**Lilium regale** Wilson WRKY3 modulates an antimicrobial peptide gene, **LrDef1**, during response to **Fusarium oxysporum**

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**Abstract**

**Background:** WRKY transcription factors (TFs) play vital roles in plant growth and development, secondary metabolite synthesis, and response to biotic and abiotic stresses. In a previous transcriptome sequencing analysis of *Lilium regale* Wilson, we identified multiple WRKY TFs that respond to exogenous methyl jasmonate treatment and lily Fusarium wilt (*Fusarium oxysporum*).

**Results:** In the present study, the WRKY TF *LrWRKY3* was further analyzed to reveal its function in defense response to *F. oxysporum*. The *LrWRKY3* protein was localized in the plant cell nucleus, and *LrWRKY3* transgenic tobacco lines showed higher resistance to *F. oxysporum* compared with wild-type (WT) tobacco. In addition, some genes related to jasmonic acid (JA) biosynthesis, salicylic acid (SA) signal transduction, and disease resistance had higher transcriptional levels in the *LrWRKY3* transgenic tobacco lines than in the WT. On the contrary, *L. regale* scales transiently expressing *LrWRKY3* RNA interference fragments showed higher sensitivity to *F. oxysporum* infection. Moreover, a *F. oxysporum*-induced defensin gene, *Def1*, was isolated from *L. regale*, and the recombinant protein LrDef1 isolated and purified from *Escherichia coli* possessed antifungal activity to several phytopathogens, including *F. oxysporum*. Furthermore, co-expression of *LrWRKY3* and the *LrDef1* promoter in tobacco enhanced the *LrDef1* promoter-driven expression activity.

**Conclusions:** These results clearly indicate that *LrWRKY3* is an important positive regulator in response to *F. oxysporum* infection, and one of its targets is the antimicrobial peptide gene *LrDef1*.

**Keywords:** *Lilium regale*, WRKY transcription factor, *Fusarium oxysporum*, Transcriptional regulation, Defensin

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**Background**

WRKY transcription factors (TFs), the key regulators of plant response to biotic and abiotic stresses, constitute one of the largest TF families in plants. WRKY TFs possess a highly conserved domain comprising approximately 60 amino acid residues that contains one or two highly conserved short peptides (‘WRKYGQK’) at the N terminal, and a conserved zinc-finger motif of CX-CX_{22-23}HXC (C_{4}HC) or CX_{2-3}CX_{22-23}HXH (C_{2}H_{2}) at the C terminal [1, 2]. The WRKY TF family can be classified into three groups based on the number of WRKY domains and the type of zinc-finger motif [1]. In general, group I contains two WRKY domains and a zinc-finger motif. Group II contains a WRKY domain and a C_{2}H_{2} zinc-finger motif, whereas group III includes one WRKY motif and the zinc-finger domain C_{2}HC. Group I WRKY TFs can be divided into two subgroups: the Ia subgroup possessing a C_{2}H_{2} zinc-finger motif, and the Ib subgroup containing a C_{2}HC zinc-finger motif. Group II WRKY TFs can be further subdivided into five subgroups, Ila,
IIb, IIC, IID, and IIE, according to their phylogenetic relationships [3, 4].

WRKY TFs have been shown to be important regulators in plant defense response against a variety of pathogens. For example, 16 CmWRKY genes exhibit distinct expression patterns in melon (Cucumis melo) during Podosphaera xanthii infection [5]. In peony (Paonia lactiflora) infected by Alternaria tenuissima, PiWRKY65 expression is significantly enhanced, whereas reduced expression of PiWRKY65 via virus-induced gene silencing enhances sensitivity to Alternaria tenuissima infection [6]. Arabidopsis thaliana plants expressing grape (Vitis labrusca × V. vinifera) WRKY3 exhibit improved salt and drought tolerance at germination, seedling, and maturity stages. Compared with wild-type (WT) plants, ViWRKY3 transgenic Arabidopsis lines also have increased resistance to Golovinomyces cichoracearum but increased sensitivity to Botrytis cinerea [7].

WRKY TFs self-regulate and cross-regulate with other TFs to control the defense response of plants to biotic stress. In rice (Oryza sativa), OsWRKY45–2 enhances resistance to Magnaporthe oryzae by transcriptionally activating OsWRKY13 [8]. The overexpression of oilseed rape (Brassica napus) WRKY15 inhibits the transcriptional activation of BnWRKY33, which leads to increased plant susceptibility to Sclerotinia sclerotiorum [9]. CaWRKY40b in pepper (Capsicum annuum) exhibits positive feedback regulation at the transcriptional level by directly targeting the W box element in its own promoter; this gene plays a negative regulatory role in the defense response of pepper against Ralstonia solanacearum, thus reducing plant immunity [10].

Lily (Lilium) is one of the most popular fresh-cut flowers but is vulnerable to Fusarium wilt disease caused by pathogenic Fusarium species, such as F. oxysporum. Fusarium wilt affects the yield and quality of lily bulbs and cut flowers and also causes withering and death of lily plants. In our previous study, we carried out a preliminary investigation of the molecular interaction mechanism between a wild lily (L. regale Wilson) and F. oxysporum and confirmed that L. regale has strong resistance to F. oxysporum [11]. Drawing on the results of our previous L. regale transcriptome sequencing analysis, 35 WRKY genes responsive to F. oxysporum were cloned from L. regale [12]. qRT-PCR analysis revealed that the expression levels of LrWRKYs were induced by the exogenous application of four signaling molecules, such as salicylic acid (SA), methyl jasmonate (MeJA), and ethylene (ETH). Furthermore, the functional analysis confirmed that LrWRKY2 is a positive regulator that mediates L. regale defense responses to F. oxysporum infection by regulating chitinase 2 expression [13]. Compared with LrWRKY2, LrWRKY3 (GenBank no. MW125548) showed higher expression levels in root, stem, leaf, and flower tissues and upon treatment with SA, MeJA, and ETH. Moreover, LrWRKY3 exhibited more transcript accumulation in the L. regale roots during F. oxysporum infection than LrWRKY2 did. WRKY TF family members regulate a complex defenses network in plants in response to biotic stress [14], and similar observations were made in L. regale.

In this study, we investigated the regulatory mechanism of LrWRKY3 in defense response of L. regale to F. oxysporum. The onion (Allium cepa) epidermis cells without the chloroplast disturbance are large and easy to observe under microscope, thus they are commonly used for subcellular localization analysis of plant proteins. The transient expression in onion epidermal cells mediated by the Agrobacterium tumefaciens was used to analyze the subcellular localization of LrWRKY3. In addition, due to the current lack of a rapid and efficient method for the lily genetic transformation [15], LrWRKY3 was heterologously expressed in tobacco (Nicotiana tabacum) for functional verification. It is widely known that tobacco is an important model plant for rapid confirmation of gene functions, and F. oxysporum can rapidly cause Fusarium wilt in tobacco [16]. In addition, the LrWRKY3 function in defense response was explored in depth by transiently expressing its RNA interference (RNAi) fragment in L. regale scales. Finally, we isolated a F. oxysporum-responsive defensin gene (LrDef1, GenBank no. MZ872924) from L. regale and confirmed the resistance of LrDef1 to F. oxysporum. Moreover, this study further verified that LrWRKY3 regulates LrDef1 expression considering that the LrDef1 promoter had a W-box and rarely known interaction between the WRKY TFs and defensin during response to phytopathogen infection in plants.

