein. The relationship between apple MdPBS3.1 and *PBS3.1* from other species also was analyzed. The results showed that the *PBS3.1* from 13 species could be divided into three classes. Class I contained *PBS3.1* from tobacco (*Nicotiana glauca*) and pear (*Pyrus × bretschneideri*), whereas class III contained *PBS3.1* from astragalus (*Herrania umbratica*), rose (*Rosa chinensis*), cassava (*Manihot esculenta*), cacao (*Theobroma cacao*), pea (*Parasponia andersonii*), and dungarunga (*Trema orientalis*). MdPBS3.1 belonged to class II, which contained *PBS3.1* from loquat (*Eriobotrya japonica*), club (*Prunus mume*), cherry (*Prunus avium*), and peach (*Prunus persica*), which all belong to the Rosaceae family. Apple MdPBS3.1 showed the strongest relationship to loquat *PBS3.1*, suggesting they may have similar functions (Fig. 1B). In addition, MdPBS3.1 primarily contained an adenosine monophosphate (AMP)-binding site (Fig. 1C), indicating that MdPBS3.1 belongs to the AMP-binding superfamily.

MdPBS3.1 confers increased resistance to *B. dothidea* in apple calli.

To investigate the role of *MdPBS3.1* in resistance to *B. dothidea*, *MdPBS3.1*-overexpressing and *MdPBS3.1*-antisense apple calli were obtained (Supplementary Fig. S2A and B). After inoculation with *B. dothidea*, *MdPBS3.1* overexpression reduced the diameter of the disease spot in apple calli compared with the wild type (WT). (Fig. 2A and B). This result was further confirmed by quantitative real-time PCR (qRT-PCR) detection of the expression of pathogen-associated genes. The expression of *MdPR1*, *MdPR5*, and *MdNPR1* was significantly induced in the *MdPBS3.1*-overexpressing calli

in response to *B. dothidea* is unclear. To further investigate the activation of *MdPBS3.1* by *B. dothidea*, the *proMdPBS3.1-GUS* vector was constructed and transiently transformed into 'Royal Gala' apple seedlings by vacuum infiltration. After the transformed plants were treated with *B. dothidea* for 6 h, glucuronidase (GUS) was intensely stained. Compared with *B. dothidea* treatment for 6 h, the apple seedlings could still be stained by

Fig. 1. The expression of *MdPBS3* genes induced by *Botryosphaeria dothidea*. **A**, Expression of *MdPBS3.1* and *MdPBS3.2* in ‘Fuji’ apples treated with or without *B. dothidea* for 0 days, 1 day, and 2 days. Untreated apples were used as the control. **B**, Phylogenetic tree between apple *MdPBS3.1* and *PBS3.1* from other species, including *Malus domestica*, *Eriobotrya japonica*, *Pyrus bretschneideri*, *Prunus avium*, *Prunus persica*, *Prunus mume*, *Rosa chinensis*, *Theobroma cacao*, *Trema orientale*, *Parasponia andersonii*, *Manihot esculenta*, *Herrania umbratica*, and *Nicotiana glauca*. **C**, Conserved domain of the *MdPBS3.1* protein.

Fig. 2. Enhanced resistance to *Botryosphaeria dothidea* of MdWRKY46 in an *MdPBS3.1*-dependent manner in apple calli. **A**, Phenotype of wild type, *MdPBS3.1* overexpressing, *MdPBS3.1* antisense apple calli, *MdWRKY46* overexpressing, and *MdWRKY46* overexpressing and *MdPBS3.1* antisense double-transgenic apple calli inoculated with *B. dothidea*. **B**, Diameters of disease spots of different apple calli in A. **C**, Expression levels of *MdPR1*, *MdPR5*, and *MdNPR1* in different types of apple calli.

GUS after 12 h and 24 h of treatment, but the color became much lighter, suggesting low GUS activity (Fig. 3A). The results of experiments that investigated *GUS* expression yielded consistent results (Fig. 3D), indicating that *MdPBS3.1* was induced by *B. dothidea*.

