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Identification and functional analysis of the *HvWRKY1* gene associated with Qingke (*Hordeum vulgare* L. var. *nudum* Hook. f.) leaf stripe disease

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Abstract: To explore the role of *WRKY* transcription factors (TFs) in the resistance process of Qingke (*Hordeum vulgare* L. var. *nudum* Hook. f.), leaves of the leaf stripe disease-resistant variety Kunlun 14 and the susceptible variety Z1141 were sequenced by transcriptome sequencing (RNA-seq). A differentially expressed gene *HvnWKRY1* was identified, and its disease-resistance function was preliminarily analysed. The result showed that the open reading frame (ORF) of the gene was 1 062 bp and encoded 354 amino acids. It contained the conserved WRKY domain (273–351) and belonged to the WRKY protein family. The phylogenetic tree results showed that HvWRKY1 was most closely related to *Hordeum vulgare* L. The WRKY family of Qingke, barley, maize and rice were divided into categories I, II, and III, among which HvWRKY1 was located in group III. Results of the quantitative real-time fluorescence PCR (qRT-PCR) showed that the expression of *HvWRKY1* was significantly ($P < 0.01$) higher in leaf stripe infected leaves of Kunlun 14 than that of Z1141. In *Arabidopsis thaliana* transformed with *HvWRKY1*, resistance to *Botrytis cinerea* was enhanced. The RNA-seq analysis showed there were 824 differentially expressed genes (DEGs). Data of the Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment indicated, that a plant-pathogen interaction pathway was enriched. This study is expected to provide a theoretical basis for further studies of functioning of the Qingke gene *HvWRKY1* in resistance to the leaf stripe disease.

Keywords: barley leaf stripe disease; *HvWRKY1* gene; Qingke; RNA-seq; transgenic

Qingke belongs to the wheat family Gramineae and is a variety of barley that can adapt to extreme harsh environments such as intense cold, hypoxia, low temperature, drought, and strong ultraviolet light in the Qinghai-Tibet Plateau and is a symbol of Tibetan agricultural civilization (Zeng et al. 2018). It is therefore an important food, economic, and feed crop on the Qinghai-Tibet Plateau, with a cultiva-

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tion history of about 3 500 years (Yao et al. 2018; Zeng et al. 2018).

The pathogen of barley leaf stripe disease is *Vermidium vermicularis* (*Drechslera graminea* (Rabenh.) Shoem), which belongs to the subphylum Hemiomyces. The sexual form is the *Pyrenophora graminea* S. Ito & Kurib, belonging to the subphylum Ascomycetes (Walters et al. 2012). Barley leaf stripe disease is mainly caused by seed fungus. During seed germination, the mycelium grows and infects the plant from the bud sheath to the young bud, invades each layer of tender leaf tissue successively, and finally invades the ear (Pecchioni et al. 1996; Si et al. 2020). In recent years, leaf stripe disease has rapidly developed into one of the main diseases in Qingke areas and northern spring wheat areas on the Qinghai-Tibet Plateau (Si et al. 2019). Leaf stripe disease has become one of the most serious diseases in barley cultivation, which seriously threatens the yield and quality of barley (Gatti et al. 1992). At present, the *Rdg1a* (Giese et al. 1993) and *Rdg2a* (Bulgarelli et al. 2004) genes have been mapped on the 2H and 7H chromosomes of barley, respectively. Yao et al. (2021a) previously obtained a series of anti-leaf stripe disease miRNAs (Hvu-miRNA-168-3p, Hvu-miRNA-159b, and Hvu-novel-91) and target genes (*HvCYP450*, *HvRGA*, *HvLIN*, and *HvWRKY*) by miRNA sequencing (miRNA-seq). Later, Yao et al. (2021b) found that the expression levels of *HvAGO1*, *HvAGO2*, and *HvAGO4* were significantly increased under barley leaf stripe disease stress through *AGO* gene family analysis. No similar new genes related to barley leaf stripe disease have been reported.

The WRKY transcription factors (TFs) are one of the most important TFs in plants, and this gene family is mainly involved in plant biotic and abiotic stress responses (Khoso et al. 2022), of which biological stresses include attacks by pathogens, fungi, and viruses (Jiang et al. 2017). For example, in rice, knock-out of the *WRKY22* gene resulted in increased susceptibility to *rice blast*. Over-expression of the *WRKY22* gene increased rice resistance to rice blast (*Piricularia oryzae*) (Cheng & Wang 2014). Abiotic stresses include drought, saline-alkali, and oxidative stress (Khoso et al. 2022). For example, a *WRKY10* gene related to drought resistance was found in wheat and was introduced into tobacco through the construction of an overexpression vector, which significantly improved the drought and salt tolerance of tobacco (*Nicotiana rustica* L.) (Wang et al. 2013). However, the response of the *WRKY* gene to barley leaf stripe disease has not been reported. Therefore, studying the signal transduction pathway and mechanism of *WRKYs* in barley is important.

In this study, a differentially expressed *WRKY* was identified in different resistant barley varieties infection with leaf stripe by RNA-seq. Then it was isolated from the leaves of varieties Kunlun 14 and Z1141 and named *HvWRKY1*. The structural, physiological, and biochemical characteristics of the HvWRKY1 protein sequence, and the consistency and evolutionary relationship between HvWRKY1 and other gramineous WRKY proteins were analysed. Subcellular localization of HvWRKY1 and expression pattern analysis of *HvWRKY1* under the barley leaf stripe disease were conducted. The disease resistance of *Arabidopsis* transformed with the *HvWRKY1* gene was increased upon infection with *Botrytis cinerea*. RNA-seq was performed using the leaves of wild and transgenic plants, and then quantitative real-time fluorescence PCR (qRT-PCR), gene ontology (GO) and the Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis were performed. We identified various differentially expressed genes (DEGs) and some pathways related to disease resistance. This study provides a theoretical foundation for further research on the anti-leaf stripe disease function and disease-resistance mechanism of the Qingke *HvWRKY1* gene.

MATERIAL AND METHODS

Test materials. The disease-resistant Qingke variety Kunlun 14 and the disease-susceptible Qingke variety Z1141 were provided by the Academy of Agriculture and Forestry Sciences, Qinghai University (Yao et al. 2021a).

Total RNA extraction and cDNA synthesis of barley streaking disease leaves. A plant RNA extraction kit (TaKaRa, Beijing, China) was used to extract RNA from the normal and susceptible leaves of Kunlun 14 and Z1141 under leaf stripe disease stress. The determination and detection of RNA concentration and purity referred to the method presented by Yao et al. (2021b). A cDNA synthesis kit (TaKaRa, Beijing, China) was used for reverse transcription of RNA to obtain cDNA, which was stored at −80 °C.

