The WRKY transcription factor PlWRKY65 enhances the resistance of *Paeonia lactiflora* (herbaceous peony) to *Alternaria tenuissima*

Xue Wang¹, Junjie Li¹, Jing Guo¹,², Qian Qiao³, Xianfeng Guo¹,² and Yan Ma¹,²

**Abstract**

In this study, the disease resistance gene *PlWRKY65* was isolated from the leaves of *Paeonia lactiflora* and analyzed by bioinformatics methods, and the localization of the encoded protein was explored. Quantitative real-time PCR (qRT-PCR) was also used to explore the response of this gene to *Alternaria tenuissima*. The results showed that the gene sequence contained multiple cis-acting elements involved in the response to hormone signaling molecules belonging to the IIe subgroup of the WRKY family, and the encoded proteins were located in the nucleus. The *PlWRKY65* gene has a positive regulatory effect on *A. tenuissima* infection. After silencing the *PlWRKY65* gene via virus-induced gene silencing (VIGS), it was found that the gene-silenced plants were more sensitive to *A. tenuissima* infection than the wild plants, exhibiting more severe infection symptoms and different degrees of changes in the expression of the pathogenesis-related (PR) genes. In addition, we showed that the endogenous jasmonic acid (JA) content of *P. lactiflora* was increased in response to *A. tenuissima* infection, whereas the salicylic acid (SA) content decreased. After *PlWRKY65* gene silencing, the levels of the two hormones changed accordingly, indicating that *PlWRKY65*, acting as a disease resistance-related transcriptional activator, exerts a regulatory effect on JA and SA signals. This study lays the foundation for functional research on WRKY genes in *P. lactiflora* and for the discovery of candidate disease resistance genes.

**Introduction**

Plant immunity is a complex, multilayered system that includes several defense mechanisms. In addition to passive defense mechanisms, such as the cuticle, cell wall, and antimicrobial compounds, plants exhibit two layers of active defense mechanisms. The first is pattern-triggered immunity (PTI), induced by pathogen-associated molecular patterns (PAMPs), and the second is effect-triggered immunity (ETI), induced by pathogen proteins. When an immune response occurs in plants, the expression of disease resistance genes in cells is regulated by transcription factors (TFs), including the WRKYs, which constitute one of the largest transcription factor families and are involved in disease resistance.

The WRKY TF family is named for the highly conserved WRKYGQK heptapeptide sequence in the N-terminal amino acid sequence of its members. Because of the presence of different numbers of WRKY domains and different zinc finger motifs, the WRKY TFs can be divided into three groups. Based on the phylogenetic tree of WRKY protein domains, group II can be divided into five subgroups (IIa–e). Studies have shown that when induced by external stimuli in plants, WRKY TFs are regulated by a cascade of defense signaling networks that bind with the promoters of downstream genes to regulate their expression and enhance plant defense. Among plant disease resistance-related responses, the most...
widely studied is the regulation of the salicylic acid (SA) and jasmonic acid (JA) signaling pathways. A great deal of scientific research has demonstrated that the WRKY family is directly or indirectly involved in these two signaling pathways. For example, GbWRKY1 mediates the disease resistance and development of cotton through the JA signaling pathway and the negative regulation of cotton resistance to Botrytis cinerea. Rice OsWRKY13 can directly or indirectly regulate the expression of upstream and downstream genes of JA and SA to inhibit JA synthesis-related and JA-responsive gene expression and activate SA synthesis-related and SA-responsive gene expression to participate in disease resistance in rice.