The promoter of *MdPBS3.1* was analyzed, and many *cis*-elements, such as MBS, W-box, AREB, and others, were found (Supplementary Table S2). Studies have reported that many WRKY transcription factors are involved in plant resistance in the SA pathway, and W-box (TTGACT/C) is considered the WRKY transcription factor-binding site. In this study, the focus was on the two W-box motifs in the *MdPBS3.1* promoter: *proMdPBS3.1-GUS* (*m1*) was constructed with the first W-box (W-box1) mutated to TACGTC, and *proMdPBS3.1-GUS* (*m2*) was constructed with the second W-box (W-box2) mutated to TACGTC. Thereafter, the two vectors were transformed to the 'Royal Gala' apple seedlings and treated with *B. dothidea*. GUS staining and *proMdPBS3.1-GUS* (*m2*) activity in apple seedlings were reduced compared with the nonmutant *MdPBS3.1* promoter. However, *proMdPBS3.1-GUS* (*m1*) GUS analysis showed no apparent changes (Fig. 3B

Fig. 3. Glucuronidase (GUS) staining of the *MdPBS3.1* promoter after its response to *Botryosphaeria dothidea*. **A**, Apple seedlings transferred to the *MdPBS3.1* promoter (*proMdPBS3.1-GUS*) and **B** and **C**, apple seedlings transferred to the mutated *MdPBS3.1* promoter (*proMdPBS3.1-GUS* (*m1* or *m2*)) were analyzed by GUS staining. **D**, GUS relative expression after treating with *B. dothidea* for 0 h, 6 h, 12 h, and 24 h, respectively. *proMdPBS3.1-GUS* (*m1* or *m2*) represents the mutant promoter of *MdPBS3.1* in which W-box1 or W-box2 (TTGACA) was mutant to TACGTC, respectively.

and D). These results indicate that the W-box2 of the *MdPBS3.1* promoter is crucial for response to *B. dothidea*.

MdWRKY46 binds the W-Box2 in the promoter sequence of *MdPBS3.1*.

To identify the potential WRKY transcription factors that regulate the expression of *MdPBS3.1*, a yeast single-hybrid screen library was prepared using the *MdPBS3.1* promoter fragment, with the approximately 739-bp W-box2 as the bait. One positive clone encoding probable WRKY transcription factor 46 (MdWRKY46, MD07G1146900) was identified.

To examine the interaction of MdWRKY46 and the promoter of *MdPBS3.1*, an electrophoretic mobility shift assay (EMSA) was carried out to detect binding of MdWRKY46 to the promoter region of *MdPBS3.1*. The results indicate MdWRKY46 was bound directly to the promoter W-box2 of *MdPBS3.1* in vitro (Fig. 4A and B). As shown in Figure 4B, after the W-box2 in the *MdPBS3.1* promoter was mutated to TACGTC, binding disappeared in the EMSA (Fig. 4B), indicating that the binding was specific. Thereafter, a yeast one-hybrid (Y1H) assay was carried out to confirm the binding of MdWRKY46 to the promoter of *MdPBS3.1*. The results showed that *MdWRKY46-AD* and *proMdPBS3.1-box2-pHIS2*-cotransformed yeast cells were able to grow in the -Trp/-Leu/-His medium supplemented with 20 mM 3-amino-1,2,4-triazole (3-AT), but the negative control cotransformed with AD and *proMdPBS3.1-box2-pHIS2* failed to grow (Fig. 4C). These findings indicate that MdWRKY46 specifically binds to the W-box2 of the *MdPBS3.1* promoter in vivo.

To further demonstrate that MdWRKY46 can bind the promoter of *MdPBS3.1*, *MdWRKY46* fused with GFP-tagged transgenic calli was obtained (Fig. 4D). Chromatin immunoprecipitation and quantitative PCR (ChIP-qPCR) assays were performed using *MdWRKY46* transgenic calli and GFP transgenic calli. The results show that the *MdPBS3.1* promoter fragments were enriched with *MdWRKY46* transgenic calli (Fig. 4E), suggesting that MdWRKY46 can directly interact with the W-box2 motif in the promoter region of *MdPBS3.1*.

Transcriptional activation of *MdPBS3.1* by MdWRKY46.

The aforementioned results indicate that MdWRKY46 was able to bind to the promoter of *MdPBS3.1*. To further examine whether the transcriptional activity of *MdPBS3.1* was induced or suppressed by MdWRKY46, a *GUS* reporter gene was fused downstream of the 739-bp *MdPBS3.1* promoter and defined as *proMdPBS3.1-box2-GUS*. Additionally, the open reading frame of *MdWRKY46* was inserted into the *pGreenII* 62-SK vector under the control of the CaMV 35S promoter. Thereafter, the recombinant *MdWRKY46-pGreenII* 62-SK and *proMdPBS3.1-box2-GUS* plasmids were used in a GUS transactivation assay using 'Orin' apple calli. GUS activity assays and western blotting analysis also were performed. The results showed that GUS staining of *MdWRKY46-pGreenII* 62-SK and *proMdPBS3.1-box2-GUS* calli produced a stronger color than in the other combinations, including *pGreenII* 62-SK + 1300-GUS, *MdWRKY46-pGreenII* 62-SK + 1300-GUS, and *pGreenII* 62-SK + *proMdPBS3.1-box2-GUS* (Fig. 5A). GUS activity also was higher in western blotting, indicating that MdWRKY46 bound to the promoter of *MdPBS3.1* to activate its transcription (Fig. 5A and B).