Isolation of the Qingke *HvWRKY1* gene. In the previous study, the barley resistant variety Kunlun 14 and susceptible variety Z1141 were infected with leaf stripe disease, then the normal and infected leaves were selected for RNA-seq by Novogene (Beijing, China; Accession No.: PRJNA872218). Based on the data, a DEG (gene ID: HORVU4Hr1G011590) was obtained

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and annotated as *HvWRKY1*. Primer 5.0 was used to design the amplification primer *HvWRKY1*-F/R for the coding domain sequence (CDS) of this gene (Table 1), and PCR amplification was performed using leaf cDNA. The PCR amplification system and agarose gel electrophoresis detection followed the methods of Yao et al. (2021b). The target bands were recovered using the DiaSpin DNA Gel Extraction Kit (Sangon Biotech, Shanghai, China), and the target fragments were connected to the vector of pEasy-Blunt (TransGen, Beijing, China) and transformed into *E. coli* Trans-T1 receptive cells. Three positive clones were selected and sent to Sangon Biotech (Sangon Biotech, Shanghai, China) for sequencing.

Bioinformatics analysis of the Qingke *HvWRKY1* gene. The CDS region of the *HvWRKY1* gene was extracted from the plant genome database Gramene (http://www.gramene.org/). The barley reference genome (ftp://ftp.ensemblgenomes.org/pub/release-63/plants/fasta/hordeum_vulgare/dna) was used to obtain the promoter regions. Expasy Protparam (http://www.expasy.org/tools/protparam.html) and Protscale (https://web.expasy.org/protscale/) were used to predict the physical and chemical properties of the hydrophilic/hydrophobic proteins. Online analysis software (http://www.cbs.dtu.dk/services/Net Phos/) was used to predict the protein phosphorylation site. SignalP 4.1 (http://www.Detaibio.com/tools/signal-peptide.html) and TMHMM-2.0 (http://www.cbs.dtu.dk/services/TMHMM-2.0/) were used to predict the protein signal peptide and membrane structure. SMART (http://SMART.embl-heidelberg.de/) was used to predict the domain. SPOMA (https://npsa-prabi.ibcp.fr/cgi-bin/npsa_automat.pl?page=/NPSA/npsa_sopma.html) and SWISS-MODEL (https://swissmodel.expasy.org/) were used to predict the secondary and tertiary structure of the HvWRKY1 protein. The online software PlantCARE was used for promoter region (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/) analysis. Using an NCBI (https://www.ncbi.nlm.nih.gov/) query, Qingke HvWRKY1 homologous protein amino acid sequence was searched against other gramineous plants, and DNAMAN 6.0 software was used for multiple sequence alignment. In the barley genome (https://ensembl.gramene.org/Hordeum_vulgare/Tools/Blast/Results?tl=qc1r7FSKnIRWWvEB-13415-2148596), there were seven *WRKY* family genes (gene ID: HORVU4HG0340900, HORVU5HG0433910, HORVU5HG0509460, HORVU2HG0192860, HORVU2HG0158690, HORVU2HG0194370, and HORVU4HG0410410) that were closely related to *HvWRKY1*. In the rice genome (https://rapdb.dna.affrc.go.jp/tools/blast/run) there were seven *WRKY* family genes (gene ID: Os12g0597700-02, Os12g0597700-01, Os03g0741400, Os04g0605100, Os04g0287400, Os05g0129800, and Os02g0698800) that were closely related to *HvWRKY1*. In the maize genome (https://ensembl.gramene.org/Zea_mays/Transcript/Summary?db=core;g=Zm00001eb240750;r=5:162419477-162420949;t=Zm00001eb240750_T001;tl=rhqMQuFJ3Dj1nlvq-13460-2153714), there were seven *WRKY* family genes (gene ID: Zm00001eb051120, Zm00001eb278090, Zm00001eb061030, Zm00001eb154170, Zm00001eb335440, Zm00001eb370960, and Zm00001eb240750) that were closely related to *HvWRKY1*. MEGA7 software was used to construct the phylogenetic tree, and iTOL (https://itol.embl.de/itol.cgi) was used to illustrate the tree.

Subcellular localization of Qingke HvWRKY1. The primer *HvWRKY1*-GFP with a specific joint was

Table 1. Primers used in this study

Primer name		Primer sequence (5'→3')	Purpose
HvWRKY1	forward	ATCTGAGCGAGTTTTCTCTCCCATT	gene cloning
	revers	TGCCTTGTGATAACTAATGTGTCAA	
HvWRKY1	forward	CGATGCCTTAATTGCTACG	qRT-PCR
	revers	TGTGATTGCCATTGCTGTA	
TC139057	forward	GAAGGATGAGCAAAAGGCCCT	internal reference gene
	revers	GGCAGGCAGACTCATTTCTTCC	
HvWRKY1-GFP	forward	GGGGACAAGTTTGTACAAAAAAGCAGGCTTCA TGGAGGAAGTGGAGGAGGCC	subcellular localization vector
	revers	GGGGACCACTTTGTACAAGAAAGCTGGGTCTTACCA ACAAAGTTGATGCAT	

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designed (Table 1), and the cDNA of Kunlun 14 leaf was used to construct the expression vector using the Gateway method. The recombinant plasmid HTC-PAST-WRKY1-GFP was subsequently used for the *Agrobacterium*-mediated tobacco transformation experiment. Fluorescent signals of green fluorescent protein (GFP) were detected using a laser confocal microscope (Nikon-A1R, Shanghai, China), following which the images were analysed.

Analysis of the expression pattern of Qingke *HvWRKY1*. According to the gene sequence of Qingke *HvWRKY1* obtained by amplification, quantitative primers *HvWRKY1*-SF /SR were designed using Primer 6.0 (Table 1). The cDNA of the normal leaves and susceptible leaves of the Kunlun 14 and Z1141 were infected with barley leaf stripe disease, and *TC139057* was used as an internal reference gene for qRT-PCR. The reaction system was based on the method presented by Yao et al. (2021b). The relative expression of the *HvWRKY1* gene was calculated using the $2^{-\Delta\Delta Ct}$ method, and the significance was detected by SPSS 26.0 (Yokotani et al. 2018).

Transformation of *HvWRKY1* gene into *Arabidopsis* and evaluation of its resistance to *Botrytis cinerea*. Gateway technology was used to construct a plant expression vector, and *HvWRKY1* PCR primers with attB sites were designed, followed by the cloned primer sequence after the joint (Table 1). Using a KOD-FX high fidelity PCR kit (ToYoBo, Shanghai, China), the plasmid with the *HvWRKY1* sequence was selected, and the primers with attB sites were used to amplify the cDNA sequence genes of the target genes. The PCR products were recovered by ethanol precipitation for the BP reaction (construct entry vector). The pdNOR ZEO intermediate vector plasmids identified as positive by recombinant PCR were mixed with the target vector pANIC6E plasmids for LR reaction (construct of expression vector). The transformation of *Arabidopsis* was performed by the *Agrobacterium*-mediated method, and the positive seedlings were identified by PCR.

Transgenic *Arabidopsis* gray mold resistance was identified by the gray mold spore infestation method, and the methods of *Botrytis cinerea* culture and inoculation and putrescine culture and infection were based on those reported by Scarboro et al. (2021).

