Knockdown of GmVQ58 encoding a VQ motif-containing protein enhances soybean resistance to the common cutworm (Spodoptera litura Fabricius)

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Abstract

Plants have evolved complex defense mechanisms to withstand insect attack. Identification of plant endogenous insect resistance genes is of great significance for understanding plant–herbivore interactions and improving crop insect resistance. Soybean (Glycine max (L.) Merr.) is an important crop that is often attacked by the common cutworm (CCW) (Spodoptera litura Fabricius). In this study, based on our transcriptomic data, the gene GmVQ58, encoding a FxxxVQxxTG (VQ) motif-containing protein, was cloned and characterized. This gene showed the highest expression in the leaves and roots and was up-regulated significantly after CCW attack. Constitutive expression of GmVQ58 rescued the susceptibility of an Arabidopsis mutant to CCW, and interference of GmVQ58 in soybean hairy roots enhanced the resistance to CCW. Furthermore, GmVQ58 was localized to the nucleus and physically interacted with the transcription factor GmWRKY32. The expression of two defense-related genes, GmNI:IFR and GmVSPβ, was up-regulated in GmVQ58-RNAi lines. Additionally, the promoter region of GmVQ58 was likely selected during domestication, resulting in different expression patterns in cultivated soybeans relative to wild soybeans. These results suggest that silencing GmVQ58 confers soybean resistance to CCW.

Keywords: Common cutworm, GmVQ58, GmWRKY32, resistance, soybean, VQ gene.

Introduction

Plants are constantly exposed to numerous abiotic and biotic stress factors during their life cycle, of which insect attack is a severe stress that reduces plant growth and development and agricultural output. Soybean (Glycine max (L.) Merr.) is one of the most important crops, providing humans with high-quality protein and abundant oils. This plant faces threats from various herbivorous insects of which the common cutworm (CCW) (Spodoptera litura Fabricius) is one of the major pests in southern China and southwestern Japan, feeding on soybean leaves, flowers, pods, and young stems (Cui et al., 1997; Komatsu et al., 2004). Under millions of years of selection pressure generated by insects, plants have evolved complex defense systems to
protect themselves from attack (Kessler and Baldwin, 2002; Erb and Reymond, 2019). Understanding the mechanism of insect resistance is important for breeding insect-resistant plants.

The rapid development of next-generation genome sequencing technologies, such as RNA sequencing (RNA-seq), has provided effective tools to explore the mechanisms of gene regulatory networks and identify relevant genes. For example, in rice a transcription factor was identified that coordinates internode elongation and photoperiodic signals, by analysing the transcriptome profiles of two varieties with different photoperiod sensitivities under short-day treatment (Gómez-Ariza et al., 2019). In tomato transcriptome analysis revealed potential mechanisms for inhibition of intumescence by ultraviolet radiation (Wu et al., 2017). In soybean, our group previously initiated a study on the defense responses of cultivated soybeans to CCW via RNA-seq (Wang et al., 2015d).

Through database analysis, GmVSPβ (encoding a vegetative storage protein), GmN:IFR (encoding an NADPH-isoflavone reductase) and GmWRKY32 (encoding a WRKY-type transcription factor) showed differential expression patterns between CCW-resistant and CCW-susceptible soybean lines. GmVSPβ and GmN:IFR positively regulate plant resistance to CCW in tobacco (Wang et al., 2015d), and GmWRKY32 strongly activated the transcription of these genes in Arabidopsis protoplasts (Wang et al., 2015a). In addition, many other differentially expressed genes were detected in our transcriptome database; these genes were involved in several important cellular processes, including primary signaling related to defense, transcriptional regulation, and secondary metabolism. Among these genes, a VQ gene showed markedly higher expression levels in a CCW-susceptible soybean accession than in a CCW-resistant soybean accession.

VQ genes are known for their proteins’ containing a conserved FxxxVQxxTG motif (VQ motif) (Cheng et al., 2012). These genes are plant-specific and form multigene families with 34, 39, 18, and 74 members in Arabidopsis, rice, grape, and soybean, respectively (Cheng et al., 2012; Kim et al., 2013; Wang et al., 2014; Wang et al., 2015b). Most of these genes are heterogeneously distributed on chromosomes and do not contain introns. To date, functional analysis of VQ members has mainly focused on the model plant Arabidopsis. Arabidopsis AtVQ21, encoding one substrate of mitogen-activated protein kinase 4, functions as a positive regulator in plant defense against Pseudomonas syringae (Petersen et al., 2010). AtVQ12 is highly responsive to Botrytis cinerea infection and has functional redundancy with AtVQ29 in the control of plant basal resistance against B. cinerea (Wang et al., 2015a). AtVQ23 and AtVQ16, encoding two sigma factor binding proteins, were also reported to regulate plant defense against B. cinerea in a redundant manner (Lai et al., 2011). Loss-of-function of AtVQ22 (JAV1) leads to increased plant resistance against a variety of insects and pathogens, such as Spodoptera exigua, Bactria impatiens, aphids, and B. cinerea (Hu et al., 2013a). In soybean, two VQ genes, GmVQ35 and GmVQ47, were reported to participate in plant resistance against B. cinerea (Zhou et al., 2016). In addition to responding to biotic stresses, VQ genes play an important role in plant responses to abiotic stress. For instance, in Arabidopsis, overexpression of AtVQ9 causes high susceptibility of transgenic Arabidopsis to salt stress (Hu et al., 2013b). AtVQ15, which encodes a calmodulin-binding protein, is necessary for osmotic stress tolerance in plant seedlings (Perruc et al., 2004). In banana fruit, MaVQ5 was reported to be cold-responsive and may function as a repressor to antagonize MaWRKY26 in relation to the methyl jasmonate-mediated cold stress response (Ye et al., 2016). Moreover, some Arabidopsis VQ genes were reported to regulate plant growth and development. AtVQ14 is strongly expressed in early endosperm development, and only small seeds are produced in vq14 mutants, suggesting that AtVQ14 not only regulates endosperm development but also affects seed size (Wang et al., 2010). Loss-of-function of AtVQ8 leads to pale-green and stunted-growth phenotypes, and the overexpression of AtVQ17 and AtVQ18 suppresses plant growth and development (Cheng et al., 2012).