Herbaceous peony is a common ornamental plant in China that has both economic and cultural value. Red spot is a serious disease of herbaceous peony caused by Alternaria alternata (A. alternata) and Alternaria tenuissima (A. tenuissima) that hinders peony production and causes serious economic loss. Moreover, the large-scale infection of peonies affects the growth and development of the underground bud, thereby affecting plant production in the second year. In recent years, the roles of WRKY TFs in disease resistance in plants have been widely reported. In Solanum pimpinellifolium, SpWRKY1, SpWRKY3, and SpWRKY6 participate in resistance against Phytophthora infestans as positive regulators. Overexpression of OsWRKY13 may improve the ability of rice to resist bacterial blight, which is a common problem in rice. However, no studies on the response of the Paeonia lactiflora WRKY family to pathogen stress have been reported. In this research, we identified a differentially expressed gene, PIWRKY65, from transcriptome data collected under infection with A. tenuissima. Studies have shown that PIWRKY65 can positively regulate the resistance of herbaceous peony to A. tenuissima and may play a role through either direct or indirect involvement in SA- and JA-mediated disease resistance signaling pathways.

Results
Cloning and sequence analysis of PIWRKY65
The genomic DNA and cDNA of P. lactiflora ‘Da Fugui’ were used as templates for amplification, and sequences with lengths of 1044 and 825 bp were obtained, respectively. The corresponding GenBank accession number is KY271096. The obtained gene was named PIWRKY65 due to its high homology with Arabidopsis AtWRKY65 (Fig. 1a). By comparing genomic DNA with cDNA sequences, PIWRKY65 was found to contain three exons and two introns (Fig. 1b), and the second intron was a typical R-type intron in the conserved WRKY domain.

Furthermore, PlantCARE software was used to analyze the PIWRKY65 gene sequence, and the results confirmed that the intron sequences of PIWRKY65 contain 14 CAAT box components, 6 TATA box components, G-box light-responsive components, several hormone signaling molecule-responsive cis-elements, such as methyl jasmonic acid (MeJA) signal elements, and cis-elements involved in the metabolic regulation of proteins.

A phylogenetic tree of PIWRKY65 and Arabidopsis WRKY family genes was constructed by referring to the standard classification of Arabidopsis WRKY members to carry out phylogenetic analysis and clustering. The results showed that PIWRKY65 and AtWRKY65 formed a branch belonging to the Ile subgroup of the WRKY family (Fig. 1c). Multiple comparative analyses indicated that the PIWRKY65 gene-coding region contains a WRKY domain structure consisting of 58 (152–209) amino acids, and this structure shares high homology with other plant WRKY65 TFs. In the N-terminal domain, the conserved structure contains the highly conserved WRKYGQK heptapeptide sequence, and the C-terminus harbors a C2H2 (CX5CX23HNH) zinc finger-type structure (Fig. 1d). The phylogenetic tree results showed that among the analyzed WRKY65 proteins, P. lactiflora PIWRKY65 was the most closely related to Nelumbo nucifera NnWRKY65 (Fig. 1e).

Subcellular localization of the PIWRKY65 protein
We transformed the recombinant vector pROKII-PIWRKY65-GFP and the vector containing only GFP into tobacco leaf epidermal cells to study the specific sites of the PIWRKY65 protein in cells. The results showed that green fluorescence in the epidermal cells of tobacco leaves transformed with the recombinant vector was present only in the nucleus, while in the leaves of the control group, the fluorescence showed a diffuse distribution and was present in both the nucleus and cytoplasm. Thus, it was inferred that the PIWRKY65 protein is present in the nucleus and may play a role there (Fig. 2).

PIWRKY65 was positively induced by A. tenuissima
Based on the differential expression of PIWRKY65 according to the transcriptome data, we further studied the expression patterns of PIWRKY65 after infection with A. tenuissima. The results showed that PIWRKY65 was positively expressed during infection with A. tenuissima. The expression of PIWRKY65 increased sharply and peaked at 24 h after infection with A. tenuissima, at a level 16.76 times higher than that of the control. Thereafter, the expression level remained higher than that of the control group until 96 h, showing an overall upward trend (Fig. 3).