Subsequently, a firefly luciferase (LUC) complementation imaging assay was carried out to verify the activation of MdWRKY46 to *MdPBS3.1* in vivo. A *LUC* gene was fused downstream of the *MdPBS3.1* promoter and defined as *proMdPBS3.1-box2-pGreenII* 0800-LUC. The luminescence intensity was significantly increased compared with the controls when *MdWRKY46-pGreenII* 62-SK and *proMdPBS3.1-box2-pGreenII* 0800-LUC were coinjected into tobacco (Fig. 5C), further demonstrating that MdWRKY46 increased *MdPBS3.1* expression.

Thereafter, *proMdPBS3.1-box2-GUS* was transformed into apple calli to generate *proMdPBS3.1-box2-GUS*-overexpressing apple calli. MdWRKY46 was then transiently transformed into *proMdPBS3.1-box2-GUS* and *1300-GUS* transgenic calli, respectively. GUS activity in the two transgenic calli was compared, which revealed that double-transgenic calli contained MdWRKY46 and that *proMdPBS3.1-box2-GUS* promoted the transcriptional activity of GUS compared with the 1300-GUS-overexpressing calli (Fig. 5D and E). In addition, MdPBS3.1 expression was significantly increased in MdWRKY46 transgenic calli (Fig. 5F). These results indicated that MdWRKY46 can transcriptionally activate the expression of MdPBS3.1 by binding to its promoter.

MdWRKY46 enhances resistance to *B. dothidea* in an MdPBS3.1-dependent manner in apple.

To confirm the biological function of MdWRKY46, the transcriptional level of MdWRKY46 after SA treatment was

examined, and SA was found to significantly induce MdWRKY46 expression, indicating its potential function in the SA signaling pathway. *B. dothidea* mainly affects the branches and fruits of apples. To examine whether MdWRKY46 regulated MdPBS3.1-modulated resistance to *B. dothidea* in apple fruits, *B. dothidea* was inoculated into VIGS-injected apple peel. Both *pIR-MdPBS3.1* and *pIR-MdWRKY46* increased resistance to *B. dothidea* compared with the control (*pIR*). In contrast, *TRV-MdPBS3.1* reduced resistance to *B. dothidea* compared with the control (*TRV*). The results of coinjecting *pIR-MdWRKY46* and *TRV-MdPBS3.1* were similar to those of *TRV-MdPBS3.1* (Fig. 6A and B), indicating the positive roles of MdWRKY46 and MdPBS3.1 in *B. dothidea* resistance. Next, the free SA and SAG levels 2 days after injection were measured. When MdPBS3.1 or MdWRKY46 were overexpressed, the free SA and SAG levels increased compared with the control. When the expression of MdPBS3.1 was inhibited, however, the free SA and SAG levels

Fig. 4. The binding of MdWRKY46 on the *MdPBS3.1* promoter. **A**, Schematic map of W-box in the promoter of *MdPBS3.1*. **B**, Electrophoretic mobility shift assays were used to detect binding of MdWRKY46 to the *MdPBS3.1* promoter. Each biotin-labeled DNA probe was incubated with MdWRKY46-GST protein. The mutated probe of P had a mutation in the W-box (TTGACA was replaced by TACGTC). **C**, Yeast one-hybrid assays were used to detect the transcriptional activation of *MdPBS3.1* by MdWRKY46. The constructs were cotransformed into yeast Y187. Yeast was cultured on a culture medium devoid of tryptophan and histidine (SD/-W/-H) and then screened for the lack of tryptophan, leucine, and histidine (SD/-W/-L/-H) with 20 mM and 80 mM 3-AT (3-amino-1,2,4-triazole). **D**, MdWRKY46 protein expression using an anti-GFP antibody in *MdWRKY46* overexpressed calli and wild-type calli. Anti-ACTIN was used as the loading control. **E**, Chromatin immunoprecipitation and quantitative PCR assays were performed to detect enriched DNA fragments of *MdWRKY46*-overexpressing calli (*MdWRKY46-GFP*) and calli transformed with an empty vector (*GFP*). *MdACTIN* served as the control.

decreased (Fig. 6C and D). These results indicate that SA may be involved in resistance to *B. dothidea* through the MdWRKY46-mediated *MdPBS3.1* pathway.