Although VQ genes have been well studied in Arabidopsis, few studies have reported the roles of soybean VQ genes, especially their function in insect resistance. In this study, we isolated and characterized a soybean VQ gene, GmVQ58. We analysed the genomic sequence of the gene and characterized its protein localization. The expression pattern of this gene in various tissues and leaves after CCW feeding was also investigated, and its protein interaction with GmWRKY32 was demonstrated in vivo. Two force-feeding trials were carried out to evaluate the resistance of GmVQ58 transgenic Arabidopsis and soybean hairy roots. Additionally, the promoter region of GmVQ58 was analysed for sequence diversity in wild soybeans, landraces and improved cultivars. Our work provides insight into the role and mechanism of GmVQ58 in plant defense against CCW, which may lay the foundations for breeding soybean for insect resistance.

**Materials and methods**

**Plant materials and common cutworm induction treatments**

The soybean cultivar Williams 82 was grown in a growth room with a 16 h–8 h day–night photoperiod, a daytime temperature of 25 °C and a night-time temperature of 24 °C. To identify the expression level of GmVQ58 in various tissues, leaves, stems, roots, flowers, pod shells, and seeds of soybean were sampled at different developmental stages: leaves, stems, and roots were sampled at the V4 stage; mature flowers were collected at the R2 stage; and pod shells and seeds were harvested 15 d after flowering. CCW induction treatments using soybean seedlings from the V4 stage were performed as reported (Fan et al., 2012). Leaves from both control and treatment plants were collected at 1, 6, 12, 24, 48, and 72 h after CCW attack for isolation of total RNA.

The Arabidopsis ecotype Columbia 0 (Col-0) plants and the jav1 mutants (SALK_146039C) used in this study were obtained from the Arabidopsis Biological Resource Center (ABRC, Columbus, OH, USA). The T-DNA was inserted into the promoter of the JAV1 gene, approximately 600 bp upstream of the ATG (translational start codon) (Ali et al., 2019). Arabidopsis plants were grown in a growth room with a 14 h day and 10 h night photoperiod, daytime temperature of 23 °C and night-time temperature of 22 °C. CCW induction treatments in 21-day-old transgenic Arabidopsis were carried out using a previously described protocol (Liu, 2015). Leaves from transgenic Arabidopsis were attacked by CCW and left for 1 h before histochemical β-glucuronidase (GUS) staining. Leaves from un-attacked transgenic Arabidopsis were used as controls.

**Analysis of differentially expressed soybean VQ genes**

Twenty-nine soybean VQ genes were present in our previously reported analysis of the transcriptome responses of two cultivated soybean lines to
CCW attack (Wang et al., 2015d). Four treatments were used in this experiment (treatment of the resistant soybean lines (R) at 5 d and susceptible soybean lines (SK) at 12 d and their corresponding controls (RCR) and (SCR)). The sequence data were downloaded from the NCBI database (https://www.ncbi.nlm.nih.gov/sra/SRR049638). We filtered the raw reads, assembled the transcriptome, blasted the reference database, and quantified the gene expression (reads per kilobase per million mapped reads (RPKM)) as described by Wang et al. (2015d). Differentially expressed GmVQs were identified by a statistical comparison using the rigorous algorithm method (Audic and Claverie, 1997). The screening conditions were set as follows: false discovery rate ≤0.001 and an absolute value of log2 fold change between susceptible and resistant lines ≥1.5. A heat map based on gene expression was generated by the R software package ‘heatmap’ (https://cran.r-project.org/web/packages/heatmap3/index.html).

Another transcriptomic dataset for wild soybeans responding to CCW attack was also used in this study (Du et al., 2019). The resistant lines were sampled at 1 d (RK-1d, RCR-1d) and 2 d (RK-2d, RCR-2d) after induction, while the susceptible lines were collected at 1 d (SK-1d, SCK-1d) and 3 d (SK-3d, SCK-3d) after induction. The sequence data were also downloaded from the NCBI database (https://www.ncbi.nlm.nih.gov/sra/PRJNA493962). The filtration of raw reads, alignment to the reference genome and quantification of gene expression were performed as reported by Du et al. (2019). Differential gene expression was determined using the DESeq R package (Anders and Huber, 2010). The screening standards for differentially expressed VQ genes in wild soybeans were consistent with those in cultivated soybeans.