RNA-seq and result analysis. Four weeks wild and transgenic *Arabidopsis* leaves after planting were used to sequenced by RNA-seq (Novogene, Beijing, China). DESeq 2 (1.20.0) software was used to conduct differential expression analysis. According to DESeq 2, the adjusted $P \leq 0.05$ were assigned as DEGs. GO enrichment analysis (http://wego.genomics.org.cn/cgi-bin/wego/index.pl) and KEGG pathway (www.kegg.jp/kegg/kegg1.html) were performed. Protein–protein interactions (PPIs) were predicted using STRING (http://version10.string-db.org/cgi/input.pl?UserId=dmZrtDdLzlbS&sessionId=AFEwAANBK10b&input_page_show_search=off). Online software (http://version10.string-db.org/cgi/input.pl?UserId=dmZrtDdLzlbS&sessionId=AFEwAANBK10b&input_page_show_search=on) was used to construct a protein interaction network.

RESULTS

Isolation and sequence analysis of the *HvWRKY1* gene. A target band of about 1 300 bp was amplified using the reverse transcription cDNA of total RNA from the leaves of Kunlun 14 and Z1141. The open reading frame (ORF) was 1 062 bp and encoded 354 amino acids (Figure S1 in Electronic Supplementary Material (ESM)). The sequence consistency between Kunlun 14 and Z1141 was 100%. Conserved domain prediction analysis of the amino acid sequences revealed that this protein had a typical WRKY domain (273–351) and annotated as WRKY1 belonging to WRKY protein family (Figures 1A).

The physicochemical properties analysis of WRKY1 showed that the molecular formula was $C_{1710}H_{2718}N_{516}O_{506}S_{19}$, the molecular weight was 39.21 kDa, the instability index was 59.52, the fat solubility index was 66.60, the theoretical isoelectric point was 9.88, and there were 25 negatively charged residues (Asp + Glu). The number of positively charged residues (Arg + Lys) was 48. The average hydrophobicity (GRAVY) of HvWRKY1 was –0.522. HvWRKY1 was a hydrophilic unstable basic protein with no transmembrane structure and no signal peptide (Figure 1B).

The prediction results of the secondary structure of WRKY1 showed that the secondary structure was mainly composed of random coil (53.82%), α-helix (28.90%), extended chain (13.60%), and β-corner (3.68%) (Figure 1C), indicating that the protein was dominated by random coils and α-helixes. These two secondary structures might play an important role in the function of proteins. The tertiary structure prediction results showed that the WRKY domain was located at the N-terminal of the amino acid sequence (Figures 1D). The Uniprot gene annotation showed that the protein was WRKY1, so the gene was named *HvWRKY1*.

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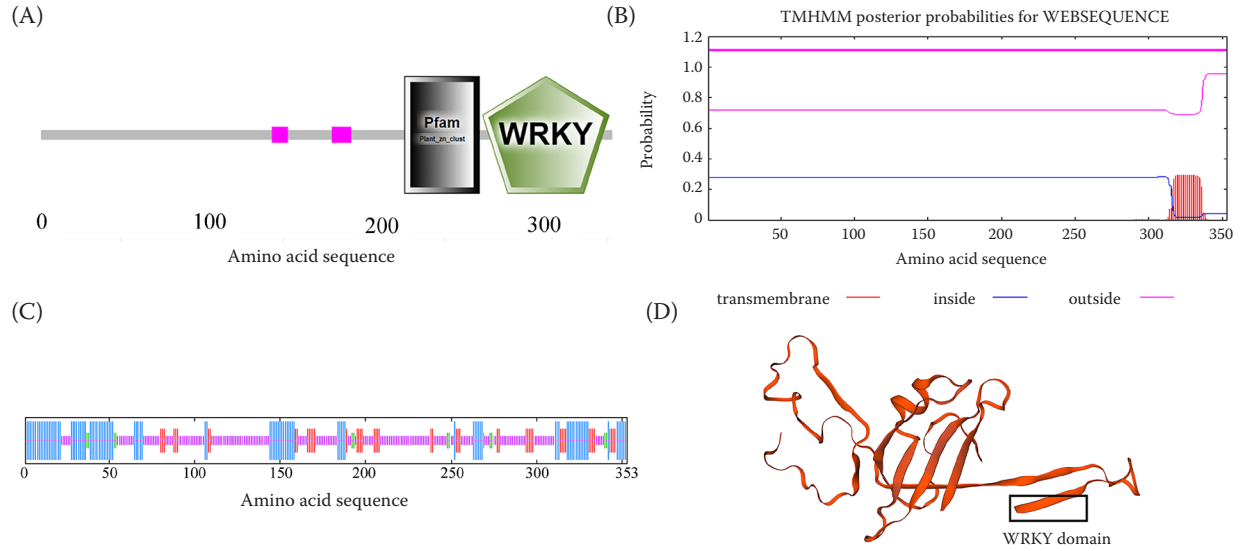

Figure 1. Protein analysis of the barley *HvWRKY1* gene: protein domain (A), transmembrane structure (B), secondary structure prediction (C), three-level structure prediction (D)
Blue represents an α-helix, red represents an extended strand, green represents a β-turn, and orange indicates random curls

The *cis*-acting element analysis of *HvWRKY1* gene. PlantCARE was used to analyse the *cis*-acting elements of the *HvWRKY1* gene, and the results showed that it contained abundant *cis*-acting elements, such as seven ABREs, one MYB family recognition site, core promoter element and other expression elements. (Table 2).

Homology comparison and phylogenetic analysis of HvWRKY1 proteins. The WRKY1 amino acid sequence consistency among Qingke, barley (*Hordeum vulgare* L.), wheat (*Triticum dicoccoides*), goat grass (*Aegilops tauschii*), brachypodium (*Brachypodium distachyon*), millet (*Panicum hallii*), foxtail millet (*Setaria italica*), and broomcorn millet (*Panicum miliaceum* L.) was 82.74%, 79.73%, 79.45%, 74.52%, 70.96%, 70.49%, and 69.40%, respectively. These sequences all had a conservative WRKY domain (Figure 2). The sequence consistency of the WRKY domain was 39.24%, 37.97%, 39.24%, 37.80%, 39.24%, 39.24%, and 38.75%. The phylogenetic tree results

Figure 2. Multiple alignments of the HvWRKY1 and WRKY domains compared with other gramineae plants
The red line represents the WRKY domain; the blue lines represents the WRKYGQK motif; and the black lines indicate $CX_5CX_{23}HX_1H$ zinc-finger motifs

https://doi.org/10.17221/14/2023-CJGPB

Table 2. Structure of the *cis*-acting elements in the promoter region of the *HvWRKY1* gene

