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ORIGINAL RESEARCH

Pathogen-induced MdWRKY1 in 'Qinguan' Apple Enhances Disease Resistance

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Abstract The WRKY transcription factors have important functions in plant-defense signalling networks. We isolated MdWRKY1 from the Chinese 'Qinguan' apple, which is resistant to *Alternaria* blotch or leaf spot. The MdWRKY1 protein was targeted to the nucleus and activated the expression of a reporter gene, consistent with the functioning of a transcription factor. When plants were infected with the pathogen *Alternaria alternata f.* sp. *mali*, MdWRKY1 was induced dramatically. Similarly, treatment with hormones SA and MeJA increased transcription significantly. Overexpression in tobacco also enhanced resistance to *Phytophthora parasitica* var. *nicotianae* Tucker. These results suggest that MdWRKY1 is a positive regulator of the defense response in higher plants.

Keywords Alternaria leaf spot · Apple · Disease resistance · Transcription factor · WRKY

Plants adapt to biotic and abiotic stresses in their natural habitats by activating multiple defense signalling pathways (Singh et al. 2002; Lagace and Matton 2004; Sujeeth et al. 2010). Transcription factors (TFs) are important for regulating plant responses to environmental conditions (Chen et al. 2002; Zhang 2003; Ulker and Somssich 2004; Zhou et al. 2008; Seo et al. 2010). The WRKY TFs are a large family with most members involved in diverse biotic and abiotic stress responses (Ulker and Somssich 2004). Those factors contain one or two conserved WRKY domains that comprise 60 amino acid residues and a conserved WRKYGQK sequence followed by a C_2H_2 or C_2HC zinc finger motif. These predominantly plant-specific proteins have been identified in a wide range of higher plants (Zheng et al. 2006; Mzid et al. 2007; Cai et al. 2008; Oh et al. 2008; Pandey and Somssich 2009; Song et al. 2009).

WRKY proteins are thought to play significant roles in response to bacterial or fungal attacks and pathogen-related hormones, such as jasmonic acid, ethylene, and salicylic acid (Journot-Catalino et al. 2006; Xu et al. 2006; Eulgem and Somssich 2007). In *Arabidopsis*, stress-induced *WRKY25* functions as a negative regulator, and mutant lines show increased resistance to *Pseudomonas syringae* (Zheng et al. 2007). In rice, overexpressed *OsWRKY89* enhances resistance against fungal blast and the white-backed plant-hopper *Sogatella furcifera* (Wang et al. 2007). In tobacco, the *NtWRKY12* TF acts synergistically in PR-1a expression, which is induced by salicylic acid and bacterial elicitors (van Verk et al. 2008).

Alternaria blotch caused by the fungal pathogen *Alternaria alternata* is one of the most damaging leaf spot diseases of cultivated apple (*Malus domestica* Borkh.) (Rotem 1994; Bulajic et al. 1996). The AM toxin synthetase cloned from the *A. alternata* apple pathotype is considered the main pathogenic factor (Johnson et al. 2000). In the course of interactions between fungi and

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plants, multiple copies of AMT2 are a prerequisite for the pathogen to produce enough toxins for full pathogenicity. The molecular mechanism of disease resistance against *A. alternata* has not been reported (Harimoto et al. 2007, 2008). Although 39 *WRKY* genes have been identified in apple (Apple Transcription Factor Database, http://planttfdb.cbi.pku.edu.cn:9010/web/index.php?sp=md), little is known about the biological roles for each protein in that plant. Here, we report the cloning, characterization, and functional analysis of a WRKY TF and MdWRKY1 related to disease resistance in the *Alternaria* leaf spot-resistant 'Qinguan' apple (Dang et al. 2006). Our goal was to determine whether this application of MdWRKY1 can be a feasible tool for plant genetic engineering.

Materials and Methods

Plant Materials, Fungal Pathogen, and Hormone Treatments

Plants of *M. domestica* Borkh. 'Qinguan' were grown at an apple experiment station at the Northwest A & F University, Shaanxi, China. They were inoculated with pathogen *A. alternata f.* sp. *mali* as described by Harimoto et al. (2008). Briefly, spores in suspension (1×10^4 spores ml^{-1}) were sprayed on young leaves, which were then covered with plastic film. Samples were collected at 0, 1, 2, 3, 4, 5, 6, and 7 days post-inoculation (dpi) and stored in liquid nitrogen. For hormone treatment, leaves were sprayed with 200 μM salicylic acid (SA) or 50 μM methyl jasmonate (MeJA).