The analysis of the two transcriptomes was conducted in cooperation with Beijing Genomics Institute (BGI), China.

Cloning of the GmVQ58 gene

The full-length cDNA of GmVQ58 (Glyma.14g002800) was PCR-amplified from the leaf cDNA of soybean cultivar Williams 82 (primers shown in see Supplementary Table S1 at JXB online). The PCR products were gel-purified (Axygen, USA) and sequenced by Thermo Fisher Scientific (Shanghai, China).

Sequence and phylogenetic analysis

For the names of the soybean and Arabidopsis VQ genes, refer to Wang et al. (2014) and Cheng et al. (2012); the protein sequences were obtained from Phytozone (https://phytozone.jgi.doe.gov/pz/portal.html). Putative Physcomitrella patens VQ protein sequences were obtained using the VQ motif as a keyword to search the Physcomitrella patens database in Phytozone. The sequence alignment was analysed using ClustalX software version 1.83 (Jeanmougin et al., 1998). A neighbor-joining (NJ) phylogenetic tree was constructed based on protein sequences with MEGA 6.0 software using the following parameters: bootstrap (1000 replications), p-distance, uniform rates, and pairwise deletion (Tamura et al., 2013). The best Arabidopsis hit for the GmVQ58 protein was determined by BLASTP analysis against the Arabidopsis proteins (http://www.arabidopsis.org/). GSDS (http://gsds.cbi.pku.edu.cn) was used to analyse the gene structure.

Gene expression analysis

Total RNA was isolated from Arabidopsis and soybean using the RNAsimple Total RNA Kit (Tiangen, Beijing, China). First-strand cDNA was reverse-transcribed with the PrimeScript™ 1st Strand cDNA Synthesis Kit (TaKaRa, Dalian, China). Quantitative real-time polymerase chain reaction (qRT-PCR) was used for gene expression analysis using an ABI 7500 system (Applied Biosystems, Carlsbad, CA, USA) with Aceq qPCR SYBR Green Master Mix (Vazyme Biotech Co., Nanjing, China), and qRT-PCR data were analysed using the 2−ΔΔCt method (Livak and Schmittgen, 2001). Three biological and three technical replicates were used in these experiments. The soybean tubulin gene (Glyma.03g124400) and Arabidopsis tubulin gene (At5g52690) were used as internal controls to normalize the samples. All specific primers used for the gene analysis are listed in Supplementary Tables S1, S2. Student’s two-tailed t-test was used for statistical analysis.

Histochemical β-glucuronidase staining

Genomic DNA was extracted from leaves of the soybean cultivar Williams 82 with a DNAsecure Plant Kit (TianGen, Beijing, China). The 2184-bp region upstream of the ATG of GmVQ58 was cloned from the leaf DNA using special primers (shown in Supplementary Table S1) and subsequently inserted into the pCAMBIA1381 vector. Then, the recombinant plasmid GmVQ58-GUS was introduced into Arabidopsis via the floral dip method (Clough and Bent, 1998). GUS staining in T1 transgenic plants was performed using a previously described protocol (Chao et al., 2014).

Subcellular localization

The ORF of GmVQ58 without a stop codon was cloned into the pFGCS5941 vector with the green fluorescent protein (GFP) gene downstream of the CaMV 35S promoter. Then, the recombinant vector 35S;GmVQ58-GFP and control (empty vector 35S;GFP) were transformed into tobacco (Nicotiana benthamiana) leaves for transient expression. The GFP signal was monitored by confocal laser scanning microscopy (Leica TCS SP2, Mannheim, Germany).

Construction of the GmVQ58 overexpression vector for Arabidopsis transformation

To construct the pMDC83–GmVQ58 overexpression vector, the coding sequence (CDS) of GmVQ58 was cloned into the pMDC83 vector by Gateway™ technology (Thermo Fisher Scientific, Shanghai). Then, the recombinant vector driven by the double CaMV 35S promoter was introduced into Agrobacterium tumefaciens strain EHA105 and subsequently transformed into Arabidopsis using the floral dip method (Clough and Bent, 1998). Since the pMDC83 vector contained the hygromycin B gene (HygB), the transgenic Arabidopsis plants were screened on Murashige and Skoog medium containing 40 μg ml−1 HygB. The HygB-resistant plants were then transferred to soil for further molecular identification at both the genomic and the transcriptional level. Three homozygous T3 transgenic lines were chosen for phenotypic analysis and evaluation of resistance to CCW. The primers used in this experiment are listed in Supplementary Table S2.

Overexpression and suppression of GmVQ58 in soybean hairy roots

The pMDC83–GmVQ58 overexpression vector was also used in this experiment. Considering that the length of the GmVQ58 CDS was less than 500 bp, to meet the length requirement of interference fragments, the full CDS of GmVQ58 was cloned into the vector pB7GTWGW2(II) to generate the pBI–GmVQ58-RNAi vector. Then, pMDC83–GmVQ58, pBI–GmVQ58-RNAi, and their separate empty vectors were transformed into Agrobacterium rhizogenes strain K599. The soybean cultivar ‘Jack’ was used as a hairy root transgenic acceptor for its high efficiency in transformation. Four different transformed Agrobacterium rhizogenes strains were separately inoculated into Jack cotyledons as described previously (Du et al., 2016). The cotyledons were grown on white medium containing 500 μg ml−1 carbenicillin disodium and 50 μg ml−1 cefotaxime disodium at 25 °C in the dark. After 25 d, transgenic hairy roots were formed from the abdomen of inoculated cotyledons and confirmed by PCR and qRT-PCR analysis (primers shown in Supplementary Table S1).

