Transcription factor ScWRKY4 in sugarcane negatively regulates the resistance to pathogen infection through the JA signaling pathway

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Highlight

Transgenic plants overexpressing the *ScWRKY4* gene negatively regulated resistance to pathogen by inhibiting the expression of the *JAZ* genes.
ABSTRACT

WRKY transcription factor, the transcriptional regulators unique to plants, plays an important role in plant defense response to pathogen infection. However, the disease resistance mechanism of WRKY gene in sugarcane remains unclear. Previously, we identified a ScWRKY4 gene, a member of class IIc of the WRKY gene family, from the sugarcane cultivar ROC22. This gene could be induced by the stresses of salicylic acid (SA) and methyl jasmonate (MeJA). Interestingly, the expression of the ScWRKY4 gene was down-regulated in smut-resistant sugarcane cultivars but up-regulated in smut-susceptible sugarcane cultivars under Sporisorium scitamineum stress. Besides, stable overexpression of the ScWRKY4 gene in Nicotiana benthamiana enhanced susceptibility to Fusarium solani var. coeruleum and caused the down-regulated expression of immune marker-related genes. Furthermore, transcriptome analysis indicated that, the expression of most JAZ genes was suppressed in plant signal transduction pathway. In addition, ScWRKY4 could interact with ScJAZ13 and repress the expression of ScJAZ13. We thus hypothesized that the ScWRKY4 gene was involved in the regulatory network of plant disease resistance, most probably through the JA signaling pathway. The present study depicted the molecular mechanism of the ScWRKY4 gene involved in sugarcane disease resistance and laid the foundation for the subsequent investigation.

Keywords: ScWRKY4, expression profile, disease resistance, transcriptome, protein interaction, sugarcane
**Abbreviations:** SA- salicylic acid, MeJA- methyl jasmonate, ROS- reactive oxygen species, JA- jasmonic acid, RT-qPCR- real-time quantitative PCR, DAB- 3,3’-diaminobenzidine, GO-gene ontology, BiFC- bimolecular fluorescent complimentary, YFP- yellow fluorescent protein, GFP- green fluorescent protein, WT- wild-type, GAPDH- glyceraldehyde-3-phosphate dehydrogenase, HR- hypersensitive response, ET- ethylene, DEGs- differentially expressed genes.
Introduction

Sugarcane (Saccharum spp.) is a cash crop grown mainly in subtropical and tropical regions and plays an important role in the agricultural economy of China (Singels et al., 2021). Like other crops, biotic and abiotic stresses, such as drought, low temperature and pathogenic fungi, are the main factors restricting sugarcane production (Rajput et al., 2021). Sugarcane smut, which seriously endangers the healthy and sustainable development of sugarcane industry, is a worldwide fungal disease caused by the fungus Sporisorium scitamineum (Bhuiyan et al., 2021).

When plants encounter stress during growth and development, they will form a complex defense regulatory network, and transcription factors perform a critical function during this period (Singh et al., 2002). WRKY transcription factors, one of the largest families of transcription factors in plants, are widely involved in plant responses to biotic, abiotic and hormonal stresses (Jiang et al., 2017), especially in the formation of plant disease resistance (Rushton et al., 2010). They have a 60 amino acid long DNA binding domain, which is characterized by a highly conserved WRKYGQK core motif at the N-terminal and a CX$_{4-5}$CX$_{22-23}$HXH zinc finger motif at the C-terminal (Bakshi and Oelmüller, 2014). According to the number of typical WRKY domains and the type of zinc finger motif, WRKY family members can be divided into three groups, group I has two WRKY domains, while group II or III has only one WRKY domain (Eulgem et al., 2000). C$_2$H$_2$ (CX$_{4-5}$CX$_{22-23}$HX$_{1-3}$), where X can be any amino acid, is the zinc finger structure possessed by members of groups I and II, however it is C$_2$H$_3$ (CX$_3$CX$_{23}$HX$_{3}$) in group III (Eulgem et al., 2000). Based on the homology, the members of group II WRKY family can be further classified as five subgroups, IIa, IIb, IIc, IId and Ile (Zhang and Wang, 2005).

Plants have evolved a complex mechanism to respond to a series of biotic stresses (Gull et al., 2019). The regulatory role of WRKY transcription factors in plant immune response to various biotic stresses has been extensively studied (Wani et al., 2021), with functions including positive and negative regulation in plant defense responses to various pathogen infection (Pandey and Somssich, 2009). In Vitis vinifera, transient silencing of VqWRKY31 reduced resistance to powdery mildew (Yin et al., 2022). In Juglans regia, silencing of JrWRKY21 significantly reduced the resistance of walnuts to Colletotrichum gloeosporioides, while the disease resistance was significantly enhanced in walnut overexpressing JrWRKY21 (Zhou et al., 2022). In Oryza sativa, knock out mutations of the OsWRKY53 transcription factor thickened sclerenchyma cell walls, thereby conferring higher resistance to rice bacterial blight (Xie et al., 2021). In Triticum aestivum,
TaWRKY19 negatively regulated stripe rust resistance through the production of reactive oxygen species (ROS) and transcriptional repression of TaNOX10 (Wang et al., 2022b). Notably, when subjected to biotic stresses, WRKY transcription factors could activate the expression levels of genes related to salicylic acid (SA) and jasmonic acid (JA) signaling pathways in plants, and then responded to different biotic stresses (Jiao et al., 2018). For example, AtWRKY70, an important member of the SA- and JA-regulated defense signaling pathway, was induced by SA and repressed by JA, thereby activating SA-inducible genes and repressing JA-responsive genes that were further involved in defense responses (Li et al., 2004). Previous research also demonstrated that overexpression of AtWRKY70 increased the resistance of Arabidopsis to Pseudomonas syringae pv tomato (Li et al., 2004). Nevertheless, GhWRKY70 negatively regulated tolerance to Verticillium dahliae in Gossypium hirsutum by up-regulating the expression of SA-related genes and repressing the expression of JA-related genes (Xiong et al., 2019). In sugarcane, Sc-WRKY gene (GeneBank accession number GQ246458) belongs to class IIc was first cloned and its expression could be induced by the stress of both S. scitamineum and SA (Liu, 2012). In addition, the expression of sugarcane class IIc ScWRKY3 gene (GeneBank accession number: MK034706) was up-regulated in smut-susceptible variety ROC22, but remained unchanged in smut-resistant variety Yacheng05-179, and its expression was inhibited by SA and MeJA treatments (Wang et al., 2018b). Li et al. identified 154 members of the SsWRKY gene family in the Saccharum spontaneum genome, and RNA-seq analysis revealed that SsWRKYS displayed different temporal and spatial expression patterns in different developmental stages, of which, 52 SsWRKY genes were expressed in all tissues, four SsWRKY genes were not expressed in any tissues, 21 SsWRKY genes may be involved in photosynthesis (Li et al., 2020). Besides, Javed et al. described 53 ShWRKY genes in Saccharum spp. hybrid R570, of which four genes, ShWRKY13-2/39-1/49-3/125-3, were significantly up-regulated in sugarcane cultivars LCP85-384 resistant to leaf scald (Javed et al., 2022). From all the above, the disease resistance regulatory mechanisms of WRKY genes had been widely reported in other species, however there were only few and superficial reports on the disease resistance functions of sugarcane WRKY genes.