Site name	Sequence	Starting position	Function
A-box	CCGTCC	–153	cis-acting regulatory element
	CCGTCC	+1 237	
	CCGTCC	+1 241	
	CCGTCC	–1 257	
	CCGTCC	+1 270	
ABRE	ACGTG	+102	cis-acting element involved in the abscisic acid responsiveness
	GACACGTACGT	–187	
	CGCACGTGTC	+188	
	ACGTG	+191	
	AACCCGG	–1 488	
	GCCGCGTGGC	–1 561	
	ACGTG	+1 979	
AE-box	AGAAACTT	–971	part of a module for light response
	AGAAACAA	+1 352	
ATCT-motif	AATCTAATCC	+1 204	part of a conserved DNA module involved in light responsiveness
CAAT-box	CAAT	+97	common cis-acting element in promoter and enhancer regions
	CAAAT	–255	
	CAAAT	–448	
	CAAAT	–510	
	CAAAT	–638	
	CAAAT	+672	
	CAAAT	+844	
	CAAAT	+1 680	
CAT-box	GCCACT	+1 198	
	GCCACT	–1 884	
CGTCA-motif	CGTCA	+15	cis-acting regulatory element involved in the MeJA-responsiveness
	CGTCA	+785	
	CGTCA	–1 149	
	CGTCA	–1 850	
	CGTCA	–1 876	
GA-motif	ATAGATAA	–61	part of a light responsive element
G-box	TACGTG	+101	cis-acting regulatory element involved in light responsiveness
	CACGTT	–190	
	CACGTC	–1 978	
GC-motif	CCCCCG	+1 046	enhancer-like element involved in anoxic specific inducibility
	CCCCCG	+1 654	
MRE	AACCTAA	–598	MYB binding site involved in light responsiveness
Sp1	GGGCGG	–1 049	light responsive element
	GGGCGG	–1 504	
	GGGCGG	+1 581	
	GGGCGG	+1 585	
TATA-box	TATA	+66	core promoter element around -30 of transcription start
	TACAAAA	+388	

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Table 2 to be continued

Site name	Sequence	Starting position	Function
	TATATAAATC	–420	
	ATTATA	+422	
	TATATAA	–423	
	TATATA	+424	
	ATATAA	+425	
	TATA	+426	
	ATTATA	+505	
	TATAA	–506	
	TATA	+507	
	TATA	+525	
	TATACA	–605	
TATA-box	TATA	+607	core promoter element around -30 of transcription start
	TATA	+661	
	ATATAA	+717	
	TATA	+718	
	TATAA	–741	
	TATA	+742	
	TATAAAA	–751	
	TATAAA	–752	
	TATAA	–753	
	TATA	+754	
	ATTATA	+1 321	
	TATAA	–1 322	
	TATA	–1 323	
TCCC-motif	TCTCCCT	+913	part of a light responsive element
TC-rich repeats	GTTTTCTTAC	–126	cis-acting element involved in defense and stress responsiveness
TCT-motif	TCTTAC	–126	part of a light responsive element
TGA-box	TGACGTAA	–782	part of an auxin-responsive element
	TGACG	–15	
	TGACG	–785	
TGACG-motif	TGACG	+1 149	cis-acting regulatory element involved in the MeJA-responsiveness
	TGACG	+1 850	
	TGACG	+1 876	
TGA-element	AACGAC	+1 359	auxin-responsive element
W box	TTGACC	–1 090	binding sites for the WRKY plant-specific transcriptional regulators
	TTGACC	+1 156	

showed that the Qingke HvWRKY1 protein was most closely with barley, followed by wheat, while most distantly with broomcorn millet (Figure 3A). The WRKY protein sequences closely related to HvWRKY1 were obtained from the reference genomes of barley, rice, and maize, which were divided into three groups I, II and III, among which group III consisted of three members, group II consisted of seven members, and group I consisted of 12 members (Figure 3B). Qingke HvWRKY1 belonged to group I. In addition, it was found that each group contained genes from barley, indicating that no significant gene loss event had

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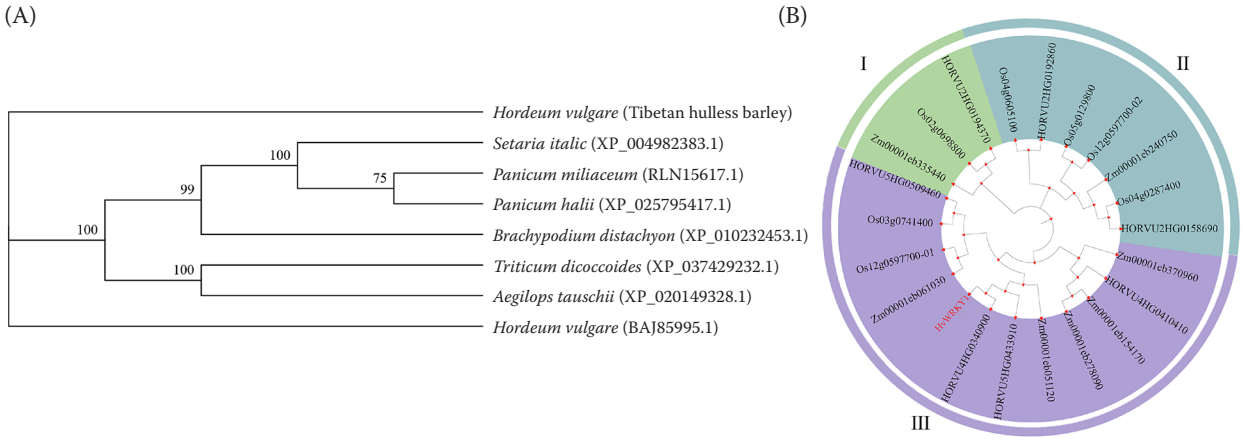

Figure 3. Phylogenetic analysis of the HvWRKY1 protein and other Gramineae plants: phylogenetic analysis of the HvWRKY1 protein and WRKY proteins of grasses (A), phylogenetic analysis of the HvWRKY1 protein and WRKY proteins that are closely related to barley, rice, and maize (B)

Green indicates group I; blue indicates group II, and purple indicates group III

occurred after the differentiation of barley, which confirmed that WRKY was a gene family with important functions in plant growth and development and that it was relatively evolutionarily conserved.

Subcellular localization of HvWRKY1. To study the specific location of the HvWRKY1 protein at the subcellular level, the *Agrobacterium* EHA105 carrying HvWRKY1 fusion GFP (HPT-PAST-HvWRKY1-GFP) was transformed into tobacco leaves through transient transformation. The fluorescence position was observed by a confocal laser-scanning microscope. The HPT-PAST-CK empty carrier was used as the control (Figure 4). The leaves transformed with the HPT-PAST-CK empty carrier showed strong green fluorescence in the nucleus, cytoplasm, and cell membrane, while the leaves transformed with HPT-PAST-HvWRKY1-GFP showed green fluorescence signals in the nucleus and cell membrane (Figure 4). These results indicated that the HvWRKY1 protein was located in the nucleus and cell membrane.