Force-feeding trial with common cutworm

Third-instar CCW larvae of a similar size were selected and starved for 12 h before participating in the force-feeding trial. In one feeding experiment, 15 mature rosette leaves from 28–day-old Arabidopsis plants for
 Yeast two-hybrid assay

A yeast two-hybrid (Y2H) assay was performed using the Matchmaker™ Gold Yeast Two-Hybrid System (Clontech, USA). The CDS of GmVQ58 was cloned into the vector pGBK7 (BD) to produce the construct BD-GmVQ58. Additionally, the CDS of GmWRKY32 (Glyma.02g115200) was PCR-amplified from the leaf cDNA of the soybean cultivar Williams 82 at 1 d after CCW attack and was subcloned into the vector pGADT7 to produce the construct AD-GmWRKY32 (primers shown in Supplementary Table S1). The transformed yeast cells were first grown on plates containing double-dropout media (−Leu/−Trp) and then transferred to higher-stringency plates containing quadruple-dropout media (−Leu/−Trp/−His/−Ade). Combinations of pGBK7-T7 (BD-53) and pGADT7-T (AD-T) were used as positive controls, while the negative controls were pGBK7-lam (BD-lam) and AD-T. Plates used for the identification of protein interactions were supplemented with X-α-Gal to visualize the interactions.

Bimolecular fluorescence complementation assay

The CDSs of GmVQ58 and GmWRKY32 were cloned into the vectors SPYNE173 and 35S-SPYCE (M) to produce the constructs GmVQ58-YFPN and GmWRKY32-YFP C, respectively, using the primers shown in Supplementary Table S1. Then, the constructs GmVQ58-YFPN and GmWRKY32-YFP C were co-transformed into tobacco (N. benthamiana) leaves for transient expression. The yellow fluorescent protein (YFP) signal was monitored by confocal laser scanning microscopy (Leica TCS SP2, Mannheim, Germany).

Promoter diversity analysis

Promoter diversity analysis was conducted by using previously published whole-genome sequencing data of 302 soybean accessions, which included 62 wild soybeans, 130 landraces and 110 improved cultivars (Zhou et al., 2015). The single-nucleotide polymorphism (SNP) data were downloaded online (https://figshare.com/articles/Soybean_resequencing_project/11761333) and then imported into the program VCFtools (Danecek et al., 2011). We used the gene name of GmVQ58 as a keyword to search Phytozome (https://phytozome.jgi.doe.gov/pz/portal.html) and obtained the gene’s physical location in the soybean reference genome (Glycine max Wm82.a1.v1) (Schmutz et al., 2010). According to the physical location, the SNP information for the 2184-bp region upstream of the ATG of GmVQ58 was obtained by VCFtools. In VCFtools, the nucleotide diversity (π) and Tajima’s D were calculated on the basis of the SNP information within the GmVQ58 promoter between accessions in each subpopulation; π represents the average number of nucleotide differences per site between two sequences (Choudhury et al., 2014). In addition, Tajima’s D test was used to evaluate the neutrality of the polymorphisms (Tajima, 1989). The visualization of aligned SNPs within the GmVQ58 promoter in 302 soybean accessions was conducted by GeneDoc software (Aguilar-Martínez et al., 2007).

The raw sequencing data containing the 2184-bp promoter region of GmVQ58 in 302 accessions (https://www.ncbi.nlm.nih.gov/sra/?term=SRP045129) were mapped to the reference genome as reported by Zhou et al. (2015) and then converted to BAM format via SAMtools software (Li et al., 2009). The BAM files were used to acquire the 2184-bp region sequence in each accession by IGTools software (Robinson et al., 2011). The relative conservation value of the GmVQ58 promoter sequences among the accessions was calculated by DnaSP 6 software (Rozas et al., 2017).

Results

Identification and phylogenetic tree analysis of differentially expressed GmVQs in the RNA-seq database

Our previous work identified the peak CCW-induced resistance time of the two cultivated soybean lines, and transcriptional analysis was carried out on samples taken during the peak resistance period of the resistant lines (5 d) and the susceptible lines (1 d), with and without CCW induction (Wang et al., 2015a). A total of 29 GmVQ genes were present in the RNA-seq database (Fig. 1A; Supplementary Table S3). After CCW induction treatment, none of the genes was detected with significant differential expression levels in the resistant lines, and only two genes (GmVQ63 and GmVQ73) were up-regulated in the susceptible lines. However, both of these genes were expressed at a very low level in all treatments (RPKM values ≤15). We then investigated the differentially expressed genes between the non-induced treatments of the resistant and susceptible lines and found that GmVQ31, GmVQ56, GmVQ58, and GmVQ69 showed differential expression levels. GmVQ31 transcription was also very low in all treatments (RPKM values ≤15), whereas GmVQ56, GmVQ58, and GmVQ69 showed markedly higher expression levels in the susceptible lines than in the resistant lines, and their absolute values of log2 ratios were 1.5627, 1.9546, and 1.5662, respectively.