VIGS of PIWRKY65 reduced the transcript abundance of endogenous PIWRKY65
To preliminarily understand the mechanism of PIWRKY65 in response to infection with A. tenuissima, we further evaluated the functions of PIWRKY65 by silencing. Leaf cDNAs obtained after 10 days of infection with the empty vector TRV::00 and recombinant vector
TRV::WRKY65 were used as templates, and pTRV1-F/R and pTRV2-F/R were used for PCR-based detection (Fig. 4a). The results showed that the target bands of 647 bp (pTRV1) and 372 bp (pTRV2) could be amplified from the P. lactiflora samples inoculated with TRV::00, and bands of 647 bp (pTRV1) and 774 bp (pTRV2::WRKY65) could be amplified from the samples inoculated with TRV::WRKY65 (Fig. 4b). These findings indicated that TRV::00 and TRV::WRKY65 were successfully inserted and expressed in the genome of P. lactiflora.

The silencing of the PlWRKY65 gene in the leaves of P. lactiflora was detected by qRT-PCR. The leaves were treated with TRV::00 or TRV::WRKY65 (with blank control), and after 16 days of treatment, the leaves were collected, and RNA was extracted. The results showed that PlWRKY65 gene expression in the leaves infected with TRV::WRKY65 was markedly lower than that in the blank control and the leaves infected with TRV::00 (Fig. 4c). These findings indicated that PlWRKY65 was effectively silenced in TRV::WRKY65-infected leaves.

**PIWRKY65-silenced plants exhibited greater sensitivity to A. tenuissima**

The control plants and the plants silenced for 20 days (silencing efficiency > 60%) were selected for the pathogen infection test. As shown in Fig. 5a, the expression of PIWRKY65 in response to infection with A. tenuissima in the PIWRKY65-silenced plants was markedly lower than that in the two control groups, but the changes in expression were consistent with those of the control plants. These findings indicated that the TRV-PIWRKY65-silencing vector effectively inhibited the expression of the PIWRKY65 gene, and in the PIWRKY65-silenced plants, PIWRKY65 still played an active regulatory role against A. tenuissima.
After infection with *A. tenuissima*, *PIWRKY65*-silenced plants exhibited higher sensitivity to the pathogens than the control plants (Fig. 5b), which was characterized by severe spreading of disease spot areas and parched perforation. After 21 days of infection, the incidence statistics of the plants showed that all plants were infected by *A. tenuissima*, and the disease indexes of the blank control plants, empty vector control plants, and *PIWRKY65*-silenced plants were 43.05, 44.10, and 62.45, respectively. The disease index of the *PIWRKY65*-silenced plants was markedly higher than that of the control (Fig. 5c) and was 19.40% and 18.35% higher than those of the blank control group and the empty vector control group, respectively. All of the above results illustrated that the *PIWRKY65* gene was involved in the resistance of peony to *A. tenuissima* and played an active role in regulating disease resistance in peony by increasing its expression.

**PIWRKY65 expression participates in JA and SA signaling**

To investigate whether endogenous hormones respond to resistance to *A. tenuissima* and whether *PIWRKY65* is associated with related hormones, we detected the endogenous levels of JA and SA in *PIWRKY65*-silenced plants and normal plants infected with *A. tenuissima* over 96 h. The results revealed (Fig. 6a) that the JA content of the control plants increased overall from 0 to 96 h, reaching the highest level at 96 h. However, SA decreased from 0 to 48 h, then increased to a peak at 72 h and decreased again thereafter. Interestingly, the JA level in the *PIWRKY65*-silenced group was lower than that in the normal group and gradually recovered to the same level as that in the normal plants at 96 h, but the SA level in the *PIWRKY65*-silenced group was higher than that in the normal group and recovered to the same level as that in the normal plants at 96 h, or even to a slightly lower level than that in the control. These data revealed that the changes in endogenous JA and SA levels are closely related to the disease resistance process after infection with *A. tenuissima* and are specifically correlated with the expression of *PIWRKY65*.