Cloning and Sequence Analysis of MdWRKY1

RNA was extracted from leaves at 0, 1, 2, 3, 4, 5, 6, and 7 dpi according to the method of Gasic et al. (2004). Briefly, 1 μg of DNase-treated total RNA was heated to 70°C for 5 min, then treated with PrimeScript™ RTase (Promega, USA) for 60 min at 42°C in a volume of 20 μl. Based on the conserved domains for apple WRKY TFs, a primer pair was designed: upstream 5′-ACCAA CAACT ACAGT GCATTA-3′ and downstream 5′-TTTTT GTGAA CTAGT AGACC C-3′. The cDNAs from eight time points were used as templates. PCR reactions consisted of 94°C for 3 min; 29 cycles of 94°C for 30 s, 60°C for 30 s, and 72°C for 2 min; then elongation at 72°C for 10 min. Target strips were extracted and cloned into pGEM-T before sequencing.

Homologous proteins were searched by BLASTX at the NCBI GenBank database (http://www.ncbi.nlm.nih.gov/BLAST/). Sequences for WRKY proteins from other plant species were also retrieved from the Bank database. A phylogenetic tree was constructed using the CLUSTALW2 program (http://www.ebi.ac.uk/Tools/clustalw2/index.html). Sequence alignment was performed via DNAman (Kyte and Doolittle 1982).

RNA Analysis

Total RNAs from apple leaves were isolated by an improved SDS/phenol method (Gasic et al. 2004), while those from tobacco were obtained as described by Ulker et al. (2007). Our semiquantitative real-time (RT)-PCR protocol included 94°C for 3 min; 25 cycles of 94°C for 30 s, 60°C for 30 s, and 72°C for 30 s; then elongation at

Table 1 Primers used in RT-PCR or qRT-PCR for analysis of expression patterns

Primer	Sequences	Tm (°C)[a]	Reference
MdWRKY1 (RT-PCR)	F 5′-AACCCAAAACCAGAGCCAAGAG-3′ R 5′-CCGGTCAACGACGATATAAACG-3′	60	
NtActin (RT-PCR)	F 5′-CTTGACGGAAAGAGGTTATT-3′ R 5′-GATCCTCCAATCCAGACACT-3′	58~60	GenBank acc. no. AB158612
MdWRKY1 (qRT-PCR)	F 5′-TCCCAGGCACAGCAGAACCA-3′ R 5′-TCAACGAGGCGGAGGAAAGC-3′	58	
MdActin (qRT-PCR)	F 5′-CCCAAAGGCTAATCGGGAGAAA-3′ R 5′-ACCACTGGCGTAGAGGGAAAGA-3′	58	GenBank acc. no. GQ339778.1
PR1 (RT-PCR)	F 5′-ACTGCAACCTCGTACATTCT-3′ R 5′-CACTTAACCCTAGCACATCC-3′	58	Matsuoka et al. 1987
PR2 (RT-PCR)	F 5′-CACAACAAGAAGCAAATCCT-3′ R 5′-TATCAAAGTGAAACCAGAGT-3′	60	Ward et al. 1991
PR3 (RT-PCR)	F 5′-AACGACGGTAGATGTCCTGC-3′ R 5′-AGATGGCTTGTTGTCCTGTG-3′	58	Linthorst et al. 1990
PR5 (RT-PCR)	F 5′-GTTTTCTTCCTCCTTGCCTT-3′ R 5′-ACATTCGCCGTATATTAGCC-3′	60	Kumar and Spencer 1992

RT-PCR real-time polymerase chain reaction, *qRT-PCR* quantitative real-time polymerase chain reaction, *Tm* temperature