Results
The L. regale subgroup-IIa WRKY3 gene was found to encode a nuclear protein

In our previous study, we identified a series of WRKY genes from a Fusarium wilt-resistant wild lily (L. regale) by transcriptome sequencing. According to the gene expression pattern data, LrWRKY3 was induced by F. oxysporum and responded to signaling molecule treatments [12]. In the present study, the function of LrWRKY3 was therefore further characterized to understand the transcriptional regulation of WRKY in L. regale in response to Fusarium wilt. The full-length cDNA of LrWRKY3, which was found to be 722 bp in length with a 537-bp open reading frame (ORF), was predicted to encode a subgroup-IIa WRKY protein with 178 amino acid residues. The calculated molecular mass of the deduced LrWRKY3 was 20.3 kDa. According to our sequence analysis, LrWRKY3 contained a highly
conserved ‘WRKYGQK’ sequence and a zinc-finger motif CX$_{4,5}$CX$_{22-23}$HXH (C$_2$H$_2$), which suggests membership in the IIa WRKY subgroup (Fig. 1a). The deduced amino acid sequence of *LrWRKY3* was highly similar to that of some WRKY proteins, including *Phoenix dactylifera* WRKY75 (GenBank no. XP_008809548.2), *Cocos nucifera* WRKY75 (KAG1370131.1), *Elaeis guineensis* WRKY75 (XP_010913024.1), and *Durio zibethinus* WRKY75 (XP_022740913.1) (Fig. 1b).

The result of PSORT prediction suggested that the protein encoded by *LrWRKY3* is localized in the plant cell nucleus. To confirm the subcellular location of *LrWRKY3*, a fusion expression vector of *LrWRKY3* and green fluorescent protein (GFP) was constructed and expressed in onion epidermal cells through *Agrobacterium tumefaciens*-mediated transformation. The green fluorescence signal of *LrWRKY3*-GFP fusion protein was exclusively distributed in the nuclei of onion epidermal cells and colocalized with the nuclear localization marker PI (Fig. 1c). In contrast, green fluorescence was detected throughout the entire onion epidermal cell in control. This result indicates that *LrWRKY3* is localized in plant cell nucleus.

**Overexpression of *LrWRKY3* in tobacco increased resistance to *F. oxysporum* and enhanced transcription levels of some JA/SA signaling pathway genes, PRs, and SODs**

To further understand the biological function of *LrWRKY3*, the plant overexpression vector pCAMBIA2300s-*LrWRKY3* was constructed and transformed into WT tobacco leaf disks. A total of 50 T$_0$ transgenic tobacco plants were obtained according to PCR analysis with *LrWRKY3*-specific primers. qRT-PCR was used to analyze the expression levels of *LrWRKY3* in leaves of 11 T$_2$ generation transgenic lines.
with WT serving as a negative control. According to the results, \textit{LrWRKY3} transcripts accumulated and were differentially expressed in the 11 transgenic tobacco lines (Fig. 2a). \textit{LrWRKY3} transcription was highest in line W3–6, with a relative expression value of 10.25. \textit{LrWRKY3} relative expression levels in W3–20, W3–35 and W3–44 were also high, and the expression value was 7.53, 8.40, and 7.25, respectively. These data indicate that \textit{LrWRKY3} was stably expressed in \textit{T2} generation transgenic tobacco.

Four transgenic lines (W3–6/20/35/44) with high \textit{LrWRKY3} expression and WT plants were inoculated with a spore suspension of \textit{F. oxysporum} to evaluate their resistance to the pathogen. The roots of WT tobacco turned black and rotted, and whole leaves had wilted 7 days after inoculation (Fig. 2b). In contrast, the roots of \textit{T2} generation transgenic tobacco lines showed no evident disease symptoms, and the leaves were healthy, bright green, and non-wilted. The results of leaf inoculation experiments were consistent with these observations. The leaves of transgenic lines exhibited only slight deterioration and local yellowing near the inoculation wound, whereas leaves of WT were obviously more yellowed and decayed, with a larger damaged area, 7 days after inoculation.
after inoculation (Fig. 2c–d). These results indicate that the overexpression of LrWRKY3 in tobacco improves disease resistance to F. oxysporum.

Transcription levels of some JA/SA signaling and defense-related genes in transgenic tobacco lines and WT tobacco were analyzed by qRT-PCR. As shown in Fig. 3, transcription levels of JA biosynthetic pathway-related genes, including NtLOX, NtAOC, NtPR1, NtAOS, NtJMT, NtKAT, and NtPACX, were obviously elevated in LrWRKY3 transgenic lines compared with WT. The expression level of NtJMT was highest in line W3–44: 16.89-fold higher than in WT tobacco. Compared with their expressions in WT, NtLOX and NtAOS were up-regulated in W3–44 by 6.49- and 2.98-fold, respectively. Relative expression levels of the SA signaling pathway-related genes NtPR1 and NtNPR1 were increased in transgenic tobacco lines. The expression of NtPR1 in line W3–44 was 24-fold that of WT, and the expression of NtNPR1 in line W3–35 was 7-fold that of WT. In W3–44, the expression levels of pathogenesis-related protein (PR) genes NtGlu2 and NtCHI1 were respectively 24- and 8.5-fold higher than that of WT. In addition, the expression level of PR gene Ntosmotin in line W3–20 was 3.4-fold higher than that in WT. Furthermore, antioxidant stress-related superoxide dismutase (SOD) genes, including NtSOD, NtCu-ZnSOD, and MnSOD, were all up-regulated in transgenic tobacco compared with WT. In conclusion, the overexpression of LrWRKY3 in tobacco up-regulated the expressions of JA/SA signaling pathway-related genes, PRs and SODs.

**Transient expression of the LrWRKY3 RNAi vector in L. regale scales increased susceptibility to F. oxysporum**

To further analyze the function of LrWRKY3, a LrWRKY3 RNAi construct was transiently expressed in L. regale scales via Agrobacterium tumefaciens mediation, and the scales were then inoculated with F. oxysporum spore suspension. Obvious blackening and decay appeared on the infected scales expressing the LrWRKY3 RNAi construct, but disease symptoms were much less evident on L. regale scales transformed with Agrobacterium tumefaciens containing an empty RNAi vector (Fig. 4a). Calculation of the damage area on F. oxysporum-infected L. regale scales confirmed that L. regale expressing the LrWRKY3 RNAi fragment was less resistant to F. oxysporum infection than the control (RNAi empty vector) (Fig. 4b). In addition, qRT-PCR analysis revealed that the expression level of LrWRKY3 in RNAi fragment-expressing L. regale was evidently lower than that in control scales, whether inoculated with F. oxysporum or not (Fig. 4c). These data clearly indicate that the decreased expression of LrWRKY3 in L. regale scales enhanced sensitivity to F. oxysporum.