The expression of *HvWRKY1* gene under Qingke leaf stripe disease. To study the expression of the *HvWRKY1* gene in different disease-resistant Qingke varieties under leaf stripe disease stress, RNA-seq (Accession No.: PRJNA872218) and qRT-PCR were used to evaluate the leaves of the resistant variety

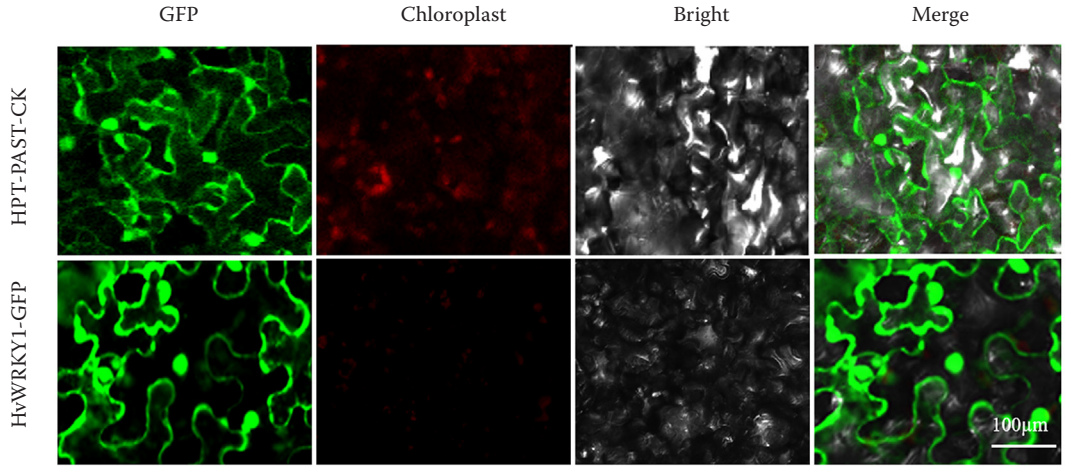

Figure 4. Subcellular localization of HvWRKY1

HPT-PAST-CK – no load; the excitation wavelength of the laser confocal microscope was set to green fluorescent protein (GFP), 448 nm (green); chloroplasts, 400 nm (red)

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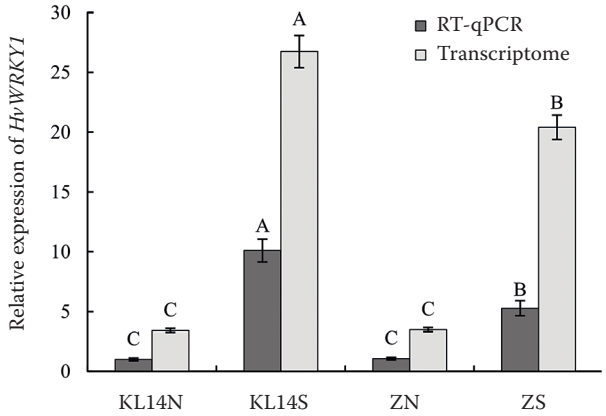

Figure 5. The relative expression of *HvWRKY1*
KL14N – Kunlun 14 without Qingke leaf stripe disease; KL14S – Kunlun 14 with Qingke leaf stripe disease; ZN – Z1141 without Qingke leaf stripe disease; ZS – Z1141 with Qingke leaf stripe disease; different capital letters represent extreme significance ($P < 0.01$)

Kunlun 14 and the susceptible variety Z1141 before and after infection with *P. graminea*. The results showed that the expression level of the *HvWRKY1* gene in the infected leaves of Kunlun 14 was 7.81 and 10.10 times significantly higher than that in the normal leaves of Kunlun 14 ($P < 0.01$). The expression of the *HvWRKY1* gene in the infected leaves of susceptible variety Z1141 was 5.84 times and 4.94 times significantly higher than that in the normal leaves of Z1141 ($P < 0.01$) (Figure 5). Meanwhile, the expression level of this gene in the infected leaves of Kunlun 14 was significantly higher than that in the infected leaves of Z1141 ($P < 0.01$). It is speculated that *HvWRKY1* plays an important positive regulatory role in the resistance of Qingke to leaf stripe disease.

Function of *Botrytis cinerea* disease resistance in *Arabidopsis* transformed with *HvWRKY1*. Transgenic *Arabidopsis* was used to identify *Botrytis cinerea* disease resistance (Figure 6). Wild-type *Arabidopsis* plants subjected to water treatment (WT-H_2O) grew normally and did not produce disease spots, while the plants in the wild-type *Botrytis cinerea* treatment (WT-*B. cinerea*) produced obvious disease spots. The transgenic *Arabidopsis* plants subjected to water treatment (*HvWRKY1*-H_2O) also grew normally and produced disease spots, while the transgenic *Arabidopsis Botrytis cinerea* treatment (*HvWRKY1-B. cinerea*) plants produced no or minor disease spots compared with WT-*B. cinerea*.

RNA-seq and differential expression gene analysis of transgenic *Arabidopsis*. The *HvWRKY1* gene was transferred into *Arabidopsis* for the RNA-seq of wild and *HvWRKY1* transferred *Arabidopsis* showed there was 824 DEGs were identified, in which 748 genes were down-regulated and 76 genes were up-regulated in transgenic *Arabidopsis* compared with the wild (Figure 7). In the KEGG analysis of the DEGs, 22 genes involved in the plant-pathogen interaction pathway were identified, and they were down-regulated in the transgenic *Arabidopsis* (Figure 9). Also, we found the expression levels of WRKY transcription factors (WRKY22 and WRKY33) and MYB

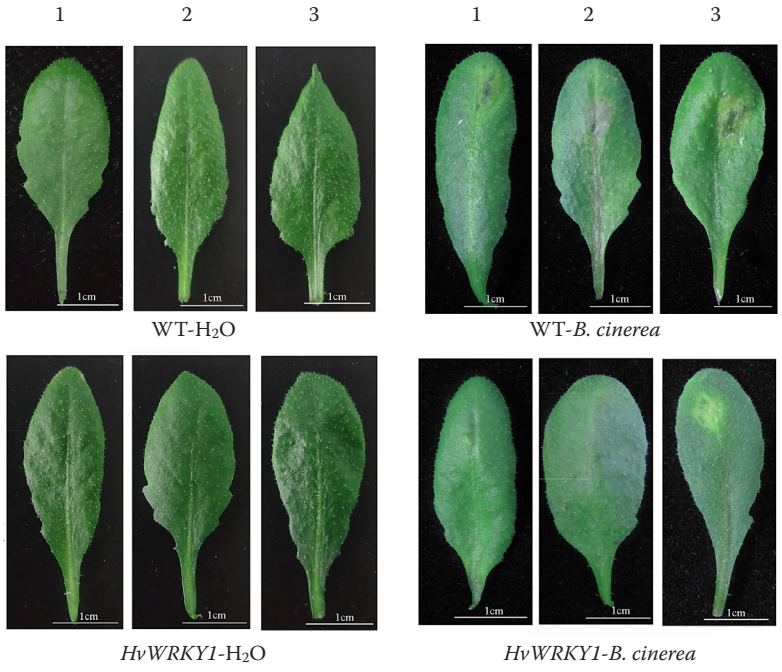

Figure 6. Evaluation of transgenic *Arabidopsis* for disease resistance
1, 2, 3 – No. of replicates; WT-*B. cinerea* – wild-type *Botrytis cinerea* treatment; WT-H_2O – wild-type water treatment; *HvWRKY1-B. cinerea* – transgenic *Arabidopsis Botrytis cinerea* treatment; *HvWRKY1*-H_2O – transgenic *Arabidopsis* water treatment