Furthermore, a NJ phylogenetic tree was generated using the protein sequences of all VQ genes from soybean, Arabidopsis, and the basal plant Physcomitrella patens (Fig. 1B). GmVQ35 and AtVQ21 were clustered together, and GmVQ47 was grouped with AtVQ12 and AtVQ29. GmVQ35, GmVQ47, AtVQ12, AtVQ21, and AtVQ29 are all disease resistance proteins (Petersen et al., 2010; Wang et al., 2015a; Zhou et al., 2016). GmVQ58 was grouped with JAV1 (best Arabidopsis hit). JAV1 functions as a negative regulator of plant defense against both insects and pathogens (Hu et al., 2013a). However, the relationship of JAV1 and GmVQ56 with GmVQ69 was distinct according to the phylogenetic analysis. Since the GmVQ58 transcript abundance differed strongly between the resistant and susceptible lines, and its protein was clustered with Arabidopsis AtJAV1, GmVQ58 was selected for further functional study.

Sequence analysis of GmVQ58

GmVQ58 is located on chromosome 14 in the soybean genome. The full-length genomic sequence of GmVQ58 is 1185 bp, including a 486-bp coding region, a 223-bp 5′-untranslated region (UTR), and a 476-bp 3′-UTR. The GmVQ58 ORF encodes a polypeptide of 161 amino acids with a molecular mass of 17.49 kDa and an isoelectric point of 10.10. Since the majority of VQ genes in Arabidopsis, soybean, rice, grape, etc., are intronless
(Cheng et al., 2012; Kim et al., 2013; Wang et al., 2015b; Zhou et al., 2016), we compared the exon–intron structures of three differentially expressed soybean VQ genes (GmVQ56, GmVQ58, and GmVQ69) and eight Arabidopsis or soybean VQ genes reported to be associated with biotic stress responses (AtVQ12, AtVQ16, AtVQ21, JAV1, AtVQ23, AtVQ29, GmVQ35, and GmVQ47) (Petersen et al., 2010; Lai et al., 2011; Hu et al., 2013a; Wang et al., 2015a; Zhou et al., 2016). With the exception of GmVQ56, these 11 genes all had no introns (Fig. 1C).

GmVQ58 was highly expressed in leaves and roots and induced by CCW attack.

We investigated the expression levels of GmVQ58 in different soybean tissues using qRT-PCR. The detection of GmVQ58 transcripts was highest in leaves and roots (Fig. 2A). To further visualize the expression pattern of GmVQ58, we generated transgenic Arabidopsis expressing the GUS reporter gene controlled by the GmVQ58 promoter. In both 7-day-old seedlings and 5-week-old plants, the GUS reporter gene was highly expressed in leaves and roots, indicating that the soybean GmVQ58 promoter displays a similar expression pattern in transgenic Arabidopsis (Fig. 2B).

Moreover, we examined GmVQ58 transcripts at multiple time points after CCW induction by qRT-PCR. As shown in Fig. 3A, the expression of GmVQ58 was strongly up-regulated after CCW feeding, peaked at 48 h, and then decreased sharply at 72 h in leaves. These results were also demonstrated by GUS staining, as GUS activities were significantly increased in the leaves of 21-day-old GmVQ58pro::GUS transgenic Arabidopsis.
GmVQ58 is involved in soybean insect resistance

at 1 h after CCW attack (Fig. 3B). Taken together, these results suggested that GmVQ58 responds to CCW attack and may be involved in plant resistance against CCW.

GmVQ58 localized in the nucleus

To investigate the subcellular localization of GmVQ58 in vivo, the CDS of GmVQ58 fused to GFP was driven by the constitutive CaMV 35S promoter. Then, the construct 35S:GmVQ58-GFP was transiently expressed in the leaves of tobacco (N. benthamiana). GFP signals showed that the GmVQ58-GFP fusion protein was only localized in the nuclei of tobacco cells, whereas the empty vector control (35S:GFP) was distributed throughout the cell (Fig. 4).

GmVQ58 overexpression suppresses resistance to common cutworm in transgenic Arabidopsis

To identify the role of GmVQ58 in plant resistance to CCW, we ectopically expressed GmVQ58 in the jav1 mutants. AtJAV1, the homologous gene of GmVQ58 in Arabidopsis, functions as a negative regulator in defense against various biotic stresses (Hu et al., 2013a). After PCR and qRT-PCR examination, five T1 generation GmVQ58-OE jav1 transgenic lines were obtained, and three independent transgenic lines (line 1, line 2, and line 3) with relatively high expression of GmVQ58 were selected for further phenotypic analysis (see Supplementary Fig. S1). The jav1 mutants and T3 generation GmVQ58-OE jav1 transgenic lines were also examined using PCR (Supplementary Fig. S2) and qRT-PCR. Arabidopsis ecotype Col-0 plants were used as controls. No unique morphological phenotype was observed in the seedlings of Col-0 plants, jav1 mutants, or three independent GmVQ58-OE jav1 transgenic lines (Fig. 5A), indicating that GmVQ58 may not play an important role in seedling growth and development. Since the T-DNA was inserted into the JAV1 gene promoter (Ali et al., 2019), the jav1 mutant was not a null mutant and displayed a significantly decreased expression of JAV1 (Fig. 5B). Additionally, GmVQ58 was expressed in three GmVQ58-OE jav1 transgenic lines (Fig. 5C).