[a] annealing temperature used in PCR

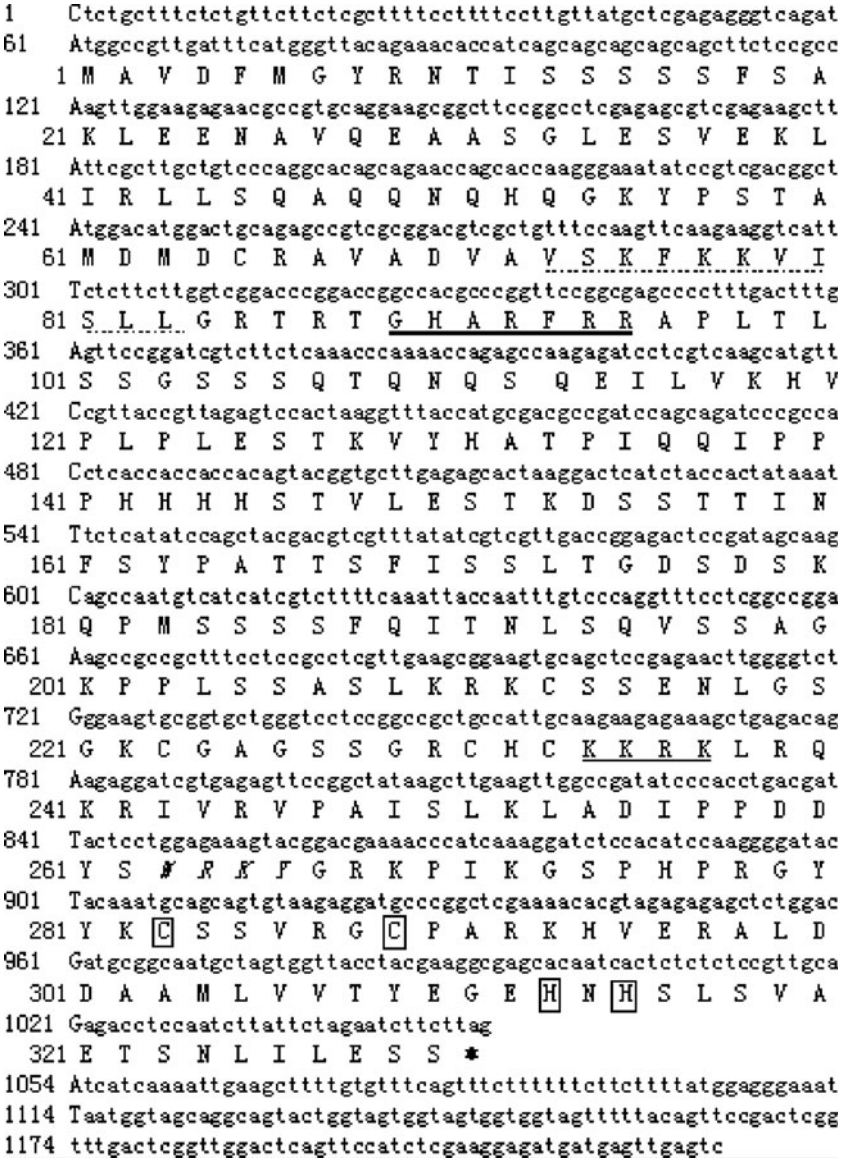

Fig. 1 Analysis of cDNA and deduced amino acid sequence from MdWRKY1. WRKY motif is in *italics*. Putative nuclear localization signal (KKRK) is *double-underlined*, GHARFRR domain is *underlined*, conserved calmodulin-binding domain is marked by *dashed lines*, and two cysteines and histidines of zinc-finger motif are *boxed*

72°C for 10 min. Quantitative RT-PCR (qRT-PCR) was performed in 96-well blocks, using an IQ5 real-time PCR cycler (Bio-Rad Laboratories, USA) and SYBR green master mix (Takara Biotechnology, Japan) in a reaction volume of 25 μl. Cycling parameters were 95°C for 30 s, then 40 cycles of 95°C for 5 s and 60°C for 30 s. To evaluate the quality of the dissociation curves, the following program was added after the 40 PCR cycles: 95°C for 15 s, followed by a constant increase in temperature from 60°C to 95°C. Apple *actin* (*MdActin*) and tobacco *actin* (*NtActin*) were amplified as internal controls. Primers are listed in Table 1. Each relative expression level was analyzed with IQ5 software per the normalized expression method.

Subcellular Localization of MdWRKY1

To determine its subcellular localization, the full-length open reading frame (ORF) of MdWRKY1 (without the termination codon) was amplified by PCR with forward primer GGGTA TAGAA TGGCC GTTGA TTTCAT G and reverse primer GGGGG TACCA GAAGA TTCTA GAATA A. After verification by sequencing, the fragment was ligated into the pBI221–GFP vector under the control of the CaMV35S promoter to generate the *35S:MdWRKY–GFP* construct. The sequenced plasmids were delivered into onion epidermal cells with a PDS-1000/He gene gun at 1,100 psi, then cultured on an Murashige and Skoog (MS) medium under darkness for 18 h at 22°C.