The *L. regale* defensin gene *Def1* was revealed to be a *F. oxysporum* resistance gene

To explore the regulation of PR gene expression by LrWRKY3, a *L. regale* defensin gene, *LrDef1*, was cloned on the basis of transcriptome sequencing data (unpublished). The full-length cDNA of *LrDef1* was 501 bp, with a 225-bp coding region, and was predicted to encode a protein containing 74 amino acid residues. The predicted molecular weight of the deduced protein LrDef1 was approximately 8.06 kDa. The *LrDef1* promoter sequence (850bp) was obtained by genome walking. The cDNA sequence of *LrDef1* and the sequence of its promoter fragment were given in the supplementary information (Additional file 1). Using the PlantCARE prediction program, we identified many cis-acting elements in the *LrDef1* promoter, including the TGACG motif (MeJA response element), W box (ET, SA, and MeJA response element), and ABRE (ABA response, hypersaline, and dark induction element), and a series of cis-elements involved in response to plant hormone signals and abiotic and biotic stresses. The prediction results of cis-acting elements in the promoter sequence of *LrDef1* were given in supplementary information (Additional file 2, Table S1).

In the qRT-PCR analysis, *LrDef1* transcripts were detected in *L. regale* roots, stems, leaves, flowers, and scales (Fig. 5a). In particular, *LrDef1* was strongly expressed in scales. After *F. oxysporum* inoculation, the expression of *LrDef1* in *L. regale* roots was rapidly induced, with a peak at 24 h (Fig. 5b) when the expression level was approximately 4.1-fold of the control. According to these results, *LrDef1* is a *F. oxysporum* infection-induced gene and is dominantly expressed in *L. regale* scales. An N-terminal signal peptide was detected in the deduced protein LrDef1, which was predicted to localize in plant cell wall, thus indicating that LrDef1 may be a secretory protein. Moreover, the subcellular localization of LrDef1 was also examined by fusion expression with GFP in onion epidermal cells. The green fluorescence signal of fusion gene of *LrDef1* and GFP was distributed in the cell wall of onion epidermal cells (Fig. 5c). This result demonstrates that LrDef1 is located in plant cell wall as an extracellular protein.

The recombinant plasmid pET-32(a)-LrDef1-His containing a His-tag was transformed into *Escherichia coli* strain BL21 (DE3) for heterologous expression. As revealed by SDS-PAGE, the molecular mass of the induced His-LrDef1 fusion protein was consistent with its predicted size, 25 kDa (Fig. 6a). The recombinant protein was purified by Ni-NTA-sepharose (Sangon Biotech, China) column affinity chromatography and imidazole elution buffer (Fig. 6b) and then used in antifungal experiments. Original image of Fig. 6a and Fig. 6b were
Fig. 3 The expression levels of JA/SA signaling pathway-related genes, PRs and SODs were up-regulated in the LrWRKY3 T2 transgenic tobacco lines. WT: wild-type tobacco; W3–6/20/35/44: LrWRKY3 T2 transgenic tobacco lines. The results were shown as the average values calculated from three replicates and calculated by the 2^{ΔΔCT} method and analyzed by the Student’s t test (** p < 0.01, * p < 0.05)
As shown in Fig. 6c–e, the LrDef1 recombinant protein had different inhibitory effects on the mycelial growth of three fungi: *F. oxysporum*, *F. solani*, and *Alternaria alternata*. The recombinant protein had the strongest inhibitory effect on *F. oxysporum*, followed by *Alternaria alternata* and *F. solani*. Moreover, the antifungal activity increased along with the mass of LrDef1 recombinant protein (Fig. 6f).

PCR analysis with LrDef1-specific primers identified 35 T₀ LrDef1 transgenic tobacco plants. In addition, qRT-PCR was used to analyze the expression levels of LrDef1 in 12 T₂ generation transgenic lines (F1/2/4/5/8/9/11/16/17/19/20/21), and the result indicated that LrDef1 was expressed in all transgenic lines (Fig. 7a). We selected four of the T₂ generation LrDef1 transgenic tobacco lines (F8/9/11/16) to study their resistance to *F. oxysporum*. Seven days after inoculation with *F. oxysporum* spore suspension (2 × 10⁶ spores/mL), WT tobacco roots were black and rotten, and some leaves had started to shrink. In contrast, the roots of transgenic tobacco lines were only slightly darkened, and the leaves were still fully extended (Fig. 7b). Overexpression of LrDef1 in tobacco thus enhanced resistance to *F. oxysporum* infection.

**The LrWRKY3 recombinant protein specifically bound to the LrDef1 promoter fragment with a W box**

The recombinant vector pET-32(a)-LrWRKY3 was constructed and transformed into *E. coli* strain BL21 (DE3) for heterologous expression to obtain a LrWRKY3 recombinant protein. An electrophoretic mobility shift assay (EMSA) was used to analyze the binding between the LrWRKY3 recombinant protein and probes to reveal whether the specific binding site of LrWRKY3 is W box. As shown in Fig. 8a, lanes 1 and 2 on the EMSA gel both contained biotin-labeled probes designed from the LeDef1 promoter fragment with a W box. Original image of Fig. 8a was shown in the supplementary information (Additional file 3). The band in lane 1, corresponding to biotin-labeled probe with no LrWRKY3 recombinant
protein added, was not retarded on the gel. The presence of retarded bands in lanes 2 and 3 after the addition of LrWRKY3 indicates that LrWRKY3 was able to bind to the probes containing W box, which slowed their migration rates on the gel. The band in lane 3 was less delayed than the band in lane 2 because of competition due to the large number of unlabeled probes, which were 50 times more abundant than the biotin-labeled ones. Moreover, the LrWRKY3 protein was unable to bind to the mutant probe in lane 4 and thus no mobility shift was observed. These data fully illustrate that LrWRKY3 specifically binds to the W box.

**LrWRKY3 transcriptionally activated LrDef1 in yeast cells**

To determine whether LrWRKY3 has transcriptional activation activity, we integrated the ORF of LrWRKY3 into the prey vector pGADT7 AD in a Y1H system followed by co-transformation with the recombinant bait vector pAbAi-pLrDef1 in Y1HGold yeast cells, with the pAbAi-p53 plasmid used as positive control and the pAbAi empty vector as negative control. Yeast cells co-transformed with pGADT7 AD-LrWRKY3 and pAbAi-pLrDef1 vectors were able to grow normally on auxotrophic SD/-Leu/AbA solid medium (Fig. 8b). In contrast, yeast cells co-transformed with pGADT7 AD-LrWRKY3 and bait empty vectors could not grow on the solid medium. These results indicate that pGADT7 AD-LrWRKY3 could be integrated into the yeast genome containing bait plasmid to produce a fusion protein, Gal4-LrWRKY3, capable of recognizing and activating the W box of pLrDef1 in yeast cells. The Gal4-LrWRKY3 protein activated the expression of UR1-C in the recombinant bait plasmid pAbAi-pLrDef1, which enabled yeast cells to grow normally on SD/-Leu/AbA medium. This outcome suggests that the LrWRKY3 protein can specifically bind to the LrDef1 promoter in yeast and has trans-activation activity.
LrWRKY3 positively regulated the expression of *F. oxysporum*-resistance gene *LrDef1* in tobacco