**Differential regulation of pathogenesis-related genes by *PIWRKY65***

We conjectured that *PIWRKY65* might be involved in the JA and SA signaling pathways because of the changes in endogenous hormones in *PIWRKY65*-silenced plants and the hormone signal-response elements in the *PIWRKY65* gene. Therefore, we used qRT-PCR to analyze the expression of the JA and SA pathogenesis-related (PR) genes PR1, PR2, PR4B, PR5, and PR10 to verify this hypothesis. The results showed that only *PlPR1* expression in *PIWRKY65*-silenced plants was markedly higher than that in control plants after 96 h of infection with *A. tenuissima*, while the expression of the other four *PlPRs* in *PIWRKY65*-silenced plants was markedly lower than that in the control plants (Fig. 6b). These results further indicated that *PIWRKY65* regulated the resistance of *P. lactiflora* to *A. tenuissima* by participating in the JA and SA signaling pathways.
Discussion

The transcriptional regulation of defense genes plays indispensable roles in plant resistance. Therefore, the identification of the components of the plant defense system and corresponding response pathways is an important step for understanding plant stress resistance. The WRKY TF family, which is involved in disease responses, was identified recently, and many of its members are involved in the pathogen resistance response, as shown for the Arabidopsis thaliana WRKY7, 33, 22, 70, and 54 TFs, which are directly involved in resistance to fungi. However, most studies on these TFs have been conducted in A. thaliana, tomato, tobacco, potato, rice, cotton, and other plants, and research on the gene functions of peony WRKY TFs is very limited. We screened a differentially expressed PIWRKY65 gene

Fig. 4 The effect of PIWRKY65 silencing in VIGS plants. a Schematic diagram of the pTRV1 and pTRV2 plasmids and primers. b The PCR identification of RNA1 and RNA2 of TRV in P. lactiflora leaves. c PIWRKY65 gene expression levels in PIWRKY65-silenced plants. S1 and S2 are both TRV:WRKY65 plants and are independent of each other.
from the transcriptome data of peony, speculated that it was related to the regulation of disease resistance, and provided strong evidence of the involvement of this gene in disease response regulation.

Regulatory elements involved in gene expression have been found in the introns of many genes. The introns of \textit{PlWRKY65} contain TATA box components, G-box light-responsive components and several hormone signaling molecule-responsive cis-elements. These characteristics indicate that the introns of this gene may increase the transcription of the gene and participate in hormone regulation or synthesis and other plant developmental processes. Theoretically, we can establish a comparison between WRKY TFs from other plants and peony WRKYs and infer the potential functions of homologous WRKY proteins according to the known functions of these proteins. Previous studies have shown that the \textit{Arabidopsis AtWRKY65} gene is involved in the immune response related to \textit{AtFLD} \textsuperscript{22}. Based on the fact that \textit{PlWRKY65} and \textit{AtWRKY65} both belong to the Ile subgroup, it is speculated that \textit{PlWRKY65} may be involved in the stress response during plant growth. The subcellular localization results implied that the \textit{PlWRKY65} protein was present in the nucleus, which agreed with the results obtained for...
WRKY proteins in other species\textsuperscript{23–25}. Therefore, we speculated that the PlWRKY65 protein acts as a transcriptional regulator, similar to most other WRKY proteins, and activates the expression of downstream target genes by binding to the W-boxes of target genes.

TFs, especially those of the WRKY family, play an important role in plant disease resistance responses as signaling pathway regulators. \textit{AtWRKY8} and \textit{AtWRKY28}, which belong to the IIA subgroup, can positively regulate the resistance of plants to \textit{B. cinerea}\textsuperscript{26,27}. Studies on the overexpression of \textit{OsWRKY45-1} and \textit{OsWRKY45-2} showed that this pair of alleles had a positive regulatory effect on resistance to \textit{Magnaporthe grisea}, a fungal pathogen of rice\textsuperscript{6}. The same conclusion was reached in this study, in which the expression of \textit{PlWRKY65} increased under induction by \textit{A. tenuissima} and positively regulated the disease resistance of peony. However, in tomato, \textit{SiWRKY70} transcription negatively regulates plant resistance to fungi\textsuperscript{28}. Therefore, WRKY TFs may be positively or negatively regulated to participate in the disease resistance-related responses of plants. In the experiment involving \textit{A. tenuissima} infection, all treatments were carried out under natural light, and because \textit{A. tenuissima} infection occurred during the day, sampling at 12 and 36 h after infection was conducted at night. The experiment revealed that \textit{PlWRKY65} expression in the blank control group was similar at 12 and 36 h but slightly higher at 24 and 48 h (Fig. 3). These results, together with the results for the G-box light-responsive components in the gene sequence, indicate that the expression of \textit{PlWRKY65} may be induced by optical signals. At 48 h after infection, the outdoor temperature plummeted, accompanied by rain, which may have been the reason for the significant increase in expression in the blank control at 72 and 96 h.