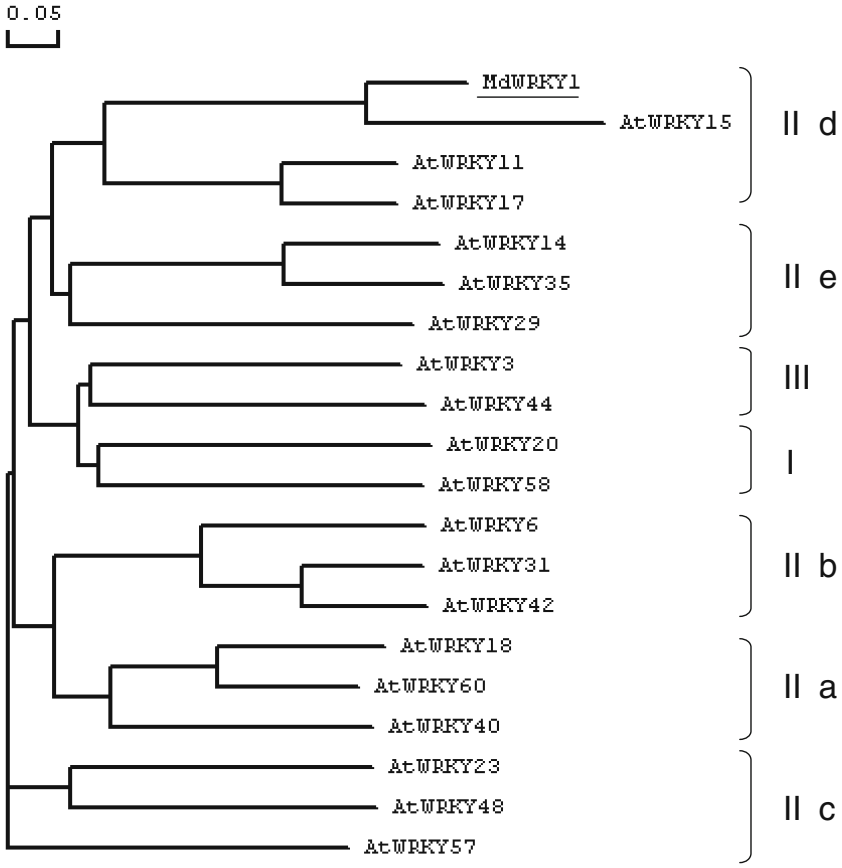

Fig. 2 Relationships among amino acid sequences from MdWRKY1 and *Arabidopsis* WRKY proteins. Phylogenetic and evolutionary analyses were performed with CLUSTALW. GenBank accession numbers are as follows: WRKY3 (AT2G03340.1), WRKY6 (AT1G62300.1), WRKY11 (NP_849559), WRKY14 (NP_564359), WRKY15 (AT2G23320.1), WRKY17 (NP_565574), WRKY18 (AT4G31800.1), WRKY20 (NP_849450), WRKY23 (NP_182248), WRKY29 (NP_194086), WRKY31 (NP_567644), WRKY35 (NP_181029), WRKY40 (NP_178199), WRKY42 (NP_192354), WRKY44 (NP_181263), WRKY48 (NP_199763), WRKY57 (NP_974112), WRKY58 (NP_186757), and WRKY60 (NP_180072)

Transactivation Functioning of MdWRKY1

To analyze the transactivational activity of MdWRKY1, we cloned its ORF into the yeast expression BD vector pGBKT7. Plasmids *pGBKT7–MdWRKY1* and pGBKT7 were transformed into *Saccharomyces cerevisiae* strain AH109 according to protocols provided by Clontech (USA). The transformed yeast was streaked onto –Trp/SD (synthetic dextrose) or –Trp/–Ade/–His/SD media plates to observe growth at 30°C for 3 to 4 days. Activity of β-galactosidase was assayed by using X-gal.

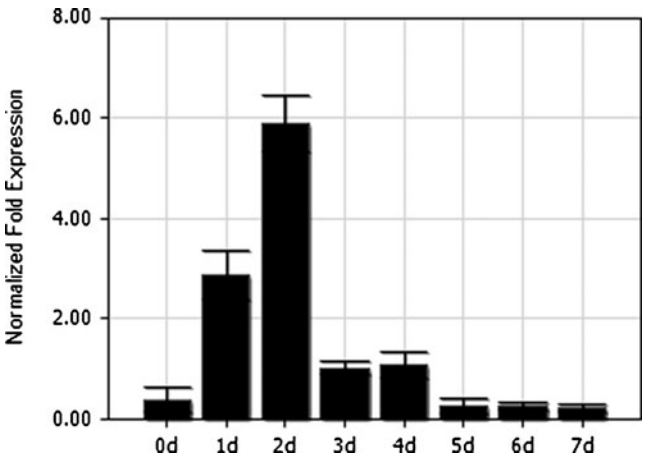

Fig. 3 Expression profile of MdWRKY1 in *M. domestica* Borkh. 'Qinguan' following induction by *A. alternata f.* sp. *mali*. MdActin was used as internal control for qRT-PCR. Mean values and standard deviations were obtained from three technical and three biological replicates

Overexpression of MdWRKY1 in Tobacco and Infection by *Phytophthora parasitica* var. *nicotianae* Tucker

To construct the overexpression vector for MdWRKY1, we subcloned the coding sequence into a pGEM-T easy vector for sequencing, then subcloned it into the binary plant transformation vector pBI121. The recombinant plasmids were introduced into *Agrobacterium* strain GV3101. Plant overexpression constructs of MdWRKY1 were transferred into tobacco NC89. Transgenic plants were selected on an MS medium containing 200 μg L^{-1} kanamycin and 400 μg L^{-1} carbenicillin.