In the force-feeding trial, 24 Arabidopsis seedlings for each line were used to evaluate insect resistance. The rosette leaves from 28-day-old Col-0 plants, jav1 mutants, and three GmVQ58-OE jav1 transgenic lines were separately fed to CCW third-instar larvae. After feeding for 4 d, the CCW larvae feeding on the jav1 mutants devoured a few leaves and grew slowly, whereas Col-0 plants and GmVQ58-OE jav1 transgenic lines promoted larval growth (Fig. 6A, C). Moreover, the RGRs of CCWs feeding on Col-0 plants or the GmVQ58-OE jav1 transgenic lines were significantly
higher than those of larvae reared on the jav1 mutants (Fig. 6B). No significant difference in resistance to CCW was observed between Col-0 plants and the jav1 mutants. Taken together, these results demonstrated that overexpression of GmVQ58 rescues the susceptibility of the jav1 mutants to CCW.

Soybean hairy root transformation confirms the role of GmVQ58 in resistance against CCW

Ectopic expression of GmVQ58 complemented the jav1 mutant to restore the Col-0 phenotype. The resistance of complemented Arabidopsis to CCW was similar to that of the Col-0
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plants (Fig. 6). Then, we evaluated the function of GmVQ58 in the soybean system. We generated two constructs, pMDC83-GmVQ58 and pBI-GmVQ58-RNAi, for overexpression (GmVQ58-OE) and suppression (GmVQ58-RNAi) of GmVQ58, respectively. Two constructs with their empty vectors (Control-OE and Control-RNAi) were transformed into soybean hairy roots. In this experiment, we obtained six dishes of transgenic hairy roots for each genotype (Fig. 7A), which were subsequently examined using PCR (Supplementary Fig. S3) and qRT-PCR. Expression analysis showed that the expression level of GmVQ58 in GmVQ58-OE hairy roots was increased by more than 14-fold over that in the Control-OE hairy roots, while the expression level of GmVQ58 in GmVQ58-RNAi hairy roots was 43% lower than that in the Control-RNAi hairy roots (Fig. 7B).

Furthermore, a 6-d force-feeding trial was performed using transgenic hairy roots. In this experiment, there was no significant difference in resistance to CCW between the GmVQ58-OE transgenic hairy roots and the Control-OE roots, although the RGRs of CCWs feeding on the GmVQ58-OE transgenic hairy roots were slightly higher than those of CCWs feeding on the Control-OE hairy roots (Fig. 8A, B). However, after feeding for 4 d, the CCW larvae feeding on the GmVQ58-RNAi transgenic hairy roots grew more slowly than those feeding on the Control-RNAi hairy roots (Fig. 8C). We compared their RGRs and found that when fed with GmVQ58-RNAi hairy roots, the RGRs of CCWs were significantly lower than those of CCWs feeding on the Control-RNAi hairy roots (Fig. 8D). Taken together, these results suggested that silencing GmVQ58 elevates soybean resistance to CCW.

GmVQ58 interacts with GmWRKY32

GmN:IFR (Glyma.01g172600) and GmVSPβ (Glyma.09g200100), two CCW-responsive genes, positively regulate plant resistance to CCW in tobacco (Wang et al., 2015d). Our previous studies showed that GmWRKY32 (Glyma.02g115200) could strongly activate the transcription of GmN:IFR (8.5-fold) and GmVSPβ (4.8-fold) in Arabidopsis protoplasts (Wang et al., 2015c). Additionally, several members of the VQ family have been reported to physically interact with WRKY transcription factors to participate in plant defense reactions (Cheng et al., 2012). We then examined the interaction between GmVQ58 and GmWRKY32 using the Y2H system. GmVQ58 was ligated with the GAL4 DNA-binding domain (BD-GmVQ58), and GmWRKY32 was fused to the GAL4 activation domain (AD-GmWRKY32). As shown in Fig. 9A, coexpression of BD-GmVQ58 with AD-GmWRKY32 caused strong activation of α-galactosidase activity, suggesting that GmVQ58 interacts with GmWRKY32 in yeast. The interaction between GmVQ58 and GmWRKY32 in plant cells was subsequently confirmed by bimolecular fluorescence complementation assay. GmVQ58 and GmWRKY32 were fused to the N-terminal and C-terminal fragments of YFP, respectively. YFP fluorescence was observed in the nuclei of tobacco cells co-transformed with the GmVQ58-YFPβ and GmWRKY32-YFPα constructs, whereas no YFP fluorescence

Fig. 6. Overexpression of GmVQ58 in the jav1 mutants attenuated plant resistance to CCW. (A) Representative rosette leaves of Col-0 plants, the jav1 mutants and three GmVQ58-OE jav1 transgenic lines after 24 h of CCW feeding without (CK) or with (CCW) CCW exposure. Scale bar: 4 mm. (B) Relative growth rates of CCW larvae feeding on leaves from Col-0 plants, jav1 mutants, or three GmVQ58-OE jav1 transgenic lines after 2, 3, and 4 d of feeding. Two-tailed t-tests were used for statistical analysis. n=3. **P<0.01. Error bars denote ±SE. (C) Representative CCW larvae taken from rosette leaves of Col-0 plants, the jav1 mutants, and three GmVQ58-OE jav1 transgenic lines at days 0 and 4. Scale bar: 1 cm. 1#, 2#, and 3# represent three independent GmVQ58-OE jav1 transgenic lines.
was detected in the controls (Fig. 9B). All of these results indicated that GmVQ58 physically interacts with GmWRKY32.