Next, *LrWRKY3* and the *LrDef1* promoter were co-expressed in tobacco to explore the effect of *LrWRKY3* on transcriptional activity of *LrDef1* promoter. According to a GUS activity analysis, the β-glucuronidase (*GUS*) gene had the highest activity in pBI121-*GUS* transgenic tobacco, approximately 88 pM 4-MU min⁻¹ µg⁻¹ (Fig. 8c). At the same time, GUS activity in *LrWRKY3/pLrDef1* transgenic tobacco was significantly higher than that in *pLrDef1* transgenic tobacco. The average GUS activity of four *pLrDef1* transgenic tobacco lines
(PD-2/3/7/10) was approximately 24, 26, 33, and 31 pM 4-MU min$^{-1}$μg$^{-1}$, respectively. The average GUS activity of four LrWRKY3/pLrDef1 co-expressing transgenic tobacco lines (Co-1/7/9/12) was higher than 40 pM 4-MU min$^{-1}$μg$^{-1}$, namely, approximately 52, 48, 46, and 42 pM 4-MU min$^{-1}$μg$^{-1}$, respectively. Noteworthily, the average activity of Co-1 was approximately 1.7-fold higher than that of PD-7. These results indicate that the specific bindings of LrWRKY3 with W box in LrDef1 promoter activated the expression of downstream reporter gene GUS driven by LrDef1 promoter, thereby enhancing GUS activity.

**Discussion**

A large amount of evidence indicates that WRKY TFs play important roles in regulating plant resistance to pathogenic fungal infection. To explore the function of WRKY TFs in L. regale, we isolated the WRKY family member gene LrWRKY3 from L. regale in the present study. Structural analysis revealed that LrWRKY3 contains a highly conserved ‘WRKYGQK’ short peptide and a C$_2$H$_2$ zinc finger, thus indicating that this gene belongs to the IIa WRKY TF group. Nuclear localization is essential for TFs to activate or inhibit expression of their target genes under stress conditions. In subcellular localization experiments, the green fluorescence signal of the LrWRKY3-GFP fusion protein was detected in the nuclei of onion epidermal cells. These results indicate that LrWRKY3 is a nuclear-localized protein that possibly regulates cellular transcriptional reprogramming under biotic stress.

Gene overexpression and gene silencing are commonly used to investigate the role of WRKY TFs in plant defense. Transgenic Arabidopsis plants overexpressing grapevine (V. davidii) WRKY53 show strong disease resistance to Coniella diplodiella, Pseudomonas syringae, and G. cichoracearum [17]. Overexpression of SpWRKY3 in currant tomato (Solanum pimpinellifolium) positively modulates defense response to Phytophthora infestans, as indicated by a reduced number of necrotic cells, lesion size, and disease index; however, the resistance is weakened by SpWRKY3 silencing [18]. The transient silencing of strawberry (Fragaria × ananassa) WRKY1 reduces sensitivity to Colletotrichum acutatum and has indicated that FaWRKY1 plays a negative regulatory role in strawberry resistance to P. scutellum. Even more interesting,
FaWRKY1 positively regulates resistance to *P. syringae* in *Arabidopsis* [19]. In the present study, reverse genetics was used to verify the function of *LrWRKY3*. Overexpression of *LrWRKY3* in tobacco enhanced resistance to *F. oxysporum*. At the same time, the transient expression of *LrWRKY3* RNAi vector in *L. regale* scales increased decay and disease area, which indicates that the decreased expression of *LrWRKY3* in *L. regale* scales increased susceptibility to *F. oxysporum*. These data clearly indicate that *LrWRKY3* is an important positive regulator in defense responses of *L. regale*.

Plant defense systems rely on a complex signal regulatory network. Phytohormones play a crucial role, with SA and JA, in particular, serving as important immune response regulators [20]. WRKY TFs are important regulatory nodes in SA, JA, and ETH signaling pathways [21]. OsWRKY4 enhances rice resistance to the necrotrophic pathogen *Rhizoctonia solani* and participates in JA-mediated pathway [22]. Overexpression of *NtWRKY50* in tobacco alters SA and JA contents, increases expression of defense-related genes, and enhances resistance to *R. solanacearum* [23]. In our previous study, SA, MeJA,
H$_2$O$_2$, ETH, and F. oxysporum treatment induced the expression of LrWRKY3 [12]. In the SA-mediated signaling pathway, PR1, which is downstream of NPR1, is positively regulated by NPR1 to acquire resistance [24]. In the present study, transcription levels of NtNPR1 and NtPR1 were significantly increased in LrWRKY3 transgenic tobacco lines. In addition, expression levels of JA biosynthetic pathway-related genes (NtLOX, NtAOC, NtOPR, NtAOS, NtKAT, NtPACX, and NtJMT) were significantly higher in LrWRKY3 transgenic tobacco lines compared with WT tobacco. These results suggest that LrWRKY3 participates in JA- and SA-mediated signal transduction pathways, which are essential in the immune response of L. regale against Fusarium wilt.

The overexpression of OsWRKY80 in rice significantly enhances resistance to sheath blight and up-regulates transcription levels of some disease resistance-related genes, including PR1a, PR1b, PR5, and PR10 in transgenic lines compared with WT plants. The RNAi of OsWRKY80 significantly reduces sheath blight resistance, however, and the above-mentioned disease resistance-related genes are depressed in OsWRKY80 RNAi lines [25]. In the present study, we also analyzed the expression levels of three PRs (NtGlu2, NtCHI, and Ntosmotin) and three SODs (NtSOD, NtCu-ZnSOD, and MnSOD). These genes were significantly up-regulated in the LrWRKY3 transgenic tobacco lines compared with WT, which suggests that LrWRKY3 modulates the expression of defense-related genes during response to F. oxysporum.

Antimicrobial peptides, including defensins, are the main components of plant innate immune system. Plant defensins, a class of small, cysteine-rich alkaline cationic peptides, are the first line of defense against pathogen invasion [26]. These compounds can bind to fungus-specific membrane components, leading to invagination of the cell membrane of pathogenic fungi; they can also be intercalated or transported into the cell membrane to give rise to a series of biochemical changes [27]. Various plant defensins have been introduced into plants to enhance their resistance. For instance, overexpression of a Panax notoginseng defensin-like gene (PnDEF1) in tobacco enhanced resistance to F. solani; moreover, the PnDEF1 recombinant protein showed antifungal activity against F. solani, F. oxysporum, Botryosphaeria dothidea, and S. sclerotiorum in vitro [28]. As another example, overexpression of the NmDef02 reduced the susceptibility of soybean (Glycine max) to Phakopsora pachyrhizi and C. truncatum and improved resistance against diseases [29]. In the present study, a F. oxysporum-responsive defensin gene, LrDef1, was isolated from L. regale. Bioinformatics analysis revealed that LrDef1 possesses a signal peptide corresponding to the characteristics of plant defensins, and subcellular localization experiments confirmed that LrDef1 encodes a cell wall protein. Further analyses revealed that LrDef1 is an antifungal protein, with the recombinant protein LrDef1 able to inhibit the mycelial growth of F. oxysporum, F. solani, and Alternaria alternata. In addition, the overexpression of LrDef1 in tobacco enhanced the resistance of tobacco to F. oxysporum. We thus conclude that LrDef1 is a F. oxysporum-resistance gene in L. regale.