For analysis of fungal resistance, mycelia of *P. parasitica* were cultured on an oat medium (30 gL^{-1} oats and 17 to 20 gL^{-1} agar) at 28°C. When the mycelia grew to the side of the plate, 0.5-cm-diameter agar discs were excised

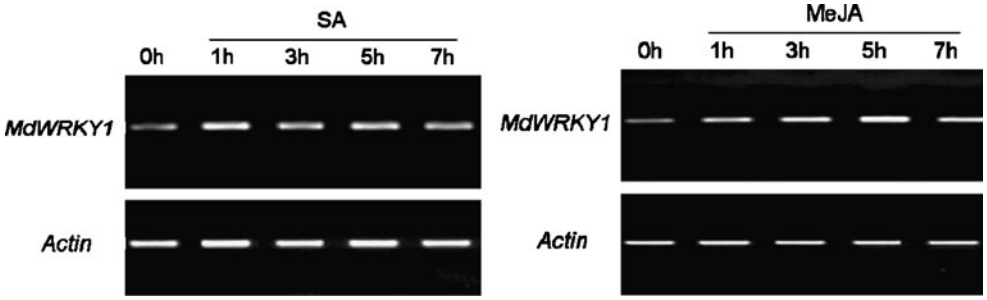

Fig. 4 Expression of MdWRKY1 in response to SA and MeJA. Actin was used as internal control for semiquantitative RT-PCR

with a punch from the edges of those colonies. They were then inverted onto leaves that had been detached from transgenic and wild-type (WT) control plants. All infected leaves were placed on a porcelain dish, covered with plastic film, and held at 28°C. Disease symptoms were photographed after 4 days of incubation. Intensity was evaluated by measuring the size and fresh weight of the lesions.

Results and Discussion

Sequence Analysis of MdWRKY1

WRKY transcription factors are vital for defense responses in plants (Eulgem and Somssich 2007). Earlier studies of biotic stresses and TFs focused mainly on *Arabidopsis*, tobacco, or rice (Pandey and Somssich 2009). In *M. domestica*, 39 TFs have been found in the apple transcription factor database, but their functions have not previously been reported. From *M. domestica* 'Qinguan', we isolated a full-length 1224-bp cDNA. MdWRKY1 (GenBank accession no. HM859901) encodes a 331 amino acid peptide. Sequence analysis showed that it contains one WRKY domain, one C_2H_2 zinc-finger motif (C-X_7-C-X_{23}-H-X_1-C), and one predicted nuclear-localization signal (KKRK). This peptide also has a conserved calmodulin-binding domain, a GHARFRR domain, and a plant-specific zinc cluster (Fig. 1). Proteins of the WRKY family are classified into three groups (I, II, and III), according to their number of WRKY domains and presence of the zinc finger-like motif (Eulgem et al. 2000). Group II can be further split into five distinct subgroups (IIa through IIe) based on additional short conserved structural motifs. Our phylogenetic tree showed that MdWRKY1 belongs to subgroup IId (Fig. 2), which includes *AtWRKY27*; members contain a unique conserved calmodulin-binding domain that can participate in calcium-signaling pathways (Dong et al. 2003). The

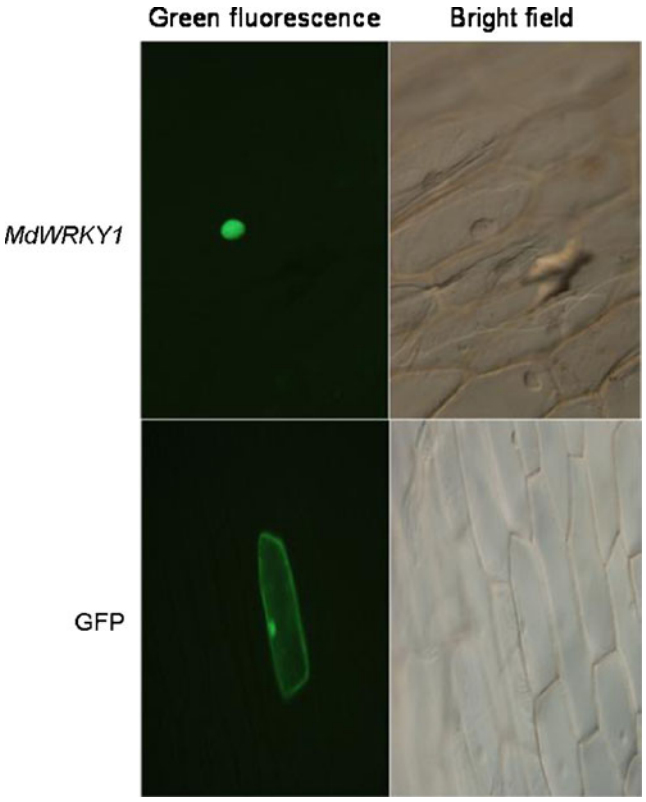

Fig. 5 Subcellular localization of MdWRKY1. Protein was localized to nucleus in onion epidermal cells whereas vector GFP was spread throughout cytosol and nucleus