In addition, the expression of *PR* genes (*MdPR1*, *MdPR5*, *MdNPR1*) after injection was examined. Compared with the control, the expression of *PR* genes was significantly upregulated after *MdWRKY46* or *MdPBS3.1* overexpression, whereas expression of *MdPBS3.1* was inhibited or expression of *PR* genes decreased after coinjection of *TRV-MdPBS3.1* and *pIR-MdWRKY46* (Fig. 6D).

The function of MdWRKY46 and *MdPBS3.1* in resistance to *B. dothidea* was further confirmed in transgenic apple calli. *MdWRKY46*-overexpressing, *MdWRKY46*-overexpressing, and *MdPBS3.1*-antisense double-transgenic apple calli were obtained. After inoculation with *B. dothidea*, *MdWRKY46* overexpression reduced the diameter of the disease spot compared with the WT, and when expression of *MdPBS3.1* was inhibited, the diameter of the disease spot increased. The disease spot was also larger in double-transgenic calli than in the WT (Fig. 2A and C). These results were further confirmed by qRT-PCR used to detect

expression of *PR* genes. The expression of *MdPR1*, *MdPR5*, and *MdNPR1* was significantly increased in *MdPBS3.1*-overexpressing and *MdWRKY46*-overexpressing calli (Fig. 2D). These results indicated that MdWRKY46 and *MdPBS3.1* are able to positively regulate resistance to *B. dothidea*. They also demonstrated that MdWRKY46 may improve resistance to *B. dothidea* through an *MdPBS3.1*-dependent pathway.

DISCUSSION

In plants, specific disease resistance (R) proteins identify pathogens and enhance resistance to disease (Jones and Dangl 2006; Wang et al. 2015). R proteins bring about a complex series of defense responses, such as the strengthening of the cell wall, bursts of ROS, and accumulation of antimicrobial proteins, known as PR proteins (Hammond-Kosack and Jones 1996; Lehmann et al. 2015). SA is considered a key second messenger involved in the induction of PR proteins and the expansion of the oxidative burst (Lu et al. 2016; Ryals et al. 1996; Shirasu et al. 1997; Yalpani et al. 1991).

Fig. 5. Transcriptional activation of *MdPBS3.1* by *MdWRKY46*. **A** and **B**, pGreenII 62-SK + 1300-GUS, *MdWRKY46*-pGreenII 62-SK + 1300-GUS, pGreenII 62-SK + pro*MdPBS3.1*-box2-GUS, and *MdWRKY46*-pGreenII 62-SK + pro*MdPBS3.1*-box2-GUS were transformed into apple calli for glucuronidase (GUS) staining, immunoblotting (**A**), and GUS activity (**B**). **C**, Dual luciferase assays in tobacco leaves. pro*MdPBS3.1*-box2-pGreenII 0800-LUC + *MdWRKY46*-pGreenII 62-SK was transiently transformed into tobacco leaves to detect luminescence, and pGreenII 0800-LUC + pGreenII 62-SK, pGreenII 0800-LUC + *MdWRKY46*-pGreenII 62-SK, and pro*MdPBS3.1*-box2-pGreenII 0800-LUC + pGreenII 62-SK were used as the controls. **D** and **E**, *MdWRKY46*-GFP was transiently transformed into pro*MdPBS3.1*-GUS transgenic calli or 1300-GUS transgenic calli to detect GUS staining (**D**) and GUS activity (**E**). **F**, Expression of *MdPBS3.1* in wild-type (WT) and *MdWRKY46* transgenic calli. WT represents wild-type 'Orin' apple calli. All experiments were repeated at least three times, and the same results were obtained each time. 1300-GUS: empty gus reporter.

The responses of *MdPBS3.1* to *B. dothidea*.