We have previously reported that, the expression of ScWRKY4 gene, which belonged to class IIc of WRKY family, could be induced under SA, methyl jasmonate (MeJA) and smut stress (Wang et al., 2018a). What interests us most is that, the expression of the ScWRKY4 gene was down-regulated in smut-resistant sugarcane cultivars but up-regulated in smut-susceptible sugarcane cultivars under S. scitamineum stress. In the present study, we also found both transient and stable overexpression of the ScWRKY4 gene in Nicotiana
benthamiana enhanced the susceptibility of tobacco plants to Fusarium solani var. coeruleum and caused down-regulated expression of immune marker genes. Strikingly, after inoculation with pathogen, the expression of most JAZ-related genes was repressed in transgenic tobacco plants stably overexpressing ScWRKY4 gene. Further experiments indicated that, ScWRKY4 interacted with ScJAZ13 and could inhibit the expression of ScJAZ13. Our study aimed to explore the regulatory network/mechanism of disease resistance for the ScWRKY4 gene and provided a theoretical basis for subsequent studies on members of the WRKY gene family in sugarcane.

Materials and methods

Plant materials, culture conditions and pathogen inoculation

The two smut-resistant sugarcane cultivars (YZ01-1413 and YT96-86), and two smut-susceptible sugarcane cultivars (YZ03-103 and FN39) used in this study were provided by the Key Laboratory of Sugarcane Biology and Genetic Breeding, Ministry of Agriculture and Rural Affairs (Fuzhou, China). The above cultivars were inoculated with S. scitamineum according to the methods of Que et al. (Que et al., 2014). Three single buds were collected at 0, 1, 3 and 7 days post inoculation (dpi), and then stored in -80°C refrigerator after freezing in liquid nitrogen.

The gateway primers (Supplementary Table S1) were designed to construct the ScWRKY4 gene into the overexpression vector pEarleyGate 203. The empty pEarleyGate 203 (35S::00) and the fusion vector pEarleyGate 203-ScWRKY4 (35S::ScWRKY4) were transiently overexpressed in N. benthamiana by the Agrobacterium-mediated method. After 1 d, two leaves were taken from each plant and used for real-time quantitative PCR (RT-qPCR) and 3,3’-diaminobenzidine (DAB) histochemical analysis, respectively (Wang et al., 2020). The fungal pathogen F. solani var. coeruleum was inoculated into the over expressed N. benthamiana leaves for 1 day, and then the symptoms (phenotype and DAB) of N. benthamiana leaves were observed and the relative transcription levels of tobacco immune related marker genes were calculated (Wang et al., 2020).

Agrobacterium tumefaciens carrying pEarleyGate 203-ScWRKY4 was stably transformed into N. benthamiana by leaf-disc method (Müller et al., 1987). The T3 generation of plants overexpressing ScWRKY4 gene was screened on MS (murashige and skoog) medium supplemented with glufosinate. When the plants grew to 5–8 leaves old, the DNA of plant leaf was extracted and diluted to 25 ng/L. The pEarleyGate203-ScWRKY4 plasmid was used as a positive control and wild-type (WT) N. benthamiana as a negative control.
Genomic DNA of transgenic plants was used as a template for PCR amplification and electrophoresis detection (Su et al., 2020). RNA was extracted from the leaves of the plants, and the expression of ScWRKY4 gene in the transgenic plants was measured using WT plants as the control (Sun et al., 2023). Transgenic plants overexpressing the ScWRKY4 gene and WT were inoculated with the F. solani var. coeruleum and the phenotypic leaves were analyzed by DAB and trypan blue staining (Su et al., 2022). Samples were taken at 0 and 2 dpi for subsequent detection of immune marker related genes, determination of physiological indicators, and transcriptome analysis.