It has been found that plants infected by pathogens tend to accumulate plant protectors, such as phytoalexins, scopolin, and scopoletin around the invasion site, and the synthesis of these protectors depends on the JA, SA, and ethylene signaling pathways in plants\textsuperscript{29–31}. However, most studies have revealed that WRKY TFs can mediate multiple disease resistance response pathways to regulate the expression of disease resistance-related genes, including the JA and SA signaling pathways\textsuperscript{6}. In \textit{Catharanthus roseus}, JA can promote the transcriptional accumulation of \textit{CrWRKY1}, \textit{CrWRKY8}, \textit{CrWRKY13}, and \textit{CrWRKY38}\textsuperscript{32}. Under MeJA treatment, \textit{GhWRKY40} gene expression is upregulated and positively regulates the resistance of cotton plants to \textit{Ralstonia solanacearum}\textsuperscript{33}. Rice \textit{OsWRKY13} is co-induced by SA and JA, and studies have surprisingly shown that \textit{OsWRKY13} in turn affects the accumulation of SA; that is, plants that overexpress \textit{OsWRKY13} accumulate more free SA\textsuperscript{8}. In this study, \textit{PlWRKY65} silencing caused a decrease in JA content and an increase in SA content, which indirectly indicated that \textit{PlWRKY65} might promote JA accumulation and inhibit SA synthesis.

In the complex network through which plants regulate their response to external injury, there is an obvious antagonistic relationship between the SA and JA signaling
pathways, and WRKY TFs mostly play a regulatory role at the junction of their signaling pathways. Rice OsWRKY13, as a TF that directly or indirectly regulates disease resistance via the SA and JA signaling pathways, activates the transcription of SA synthesis-related genes and SA-induced genes and inhibits the expression of JA synthesis-related genes and JA-induced genes. Erwinia carotovora infection in wild-type Arabidopsis can induce increased AtWRKY70 gene expression, which has been proven to be associated with elevated levels of endogenous SA. At the initial stage of infection, the transient increase in the JA level inhibits the expression of AtWRKY70. Studies have concluded that AtWRKY70 is a transcriptional activator of SA-inducing genes and a transcriptional repressor of JA-inducing genes, leading to the intersection of the SA- and JA-mediated signal defense pathways. Our research clearly showed that after infection with A. tenuissima, the endogenous JA content increased, but the content of endogenous SA decreased, and the two hormones showed opposite trends 96 h after infection. These results not only confirmed the mutual inhibition of the two hormones but also suggested that PIWRY65 may be positively regulated in the JA-mediated signaling pathway and may inhibit SA signaling pathway regulation. SA is an indispensable regulator that mainly combats infections involving biotrophic pathogens, such as Hyaloperonospora parasitica and Oidium neolycopersici; however, JA-related defense mechanisms protect against necrotrophic pathogens such as Alternaria brassicicola and Botrytis cinerea. As a necrotrophic pathogen, A. tenuissima is likely to cause the activation of JA signaling when it infects plants, which further explains the increase in JA content after infection with A. tenuissima.