SA plays important roles in plant disease resistance (Lu et al. 2016; Shirasu et al. 1997; White 1979). A major portion of SA produced in plants is converted into SAG, which is the dominant form of SA (Barman et al. 2016; Enyedi and Raskin 1993; Malamy et al. 1992). PBS3 belongs to the GH3 family of acyl adenylases/thioester-forming enzymes that contribute to the accumulation of SAG (Nobuta et al. 2007; Staswick et al. 2002). A *pbs3-1* mutant has been shown to decrease the resistance to *P. syringae* (*avrRpt2*) infection (Nobuta et al. 2007). PBS3 has an AMP-binding motif that is necessary for adenylation (Nobuta et al. 2007). AMP-binding and phylogenetic tree analysis of *MdPBS3.1* revealed that the function of *MdPBS3.1* was conserved among different plant species (Fig. 1). Based on the results, *MdPBS3.1* could be induced by *B. dothidea* (Fig. 1), suggesting that *MdPBS3.1* may be associated with resistance to *B. dothidea*. The response of the *MdPBS3.1* promoter to *B. dothidea* (Fig. 3) further implied that *MdPBS3.1* plays a vital role in the disease resistance of apple.

The increased SA content influenced by *MdWRKY46* and *MdPBS3.1*.

The GH3 family was first found in soybean plants (Hagen et al. 1984). In *Arabidopsis*, 19 GH3 genes have been identified

and divided into three distinct groups based on protein analysis (Staswick et al. 2005; Westfall et al. 2010). PBS3 belongs to group III of the GH3 proteins. A benzoyl-amino acid synthetase (*AtPBS3*) is involved in the SA pathway (Nobuta et al. 2007; Okrent et al. 2009). In the process of plant immunity, PBS3 protects EDS1 from proteasome-mediated degradation (Chang et al. 2019). *MdPBS3.1* can enhance resistance to *B. dothidea* and the content of free SA and SAG (Fig. 2). Under normal circumstances, however, free SA remains at its basal level, at which it does not affect the growth and development of plants (Hao et al. 2018). When inoculated with *B. dothidea*, SAG may convert to SA to activate resistance. During the conversion of SAG and free SA, *MdPBS3.1* plays an indispensable role. In addition, the weak response of the promoter of *MdPBS3.1* to *B. dothidea*, in which the W-box1 motif was mutated, indicates there may exist vital WRKY transcription factors that are related to resistance to *B. dothidea*.

A growing body of research indicates that WRKY proteins are involved in responses to biotic stress in a manner that involves transcriptional activation or inhibition (Dong et al. 2003; Kim et al. 2006; Li et al. 2006). In tobacco, *NtWRKY12* binds to the *PR1* promoter and activates the transcription of *PR1* by SA and bacterial elicitors (van Verk et al. 2008). *Arabidopsis*

Fig. 6. Increased resistance of *MdWRKY46* against *Botryosphaeria dothidea* in an *MdPBS3.1*-dependent manner in apple. **A**, *pIR-MdWRKY46*, *TRV-MdPBS3.1*, *pIR-MdPBS3.1*, *pIR-MdWRKY46*, and *TRV-MdPBS3.1* were transiently transformed into 'Fuji' apple, and phenotypic changes were observed after inoculation with *B. dothidea*. **B**, Lesion diameter; **C**, free salicylic acid content; and **D**, *MdPR1*, *MdPR5*, and *MdNPR1* expression levels were determined. *pIR* and *TRV* were used as the controls.

AtWRKY44 is able to activate SA-induced disease resistance by directly binding to the promoter of *AtWRKY70* (Shim et al. 2013). In *Brassica napus*, *BnWRKY33* can positively regulate *Sclerotinia sclerotiorum*, whereas *BnWRKY15* binds to the *BnWRKY33* promoter, inhibits the transcriptional activation of *BnWRKY33*, and reduces the resistance of plants to infection by *S. sclerotiorum* (Liu et al. 2018a). In rice, OsWRKY67 can bind to the promoters of *PR1a* and *PR10* and activate their expression to enhance resistance to leaf blast, panicle blast, and bacterial blight and accumulate endogenous SA (Liu et al. 2018b). In this study, a WRKY transcription factor (MdWRKY46) interacting with the *MdPBS3.1* promoter was identified (Fig. 4). In addition, experiments demonstrated that *MdPBS3.1* could be activated by MdWRKY46 to enhance resistance to infection by *B. dothidea* (Figs. 5 and 6). Upon repression of *MdPBS3.1* and *MdWRKY46* in apple peel, the increased SA content suggested that MdWRKY46 contributes to the accumulation to SA partially in an *MdPBS3.1*-dependent

way. When the W-box2 motif was mutated, the promoter of *MdPBS3.1* also responded to *B. dothidea* (Fig. 3), suggesting that other transcription factors may mediate the activity of MdPBS3.1 during SA signaling and disease resistance.