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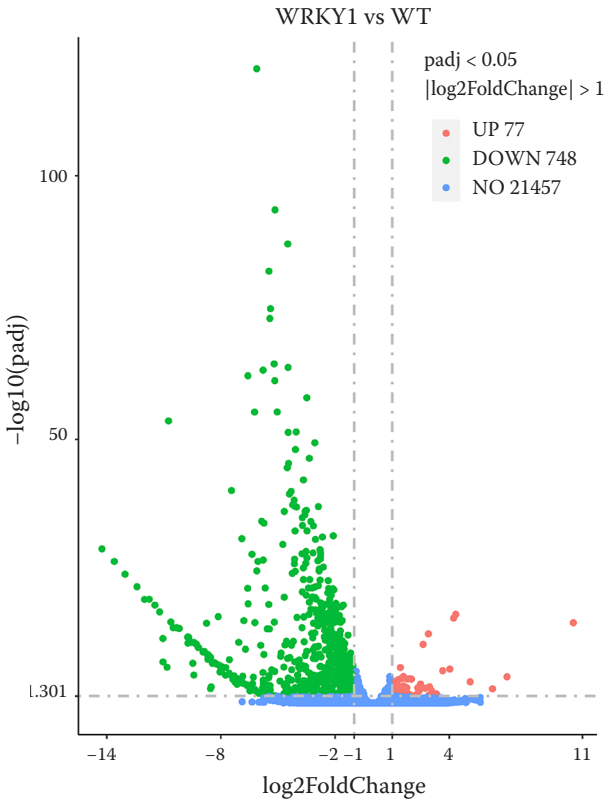

Figure 7. Differential gene volcano map
The abscissa is the log2FoldChange value, the ordinate is –log10padj or -log10pvalue, and the blue dotted line indicates the threshold line of the differential gene screening criteria

transcription factor (MYB30) were higher in wild than in the *HvWRKY1* transfered *Arabidopsis* (Figure 8).

GO enrichment was used to explore the functional classes of the annotated genes and classify 824 known annotated proteins. The 30 GO enrichments indicated that these genes fell into three broad categories: biological processes, cell components, and molecular functions (Figure S2 in ESM). In biological processes, signal transduction (GO: 0007165, GO: 003052) and cell stimulus response (GO: 0051716) were significantly enriched ($P < 0.05$), including 28 genes. The cellular components of the extracellular region (GO: 0005576) and extracellular body (GO: 0048046) were significantly enriched, including six and five genes. The molecular functions of transcriptional regulatory activity (GO: 0140110), DNA-binding transcription factor activity (GO: 0003700) were significantly enriched, containing 52 and 50 genes, respectively (Figure 10A). The KEGG enrichment analysis revealed that 824 DEGs were involved and significantly enriched in five pathways: plant-pathogen interaction, MAPK signaling pathway, α-linolenic acid metabolism, and plant hormone signal transduction. Among the top-20 enriched KEGG pathways, one plant-pathogen interaction pathway was identified, comprising 22 genes (Figure 10B). These included calmodulin CML23 (AT1G66400.1), CML37 (AT5G42380.1), CML38 (AT1G7665.1), Calcium-dependent protein kinases CPK15 (AT4G2194.2), CPK28 (AT5G6622.1), CPK29 (AT1G76040.2), and CPK32 (AT3G57530.1). In particular, there were WRKY transcription factors (*WRKY22* and *WRKY33*) and MYB transcription factors (*MYB30*), and *WRKY22*, *WRKY33,* and *MYB30* play an important role in plant disease resistance. These results provide valuable information for the study of the resistance mechanism of *HvWRKY1*.

Analysis of the protein interaction network. In the KEGG pathway enrichment analysis of the RNA-seq data, 22 genes significantly enriched in the plant-pathogen interaction pathway were selected. Genes interacting with these genes were then identified from the protein–protein interaction (PPI) results of the RNA-seq analysis, from which a protein interaction network analysis was constructed (Figure 11). In this protein interac-

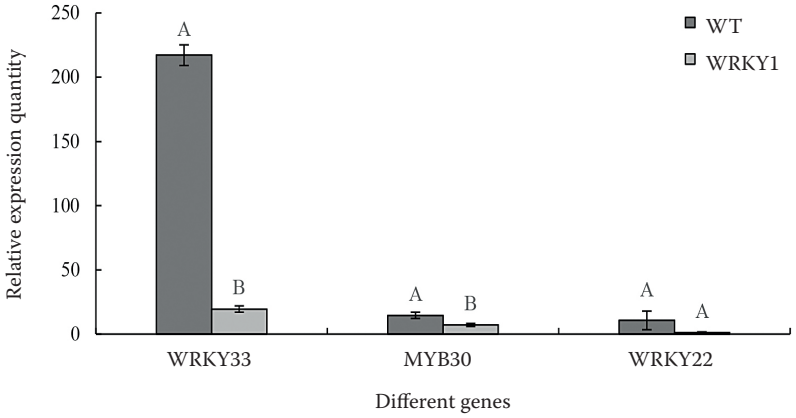

Figure 8. Relative expression levels of WRKY22, WRKY33 and MYB30 in overexpressed *Arabidopsis* and wild type *Arabidopsis*.
WT – wild type *Arabidopsis*; WRKY1 – transgenic *Arabidopsis*; different capital letters represent extreme significance ($P < 0.01$)

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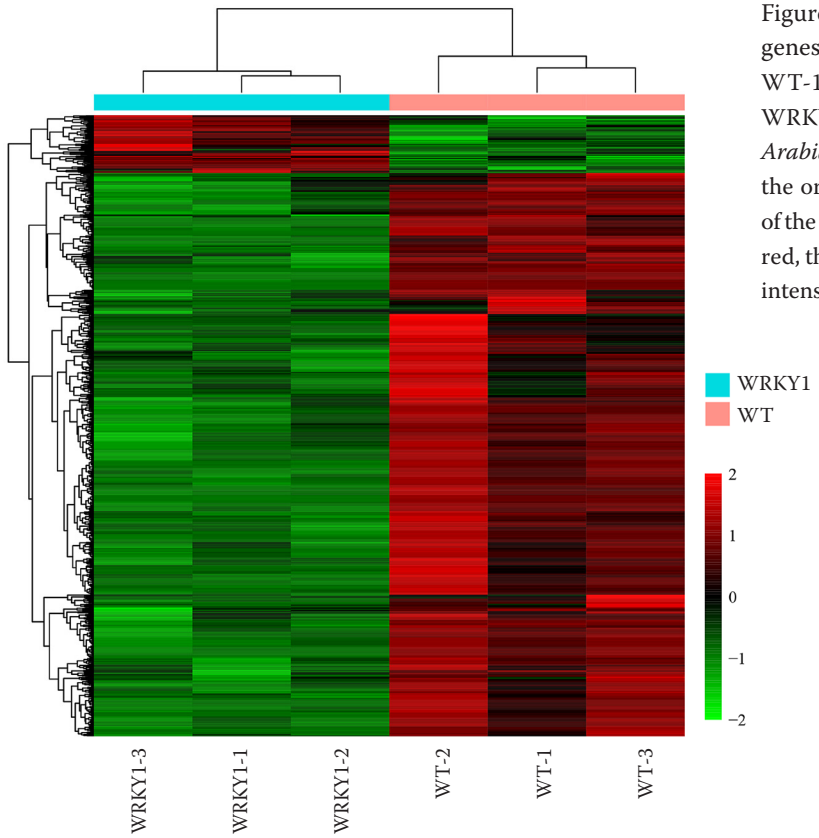