The sudden onset of PTI and ETI in plants is usually regulated by hormone signaling pathways. The best studied of these pathways are those involving SA, JA, and ethylene, and these endogenous hormones can induce the expression of PR genes. In Arabidopsis, SA activates the expression of the AtPR-1, AtPR-2, and AtPR-5 genes, while JA activates the expression of the AtPR-3, AtPR-4, and AtPR-12 genes. MeJA can induce the expression of MaPR5-2 and MaPR5-3, while both SA and MeJA can induce high MaPR1-1, MaPR2, and MaPR10c expression in banana fruit. Signal transduction in the plant immune response is dependent on TFs. It has been reported that some pathogens and pathogen-derived elicitors, including SA and JA, can induce the expression of WRKY TFs, and some WRKY TFs are in turn involved in both the SA and JA pathways, showing the intersection of these effects. Furthermore, WRKY TFs can regulate the expression of some PR genes by binding to their promoters. Rice OsWRKY3 can promote the expression of OsPR1 downstream as a transcriptional activator. In rice, transgenic lines overexpressing OsWRKY28, OsWRKY71, OsWRKY76, and OsWRKY62 activate the pathogenesis-related gene OsPR10, contributing to resistance against Xanthomonas oryzae pv. oryzae (Xoo). In our study, decreased PIWRY65 expression resulted in significantly decreased PIPR2, PIPR4B, PIPR5, and PIPR10 expression and upregulated the transcriptional abundance of PIPR1. This illustrates that PIWRY65 participates in the regulation of these PIPRs and may be involved in JA-mediated and SA-mediated signaling pathways. An important finding is that PIPR1 is likely to be highly induced by SA and participate in the SA signaling pathway.

It can be concluded that PIWRY65, acting as a transcriptional activator responding to pathogen induction, can mediate pathogen resistance by regulating PIPR gene expression, which may be partly due to SA-induced and JA-induced resistance. This study broadens our knowledge of the involvement of peony WRKY TFs in pathogen resistance (Fig. 7).

**Materials and methods**

**Plant materials and treatments**

P. lactiflora ‘Da Fugui’ was potted at the experimental forestry station of Shandong Agricultural University, Tai’an, Shandong, China, and plants were selected as experimental materials when they were in the middle stage of leaf development. The wound infection method was adopted for pathogen infection. Strain W23 of A. tenuissima preserved in our laboratory was cultured on solid PDA medium for 7 days, and the fungal colony edge
was collected as the infection source and transferred to microwounds on leaves punctured with a sterilized insect needle. Leaves were collected at 0, 12, 24, 36, 48, 72, and 96 h after infection. Infection was observed and recorded in 20–30 healthy leaves showing consistent growth for each plant. The incidence grade for peony was calculated according to Li, and the corresponding disease index was calculated as follows: disease index = \( \Sigma \) (number of disease-grade plants \( \times \) the representative value) \( \times \) 100%/plant number \( \times \) the representative value for the most severe disease. All samples were stored at \(-80^\circ\text{C}\) after quick freezing with liquid nitrogen. The control group was set up as described above, and each treatment was repeated three times.

**Nicotiana benthamiana** was cultured on an appropriate substrate and placed in a light incubator (25 \(^\circ\text{C}\), 120 mol m\(^{-2}\) s\(^{-1}\), with a light/dark period of 16 h/8 h) for constant-temperature cultivation. When the plants had produced eight true leaves, the subcellular localization test was carried out.

### Total RNA extraction and cDNA synthesis

The total RNA of the plant materials was extracted according to the instructions of the Aidlab EASYspin Rapid RNA Extraction Kit (Aidlab Biotech, Beijing, China). First-strand cDNA synthesis was performed by using the ComWin Biotech Reverse Transcription Kit (ComWin Biotech, Beijing, China).

### Cloning and sequence analysis of the *PIWRKY65* gene

The *PIWRKY65* gene was screened according to the unigene functional annotation of the WRKY genes in the transcriptome data of *P. lacti* 'Da Fugui'; its open-reading frame (ORF) sequence was predicted, and specific primers were designed (Table 1). The total DNA of *P. lacti* 'Da Fugui' was extracted via the improved CTAB method, and the full-length *PIWRKY65* gene was cloned using total DNA as a template. The NCBI BLAST program ([https://blast.ncbi.nlm.nih.gov/Blast.cgi](https://blast.ncbi.nlm.nih.gov/Blast.cgi)) was used to screen out some amino acid sequences sharing high homology with *P. lactiflora* *PIWRKY65*, and DNAMAN 5.0 software was then used for multiple-sequence alignment to analyze the structure of the gene domains and conservation. MEGA 5.0 software was used to generate the evolutionary tree of the system for homology analysis.