The expression of PR genes induced by MdWRKY46 in a *MdPBS3.1*-dependent way against *B. dothidea*.

SA-mediated plant resistance is mainly regulated by PR proteins. PR genes strictly control plant growth and defense (Linthorst 1991; van Verk et al. 2008). In *Arabidopsis*, *PR1* is an SA-inducible marker gene regulated by WRKY50 and TGA transcription factors synergistically (Hussain et al. 2018). As an SA receptor, NPR1 has a indispensable function in the transcriptional regulation of plant immunity (Ding et al. 2018). When a plant is infected with a pathogen, it often accumulates SA, and the expression of disease-resistant genes for immunity increases (Zheng et al. 2015). Moreover, *MdPBS3.1* and *MdWRKY46* can positively regulate *B. dothidea*; positively regulate the expression of *MdPR1*, *MdPR5*, and *MdNPR1*; and accumulate SA (Figs. 2 and 6). Thus, it is likely that MdWRKY46 can positively regulate resistance to *B. dothidea* by transcriptionally activating *MdPBS3.1* through the SA-mediated pathway.

Conclusion.

These results reveal that the expression of *MdWRKY46* was induced by SA treatment. MdWRKY46 activated the expression of *MdPBS3.1* by directly binding to its promoter, resulting in the increase of *MdPBS3.1* transcription. Although the over-expression of *MdPBS3.1* promoted the accumulation of SA, the expression of PR genes, including *MdPR1*, *MdPR5*, and *MdNPR1*, resulted in enhanced resistance to *B. dothidea* in apple. Therefore, we hypothesize that there is a positive loop in the regulation pathway of SA-MdWRKY46-MdPBS3.1-SA during biotic stress induced by agents such as *B. dothidea* (Fig. 7). In addition, there may be other mechanisms that regulate the expression of *MdPBS3.1*, which requires further investigation.

MATERIALS AND METHODS

Plant materials and growth conditions.

Plant materials studied included bagged 'Fuji' apples (*Malus domestica*), 'Orin' apple calli, and 'Royal Gala' apple seedlings. 'Fuji' apples were used for apple injection assays and pathogen infection assays. 'Fuji' apples were picked 150 days after flowering and stored at 28°C in darkness for 4 days after infection with *B. dothidea*. 'Orin' apple calli were used for GUS staining and activity detection, genetic transformation, and pathogen infection assays. Apple calli were cultured on Murashige and Skoog (MS) medium supplemented with 2, 4-dichlorophenoxyacetic acid at 0.5 mg·L⁻¹, 6-benzyl adenine (6-BA) at 1.5 mg·L⁻¹, sucrose at 30 g·L⁻¹, and agar at 8 g·L⁻¹ at 25°C in darkness. 'Royal Gala' apple seedlings were used for assays that tested the response to *B. dothidea*. 'Royal Gala' apple seedlings were treated with SA at 150 μmol·L⁻¹ for 0 h, 1 h, 3 h, 6 h, 12 h, and 24 h. 'Royal Gala' apple seedlings were cultured on MS medium supplemented with 6-BA at 1.0 mg·L⁻¹, naphthylacetic acid at 0.2 mg·L⁻¹, sucrose at 30 g·L⁻¹, and agar at 8 g·L⁻¹ at 25°C under 16 h of light.

Vector construction and transformation.

The promoter sequence of *MdPBS3.1* was used to construct the recombinant plasmids, including *proMdPBS3.1-GUS*, *proMdPBS3.1-box2-pHIS2*, and *proMdPBS3.1-box2-pGreenII 0800-LUC*. The vector construction of the mutant promoter of *MdPBS3.1* (*proMdPBS3.1-GUS(m1 and m2)*) was as follows: i) the recombinant *proMdPBS3.1-GUS* plasmid was used as the

Fig. 7. Model demonstrating the regulatory mechanism of resistance to *Botryosphaeria dothidea* in the salicylic acid signaling pathway by MdWRKY46 through its interaction with and activation by the *MdPBS3.1* promoter.

template; ii) two DNA fragments were amplified using primers F1/R1 and F2/R2 of *proMdPBS3.1-GUS(m1)* or *proMdPBS3.1-GUS(m2)*, respectively; and iii) the *proMdPBS3.1-GUS(m1)* or *proMdPBS3.1-GUS(m2)* fragment was obtained using primers F1/R2 of *proMdPBS3.1-GUS(m1)* or *proMdPBS3.1-GUS(m2)* after combining the two DNA fragments, which served as the template.