The DNA binding domain can bind to the W box element [(C/T) TGAC (T/C)] in promoter of target genes of WRKYSs, with WRKYSs in turn activating or inhibiting the expression of downstream genes [30]. The current tomato WRKY1 activates the non-coding RNA lncrna33732 through sequential-specific interaction with the W box element in promoter; moreover, lncrna33732 can induce the expression of respiratory oxidase and increase the accumulation of H$_2$O$_2$, thereby enhancing plant resistance to P. infestans [31]. In the present study, we obtained the LrDef1 promoter sequence (850 bp) by TAIL-PCR, and bioinformatics analysis indicated that the LrDef1 promoter sequence had a binding site specific to WRKYSs, the W box. The results of subsequent EMSA and yeast one-hybrid experiments leave little doubt that LrWRKY3 can specifically bind to the W box of LrDef1 promoter and has transcriptional activation activity in yeast cells. In addition, GUS activity was significantly enhanced in pLrDef1-GUS/LrWRKY3 co-expressed tobacco compared with transgenic tobacco expressing pLrDef1-GUS alone. All these data strongly support the hypothesis that LrWRKY3 has a transcriptional regulatory effect on the defensin gene LrDef1, an important resistance gene in response to F. oxysporum infection.

The present work will be subsequently explored for further research. Firstly, the LrWRKY3 transgenic tobacco lines will be subjected to transcriptome sequencing and determination of hormone content in order to further reveal the molecular net regulated by LrWRKY3 and verify the crosstalk between LrWRKY3 and hormone-mediated signaling pathways. In addition, it is urgent to establish a rapid genetic transformation method of L. regale, and we will be able to thoroughly understanding the regulatory mechanism of WRKY TFs in L. regale during response to Fusarium wilt in the near future.

Conclusion
A nuclear-localized IIa WRKY TF gene, WRKY3, was isolated from L. regale. Overexpression of LrWRKY3 in tobacco improved resistance to F. oxysporum; however, RNAi of LrWRKY3 in L. regale scales conferred sensitivity to F. oxysporum infection. Moreover, LrWRKY3 activated JA and SA signal pathways and up-regulated
the expression levels of PRs and SODs in the transgenic tobacco lines. In addition, LrWRKY3 showed transactivation on a F. oxysporum-resistance gene, LrDef1. We thus conclude that LrWRKY3 modulates the antimicrobial peptide gene LrDef1 and JA/SA signal pathways in L. regale during response to Fusarium wilt.

**Experimental procedures**

**Fungal and plant materials**

Wild L. regale was collected in Wenchuan County, Sichuan Province, China, and was identified by our research group through referring to a L. regale specimen (Kun 222,503) preserved in the herbarium of Kunming Institute of Botany, Chinese Academy of Sciences, and then was further confirmed by Prof. Zhineng Li from Southwest University. The L. regale was planted in a greenhouse (25°C, 60% relative humidity) at Kunming University of Science and Technology. Wild type tobacco (N. tabacum) seeds were provided by Dr. Guanzhe Liu from Yunnan Agricultural University, Kunming, China. Sterile tobacco seedlings were cultured in a climatic cabinet (25°C, 16 h light/8 h dark cycle) and used for genetic transformations. A F. oxysporum strain from a diseased lily with typical symptoms of root rot was characterized and preserved by our research group through referring to a Sichuan Province, China, and was identified by our research group through referring to a plant growth conditions were collected to analyze the expression pattern of LrDef1. Total RNA was extracted from each sample and used for cDNA synthesis. qRT-PCR amplification conditions and instrumentation were the same as in Liu et al. [32]. The expression level of L. regale glyceraldehyde-3-phosphate dehydrogenase (LrGAPDH, GenBank no. JZ391059) was used as an internal control to standardize different RNA samples. The qRT-PCR gene-specific primers sequences were given in the supplementary information (Additional file 2, Table S3). Three biological replicates were performed for each qRT-PCR assay, and the 2−ΔΔCt method was used to calculate relative gene expression levels.

**Subcellular localization analysis**

The subcellular locations of LrWRKY3 and LrDef1 were predicted using the PSORT online program (https://www.genscript.com/psort.html) and verified by transient expression of a GFP-tagged fusion protein in onion epidermal cells. The ORFs of LrWRKY3 and LrDef1 without stop codons were digested by restriction enzymes and then ligated to a pBIN m-gfp5-ER vector using T4 ligase for fusion with GFP. The pBIN m-gfp5-ER-LrWRKY3 and pBIN m-gfp5-ER-LrDef1 recombinant plasmids were individually transferred into Agrobacterium tumefaciens EHA105 via the freeze-thaw method. An empty pBIN m-gfp5-ER vector served as a control. The transformation protocols into onion epidermis are detailed in Qiu et al. [33]. Confirmation of LrWRKY3 and LrDef1 subcellular localizations was performed with a confocal laser scanning microscope (Nikon, Japan). The GFP fluorescence was detected using FITC channel with excitation wavelength of 488 nm, and the red fluorescence of PI was detected using Cy5 channel with excitation wavelength of 655 nm. The localizations of LrWRKY3 and LrDef1 were determined according to the GFP fluorescence distribution of two fusion proteins in the onion cells.

**Construction of LrWRKY3 and LrDef1 plant overexpression vectors and genetic transformation of tobacco**

The pCAMBIA2300s vector was used to construct plant overexpression vectors for LrWRKY3 and LrDef1. The ORFs of LrWRKY3 and LrDef1 were respectively obtained from pGEM-T-LrWRKY3 and pGEM-T-LrDef1 by double enzyme digestion. The enzyme digestion products were then ligated with the pCAMBIA2300s vector, which was digested with the same restriction enzyme. The ligation products were transferred into E. coli DH5α competent cells, and positive clones were screened by PCR. The pCAMBIA2300s-LrWRKY3 and pCAMBIA2300s-LrDef1 plasmids were separately transferred into A. tumefaciens LBA4404. Positive clones were identified by PCR and used for genetic transformation of tobacco according to Horsch et al. [34]. The positive transgenic tobacco seedlings were confirmed by PCR with two pairs of

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**Cloning of LrWRKY3 and LrDef1**

Total RNA was extracted from L. regale roots, and cDNA was synthesized with a reverse transcription kit (Promega, USA) to isolate LrWRKY3 and LrDef1. The methods used for RNA extraction and cDNA synthesis were the same as those detailed in Liu et al. [32]. The ORFs of LrWRKY3 and LrDef1 were cloned by PCR with specific primers (Table S1), and the PCR products were cloned into pGEM-T vector (Promega). The gene-specific primer sequences were given in the supplementary information (Additional file 2, Table S2). Positive E. coli clones containing pGEM-T-LrWRKY3 and pGEM-T-LrDef1 were confirmed by PCR.