**RNA extraction and RT-qPCR analysis**

The total RNA was extracted by TRIzol method (Connolly et al., 2006). Refer to the kit instructions of Hifair® III 1st Strand cDNA Synthesis SuperMix for qPCR (gDNA digester plus), RNA was reverse transcribed from extracted treated material into first strand cDNA, which was used as a template for quantitative detection of target gene expression levels. The obtained RNA and cDNA were tested for quality by 1.0% agarose gel electrophoresis. Primer Premier 5 software was used to design specific quantitative PCR primers (primer pair: ScWRKY4-Q-F/R) for the ScWRKY4 gene, using glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (GenBank Accession Number. CA254672) as the internal reference gene (primer pair: GAPDH-Q-F/R) (Supplementary Table S1) (Que et al., 2009). Ten tobacco immune-related marker genes including hypersensitive response (HR) marker genes NbHSR201, NbHSR203 and NbHSR515, ethylene (ET) synthesis-dependent genes NbACO-like and NbACO, SA signaling pathway-related genes NbPR2 and NbPR3, JA signaling pathway-related genes NbLOX1 and NbDEF1, and ROS related genes NbCAT1 and NbGST1 (Supplementary Table S1) were selected and their expression in tobacco leaves was examined (Sun et al., 2023). The ABI 7500 Real-time PCR System (USA) was used for RT-qPCR detection, and the quantitative reaction system was prepared and the program was set up according to the SYBR Green PCR Master Mix Kit instructions. Three technical replicates were set up for each sample, and negative controls were used as templates with sterile water. The relative gene expression was calculated using $2^{-\Delta\Delta CT}$ (Livak and Schmittgen, 2001), and the significance level of the experimental data was analyzed using DPS 9.50 software and histograms were plotted using Origin 2022 software.

**Gene ontology (GO) enrichment analysis of ShWRKY gene family members**

GO annotate our previously identified ShWRKY gene family members (Wang et al., 2022a) based on the eggNOG-mapper v2 database (http://eggnog-mapper.celbl.de/) with parameters set to default parameters.
The obtained data were then analyzed by GO enrichment using Tbtools software (Chen et al., 2020) and visualized by the online software chiplot (https://www.chiplot.online/).

**Bioinformatics analysis of ScWRKY4**

Structural domain prediction of the ScWRKY4 protein was conducted using the SMART website (https://smart.embl.de/). Protein sequence alignment was performed using MUSCLE v3.7 (Edgar, 2004), and the phylogenetic evolutionary tree of ShWRKY proteins and ScWRKY4 protein was constructed using IQ-TREE in PhyloSuite software, and the replicate value was set to 1000 times (Zhang et al., 2020). Beautify the evolutionary tree with the help of EvolView online website (https://evolgenius.info//evolview-v2) (Subramanian et al., 2019). The conserved motif information of ScWRKY4 gene was obtained through the analysis of MEME online website (http://meme-suite.org/index.html), and the parameter settings showed 10 conserved motifs and the rest with default parameters (Bailey et al., 2009). Multiple sequence alignment of the ScWRKY4 gene with the ShWRKY genes was performed by DNAMAN software to obtain exon-intron structure information of the ScWRKY4 homologous gene from the S. spp hybrid R570 genome (Garsmeur et al., 2018) GFF3 file and gene structure of the ScWRKY4 homologous gene were visualized using TBtools software (Chen et al., 2020). Retrieved 2000 bp promoter sequences upstream of homologous genes were retrieved and cis-acting regulatory elements in promoter sequences were predicted using PlantCARE online website (https://bioinformatics.psb.ugent.be/webtools/plantcare/html/) (Lescot et al., 2002).

**Hormone content and enzyme activity determination**

The contents of endogenous hormones SA and JA were determined in leaves of WT and transgenic tobacco plants at 0 dpi and 2 dpi respectively, according to the instructions of the plant SA ELISA kit and JA ELISA kit (Enzyme-linked Biotechnology, Shanghai, China). Similarly, the enzymatic activities of catalase (CAT) and glutathione S transferases (GST) were measured in leaves of WT and transgenic tobacco plants at 0 dpi and 2 dpi respectively, according to the CAT ELISA kit and GST ELISA kit (Enzyme-linked Biotechnology, Shanghai, China).

**Transcriptome data analysis**

RNA samples from WT plants inoculated with *F. solani* var. *coeruleum* at 0 d and 2 d were named WT-CK and WT-T, respectively. And RNA samples from ScWRKY4-overexpressed transgenic tobacco plants inoculated with *F. solani* var. *coeruleum* at 0 d and 2 d were named ScWRKY4-CK and ScWRKY4-T, respectively. Three biological replicates were set up for each of the above samples, with a total of 12 samples (WT-CK1, WT-CK2,
WT-CK3, WT-T1, WT-T2, WT-T3, ScWRKY4-CK1, ScWRKY4-CK2, ScWRKY4-CK3, ScWRKY4-T1, ScWRKY4-T2 and ScWRKY4-T3). RNA-Seq sequencing was entrusted to Genedenovo. The raw reads were quality controlled using fastp to filter low quality data and get clean reads (Chen et al., 2018). A comparative analysis based on the N. benthamiana (https://sefapps02.qut.edu.au/benWeb/subpages/downloads.php) database was carried out and annotated using the HISAT2 software (Langmead and Salzberg, 2012). Bioinformatic analysis was performed using Omicsmart (http://www.omicsmart.com), a dynamic real-time interactive online platform for data analysis.

**Yeast two-hybrid**

The recombinant plasmids pGBK7-ScWRKY4 and pGADT7-ScJAZs (JAZ6, 8, 9, 10, 11, 13 and 14) constructed earlier by our group were used in the yeast two-hybrid system. The positive control pGADT7-T+pGBK7-p53, negative control pGADT7-T+pGBK7-lam and combination of plasmid pGBK7-ScWRKY4+pGADT7-ScJAZs were co-transformed into Y2HGold yeast strain, respectively. The transformation method was carried out according to the instructions of Y2HGold Chemically Competent Cell. After transformation, the Y2HGold strain containing the above plasmid combination was spread on tryptophan-leucine (SD/-Trp-Leu) deficient medium plates, and cultured in a 28°C incubator for 2–3 d. Single colony was selected and culture in SD/-Trp-Leu liquid medium for about 18 h, and then diluted four gradients (10⁻¹, 10⁻², 10⁻³, 10⁻⁴) with sterile water, with 10 µL to spotted on SD/-Trp-Leu and tryptophan-leucine-histidine-adenine (SD/-Trp-Leu-His-Ade) deficient medium plates, respectively. After that, it was cultivated in a 28°C incubator for 2–3 d until the yeast colonies grew, and the growth of the yeast colonies was recorded by taking pictures. The interaction of proteins in the yeast was judged according to whether the yeast colonies could grow normally.