Full-length *MdPBS3.1* cDNA was used to generate *pIR-MdPBS3.1* and *MdPBS3.1-GFP*. Approximately 250 bp of the nonconserved domain sequence of *MdPBS3.1* was used to construct *TRV-MdPBS3.1*, and the reverse complement of *MdPBS3.1* was used to construct *MdPBS3.1-Anti*. Full-length *MdWRKY46* cDNA was used to generate the recombinant plasmids, including *MdWRKY46-AD*, *MdWRKY46-GFP*, *MdWRKY46-GST*, *MdWRKY46-pGreenII 62-SK*, and *pIR-MdWRKY46*. All primers in this study are listed in Supplementary Table S1.

MdWRKY46-GFP, *MdPBS3.1-GFP*, and *MdPBS3.1-Anti* were transformed into apple calli by *Agrobacterium tumefaciens*-mediated genetic transformation as previously described (An et al. 2018a). After incubation of *Agrobacterium tumefaciens*-containing recombinant plasmids or WT calli for 30 min, the infected calli were cultured on 'Orin' calli medium without antibiotics in darkness for 2 days, and the infected calli were transferred to the 'Orin' calli medium containing antibiotics for screening.

qRT-PCR assays.

qRT-PCR was performed as previously described (Qi et al. 2018). *MdACTIN* was used as the reference gene. qRT-PCR reaction conditions were as follows: predenaturation at 95°C for 10 min, followed by 40 cycles of denaturation at 95°C for 15 s, annealing at 56°C for 15 s, and extension at 65°C for 10 s. The fluorescence signal was obtained in step 3 of each cycle. The data were analyzed using the $2^{-\Delta\Delta Ct}$ method.

EMSA.

The relationship between *MdWRKY46* and *MdPBS3.1* was investigated using the LightShift Chemiluminescent EMSA kit (Thermo Fisher Scientific). The *MdWRKY46-GST* fusion protein, unlabeled probe, biotin-labeled probe, and mutant *MdPBS3.1* probe or WT *MdPBS3.1* probe were incubated in binding buffer at 24°C for 20 min. The samples were separated by polyacrylamide gel electrophoresis. The GST protein was used as a negative control.

ChIP-qPCR assays.

ChIP-PCR assays were performed using the EpiTect ChIP OneDay kit (SinaSun), according to manufacturers' instructions. The overexpression of *MdWRKY46-GFP* and the GFP-labeled empty vector in calli were used for ChIP-PCR assays. The GFP antibody was used in this assay. Enriched DNA fragments were detected by qPCR.

Y1H assays.

The MATCHMAKER One-Hybrid System (Clontech) was used to conduct Y1H assays, according to manufacturers' instructions. The *PGADT7-Rec2* yeast expression vector containing the GAL4 activation domain and the cDNA library of apple peel cells, and the bait, *pHIS2* reporter vector, which contained the critical positive regulatory elements of the *MdPBS3.1* promoter, were cotransformed into the Y187 strain. The transformants were screened on Leu-, Trp-, and His-deficient medium supplemented with 20 mM 3-AT for 3 to 4 days. The positive clones were extracted and transformed into *Escherichia coli* DH5 α for sequence analysis. The amino acid sequences of the positive clones were checked using the NCBI website.

MdWRKY46-AD and *proMdPBS3.1-box2-pHIS2* were cotransformed to the Y187 yeast strain. The cotransformed

Y187 yeast strain was cultured on Leu-, Trp-, and His-deficient medium supplemented with 20 mM and 80 mM 3-AT.

Transient expression assays.

Transient expression assays were performed as previously described (An et al. 2018b). *Agrobacterium tumefaciens*-containing *proMdPBS3.1-box2-pGreenII 0800-LUC* and *MdWRKY46-pGreenII 62-SK* were resuspended in MgCl₂ at 2 g·L⁻¹ and MES at 2.132 g·L⁻¹ and coinjected into the leaves of *Nicotiana benthamiana*. After 3 days, luminescence was detected using a live-cell imager.

Apple injection assays.