Furthermore, we examined the transcript levels of two GmWRKY32 downstream genes (GmN:IFR and GmVSPβ) in GmVQ58 transgenic soybean hairy roots by qRT-PCR (Fig. 9C, D). No significant change in their expression was detected between GmVQ58-OE roots and Control-OE roots. However, the expression levels of GmN:IFR and GmVSPβ were higher in the GmVQ58-RNAi roots than in the Control-RNAi roots, indicating that the interference of GmVQ58 increased the expression of GmN:IFR and GmVSPβ in soybean. Additionally, up-regulated expression of the two genes in GmVQ58-RNAi lines suggested that disruption of GmVQ58 enhances soybean resistance to CCW.

**GmVQ58 underwent selection during domestication**

Insect resistance is a key domestication trait for artificial selection, and related regulatory genes may have been selected during soybean domestication (Zhou et al., 2015). Thus, the expression patterns of GmVQ58 in our previous RNA-seq data for cultivated
soybeans and wild soybeans were compared and are listed in Supplementary Tables S3, S4. Unlike the expression of GmVQ58 in cultivated soybeans, there was no significant difference in the expression level of its homologous gene Glysoja.14g037272 between the resistant and susceptible wild soybean lines under non-induced treatments, although Glysoja.14g037272 was up-regulated at 3 d after CCW attack in resistant wild soybean lines (Du et al., 2019).

Variation in promoter regions may affect gene expression patterns. GUS staining showed that the 2184-bp region upstream of the ATG of GmVQ58 had promoter activity (Figs 2B, 3B). Eight SNPs were detected in the 2184-bp upstream region of GmVQ58 in 302 soybean accessions, including 62 wild soybeans, 130 landraces, and 110 cultivars (Supplementary Fig. S4), and the relative conservation value of the GmVQ58 promoter sequences among accessions was 94% (Supplementary Table S5; Zhou et al., 2015). By detecting the sequence diversity within the GmVQ58 promoter between accessions in each subpopulation, we found that the nucleotide variation (π) in wild soybeans was 0.000967, whereas the π values of the landraces (0.000591) and cultivars (0.000608) were relatively low (Table 1), suggesting that wild soybeans had higher promoter diversity than did the others. Moreover, Tajima’s D values of the wild soybeans, landraces, and cultivars were 1.594, 0.367, and 0.002, respectively, implying that there were more rare alleles in the cultivated soybeans than in the wild ones. Taken together, these results suggested that the GmVQ58 promoter might have been selected during soybean domestication.

Discussion
The discovery of plant endogenous insect resistance genes is undeniably of great significance for understanding the co-evolution of plants and pests and improving crop resistance to insects. Transcriptome sequencing has been successfully used for the identification of new plant resistance genes (Huang et al., 2015; Hickman et al., 2017). In this study, a new VQ motif-containing gene, GmVQ58, was analysed via various molecular and genetic approaches.

GmVQ58 is highly responsive to CCW attack, and silencing the gene elevates soybean defense against CCW
VQ genes play critical roles in plant defense against biotic stresses. Here, we analysed a soybean VQ gene, GmVQ58. The gene was highly responsive to CCW attack (Fig. 3).
Constitutive expression of the gene rescued the susceptibility of the jay1 mutants to CCW in Arabidopsis (Fig. 6). Overexpression of the gene did not significantly affect soybean resistance to CCW, whereas resistance was significantly enhanced in GmVQ58-RNAi lines (Fig. 8). Taken together, these results suggested that GmVQ58 plays a role in plant defense against CCW.

VQ genes belong to a polygenic family. Most VQ genes participate in plant pathogen resistance, such as Arabidopsis AtVQ12, AtVQ16, AtVQ21, AtVQ23, and AtVQ29 (Petersen et al., 2010; Lai et al., 2011; Wang et al., 2015a) and 13 rice VQ genes (Li et al., 2014). Seventy-four genes of the soybean VQ gene family have been predicted (Wang et al., 2014). Similar to their homologs in other plants, GmVQ35 and GmVQ47 regulate plant resistance against B. cinerea in transgenic Arabidopsis (Zhou et al., 2016). In this study, the two genes were not present in soybean transcriptomic data (Fig. 1A; Supplementary Table S3), and their proteins were respectively grouped with different Arabidopsis VQ proteins related to resistance against pathogens (Fig. 1B). However, GmVQ58 as well as GmVQ52 and GmVQ73 was classified with AtVQ22 (JAV1) (Fig. 1B). GmVQ52 did not show differential transcript abundance between resistant and susceptible soybean lines, and GmVQ73 displayed low expression in all treatments (Fig. 1A; Supplementary Table S3). These results also indicated that the function of GmVQ58 might be different from that of its homologs in soybean. Furthermore, in addition to playing a role in plant resistance against several insects, JAV1 negatively regulates plant resistance against pathogens (Hu et al., 2013a). Whether GmVQ58 has the same function as JAV1 needs to be studied in the future.