**Bimolecular fluorescent complimentary (BiFC)**

Using the gateway method to construct ScWRKY4 gene and ScJAZ13 gene into pEarlyGate201 and pEarlyGate202 vectors, respectively. The BiFC recombinant vectors pEarlyGate201-ScWRKY4, pEarlyGate202-ScJAZ13, the empty-loaded pEarlyGate201-YN and pEarlyGate202-YC were transformed into Agrobacterium GV3101 strains, respectively, according to the principle of equal proportion pairing. Injected into 5–8 leaf-aged N. benthamiana leaves with the same growth, and after culturing for 3 d at room temperature, about 1 cm² of tobacco leaves were cut to make glass slides, and the specimens were examined under a Leica TCS SP8 laser confocal microscope (Leica Microsystems (Shanghai) Trading Co., Ltd., Mannheim, Germany),
and the yellow fluorescent protein (YFP) was observed with a 10× lens and a YFP filter (561 nm excitation wavelength).

Detection of protein inhibition using green fluorescent protein (GFP)
The ScJAZ13 gene was constructed into the vector pCAMBIA2300 with GFP tag, and the fusion vector pCAMBIA2300-ScJAZ13 was then transformed into Agrobacterium tumefaciens strain GV3101. Agrobacterium containing the target fragment and H2B-mCherry (Howe et al., 2012) were subsequently collected and diluted with MS blank medium and mixed in equal proportions to OD = 0.8. The 1.0 mL bacterial solution (containing 200 μmol-L⁻¹ acetosyringone) was injected with a sterile syringe into 5–8 leaf old WT plants and transgenic plants overexpressing ScWRKY4 gene, respectively. After incubation for 2 d at 28°C under light for 16 h/dark for 8 h, the leaves were collected and the GFP was observed under a Leica TCS SP8 laser confocal microscope (Leica Microsystems (Shanghai) Trading Co., Ltd., Mannheim, Germany) using a 10× lens, 488 nm green fluorescence excitation wavelength.

Results
GO annotation of ShWRKY gene family
In previous study, we identified 60 ShWRKY genes from the genome of S. spp. hybrid cultivar R570 (Wang et al., 2022a). These ShWRKY genes were constitutively expressed in sugarcane tissues and their expression could be induced by the smut pathogen (Wang et al., 2022a). To further explore the function of ShWRKY genes, the GO annotation analysis was performed. The results showed that ShWRKY genes were annotated in the biological process (38%), molecular function (36%), and cellular component (26%) categories (Fig. 1A and Supplementary Table S2). In the biological process, ShWRKY genes were mainly enriched in biotic stress response and defense response items (Fig. 1B and Supplementary Table S2). For molecular function, 26 ShWRKY genes were enriched in transcriptional regulatory activity and DNA-binding transcription factor activity items (Fig. 1C and Supplementary Table S2). While in the cellular component, most of the ShWRKY genes were enriched in the nucleus, the cellular component items (Fig. 1D and Supplementary Table S2).

Characteristics of the ScWRKY4 in sugarcane
Using the leaf cDNA of sugarcane ROC22 as a template, the ScWRKY4 gene was cloned. The ORF (137–877 bp) of this gene was 741 bp in length, encoding 246 amino acids, and with a typical WRKY structural domain
(Fig. 2A). A phylogenetic tree of ScWRKY4 protein was constructed using the ShWRKY proteins as a reference, and the structure showed that ScWRKY4 protein belongs to subgroup IIc (Fig. 2B). The results of multiple sequence comparison indicated that ScWRKY4 had the highest similarity of 99.19% with ShWRKY36 (Supplementary Table S3), suggesting their functional similarity. A total of 10 conserved motifs of ScWRKY4 protein were observed using MEME software. The ScWRKY4 protein contained only Motif 1, Motif 2, Motif 3, and Motif 6 (Fig. 2C). Gene structure analysis revealed that the ScWRKY4 gene had four exons and three introns (Fig. 2D). Prediction of promoter element in the first 2000 bp upstream of ScWRKY4 gene suggested that several cis-acting regulatory elements were associated with stress, growth and hormone response (Fig. 2E and Supplementary Table S4). Of these, one stress-related cis-acting regulatory element LTR, was involved in the low temperature response. Two growth and development related cis-acting regulatory elements, such as the ARE, was essential for anaerobic induction, and the RY-element acted as a component involved in seed specificity. There were also four hormonal response related cis-acting regulatory elements, included MeJA-responsive element TGACG-motif, auxin-responsive element TGA-element, ABA-responsive element ABRE and gibberellin-responsive element P-box (Fig. 2E and Supplementary Table S4). It is thus hypothesized that ScWRKY4 gene may be involved in sugarcane growth and development and in the defence process against biotic stresses.

The expression profile of ScWRKY4 gene

Previous study showed that, the expression level of ScWRKY4 gene reached a peak value at 12 h under both SA and MeJA treatments, which was 2.69-folds and 2.38-folds that of the control group, respectively (Fig. 3A). Fig. 3A demonstrated the expression level of ScWRKY4 gene in four different sugarcane genotypes interacting with smut pathogen. As a result, the ScWRKY4 gene was down-regulated in two smut-resistant sugarcane cultivars (YZ01-1413 and YT96-86), while up-regulated in two smut-susceptible sugarcane cultivars (YZ03-103 and FN39) at 7 dpi. Therefore, it is speculated that the ScWRKY4 gene may negatively regulate the defence of sugarcane against smut fungus infection.