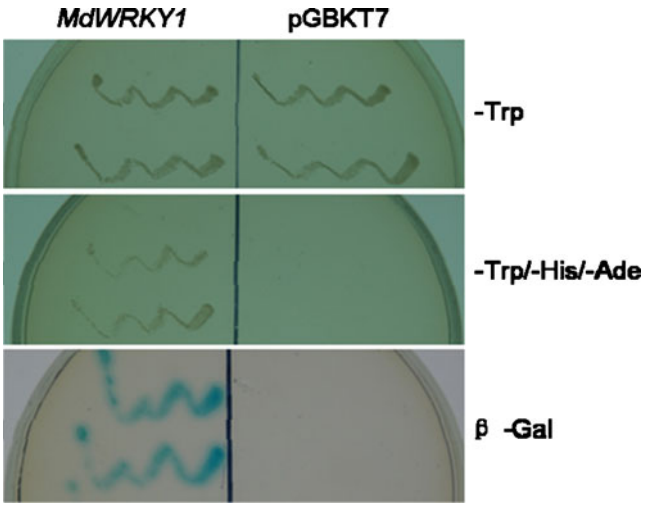

Fig. 6 Transcriptional activation by MdWRKY1 in yeast. Constructs were fused in-frame to GAL4 DNA-binding domain expression vector, then transformed into yeast strain Y190. Transformants were selected based on growth on –Trp/SD or –Trp/–Ade/–His/SD medium at 3°C for 3 days. β-galactopyranoside staining was performed to determine ability of translation product to activate transcription

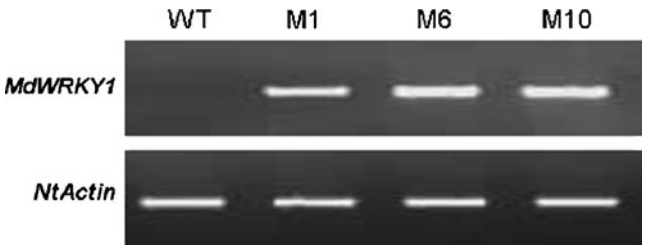

Fig. 7 Semiquantitative RT-PCR analysis of MdWRKY1 expression in wild-type and transgenic lines. NtActin was amplified as control

MdWRKY1 protein is a WRKY TF that shares 39.50% and 40.61% amino acid similarity with *AtWRKY17* and *AtWRKY11*, respectively (Eulgem et al. 2000).

MdWRKY1 is Induced by *A. alternata f.* sp. *mali* and Exogenous Hormones

Most known *WRKY* genes are responsive to pathogens. The expression of TFs is generally upregulated in infected plants and is correlated with pathogen resistance (Eulgem and Somssich 2007). For example, transcripts of *WRKY33* are substantially elevated within 24 h after inoculation with the *Botrytis* fungus (Zheng et al. 2006). Moreover, infection with *P. syringae* or *Botrytis cinerea* leads to strongly induced expression of *WRKY40* and *WRKY60* in *Arabidopsis* (Xu et al. 2006). *AtWRKY3* responds rapidly to infection by *Botrytis* and *P. syringae pv. tomato* (Pst) strain DC3000 (Lai et al. 2008). To investigate whether MdWRKY1 responds to *A. alternata f.* sp. *mali* in apple, we conducted qRT-PCR. Before inoculation, expression of MdWRKY1was low, but it then increased dramatically, peaking at 2 dpi before returning to the baseline (Fig. 3). Therefore, we propose that MdWRKY1 is involved in the defense response to this pathogen.

Such defense mechanisms are regulated by multiple signal transduction pathways, in which SA and MeJA function as key signalling molecules (Kunkel and Brooks 2002). Here, we elucidated the role of those exogenous hormones in apple. Transcription of MdWRKY1 was