Interference of GmVQ58 enhances the expression of two resistance genes downstream of GmWRKY32

To date, several members of the VQ family have been reported to physically interact with a number of proteins, especially WRKYs. Many studies have shown that WRKYs play multiple roles in responses to biotic and abiotic stresses. In Arabidopsis, AtVQ23 and AtVQ16 function as regulators of the transcription factor AtWRKY33 and positively control plant resistance against B. cinerea (Lai et al., 2011). AtVQ21 was also found to interact with AtWRKY33 and may affect WRKY-regulated resistance gene expression (Andresson et al., 2005). In banana fruit, MaVQ5 physically interacted with MaWRKY26 to regulate its transactivation of jasmonic acid biosynthetic genes (Ye et al., 2016).

In our previous studies, GmN:IFR and GmVSPβ positively regulated plant defense against CCW in tobacco, and GmWRKY32 could strongly activate the transcription of GmN:IFR and GmVSPβ in Arabidopsis protoplasts (Wang et al., 2015c,d). A total of 176 WRKY genes have been identified in the soybean genome and can be divided into three groups, namely groups I, II, and III. The group II genes are further divided into five subgroups, namely Ia, Ib, Ic, IIa, and Ie (Song et al., 2016). GmWRKY32 is a member of soybean WRKY group Ic, and its Arabidopsis ortholog, AtWRKY57 (At1g63910), has been implicated in plant biotic stress (B. cinerea resistance) and abiotic stress (drought tolerance) (Jiang et al., 2016; Jiang and Yu, 2016). In this study, we showed that GmVQ58 physically interacted with GmWRKY32, and the expression of GmN:IFR and GmVSPβ was up-regulated in GmVQ58-RNAi lines (Fig. 9). Based on these results, we hypothesize that the interaction between GmVQ58 and GmWRKY32 might repress the transcriptional activation activity of GmWRKY32 on GmN:IFR and GmVSPβ. In addition, the GmVQ58 gene might function in soybean insect resistance in other ways. To better understand the insect resistance mechanism of the GmVQ58 gene in soybean, more studies need to be considered in the future, such as the construction of GmVQ58 overexpression and silent soybean lines.

The promoter diversity of GmVQ58 is relatively high in wild soybeans

We cloned the 2184-bp region upstream of the ATG of GmVQ58. The GmVQ58 promoter could activate the expression of the GUS gene in multiple tissues in transgenic Arabidopsis (Fig. 2B). After CCW attack, the GUS gene controlled by the GmVQ58 promoter was up-regulated in transgenic Arabidopsis (Fig. 3B). These results indicated that the GmVQ58 promoter has transcriptional activity. In addition, the expression patterns of GmVQ58 in cultivated soybeans and wild soybeans were different (Supplementary Tables S3, S4). Therefore, sequence diversity in the promoter of GmVQ58 was analysed in 302 soybean accessions (Table 1). In the population, wild soybeans had the highest variation, followed by landraces and improved cultivars, suggesting that the promoter region of GmVQ58 might have been selected during soybean domestication. Domestication is a complex process that includes both natural and artificial selection. The diversity of the resistance-related genes decreased in this process (Zhao et al., 2015). It is unknown whether beneficial GmVQ58 alleles were retained or lost in cultivated soybeans, and additional work is needed, such as the evaluation on CCW resistance of the 302 soybean accessions.

### Supplementary data

Supplementary data are available at JXB online.

### Table 1. Summary of the sequence diversity of GmVQ58 promoter in wild, landrace, and cultivated soybeans

| Sequence locus | Wild | Landrace | Cultivar |
|---------------|------|----------|----------|
| Promoter      | 0.000967 | 0.000591 | 0.000608 |
| Tajima’s D    | 1.594 | 0.367    | 0.002    |

The summary of the sequence diversity of GmVQ58 promoter in wild, landrace, and cultivated soybeans.
Fig. S1. Identification of T1 generation transgenic Arabidopsis plants by PCR and qRT-PCR.

Fig. S2. Identification of T3 generation transgenic Arabidopsis plants by PCR.

Fig. S3. Identification of transgenic soybean hairy roots by PCR.

Fig. S4. Polymorphisms detected within the promoter region of GmVQ58 in 302 soybean accessions.

Table S1. Primer pairs used in soybean.

Table S2. Primer pairs used in Arabidopsis.

Table S3. List of all VQ genes present in the RNA-seq database for cultivated soybeans and their expression patterns.

Table S4. List of all VQ genes present in the RNA-seq database for wild soybeans and their expression patterns.

Table S5. Sequence alignment results of the promoter region of GmVQ58 in 302 soybean accessions.

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Author contributions

HW and DY designed the research. XL and DH conducted the experiments. RQ, QD, and LC assisted with performing the experiments. HD, HY, JW, and FH analysed the data. XL wrote the manuscript. HW and DY revised the manuscript. All authors read and approved the final manuscript.

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