Next, we transiently transformed ScWRKY4 gene into N. benthamiana leaves by Agrobacterium mediated method, and the ScWRKY4 protein was successfully detected in tobacco leaves at 1 d (Fig. 3B). DAB staining revealed, that tobacco leaves transiently overexpressing the ScWRKY4 gene (35S::ScWRKY4) had darker browning and a more pronounced allergic response than the control group (35S::00) (Fig. 3C). The expression of seven HR marker gene was significantly up-regulated except for NbHSR201 (Fig. 3D). After inoculation...
with *F. solani* var. *coeruleum* for 7 d, **35S::ScWRKY4** tobacco leaves began to wilting, and the degree was more serious than that of the **35S::00** (Fig. 3E). Compared with the **35S::00** plants, in the **35S::ScWRKY4** tobacco leaves at 2 dpi, the expression of seven immune related genes were down regulated, except that the expression of *NbPR-1a/c* did not change (Fig. 3F). The results suggest that ScWRKY4 may be a negatively regulated transcription factor.

**Stable overexpression of ScWRKY4 negatively regulates resistance to pathogen infection**

To further examine the disease resistance of *ScWRKY4* gene, we genetically transformed it into *N. benthamiana* and cultured it to T3 generation by screening on MS plates supplemented with herbicide, and finally obtained the transgenic positive lines of *ScWRKY4* (OE) (Supplementary Fig. S1A). A target band consistent with the size of the overexpression vector plasmid was identified in all transgenic plants, indicating that the *ScWRKY4* gene was successfully inserted into the genome of *N. benthamiana*, which could be used for the next step of disease resistance verification (Supplementary Fig. S1B).

From the perspective of phenotype, after inoculation with *F. solani* var. *coeruleum*, the leaves of OE plants showed significant wilting and yellowing with obvious disease spot symptoms compared to the WT at 7 dpi (Fig. 4A). Compared with WT, the DAB (H2O2 accumulation) and trypan blue staining (cell death) of OE leaves were deeper. Besides, the SA and JA contents of WT and transgenic lines plants were measured at 0 dpi and 2 dpi. There was no significant difference in the content of SA in WT and OE, while the content of JA in OE was significantly higher than the control group, 1.28-folds higher than the control group at 0 dpi. At 2 dpi, the content of SA and JA in OE plants was significantly lower than that in WT plants, 0.93-folds and 0.89-folds respectively (Fig. 4B). Enzyme activity assay revealed that the CAT and GSTs activities of the OE plants were significantly higher than the control before inoculation, 2.44- and 1.16-folds higher than the control, respectively. However, the activities of both enzymes decreased to the control level at 2 dpi (Fig. 4C). Finally, when the expression of immune marker-related genes was examined at 0 and 2dpi, the expression of the HR marker gene (*NbHSR201* and *NbHSR515*), the SA signalling pathway-related genes (*NbPR2* and *NbPR3*), the JA signalling pathway-related genes (*NbLOX1* and *NbDEF1*), and the ROS-related gene (*NbCAT1*) were all suppressed in the OE plants compared to the WT (Fig. 4D–G). The above results demonstrated that overexpression of *ScWRKY4* gene in *N. benthamiana* enhanced the susceptibility of transgenic tobacco plants to *F. solani* var. *coeruleum* infection.
Differentially expressed genes (DEGs) involved in disease resistance regulatory network of the ScWRKY4 gene

To obtain the DEGs involved in disease resistance, the WT and ScWRKY4-OE samples inoculated with *F. solani* var. *coeruleum* (WT-CK, WT-T, ScWRKY4-CK and ScWRKY4-T) at 0 d and 2 d were sequenced. The comparison combination was set as control group WT-CK-vs-WT-T and treatment group ScWRKY4-CK-vs-ScWRKY4-T. In clean reads of all samples, Q20 and Q30 were all greater than 91%, and GC content was higher than 42%, indicating that the sequencing quality was well, and the data could be used for subsequent sample analysis (Supplementary Table. S5). The regions aligned to the genome were divided into exon regions, intron regions and intergenic regions, that most of the reads could be aligned to exon regions (Supplementary Fig. S2A). The Pearson correlation coefficient between replicate samples within a group were all close to 1 (Supplementary Fig. S2B), indicating good inter-sample repeatability for the next step of between-group difference analysis.

The up-regulated DEGs in WT were significantly lower than those in OE plants when inoculated with *F. solani* var. *coeruleum* for 0 d (Fig. S3A). There were increased up-regulated DEGs in WT and decreased up-regulated DEGs in OE plants at 2 dpi (Fig. S3B). A total of 4944 up-regulated DEGs and 3903 down-regulated DEGs were identified in WT-CK-vs-WT-T, whereas a total of 8043 up-regulated DEGs and 8013 down-regulated DEGs were identified in ScWRKY4-CK-vs-ScWRKY4-T (Fig. S3C). The down-regulated DEGs in ScWRKY4-CK-vs-ScWRKY4-T was 2.05-fold higher than in WT-CK-vs-WT-T (Fig. S3C). Among the DEGs, 5488 were unique to WT-CK-vs-WT-T and 12697 to ScWRKY4-CK-vs-ScWRKY4-T (Fig. S3D). Subsequently, GO and KEGG analyses were performed to explore the disease resistance regulatory network of the ScWRKY4 gene. We found that 10.23% of the specific DEGs in WT-CK-vs-WT-T were enriched in the response to hormone item and 10.62% of the specific DEGs were enriched in the response to endogenous stimulus item (Fig. 5A, Supplementary Table. S6). In ScWRKY4-CK-vs-ScWRKY4-T, 32.34% specific DEGs were enriched in response to stimulus item, 18.73% in response to stress item, and 6.51% in response to external biotic stimulus item (Fig. 5B, Supplementary Table. S7). Surprisingly, DEGs specific to both WT-CK-vs-WT-T and ScWRKY4-CK-vs-ScWRKY4-T were significantly enriched in the plant hormone signal transduction pathway, MAPK signaling pathway-plant pathway and plant-pathogen interaction pathway (Fig. 5C, D). By contrast, the specific DEGs in WT-CK-vs-WT-T were significantly enriched in the plant hormone signal transduction pathway (Fig. 5C), whereas the specific DEGs in ScWRKY4-CK-vs-ScWRKY4-T were significantly enriched in the plant-pathogen interaction pathway (Fig. 5D).
**ScWRKY4 mediated the regulation of disease resistance-related pathway genes during pathogenic stress responses**