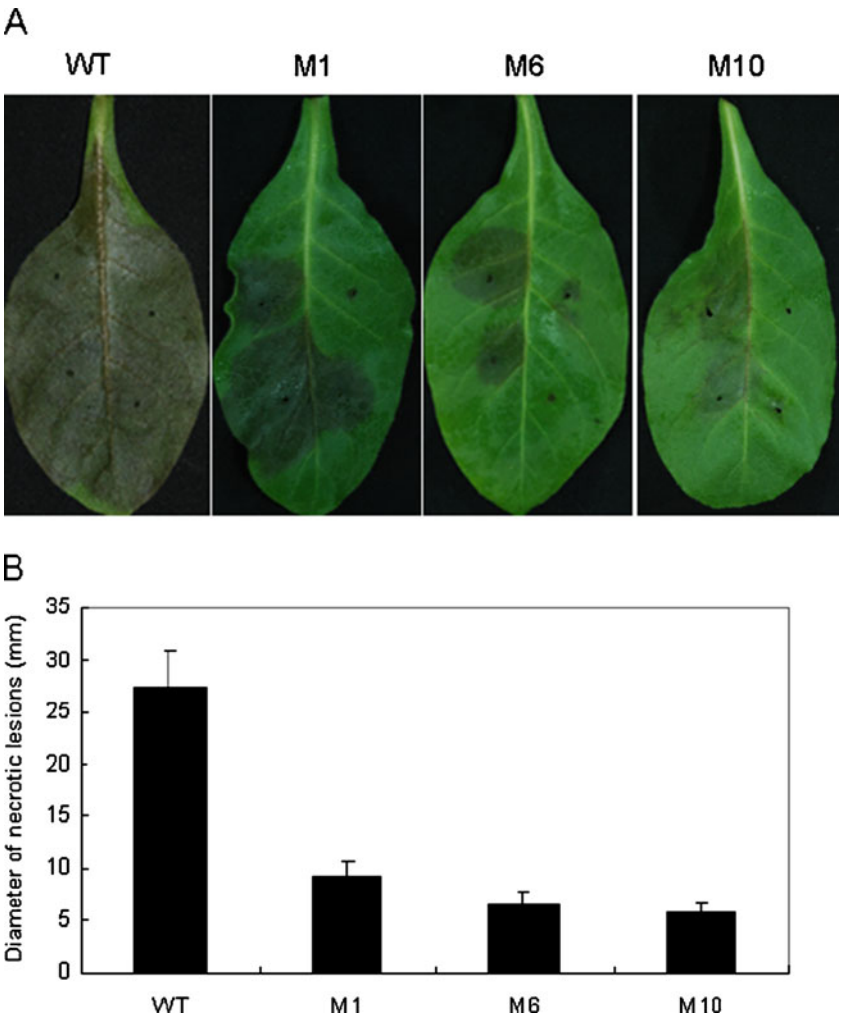

Fig. 8 MdWRKY1-enhanced resistance to *P. parasitica* in tobacco. **a** Disease-reaction phenotypes of representative wild-type and transgenic tobacco at 4 dpi. **b** Disease intensity was evaluated at 4 dpi by measuring lesions

induced significantly by SA or MeJA, reaching a peak at 1 h or 5 h, respectively, and then decreasing toward the baseline (Fig. 4). In *Arabidopsis*, *AtWRKY25* enhances resistance to *P. syringae*, being positively regulated by the SA signalling pathway but negatively regulated by MeJA (Zheng et al. 2007). *Arabidopsis WRKY33* improves resistance against necrotrophic pathogens and is a positive regulator of JA-mediated defense response signalling (Zheng et al. 2006). Based on the results from our assays, we suggest that MdWRKY1 also is involved in SA- and MeJA-mediated signalling pathways for plant responses to infection.

The Protein of MdWRKY1 is Nuclear

Sequence analysis revealed that the MdWRKY1 protein contains a putative nuclear signal. To investigate the localization of that gene product in plant cells, we used particle bombardment to transfer the *MdWRKY1–GFP* fusion gene and the control GFP driven by the 35S promoter into onion epidermal cells. The *MdWRKY1–GFP* protein was targeted to the nucleus. In contrast, the control GFP protein was distributed throughout the cytosol and the nucleus (Fig. 5), indicating that MdWRKY1 is a nuclear protein.

MdWRKY1 Functions as a Potential Transcriptional Activator

Our transactivation results showed that only yeast cells carrying *pGBKT7–MdWRKY1* could grow on an SD medium that lacked histidine. In contrast, yeast cells containing pGBKT7 (the negative control) did not grow on the medium without histidine (Fig. 6). Assays for β-galactosidase activity tested a second reporter LacZ (β-galactosidase) activity for *pGBKT7–MdWRKY1*. The MdWRKY1 protein activated β-galactosidase but the control did not, suggesting that ectopic expression of MdWRKY1 does activate reporter genes in yeast.

MdWRKY1 Enhances Resistance to *P. parasitica* and Activates the Expression of *PR* Genes in Tobacco Plants

WRKY TFs are critical to the plant response against biotic stresses (Ulker and Somssich 2004). In *Arabidopsis*, some WRKY factors are positive regulators of that response. For example, *AtWRKY33* confers resistance to the necrotrophic fungi *Alternaria brassicicola* and *B. cinerea* (Zheng et al. 2006), while *AtWRKY3* and *AtWRKY4* play positive roles in plant resistance to *B. cinerea* (Lai et al. 2008).