**Quantitative reverse transcription PCR (qRT-PCR)**

The expression patterns of LrDef1 in L. regale were analyzed by qRT-PCR. The roots of healthy L. regale plants were injured and then infected with F. oxysporum (2 × 10⁶ spores/mL) for 30 min. The roots were collected 24, 48, and 72 h after inoculation. The mimic inoculation with sterile distilled water was used as a control. Roots, leaves, stems, flowers, and scales of L. regale under normal growth conditions were collected to analyze the expression pattern of LrDef1. Total RNA was extracted from

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**Experimental procedures**

**Fungal and plant materials**

Wild L. regale was collected in Wenchuan County, Sichuan Province, China, and was identified by our research group through referring to a L. regale specimen (Kun 222,503) preserved in the herbarium of Kunming Institute of Botany, Chinese Academy of Sciences, and then was further confirmed by Prof. Zhineng Li from Southwest University. The L. regale was planted in a greenhouse (25°C, 60% relative humidity) at Kunming University of Science and Technology. Wild type tobacco (N. tabacum) seeds were provided by Dr. Guanzhe Liu from Yunnan Agricultural University, Kunming, China. Sterile tobacco seedlings were cultured in a climatic cabinet (25°C, 16 h light/8 h dark cycle) and used for genetic transformations. A F. oxysporum strain from a diseased lily with typical symptoms of root rot was characterized and preserved by our research group. F. oxysporum, F. solani, and A. alternata were conserved at 4°C and activated through culturing on potato dextrose agar medium plate for one week at 28°C before use.

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The expression patterns of LrDef1 in L. regale were analyzed by qRT-PCR. The roots of healthy L. regale plants were injured and then infected with F. oxysporum (2 × 10⁶ spores/mL) for 30 min. The roots were collected 24, 48, and 72 h after inoculation. The mimic inoculation with sterile distilled water was used as a control. Roots, leaves, stems, flowers, and scales of L. regale under normal growth conditions were collected to analyze the expression pattern of LrDef1. Total RNA was extracted from
gene-specific primers, with the WT included in the PCR analysis as a negative control. In addition, some transgenic tobacco plants were randomly selected for self-breeding cultivation T2 generation in a greenhouse.

**Gene expression and disease resistance analyses of transgenic tobacco**

Transcription levels of LrWRKY3 and LrDef1 in young leaves of T2 generation transgenic tobacco were analyzed by qRT-PCR, with the tobacco actin gene (NtACT, GenBank no. AB158612.1) used as an internal reference. Four lines of T2 generation transgenic tobacco were selected to study their resistance to F. oxysporum infection. The leaves of WT tobacco and T2 generation transgenic tobacco seedlings were injured with sandpaper and inoculated with 100 μL F. oxysporum spore suspension (2 × 10⁶ spores/mL). The infected leaves were laid flat on wet filter paper to maintain humidity and placed in an illumination incubator. The injured roots of T2 generation LrWRKY3 transgenic tobacco and WT tobacco seedlings were immersed in F. oxysporum spore suspension (2 × 10⁶ spores/mL) for 30 min and then transferred to half-strength Murashige-Skoog liquid culture medium. The inoculated tobacco leaves were collected after 7 days, and disease symptoms were recorded with a digital camera (Nikon). Lesion areas were measured with Photoshop. The leaves of LrDef1 T2 generation transgenic tobacco were inoculated with F. oxysporum for disease resistance analysis using the above-mentioned approaches.

**Measurement of expression levels of JA biosynthesis, SA signaling pathway, and defense-related genes in LrWRKY3 transgenic tobacco**

qRT-PCR was used to analyze transcription levels of JA biosynthetic pathway-related genes (NtLOX, NtAOC, NtOPR, NtAOS, NtKAT, NtPACX, and NtJMT), SA signaling pathway-related genes (NtPR1 and NtNPR1), PRs (NtGlu2, NtChi, and Ntosmotin), and SODs (NtSOD, NtCu-ZnSOD and MnSOD) in four LrWRKY3 transgenic tobacco lines. The tobacco actin was used as a reference gene. Gene-specific primers were designed according to sequences downloaded from NCBI (https://www.ncbi.nlm.nih.gov/) and are listed in Table S2.

**Transient expression of LrWRKY3 RNAi construct in L. regale scales**

Gene-specific primers (Table S1) of LrWRKY3 with an attB linker were designed to amplify a RNAi fragment (410 bp). The LrWRKY3 RNAi PCR products were recombined with pHellsgate2 vectors using Gateway BP Clonase II enzyme mixture (Invitrogen, USA). The BP reaction mixture was then transformed into E. coli DH10B competent cells. The pHellsgate2-LrWRKY3 recombinant plasmids were further transformed into A. tumefaciens EHA105, and the positive clones were identified by PCR. The scales of healthy L. regale plants were wounded with sandpaper, and A. tumefaciens suspension (50 μL) containing pHellsgate2-LrWRKY3 or pHellsgate2 empty vector was then added. The infected scales were placed in a climate chamber at 28 °C for 24 h for the transient expression of RNAi vectors in L. regale scales, with subsequent F. oxysporum inoculation with spore suspension (2 × 10⁶ spores/mL) at the wound sites. Symptoms and lesion areas of lily scales were recorded and measured after inoculation for 72 h. In addition, the expression levels of LrWRKY3 in scales were measured by qRT-PCR.

**Prokaryotic expression and antifungal activity analysis of LrDef1 recombinant protein**

The SignalP-5.0 program (https://www.cbs.dtu.dk/services/SignalP/) was used to predict the signal peptide in LrDef1. The PCR-amplified LrDef1 ORF without the signal peptide-encoding sequence but containing the restriction sites of EcoRV and EcoRI was subcloned into the prokaryotic expression vector pET-32(a) to produce 6 × His-labeled fusion protein. The pET-32(a)-LrDef1 construct was then transformed into E. coli BL21 (DE3) for heterogenic expression. The LrDef1 recombinant protein was purified by Ni-NTA-sepharose affinity chromatography. F. oxysporum, F. solani, and A. alternata were used to analyze the antifungal effects of the LrDef1 recombinant protein using the method described by Taif et al. [35].

**Isolation of the LrDef1 promoter fragment**

The LrDef1 promoter fragment was isolated using a Genome Walking kit (Takara, Japan) according to the kit instructions. Two nested gene-specific primers and a degenerate primer were used in two successive PCR rounds to amplify the LrDef1 promoter region. The secondary PCR products were cloned into pGEM-T vector and then transformed into E. coli DH5α. Positive clones were selected for sequencing. In addition, the cis-elements in promoter were predicted using the PlantCARE program (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/).

**Prokaryotic expression of the LrWRKY3 recombinant protein and EMSA**

A pair of gene-specific primers (Table S1) were designed to amplify the LrWRKY3 ORF, which was then subcloned into the pET-32(a) vector. The recombinant pET-32(a)-LrWRKY3 plasmids were further transformed into E. coli BL21 (DE3) to express the recombinant protein containing a 6 × His tag. Detailed protocols on the recombinant protein were carried out according to the method described by Taif et al. [35].
protein denaturation and renaturation of inclusion body protein are given in Zhao et al. [36].

The EMSA was used to confirm the specific binding of LrWRKY3 recombinant protein with the LrDef1 promoter fragment. A probe containing the W box sequence of LrDef1 was designed and labeled with biotin (Sangon Biotech) and is hence referred to as the biotin-labeled probe (Table S1). In addition, a W box sequence containing a mutation was labeled with biotin and used as a mutant probe. Moreover, a probe without biotin labeling, designated the unlabeled probe, was used as the competitor. The biotin-labeled and mutant probes were diluted to a concentration of 0.01 μM, and the final concentration of unlabeled probe was 0.5 μM. Equivalent amounts of sense and antisense fragments of these probes were annealed to synthesize double-stranded DNA. After addition of the biotin-labeled probes, the EMSA reaction mixture including unlabeled probes, the EMSA reaction mixture including double-stranded DNA. After incubation for 10 min, the reaction mixture was incubated at room temperature for another 15 min prior to loading onto the gel. EMSA was performed according to the standard procedures of a Light Shift Chemiluminescent EMSA kit (Pierce, USA).