To investigate the disease resistance regulatory mechanism of ScWRKY4 gene, we further analyzed the expression of key DEGs in the disease resistance-related pathway identified above. In the plant hormone signal transduction pathway, JA and SA pathway related to stress response and disease resistance, respectively. The specific DEGs TIFY10A (Nbv5tr6373992, Nbv5tr6373993, Nbv5tr6204331 and Nbv5tr6233370), TIFY10B (Nbv5tr6348185, Nbv5tr6349145, Nbv5tr6349147, Nbv5tr6374864 and Nbv5tr6405095) and TIFY11B (Nbv5tr6349144 and Nbv5tr6349145) in WT-CK-vs-WT-T were up-regulated (Fig. 6A, Supplementary Table. S8). However, the expression of these DEGs were inhibited in ScWRKY4-CK-vs-ScWRKY4-T (Fig. 6A, Supplementary Table. S8). In the plant-pathogen interaction pathway, CNGCs and CDPK were involved in the HR process of plants (Fig. 6B). CNGCs-related DEGs CNGC1 (Nbv5tr6233756 and Nbv5tr6399602) were up-regulated, and CDPK-related DEGs CPK2 (Nbv5tr6321646) and CPK5 (Nbv5tr6397003) were down-regulated (Fig. 6B, Supplementary Table. S8). In the MAPK signaling pathway-plant pathway, most of the DEGs in WT-CK-vs-WT-T were inhibited (Fig. 6C). Among them, the FLS2-related DEGs XA21 (Nbv5tr6263859), VIP1-related DEGs VIP1 (Nbv5tr6405992) and BZIP18 (Nbv5tr6405991) were up-regulated and involved in defense response and late defense response for pathogen (Fig. 6C, Supplementary Table. S8). Notably, most of the DEGs in the ScWRKY4-CK-vs-ScWRKY4-T were down-regulated, among which the pathogen attack-related DEGs (Nbv5tr6231425, Nbv5tr6339859 and Nbv5tr6343569) in pathogen attack were significantly down-regulated, and participated in the H2O2 production (Fig. 6C, Supplementary Table. S8).

**ScWRKY4 interacted with ScJAZ13 and inhibited its expression**

Transcriptome analysis revealed that JAZ genes were mostly up-regulated in WT-CK-vs-WT-T and their expression was repressed in ScWRKY4-CK-vs-ScWRKY4-T. Therefore, we analyzed the interactions between ScWRKY4 and the seven JAZ proteins previously cloned by the group. The results of the point-to-point yeast two-hybrid indicated that all plasmid combinations grew normally on SD/-Trp-Leu medium, implying that all plasmids were successfully transformed into the yeast strain Y2HGold (Fig. 7A). However, when transferred to SD/-Trp-Leu-His-Ade medium, only the BD-ScWRKY4+AD-ScJAZ13 combination was able to grow normally, suggesting that ScWRKY4 and ScJAZ13 had a reciprocal relationship (Fig. 7A). The BiFC approach was further used to verify the interaction between ScWRKY4 and ScJAZ13. When ScWRKY4 was fused to
nYFP (nYFP-ScWRKY4), and ScJAZ13 was fused to cYFP (cYFP-ScJAZ13), fluorescent complexes were formed and observed in the nucleus of *N. benthamiana* leaf cells (Fig. 7B). It is demonstrated that there is an interaction between ScWRKY4 and ScJAZ13 and that the specific protein complex is produced in the nucleus. After that, we constructed ScJAZ13 into GFP tagged vector (ScJAZ13-GFP) and transiently overexpressed it in the leaves of WT and OE plants, respectively. Two days later, the fluorescence intensity of ScJAZ13-GFP could be seen to be significantly higher in the leaves of WT plants than in the leaves of OE plants (Fig. 7C). Consequently, it is hypothesized that ScWRKY4 may repress the expression of ScJAZ13 protein and thus negatively regulated the disease resistance to pathogen infection.

**Discussion**

WRKY is one of the largest families of transcription factor in plants (Eulgem *et al.*, 2000). Its function has been successively reported in different plant species (Wani *et al.*, 2021), however, there were few reports on the function of WRKY in sugarcane (Javed *et al.*, 2022; Li *et al.*, 2020; Liu, 2012; Wang *et al.*, 2022a; Wang *et al.*, 2018a). Here in our study, *ScWRKY4* belonged to the class IIc WRKY family member, and could be induced by the stress of SA and MeJA (Fig. 3A). After inoculation with *S. scitamineum*, the expression was up-regulated in smut-susceptible sugarcane cultivars (YZ03-103 and FN39) and down-regulated in smut-resistant cultivars (YZ01-1413 and YT96-86) (Fig. 3A). It is thus speculated that *ScWRKY4* negatively regulated the resistance of sugarcane to smut pathogen infection.