### Subcellular localization

The full-length cDNA of *PIWRKY65* with the termination codon removed was used as a template, and specific primers with restriction sites (BamHI and KpnI) were designed for PCR amplification (Table 1). After enzyme digestion, the obtained product was ligated to the pROKII-GFP vector, which was also double digested, and the fusion expression vector pROKII-*PIWRKY65*-GFP was verified by sequencing. Through *Agrobacterium* mediated infection, the recombinant vector and the empty vector without *PIWRKY65* were introduced into tobacco leaves. After cultivation for 3 days, fluorescent sites were observed under a confocal laser scanning microscope (Nikon, Tokyo, Japan).

### VIGS in *P. lactiflora*

To specifically silence the *PIWRKY65* gene, we amplified a 402-bp fragment of the gene and cloned it into the pTRV2 vector. The correct recombinant vector was verified by PCR and sequencing.

The pTRV1, pTRV2, and pTRV2-*WRKY65* plasmids were transformed into *Agrobacterium tumefaciens* GV3101.

### Table 1 Primers used in the tests and their sequences

| Primer name       | Nucleotide sequence (5’-3’)                  | Purpose               |
|-------------------|---------------------------------------------|-----------------------|
| piActionF         | ACTGCTGAACGGGAATT                         | Actin primers         |
| piActionR         | ATGGCTGAACGGACCT                         |                       |
| piWRKY65qF        | TTTGCCGAAGAGAGAACT                         | Specific primer for qRT-PCR |
| piWRKY65qR        | TACACCCCTGCGTCCTCC                         |                       |
| piWRKY65F         | ATGGAACAGTCTACATAC                         | Full-length DNA and cDNA amplification |
| piWRKY65R         | TACAGGTTGTGGCACCC                         | Vector construction   |
| piWRKY65(B)F      | CCGATTCCATCGAGACCT                         |                       |
| piWRKY65(K)R      | GGGTACCCGGATTGTTGCCAC                      | Molecular detection of VIGS |
| pTRV1R            | CCGGGTTCAAATCTCC                          | Vector construction   |
| pTRV2F            | TTTATGTTCAAGGCGTCTTGTG                     |                       |
| pTRV2R            | CAAACCCGCGATCTCAAACACGTC                   |                       |
| PR1F              | TACCCAGAGACGGTTGAC                        | Primers for pathogenesis-related genes |
| PR1R              | CACACGAGTTGGCCAGTTAA                       |                       |
| PR2F              | TGCCCAAGGGGCTCTAGA                        |                       |
| PR2R              | TCCATTTCGCCGCAAGCTAA                       |                       |
| PR4BF             | ATGCCGGCTCAACACTCTGG                      |                       |
| PR4BR             | TCCACAAGAAACGCTCAC                        |                       |
| PR5F              | CAGTCTCCCTACGGCAAGG                       |                       |
| PR5R              | GGTTCACATCGCGGTTC                          |                       |
| PR10F             | CCGGCAAGATTTCAACACG                       |                       |
| PR10R             | TTATCTTGATGTTCCGACC                       |                       |