Apple injection assays were performed as previously described (An et al. 2018c). Briefly, 'Fuji' apples were picked 150 days after flowering, and *TRV-MdPBS3.1* and *pIR-MdWRKY46* were then mixed and coinjected into apple peels. *pIR-MdWRKY46* was also injected into apple peels. After 2 days, the free SA content in the apple peels was quantified by high-performance liquid chromatography, related gene expression was measured by qRT-PCR assay, and the injection holes were inoculated with *B. dothidea* mycelia. The injected apples were maintained at 28°C in darkness for 4 days.

Pathogen infection assays.

Transgenic calli (*MdWRKY46-GFP*, *MdPBS3.1-Anti*, and *MdWRKY46-GFP/MdPBS3.1-Anti*) and WT calli were grown on calli medium for 10 days. Subsequently, these calli and *B. dothidea* mycelia were cocultured for 4 days at 28°C in darkness.

B. dothidea was inoculated into 'Fuji' apples and maintained in darkness at 28°C for 0 days, 1 day, and 2 days. 'Fuji' apples picked at the same time were drilled and maintained in darkness at 28°C for 0 days, 1 day, and 2 days as a control. The inoculated peel was excised and frozen in liquid nitrogen for qRT-PCR.

As described by He et al. (2018), the samples were infected with 0.5-cm-diameter agar discs containing uniformly dispersed *B. dothidea* mycelia.

GUS staining and activity detection.

pGreenII 62-SK and 1300-GUS, *MdWRKY46-pGreenII 62-SK* and 1300-GUS, *pGreenII 62-SK* and *proMdPBS3.1-box2-GUS*, and *MdWRKY46-pGreenII 62-SK* and *proMdPBS3.1-box2-GUS* were transformed into calli by vacuum infiltration. Histochemical staining was carried out to measure GUS activity in the treated calli as previously described (Zhao et al. 2016). Briefly, the transgenic apple calli were added to a GUS-staining buffer and maintained at 37°C in darkness for 8 h. For quantitative testing of GUS activity, the transgenic apple calli were added to extraction buffer, and the protein was quantified using the RC DC Protein Assay kit (Bio-Rad Laboratories). GUS activity was quantified using a spectrofluorometer. In addition, the calli that underwent treatment with vacuum infiltration were used for protein extraction, and western blot assays were performed to measure the GUS protein level using an anti-GUS antibody (BGI).

proMdPBS3.1-box2-GUS was transformed into calli by an *Agrobacterium* spp.-mediated method. Subsequently, *MdWRKY46-GFP* was transformed by vacuum infiltration into the calli described above. GUS staining and GUS activity were performed as previously described (Zhao et al. 2016).

GUS-staining assays of 'Royal Gala' apple seedlings were performed as previously described (Zhang et al. 2018). Briefly, 'Royal Gala' apple seedlings were infiltrated in liquid medium containing *proMdPBS3.1-GUS* (*proMdPBS3.1-GUS (m1)* or *proMdPBS3.1-GUS (m1)*) under a 65 kPa vacuum for 20 min.

'Royal Gala' apple seedlings infiltrated with proMdPBS3.1-GUS were inoculated with a pathogenic spore (optical density at 600 nm of 0.6) under a 65 kPa vacuum for 10 min. After infiltration, the 'Royal Gala' apple seedlings were incubated in GUS-staining solution for 12 h, followed by destaining with 70% ethyl alcohol.

Western blot.

Calli were combined with sodium dodecyl sulfate-polyacrylamide gel electrophoresis loading buffer, boiled, and centrifuged. After collecting the supernatant, polypropylene gel electrophoresis was performed, and the proteins were transferred to polyvinylidene difluoride membranes (Millipore) for immunoblotting with an appropriate antibody.

Free SA and SAG detection assays.

As described previously by Verberne et al. (2002), free SA and SAG extracted from apple peels (approximately 0.1 g of tissue) were quantified by high-performance liquid chromatography. This experiment was performed independently three times.

Bioinformatics analysis.

The conserved domain of the MdPBS3.1 protein was analyzed using the NCBI conserved-domain online software. Phylogenetic tree analysis was performed using Mega 7.0.2 software (NCBI) as previously described (Qi et al. 2019).

Statistical analysis.

Appropriate methods, using R (3.0.2) with the R Commander package, were employed to analyze three parallel experiments statistically. The mean differences in the bars were significant at the $P_{0.05}$ level, with different letters, and not significant at the $P_{0.05}$ level, with the same letters.

AUTHOR-RECOMMENDED INTERNET RESOURCES

Apple Genome and Epigenome database: <https://iris.angers.inra.fr/gddh13>
NCBI website: <https://www.ncbi.nlm.nih.gov>

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