Previous studies have shown that plant *WRKY* gene could respond to infection caused by pathogenic fungi (Abbruscato *et al.*, 2012; Li *et al.*, 2006). Interesting but not surprising is that ROS levels in plants altered when they were infected by pathogens (Li *et al.*, 2015). After being stressed by pathogenic bacteria, the expression of those genes related to ROS scavenging system-related genes such as *CAT* and *GST*, was closely related to plant disease resistance (Yan *et al.*, 2015). *GhWRKY27a* inhibited the expression of *CAT* and *GST* genes and negatively regulated tobacco resistance to *Rhizoctonia solani* (Yan *et al.*, 2015). HR is a defense response of plants to pathogens in host-parasite incompatibility (Klement and Goodman, 1967). Overexpression of *CaWRKY40* in tobacco altered the expression of HR-related genes (*NtHSR201, NtHSR203* and *NtHSR515*) and pathogenicity-related genes, thereby regulating pepper response to *R. solanacearum* stress (Dang *et al.*, 2013). In this study, the *CAT* enzyme activity and GSTs enzyme activity of OE were significantly higher than those of WT before inoculation, but decreased to the control level after inoculation (Fig. 4C). Besides, the expression of HR marker genes *NbHSR201* and *NbHSR515*, and the ROS-related genes *NbCAT1*,
was significantly decreased (Fig. 4D, G). It is speculated that *N. benthamiana* plants overexpressing *ScWRKY4* may reduce tolerance to oxidative stress after inoculation with *F. solani* var. *coeruleum*. Reasonably, the inhibition of HR occurrence was associated with the decreased enzymatic activities of CAT and GSTs and the down-regulated expression levels of ROS and HR-related genes. SA and JA are two widely studied signaling pathways that play important regulatory roles in plant defense responses to pathogenic infection (Peng *et al.*, 2012; Spoel and Dong, 2008). WRKY25 was a negative regulator of SA-mediated defense responses against *Pseudomonas syringae* in Arabidopsis WRKY25 T-DNA insertion mutants and transgenic plants overexpressing (Zheng *et al.*, 2007). In rice, JA played an important role in *OsWRKY30* gene mediated defense responses to fungal pathogens (Peng *et al.*, 2012). Here, the contents of SA and JA in OE were lower than those in the control at 2 dpi (Fig. 4B), and genes related to SA and JA signaling pathways (*NbPR2*, *NbPR3*, *NbLOX1* and *NbDEF1*) were down-regulated in OE (Fig. 4E, F). Therefore, it is assumed that ScWRKY4 is a negative regulatory transcription factor, which may suppress the SA and JA signaling pathways by inhibiting the expression of *NbPR2*, *NbPR3*, *NbLOX1* and *NbDEF1* genes, and at the same time repress the expression of HR and ROS-related genes, *NbHSR201*, *NbHSR515* and *NbCAT1*, thereby weakening the resistance of *N. benthamiana* to *F. solani* var. *coeruleum*.

What's exciting is that plant WRKY genes are involved in different defense signaling pathways (Birkenbihl *et al.*, 2012; Peng *et al.*, 2012). Overexpression of rice *OsWRKY03* enhanced the resistance of transgenic plants to bacterial blight and induced the expression of several pathogenic related genes in transgenic plants (Liu *et al.*, 2005). Further studies revealed that *OsWRKY03*, located upstream of *OsNPR1*, acts as a transcriptional activator of SA-related or JA-related defense signaling pathways (Liu *et al.*, 2005). Besides, AtWRKY57 competed with AtWRKY33 to interact with VQ proteins SIB1 and SIB2, and competitively regulated the expression of key repressors JAZ1 and JAZ5 of the JA signaling pathway, thereby blocking JA signaling to a certain extent and attenuating the effect of WRKY33 on *Botrytis cinerea* resistance (Jiang and Yu, 2016). *OsWRKY13* enhanced rice defense responses against *R. solani* and *Sarocladium oryzae*, which may affect the TIFY9-mediated MAPK cascade signaling pathway (Lilly and Subramanian, 2019). In the present study, transcriptome data showed that in the plant hormone signal transduction pathway, after inoculation of *F. solani* var. *coeruleum*, the expression of most JAZ genes was significantly suppressed in *N. benthamiana* plants overexpressing the *ScWRKY4* gene (Fig. 6A, Supplementary Table S8). We also confirmed that ScWRKY4 interacted with ScJAZ13 and could repress the expression of ScJAZ13 (Fig. 7A–C). Therefore, we reasonably deduced that ScWRKY4 may negatively regulate resistance to pathogens by repressing the expression of...
ScJAZ13.

In conclusion, we depicted here a model for the disease resistance regulatory mechanism of the *ScWRKY4* gene (Fig. 8). That is, stable overexpression of *ScWRKY4* negatively regulated the resistance of transgenic plants to pathogen infection and caused the down-regulated expression of JA-related genes. During the transcription process, most of the JAZ genes could be repressively expressed in *ScWRKY4* transgenic plants. In addition, ScJAZ13 protein interacted with ScWRKY4 protein and was repressed by ScWRKY4. We thus proposed that ScWRKY4 may enhance susceptibility to pathogen by repressing the expression of ScJAZ13. This work is expected to lay the foundation for in-depth analysis of the biological function and mechanism of sugarcane WRKY transcription factors.