Figure 9. Heatmap of differentially expressed genes (DEGs)
WT-1, WT-2, WT-3 – wild type *Arabidopsis*; WRKY1-1, WRKY1-2, WRKY1-3 – transgenic *Arabidopsis*; the abscissa is the sample name, and the ordinate is the value after the normalization of the FPKM differential gene; the more intense the red, the higher the expression level, and the more intense the green, the lower the expression level

tion network, a total of six resistance genes were found. They were SYP121 (AT3G11820.1), NAC062 (AT3G49530.1), AT1G78410, XLG2 (AT4G34390.1), PAD3 (AT3G26830.1), and APK2A (AT1G14370.1), respectively, in which there were three transcription factors, namely MYB51 (AT1G18570.1), SCL13

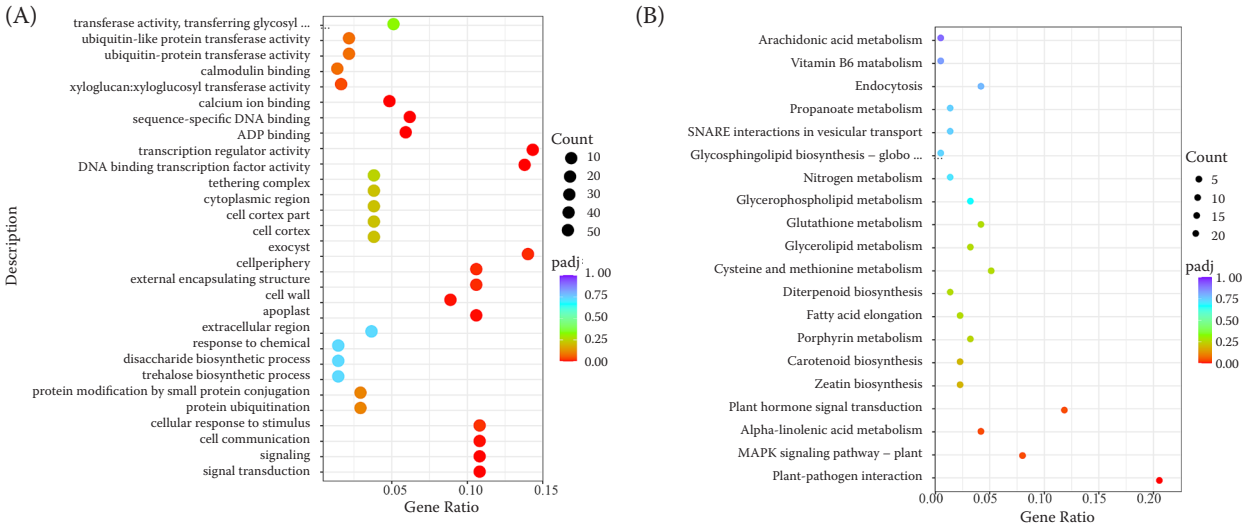

Figure 10. Gene ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analysis of 824 differentially expressed genes (DEGs): GO enrichment bubble graph; the *Y* axis is the GO term, and the *X* axis is the enrichment factor (A), KEGG enrichment bubble graph; the *Y* axis is the enrichment pathway and the *X* axis is the enrichment factors (B) The bubble colour represents the *P*-value, the redder the colour is, the smaller the *P*-value is, the higher the enrichment degree is, and the size of the bubble indicates the quantity

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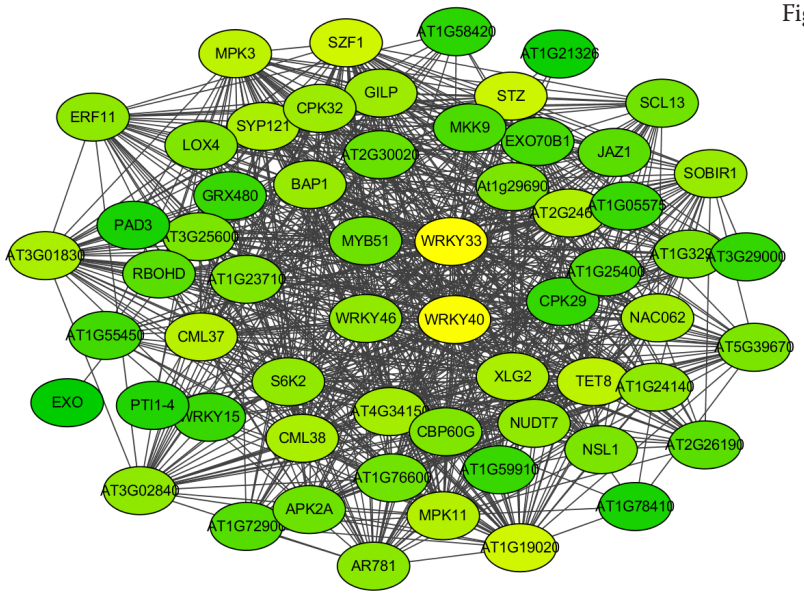

Figure 11. Prediction of protein interactions

(AT4G17230.1) and ERF11 (AT1G28370/1), and four WRKY protein (WRKY15, WRKY33, WRKY40, and WRKY46), and MYB51 interacted strongly with the WRKY transcription factors.

DISCUSSION

Studies have shown that WRKY proteins belong to the plant TF family and are involved in regulating plant stress resistance (Bahrini et al. 2011). The WRKY domain consists of 60 highly conserved amino acids, and its N-terminal is a highly conserved heptapeptide sequence WRKYGQK, which is the reason for the name WRKY. The C-end is the conservative zinc-finger structure of C2H2 (CX4-5CX22-23HX1H) or C2HC (C-X7-C-X23-HX1-C) (Pecchioni et al. 1996; Rushton et al. 2010). The specific recognition site of WRKY transcription factors is the W-box(T)(T) TGAC(C/T), the core sequence of which is TGAC, which is also the minimum recognition site required for specific DNA binding (Rushton et al. 2012). The *HvWRKY1* gene isolated in this study had a typical WRKY domain (amino acids 273–351) and was located in the nucleus and cell membrane, conforming to the characteristics of transcription factors, so this gene belongs to the WRKY gene family. The conserved region of WRKY transcription factor binds to the upstream promoter region of the gene to regulate the expression of downstream genes, enhancing or inhibiting biological and abiotic stress responses (Eulgem et al. 2000). Therefore, the conserved WRKY domain may play an important role in regulating the expression of functional genes. In this study, homology analysis was conducted between the highland barley *HvWRKY1* domain and seven other gramineous plants, and the consistency of the domain was 76.68%. Therefore, it is speculated that the WRKY domain of *HvWRKY1* plays an important role in the resistance of Qingke to leaf stripe disease.