Yeast one-hybrid assay

A yeast one-hybrid system (Takara) was used to analyze the transactivation of LrDef1 by LrWRKY3. The 500-bp LrDef1 promoter fragment containing a W box was ligated to the bait vector pAbAi, and the ligation product pAbAi-pLrDef1 was then transformed into E. coli DH5α. Positive clones containing the pAbAi-pLrDef1 recombinant plasmids were selected by PCR, and the recombinant plasmids were transformed into yeast cell Y1Hgold to obtain the yeast bait strains according to the method described in Li et al. [12]. In addition, the LrWRKY3 ORF was ligated to the prey vector pGADT7 AD and transferred into E. coli DH5α. The yeast prey recombinant vector pGADT7 AD-LrWRKY3 was then transferred into bait strain pAbAi-pLrDef1-Y1HGOLD competent cells. Moreover, the yeast prey empty vector pGADT7 AD was transferred into bait strain pAbAi-pLrDef1-Y1HGOLD competent cells as a negative control, and the pGADT7 AD-P53 vector (kit provided, Takara) was transferred into bait vector pAbAi-p53-Y1HGOLD competent cells as a positive control. The appropriate inhibitory concentration of aureobasidin A (AbA) against the bait reporter strains was determined according to the manufacturer’s instructions (Takara). The resulting three groups of yeast cells were streaked onto SD/-Leu/AbA solid medium, and the plates were placed in a 30°C incubator for 2–3 days to observe whether LrWRKY3 activated the expression of LrDef1 promoter.

Co-expression of the LrDef1 promoter and LrWRKY3 in tobacco

To verify whether the LrWRKY3 TF has a regulatory effect on LrDef1 in plants, the LrDef1 promoter was transformed into LrWRKY3 transgenic tobacco to obtain the co-expression tobacco. The CaMV 35s promoter in pBI121 vector was replaced by LrDef1 promoter to construct a plant expression vector that included a GUS gene driven by LrDef1 promoter. The pBI121-pLrDef1-GUS plasmids were transformed into A. tumefaciens LBA4404 competent cells and then transformed into WT or LrWRKY3 T2 generation transgenic tobacco leaf discs. In addition, the WT tobacco leaf disks were transformed with the empty pBI121-GUS vector as a control. Positive transgenic tobacco plants were identified by PCR with GUS-specific primers. A fluorescence spectrophotometer (Hitachi f-4600, Japan) was used to determine the GUS enzyme activity (pM 4-MU min⁻¹ μg⁻¹ protein) of positive transgenic tobacco as described by Chen et al. [37].

Data analyses

Gene expression levels, lesion sizes, fungal growth inhibition zones, and GUS activities were calculated as means plus standard deviations. Student’s t-test was performed using SPSS software (version 17.0) to reveal statistical differences between treatments, inoculations, and controls.

Abbreviations

TFs: Transcription factors; SA: Salicylic acid; MeJA: Methyl jasmonate; ETH: Ethylene; H2O2: Hydrogen peroxide; JA: Jasmonic acid; RNAi: RNA interference; ORF: Open reading frame; GFP: Green fluorescent protein; WT: Wild type; PR: Pathogenesis-related protein; SOD: Antioxidant stress-related superoxide dismutase; GUS: β-glucuronidase; Co: Co-expression; EMSA: Electrophoretic mobility shift assay; AbA: Aureobasidin A.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12870-022-03649-y.

Additional file 1: The gene sequences of LrDef1 and pLrDef1.

Additional file 2: Table S1. Prediction results of cis-acting elements in the promoter sequence of LrDef1. Table S2. The primers sequences used for function analysis of LrWRKY3 and LrDef1. Table S3. The qRT-PCR gene-specific primers sequence.

Additional file 3: Fig. S1. The original gel image of Fig. 6a. Fig. S2. The original gel image of Fig. 6b. Fig. S3. The original gel image of Fig. 8a.
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Authors’ contributions
ZW: methodology, data curation, experiment execution, writing-original draft preparation; JD: methodology, material collection, supervision; TL: investigation, software; LS: validation; LZ: supervision; HC: conceptualization; DL: methodology, supervision, writing, reviewing and editing. All of the authors read and approved the final manuscript.

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Availability of data and materials
The original contributions presented in the study are publicly available. The data can be found in the NCBI (https://www.ncbi.nlm.nih.gov/): LrWRKY3 accession number: MZ872948, LrDef1 accession number: MZ872924, pLrDef1 accession number: MZ872925.

Declarations

Ethics approval and consent to participate
All our experiments have complied with relevant institutional, national, and international guidelines and legislation. In addition, the collection of wild plant materials complied with the regulations of the People’s Republic of China on Wild Plants Protection promulgated by the National Forestry and Grassland Administration of China.

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no conflict of interest.