In contrast, many WRKY TFs act as negative regulators of defense signalling, including *AtWRKY7*, *−11*, *−17*, *−18*, *−23*, *−25*, *−27*, *−38*, *−40*, *−41*, *−48*, *−53*, *−58*, *−60*, and *−62*. Gene products from *AtWRKY18*, *AtWRKY40*, and *AtWRKY60* function as partially redundant negative regulators in resistance to *P. syringae* and *B. cinerea* (Xu et al. 2006). Overexpression of *AtWRKY18* and *AtWRKY70* in *Arabidopsis* results in constitutive expression of protective response (PR) genes and enhanced resistance to *P. syringae* (Chen and Chen 2002; Chen et al. 2002; Li et al. 2004). In rice, *OsWRKY13* improves resistance to the bacterial blight *Xanthomonas oryzae* pv.*oryzae* and the fungal blast *Magnaportha grisea* (Qiu et al. 2008). Transgenic rice overexpressing *OsWRKY53* are more resistant to *M. grisea*, thus demonstrating that this gene is a positive regulator of basal defenses (Chujo et al. 2007). Overexpression of *VvWRKY1* in tobacco is manifested by reduced susceptibility to fungi *Peronospora tabacina* and *Erysiphe cichoracearum* (Marchive et al. 2007). Constitutive expression of *VvWRKY2* in tobacco heightens resistance to *Alternaria tenuis*, *B. cinerea*, and *Pythium* (Mzid et al. 2007). Finally, in barley, *HvWRKY1* and *HvWRKY2* suppress basal defenses against virulent *Blumeria graminis* (Shen et al. 2007).

To evaluate the biological functions of MdWRKY1, we transformed its coding sequence under the control of the 35S promoter into tobacco (Fig. 7), and inoculated plants with *P. parasitica*. Once infected, WT tobacco exhibited typical necrotic lesions, especially enlarged by 4 dpi, whereas transgenic leaves appeared only partially necrotic

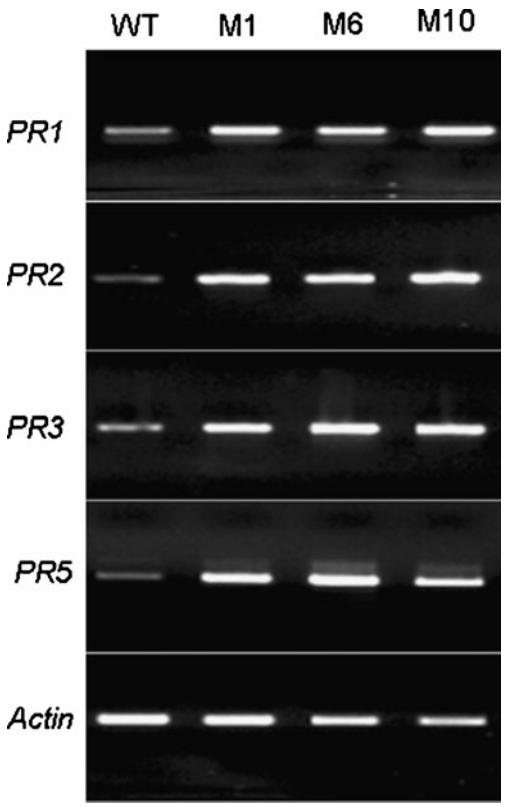

Fig. 9 Expression of *PR1*, *PR2*, *PR3*, and *PR5* in transgenic and wild tobacco lines by RT-PCR. Actin was used as internal control

in spots (Fig. 8a). In particular, those from lines M1, M6, and M10 were smaller by 2.95-, 3.96-, and 4.69-fold, respectively, compared with WT lesions (Fig. 8b). These results clearly demonstrate that MdWRKY1 enhances resistance to *P. parasitica* in tobacco.

PR proteins play a vital role in pathogen defenses. Overexpression of *PR1* in tobacco increases resistance to *P. parasitica* and *P. tabacina* (Alexander et al. 1993), while overexpression of tobacco *PR5* in potato plants improves resistance to *Phytophthora infestans* (Liu et al. 1994). The genes for *PR2* and *PR3* encode glucanases and chitinases, respectively, and these enzymes may function in cell wall degradation. To confirm the role of MdWRKY1, we performed RT-PCR to analyze the expression of four PR genes. Transcription levels of *PR1, PR2, PR3*, and *PR5* were higher in three transgenic tobacco lines than in the WT (Fig. 9), again indicating that MdWRKY1 acts as a positive regulator of plant defenses.

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