Note: The underlined 'GGATCC', 'GAATTC', and 'GGTACC' nucleotides are the added restriction enzyme recognition sites for BstHI, EcoRI, and KpnI, respectively. PR1 primers for the pTRV1 vector were designed within RNA-dependent RNA polymerase elements (PCR product size of 647 bp in theory), and primers for the pTRV2 vector were designed between MCS elements (PCR product size of 372 bp in theory).
A 1 mL aliquot of *A. tumefaciens* GV3101-pTRV1, GV3101-pTRV2, and GV3101-pTRV2-WRKY65 was cultured in 10 mL of YEP liquid medium (including 50 µg/mL Kan and 100 µg/mL Rif) at 28 °C for 24 h at 200 rpm⁻¹. Then, 10 mL of the bacterial liquid was transferred to 400 mL of YEP liquid medium (including 50 µg/mL Kan, 100 µg/mL Rif, and 200 µM acetosyringone (AS)) followed by culture at 28 °C for 5–6 days at 200 rpm⁻¹. When the OD₆₀₀ of the bacterial liquid was ~1.5, the cells were centrifuged at 4 °C at 12,000 rpm/min for 2 min, collected, and resuspended (10 mmol/L MES, 10 mmol/L MgCl₂, 150 µM AS, and aseptic water as solvent). The OD₆₀₀ of the suspension was adjusted to ~1.5. GV3101-pTRV1 and GV3101-pTRV2-WRKY65 were mixed with GV3101-pTRV1 at a 1:1 ratio, and the mixture was allowed to rest for 3–5 h at room temperature in darkness⁴⁵.

Peonies with strong and consistent growth were selected, and VIGS was carried out via the negative-pressure vacuum filtration method when the underground buds had broken through the soil and were about to produce leaves. The plants were carefully dug out of the pot and placed in a vacuum bucket containing the infection solution described above, ensuring that the plant was completely immersed in the infection solution and that the root system was not destroyed. The treated plants were returned the pots and bagged, then kept in the dark for 24 h. Thereafter, the treated plants and control plants were subjected to normal field management procedures.

**Quantitative real-time PCR (qRT-PCR)**

In this experiment, qRT-PCR was used to determine gene expression. The instrument was a Bio-Rad CFX96™ real-time system (Bio-Rad, Hercules, CA, USA), and the qRT-PCR mixture (total volume of 20 µL) contained 10 µL of SYBR® Premix Ex Taq™ (TakaRa, Inc., Japan), 8 µL of ddH₂O, 0.5 µL of each primer, and 1 µL of cDNA. The reaction procedure was as follows: 95 °C for 30 s; 40 cycles of 95 °C for 5 s and 60 °C for 30 s; and a dissociation stage of 95 °C for 10 s, 65 °C for 5 s, and 95 °C for 5 s. For each test sample, three biological repeats were performed, and the data were analyzed using the 2⁻ΔΔCT method⁴⁸. The *PlActin* gene was used as the housekeeping gene to detect the expression levels of other genes.

**Determination of endogenous hormones**

The leaves of *P. lactiflora* were sampled at 0, 12, 24, 48, 72, and 96 h after pathogen infection, and the levels of the endogenous hormones JA and SA were determined by high-performance liquid chromatography⁴⁷. The chromatographic conditions were as follows: for JA, a RIGOL L3000 high-performance liquid chromatography instrument (RIGOL, Suzhou, China) was used with a wavelength of 210 nm and a Kromasil C18 reversed-phase chromatographic column (250 mm × 4.6 mm, 5 micron); the flow rate was 0.8 mL/min, the mobile phase was 1% phosphoric acid (aqueous solution):acetonitrile = 45:55 (V/V), and the sample volume was 10 µL. For SA, a Waters 1525 high-performance liquid chromatography instrument (Waters, Shanghai, China) was used with the fluorescence detector set at an excitation wavelength of 294 nm and emission wavelength of 426 nm and a Kromasil C18 reversed-phase chromatographic column (250 mm×4.6 mm, 5 micron); the flow rate was 0.8 mL/min; the mobile phase was 1% acetic acid solution: methanol = 2:3 (V/V); and the sample volume was 10 µL. Three biological repeats were performed for each sample.

**Statistical analysis**

At least three biological replicates were included in the data, and all data were analyzed using ANOVA and Student’s t-test for the determination of significant differences by using SPSS 24.0 software.

**Acknowledgements**

This study was funded by the Shandong Provincial Agricultural Elite Varieties Project (2019 LZGC018), China.

**Conflict of interest**

The authors declare that they have no conflict of interest.

Received: 30 July 2019 Revised: 3 February 2020 Accepted: 7 February 2020

Published online: 01 April 2020

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