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References
1. Eulgem T, Rushton PJ, Robatzek S, Somsisch IE. The WRKY superfamily of plant transcription factors. Trends Plant Sci. 2000;5(5):199–206.
2. Ulker B, Somsisch IE. WRKY transcription factors: from DNA binding towards biological function. Curr Opin Plant Biol. 2004;7(5):491–8.
3. Yamakami K, Kigawa T, Inoue M, Watanabe S, Tateno M, Seki M, et al. Structures and evolutionary origins of plant-specific transcription factor DNA-binding domains. Plant Physiol Biochem. 2008;46(3):395–401.
4. Chen Y, Zhang ZL, Zou X, Huang J, Ruan P, Thompson D, et al.Annotations and functional analyses of the rice WRKY gene superfamily reveal positive and negative regulators of abscisic acid signaling in aleurone cells. Plant Physiol. 2005;137(1):76–89.
5. Chen YY, Jing X, Wang SS, Wang JQ, Zhang S, Shi QH. Genome-wide analysis of WRKY transcription factor family in melon and their response to powdery mildew. Plant Mol Biol. Report. 2021;39(4):686–99.
6. Wang X, Li J, Guo JJ, Qiao Q, Guo XF, Ma Y. The WRKY transcription factor PIWRKY6 enhances the resistance of Paecilomyces fumosoroseus (herbaceous powdery mildew) to Alternaria tenuissima. Hortic Res. 2020;7(1):720–37.
7. Guo RR, Qiao HB, Zhao J, Wang XH, Tu MX, Guo CL, et al. The grape VLVWRKY3 gene promotes abiotic and biotic stress tolerance in transgenic Arabidopsis thaliana. Front Plant Sci. 2018;9:545.
8. Viana VE, Busanello C, da Maia LC, Pegoraro C, Costa de Oliveira A. Activation of rice WRKY transcription factors: an army of stress fighting soldiers? Curr Opin Plant Biol. 2018;45:268–75.
9. Liu F, Li XX, Wang MR, Wen JY, B Shen, JX, et al. Interactions of WRKY51 and WRKY33 transcription factors and their roles in the resistance of oilseed rape to Sclerotinia infection. Plant Biotechnol J. 2018;16(4):911–25.
10. Khan MI, Zhang YW, Liu ZQ, Hu J, Liu CL, Yang S, et al. CaWRKY40b in pepper acts as a negative regulator in response to Ralstonia solanacearum by directly modulating defense genes including CaWRKY40. Int J Mol Sci. 2018;19(S5):1403.
11. Rao J, Liu DQ, Zhang NN, He H, Ge F, Chen CY. Differential gene expression in incompatible interaction between Lilium regale Wilson and Fusarium oxysporum f. sp. lili revealed by combined SSH and microarray analysis. Mol Biol. 2014;48(6):802–12.
12. Li S, Liu GZ, Pu LW, Liu XY, Wang Z, Zhao Q, et al. WRKY transcription factors actively respond to fusarium oxysporum in Lilium regale Wilson. Phytopathology. 2021;111:1625–37.
13. Li S, Hai J, Wang Z, Deng J, Liang TT, Su LL, et al. Lilium regale Wilson WRKY2 regulates chitinase gene expression during the response to the root rot pathogen fusarium oxysporum. Front Plant Sci. 2021;12:741463.
14. Jiang JJ, Ma SH, Ye NH, Jiang M, Cao JS, Zhang JH. WRKY transcription factors in plant responses to stresses. J Integr Plant Biol. 2017;59(2):86–101.
15. Azadi F, Chen DP, Kuroda K, Khan RS, Mibo M. Macro elements in inoculation and co-cultivation medium strongly affect the efficiency of Agrobacterium-mediated transformation in Lilium. Plant Cell Tiss Org. 2020;101(2):201–9.
16. Ding Y, Chen YL, Lin ZQ, Tuo YY, Li HL, Wang Y. Differences in soil microbial community composition between suppressive and root-rot-conductive in tobacco fields. Curr Microbiol. 2021;78:624–33.
17. Zhang Y, Xiao JL, Feng H, Jiang J, Fan X, Jia YF, et al. Identification of the defense-related gene VdWRKY33 from the wild grapevine Vitis davidii using RNA sequencing and ectopic expression analysis in Arabidopsis. Hereditas. 2019;156(1):14.
18. Cui J, Xu PS, Meng J, Li JB, Jiang N, Luan YS. Transcriptome signatures of tomato leaf induced by Phytophthora infestans and functional identification of transcription factor SpiWRKY3. Theor Appl Genet. 2018;131(4):784–900.
19. Higuera JJ, Garrido-Gala J, Lekhoubou A, Arjona-Girona I, Amil-Ruiz F, Mercado JA, et al. The strawberry FaWRKY1 transcription factor negatively regulates resistance to Colletotrichum acutatum in fruit upon infection. Front Plant Sci. 2019;10:490.
20. Liu LJ, Sonbol FM, Huot B, Gu YN, Wither S, Mwimba M, et al. Salicylic acid receptors activate jasmonic acid signalling through a non-canonical pathway to promote effector-triggered immunity. Nat Commun. 2016;7:13099.
21. Banerjee A, Roychoudhury A. WRKY proteins: signaling and regulation of expression during abiotic stress responses. Sci World J. 2015;2015:807560.
22. Wang HH, Meng J, Peng XX, Tang XH, Zhou PL, Xiang JH, et al. Rice WRKY14 acts as a transcriptional activator mediating defense responses toward Rhizoctonia solani, the causing agent of rice sheath blight. Plant Mol Biol. 2015;80(1–2):157–71.
23. Liu QP, Liu Y, Tang YM, Chen JN, Ding W. Overexpression of NiWRKY50 increases resistance to Ralstonia solanacearum and alters salicylic acid and jasmonic acid production in tobacco. Front Plant Sci. 2017;8:1710.
24. Cazafis L, Pieterse CMJ, Van Wees SCM. How salicylic acid takes transcriptional control over jasmonic acid signaling. Front Plant Sci. 2015;6(170):170.
25. Peng XX, Wang HH, Jang JC, Xiao T, He HH, Jiang D, et al. OsWRKY80–OsWRKY4 module as a positive regulatory circuit in rice resistance against Rhizoctonia solani. Rice. 2016;9(1):1–14.
26. Broekaert WF, Terras FRG, Cammue BPA, Osborn RW. Plant defenses: novel antimicrobial peptides as components of the host defense system. Plant Physiol. 1995;108(4):1353–8.
27. Thevissen K, Terras FRG, Broekaert WF. Permeabilization of fungal membranes by plant defenses inhibits fungal growth. Appl Environ Microbiol. 1999;65(12):5451–8.
28. Wang Q, Qiu BL, Li S, Zhang YP, Cui XM, Guo CL, et al. A methyl jasmonate induced defense-like protein from Panax notoginseng confers resistance against fusarium solani in transgenic tobacco. Biol Plant. 2010;63(1):797–807.
29. Soto N, Hernández Y, Delgado C, Rosabal Y, Ortiz R, Valencia L, et al. Field resistance to Phakopsora pachyrhizi and Colletotrichum truncatum of transgenic soybean expressing the NmDef20 plant defense gene. Front Plant Sci. 2020;11:562.
30. Liang QF, Wu YH, Wang K, Bai ZY, Liu QL, Pan YZ, et al. Chrysanthemum WRKY gene DgWRKY5 enhances tolerance to salt stress in transgenic chrysanthemum. Sci Rep. 2017;7(1):85–194.
31. Cui J, Jiang N, Meng J, Yang GL, Liu WW, Zhou XX, et al. LncRNA33732-respiratory burst oxidase module associated with WRKY1 in tomato-Phytophthora infestans interactions. Plant J. 2019;97:933–46.
32. Liu DQ, Zhao Q, Cui XM, Chen R, Li X, Qiu BL, et al. A transcriptome analysis uncovers Panax notoginseng resistance to fusarium solani induced by methyl jasmonate. Genes Genomics. 2019;41(12):1383–96.
33. Qiu BL, Zhang Y, Wang Q, Wang Z, Chen HJ, Cui XM, et al. Panax notoginseng snakin gene increases resistance to fusarium solani in transgenic tobacco. Ind Crop Prod. 2020;157:112902.
34. Horsch RB, Fry JE, Hoffmann NL, Eichholtz D, Rogers SG, Fraley RT. A simple and general method for transferring genes into plants. Science. 1985;227(4691):1229–31.
35. Taif S, Zhao Q, Pu LM, Li X, Liu DQ, Cui XM. A β-1,3-glucanase gene from Panax notoginseng confers resistance in tobacco to fusarium solani. Ind Crop Prod. 2020;143:111947.
36. Zhao Q, Qiu BL, Li S, Zhang YF, Cui XM, Liu DQ. Osmotin-like protein gene from Panax notoginseng is regulated by jasmonic acid and involved in defense responses to fusarium solani. Phytopathology. 2020;110(8):1419–27.
37. Chen R, He H, Yang Y, Qu Y, Ge F, Liu DQ. Functional characterization of a pathogenesis-related protein family 10 gene, LpPR10-5, from Lilium regale Wilson. Australas Plant Pathol. 2017;46(3):251–9.

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