**Supplementary Data**

- **Table S1.** Primers used in the experiment.
- **Table S2.** GO annotation of ShWRKY gene family in *Saccharum* spp. hybrid cultivar R570.
- **Table S3.** The percentage between 60 ShWRKYs and ScWRKY4.
- **Table S4.** Function prediction of *cis*-acting regulatory element of in the promoters of sugarcane ScWRKY4.
- **Table S5.** The statistics information of RNA-seq alignment.
- **Table S6.** The 30 most significant GO enrichment term for differentially expressed genes specific to the WT-CK-vs-WT-T.
- **Table S7.** The 30 most significant GO enrichment term for differentially expressed genes specific to the *ScWRKY4*-CK-vs-*ScWRKY4*.
- **Table S8.** Differential gene expression in disease resistance related pathways.
- **Fig. S1.** Screening of the T3 generation of transgenic *N. benthamiana* plants overexpressing *ScWRKY4*.
- **Fig. S2.** The analysis on the RNA-seq data.
- **Fig. S3.** The differentially expressed genes in WT and *ScWRKY4* gene overexpressing tobacco plants after inoculated with *F. solani* var. coeruleum at 0 dpi and 2 dpi.

**Author contributions**

DJW and YXQ conceived and designed the experiments. DJW, WW, SJZ, LQQ, YLL, and PXL performed the experiments. DJW analyzed the data and wrote the paper. YCS and YXQ revised the final version of the paper. All authors read and approved the final manuscript.

**Conflict of interest**

The authors declare no conflict of interest.

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Figure legends

**Fig. 1.** GO annotation of ShWRKY gene family in Saccharum spp. hybrid cultivar R570. (A) The category of GO function. (B) GO function of ShWRKY gene family in molecular function. (C) GO function of ShWRKY gene family in biological process. (D) GO function of ShWRKY gene family in cellular component.

**Fig. 2** Characterization of ScWRKY4 in sugarcane. (A) Conserved domains of ScWRKY4 protein. (B) A phylogenetic tree of ScWRKY4 protein. ScWRKY4 protein was shown in red bold font. (C) Conserved motifs of ScWRKY4 protein. (D) Structure of ScWRKY4 gene. (E) Distribution and functional prediction of cis-acting regulatory elements of ScWRKY4 gene promoter. The numbers in parentheses in the figure represented the number of cis-acting regulatory element.

**Fig. 3.** The function of ScWRKY4 gene. (A) Relative expression of ScWRKY4 genes in sugarcane under SA, MeJA and smut stress (YZ01-1413 and YT96-86 were smut-resistant sugarcane cultivars, YZ03-103 and FN39 were smut-susceptible sugarcane cultivars). (B) Semi-quantitative PCR amplification of ScWRKY4 in N. benthamiana leaves. 35S::00, the empty vector pEarleyGate 203. 35S::ScWRKY4, pEarleyGate 203-ScWRKY4. (C) DAB staining. (D) RT-qPCR expression of eight immunity-associated marker genes in the N. benthamiana leaves. (E) Phenotype of N. benthamiana leaves after inoculation with F. solani var. coeruleum. (F) The transcripts of immunity-associated marker genes in the N. benthamiana leaves after inoculation with F. solani var. coeruleum at 2 dpi. Data were normalized to the NbEF-1α expression level. All data points were means ± standard errors (n = 3). Different letters on the columns represented significant differences calculated by Duncan’s new multiple range test (P<0.05).

**Fig. 4.** Disease resistance of ScWRKY4 transgenic N. benthamiana inoculated with F. solani var. coeruleum. (A) Phenotype observation at 7 dpi. Use the red circle to mark lesion. (B) Determination of SA and JA contents in WT and OE plants at 0 dpi and 2 dpi. (C) CAT and GSTs enzyme activity assay in WT and OE plants at 0 dpi and 2 dpi. (D–G) Expression of HR marker genes, SA and JA signaling pathways, and ROS defense response related genes. WT, wild type N. benthamiana, OE, transgenic N. benthamiana plants overexpressing ScWRKY4 gene. Data were normalized to the NbEF-1α expression level. All data points were means ± standard errors (n = 3). Different letters on the columns represented significant differences calculated by Duncan’s new multiple range test (P<0.05).

**Fig. 5.** GO and KEGG enrichment of differentially expressed genes. (A) The bar graph of DEGs in the control group. (B) The bar graph of DEGs in the treatment group. (A) and (B) showed only the first 30 GO terms that were significantly enriched. (C) Bubble plot of DEGs enriched in disease resistance-related pathways in the control group. (D) Bubble plot of DEGs enriched in disease resistance-related pathways in the treatment group. Control group: WT-CK-vs-WT-T, treatment group: ScWRKY4-CK-vs-ScWRKY4-T.
Fig. 6 Pathways related to disease resistance and the expression levels of key genes. (A–C) The expression patterns of specific DEGs in the plant hormone signal transduction pathway, plant-pathogen interaction pathway and MAPK signaling pathway-plant pathway in the control group and the treatment group, respectively. Control group: WT-CK-vs-WT-T, treatment group: ScWRKY4-CK-vs-ScWRKY4-T. Gray box represents no expression.

Fig. 7. Sugarcane ScWRKY4 interacted with ScJAZ13 and inhibited its expression. (A) ScWRKY4 interacted with seven ScJAZs in yeast. BD: pGBK7 vector, AD: pGADT7 vector. (B) BiFC to detect the interaction between ScWRKY4 and ScJAZ13 in *N. benthamiana* epidermal cells. Images were obtained with a confocal microscope at 2 dpi. Bars, 25 μm. (C) ScWRKY4 inhibited the expression of ScJAZ13 in *N. benthamiana* epidermal cells. Images were obtained with a confocal microscope at 2 dpi. Bars, 25 μm. WT, wild type *N. benthamiana*; ScWRKY4-OE, transgenic plants overexpressing ScWRKY4.

Fig. 8. A model for disease resistance regulation of the transcription factor ScWRKY4. Stable overexpression of ScWRKY4 caused down-regulated expression of JAZ-related genes in transgenic plants. ScWRKY4 interacted with ScJAZ13 and repressed the expression of ScJAZ13. Thus, it is hypothesized that ScWRKY4 enhances susceptibility to pathogen and is a negatively regulated transcription factor.
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