Promoters can regulate DNA sequences to guide RNA polymerase to initiate gene transcription (Hoskins et al. 2011), while the epigenetic characteristics of regulatory sequences can enable different transcription factors and regulatory proteins to be aggregated into *cis*-element patterns (CRM: *cis*-regulatory module), thus determining their different functions (van Leeuwen et al. 2005). In addition, transcription factors can bind to *cis*-acting elements in the promoter regions of eukaryotic genes (Ulmasov et al. 1997). Therefore, identification and analysis of *cis*-acting elements in the promoter region can further elucidate the regulatory mechanism of gene expression molecules. *Cis*-regulatory elements control a variety of biological processes, such as growth and development, hormonal responses, and abiotic and biological stresses. Zhu et al. (2016) demonstrated that *AtWRKY1* in the guard cells of *Arabidopsis* is a negative regulator in abscisic acid (ABA) signaling that is localized in the nucleus and can bind to the W-box domain in the promoter of *MYB2*, *ABCG40*, *DREB1A*, and *ABI5* to control their transcription in response to drought stress or ABA. Turck et al. (2004) used chromatin immunoprecipitation (ChIP) technology in cultured cells and show that parsley

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(*Petroselinum crispum*) WRKY1 protein binds to the W boxes of its native promoter as well as to that of *PcWRKY3* and the defense-related PR10-class marker gene *Pathogenesis-Related1-1* (*PcPR1-1*). Chen et al. (2013) that the expression of WRKY DNA-binding protein 8 (*WRKY8*) was down-regulated after infection of crucifer-infecting tobacco mosaic virus (TMV-cg) in *Arabidopsis*. However, WRKY8 regulates the expression of *ABI4* to participate in the defense response of TMV-cg under ABA treatment, and can mediate ABA signal transduction during the interaction between TMV-cg and *Arabidopsis*. In this study, through the analysis of the cis-element of HvWRKY1 promoter, it was found that there were two W-boxes in its own promoter, and the cis-acting element was the specific binding site of WRKY. In parsley, W-box elements were also bound in the promoter of *PcWRKY3* gene, so the results of this study were similar to the results. AtWRKY1 can bind to the W-box domain in the promoter of other genes. HvWRKY1 also includes seven ABRE elements, which are associated with ABA; There are 26 TATA elements that may have transcriptional activation roles. In conclusion, it is speculated that in this study, HvWRKY1 can also bind to its own W-box element and may be involved in controlling ABA transcription.

Transcriptional regulation is a key biological process that enables cells or organisms to respond to a variety of intracellular and extracellular signals during development and determines cell characteristics (Casamassimi & Ciccodicola 2019). Transcription factors are DNA-binding proteins that regulate gene expression by interacting with the pre-initiation complex of transcription and binding to cis-regulatory elements present in each gene promoter region in a sequence-specific manner (Manna et al. 2021). In this study, GO results showed that transcriptional regulatory activity (GO: 0140110) was significantly enriched in 52 genes, and DNA-binding transcription factor activity (GO: 0003700) was significantly enriched in 50 genes. Therefore, this gene may cause changes in transcriptional regulatory activity and DNA-binding transcription factor activity in *Arabidopsis*, and it is speculated that *HvWRKY1* may play a similar role in resistance to leaf stripe disease in Qingke. Frerigmann et al. (2015) showed that the transcription factors *MYB34*, *MYB51*, and *MYB122* are precursors of plants protectin that protect against infection by specific pathogens and regulate indole-3-acetaldoxime (IAOx). In *Botrytis cinerea*, Wang et al. (2022) found that *BcWRKY33A* directly activated the expression of *BcMYB51-3* and downstream IGS biosynthetic genes, thereby improving plant tolerance to *Botrytis cinerea* infection. In *Arabidopsis*, the role of *AtMYB44* in salicylic acid (SA) and jasmonic acid (JA) -mediated defense responses was realized through direct regulation of *WRKY70* expression (Shim & Choi 2013). Zhu et al. (2022) isolated a new R2R3 type MYB transcription factor *GhODO1* from cotton (*cotton plum*). After the gene was knocked out, cotton resistance to *Verticillium dahliae* was reduced. However, overexpression of this gene increased resistance to *Verticillium dahliae* in *Arabidopsis*. In addition, MYB and WRKY transcription factors were found to interact with each other in protein interaction prediction of transgenic *Arabidopsis*. In this study, according to KEGG results in the transcriptus, there were 22 resistance genes involved in the pathway of plant-pathogen interaction. PPI results using *Arabidopsis* as the interaction model showed that there was interaction between resistance proteins and WRKY proteins, and MYB51 had strong interaction with WRKY33, WRKY40 and WRKY46 proteins. It is speculated that WRKY transcription factor and MYB transcription factor interact with each other in Qingke, and jointly regulate the disease resistance pathway of Qingke.

WRKY transcription factors can directly regulate the expression of resistance genes or overregulate the interaction between the encoding proteins of resistance genes and other proteins to achieve a resistance response. Deslandes et al. (2003) found that binding of the W-box element of the promoter of the *RRS1-R* gene in *Arabidopsis* with the WRKY transcription factors could increase gene expression, thereby producing a resistance response to bacterial wilt. Huang et al. (2016) showed that six WRKY transcription factors (*SolyWRKY41 SolyWRKY42*, *SolyWRKY53*, *SolyWRKY54*, *SolyWRKY80*, and *SolyWRKY81*) in tomato could bind to the W-box in the promoters of other genes, thereby influencing the interaction between resistance proteins and MAPK proteins to positively or negatively regulated tomato yellow leaf curl disease. Zeng et al. (2009) overexpressed the *OsWRKY45-2* gene in rice and found that it could enhance disease resistance to *M. grisea*. In this study, qRT-PCR results showed that the expression of *HvWRKY1* gene in the resistant variety Kunlun 14 and the susceptible variety Z1141 was extremely significantly increased under leaf stripe disease stress, and the expression of *HVWRKY1* gene in the resist-

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ant variety Kunlun 14 was extremely significantly higher than the susceptible variety Z1141. At the same time, *HvWRKY1* gene overexpression analysis showed that the expression pattern of *HvWRKY1* gene was similar to that of qRT-PCR, and the expression level of *HvWRKY1* gene was higher than that of qRT-PCR. Similar to the results of Zeng et al. (2009), it is speculated that this gene plays a positive regulatory role in the anti-leaf stripe disease pathway of Qingke. Therefore, it is speculated that *HvWRKY1* gene had a similar action mechanism in Qingke against leaf stripe disease, but its specific action mechanism needs to be further study.

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