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GmERF54, an ERF Transcription Factor, Negatively Regulates the Resistance of Soybean to the Common Cutworm (*Spodoptera litura* Fabricius)

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Abstract: Soybean is attacked by various herbivorous insect pest species during the whole course of its life cycle in the field. It is important for soybean production to improve insect resistance by identifying and utilizing soybean endogenous insect-resistant genes. The ethylene-responsive transcription factor (ERF) plays a significant role in plant biotic and abiotic stresses; however, few studies focus on its role in insect resistance in soybean. Here, based on our previous common cutworm (CCW)-induced soybean transcriptome data, a soybean ERF gene *GmERF54* was cloned, which responded to CCW feeding. Transcriptional analysis revealed that *GmERF54* was ubiquitous in all soybean tissues and was expressed differently in insect-resistant and insect-susceptible soybean cultivars. RNA interference of *GmERF54* increased the resistance to CCW, while the overexpression of *GmERF54* decreased the resistance to CCW in transgenic soybean hairy roots compared with their controls. GmERF54 was localized to the nucleus, had transcriptional activation activity, and interacted with AP2/ERF GmPLT2. Several putative hormone response elements were predicted in the promoter sequence of *GmERF54*. Four putative elements were only found in the *GmERF54* promoter sequence of insect-resistant cultivar Wanxianbaidongdou (WX), but not in the insect-susceptible cultivar Nannong 99-10 (99-10). *GmERF54* promoter sequences of WX and 99-10 were cloned into the pCAMBIA1381z vector containing the β-glucuronidase (*GUS*) gene to generate $GmERF54_{WX}$*:GUS* and $GmERF54_{99\text{-}10}$*:GUS* recombinant vectors, respectively. GUS staining of soybean hairy roots containing $GmERF54_{WX}$*:GUS* and $GmERF54_{99\text{-}10}$*:GUS* showed that *GmERF54* was induced by CCW attack and both MeJA (methyl jasmonate) and IAA (indole-3-acetic acid) treatments. Alleles in insect-resistant and insect-sensitive cultivars responded to these inductions differently. Overall, our results reveal that *GmERF54* may be involved in the regulation of soybean resistance to CCW.

Keywords: soybean; common cutworm; *GmERF54*; soybean hairy roots; resistance

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1. Introduction

Soybean (*Glycine max* (L.) Merr.) is an important crop that provides protein and oil for humans and other animals. The yield and quality of soybean are affected by almost all kinds of herbivorous insect pest species [1,2]. In China, the annual loss of soybean due to insect pests is approximately 10–15% [3]. Soybean can even have no yield after severe outbreaks of herbivorous insect pests [4]. Multi-host common cutworm (CCW; *Spodoptera litura* Fabricius) is a major herbivore in low latitudes of China which eats almost all soybean tissues, including leaves, stems and young pods [5]. At present, pest control relies mainly on the use of chemical pesticides, but they are expensive and harmful to the environment [6]. Therefore, in order to manage soybean pests effectively, researchers are working, identifying and using insect resistance genes in soybean breeding.

Plants have evolved a precise and complex defense system, including trichomes, toxic compounds and regeneration, under the selective pressure of long-term leaf feeding behav-

ior by pests [7–9]. Plant trichomes prevent the insects from obtaining nutrients, damage the larval gut membranes, perceive pest attacks and initiate plant defense signals [10,11]. Toxic secondary metabolites in plants negatively affect the insect feeding and oviposition behavior, the enzyme activity of protease in the gut and metabolic homeostasis [12]. Plant regeneration and reproduction after herbivore attack are considered complementary strategies in the defense response [13–15]. Plant defense systems depend on the regulation of hormones, including ethylene [16–18]. APETALA2/ethylene-responsive factors (AP2/ERF) have an important role in the ethylene pathway and are also the subject of insect resistance studies. In herbivore-induced defense responses, the expression of *Pti5* is induced by potato aphid (*Macrosiphum euphorbiae*), and the ERF *Pti5* gene contributes to the resistance of tomato to the potato aphid [19]. In addition, the mutations in *JRE4* increase the sensitivity of tomato to the generalist herbivore *Spodoptera litura* [20].

In plants, the APETALA2/ethylene-responsive factor (AP2/ERF) superfamily is large and widespread [21], and plays a significant role in regulating stress response gene expression. Each *AP2/ERF* gene has at least one AP2/ERF domain, which consists of approximately 60 amino acids [22,23]. The AP2/ERF superfamily includes five subfamilies, DREB, ERF, AP2, RAV and Soloist, which are different in the characteristics and number of the conserved AP2/ERF domains [24,25]. Many studies have shown that the members of the AP2/ERF are vital regulators of multiple stages of plant growth and development, such as flower development [22,26], flower senescence [27], leaf epidermal cell identity [28], spikelet meristem determinacy [29–31], embryo development [32], growth of internode meristem [33], leaf size [34], root initiation and development [35–37] and grain development [38–40]. In addition, AP2/ERF TFs also respond to various biotic stresses [41]. The overexpression of *AtERF1* enhances *Arabidopsis* resistance to necrotrophic fungi by upregulating the expression of *PDF1.2*, a plant defense gene [42]. The overexpression of *AtERF5* and *BrERF11* genes increases plant resistance to bacteria and fungi, respectively [43,44]. Similarly, the constitutive expression of *Rap2.2*, an *ERF* gene, increases the resistance of transgenic *Arabidopsis thaliana* to gray fungus [45]. In soybean, 301 AP2/ERF members were identified [46]. Recently, the number of studies on the family in soybean has gradually increased [47,48]. The overexpression of *GmERF113* increases the expression of *PR1* and *PR10-1*, and improves the resistance of soybean to *Phytophthora sojae* infection [49]. In addition, the overexpression of the *GmAP2/ERF144* gene in soybean significantly improves the drought resistance of transgenic lines by increasing the relative water content and decreasing the malondialdehyde content and electrical conductivity of the transgenic soybean leaves under drought conditions [46]. Although the *AP2/ERF* genes have been studied well in many plants, few studies focus on the role of *AP2/ERF* genes in soybean insect resistance.

As a time-efficient and highly effective system, the hairy root system has been widely used for the preliminary identification of genes in soybean [50]. It has been reported that insect pest species, including CCW [51,52] and aphids [53], can feed on soybean hairy roots, so the hairy roots have been used to initially identify genes for insect resistance. In addition, the function of quality-related genes or abiotic stress-related genes, such as the isoflavone-metabolism-related gene *GmMYB29* [54] and salt-tolerance-related gene *GmCDF1* [55], has also been evaluated using the soybean hairy root system.

In our previous study, the resistant cultivar Wanxianbaidongdou (WX) and susceptible cultivar Nannong 99-10 (99-10) were used as materials to analyze the CCW-induced soybean transcriptome [56]. An *ERF* gene, *GmERF54*, responding to common cutworm (CCW) feeding, was obtained by screening the transcriptome data. The aim of this study was to evaluate the role of *GmERF54* in soybean resistance to CCW. Soybean hairy roots with the overexpression or interference of *GmERF54* were used for a CCW force-feeding experiment, and the results preliminarily suggested that *GmERF54* negatively regulated soybean resistance to CCW. GUS staining of transgenic hairy roots showed that MeJA and IAA may be involved in regulating the expression of *GmERF54*, but *GmERF54* promoters from resistant and susceptible cultivars reacted differently to exogenous MeJA and IAA.

These studies provide insights into the role of the *ERF* gene in soybean insect resistance and help to further improve soybean resistance to CCW.

2. Materials and Methods

2.1. Plant Materials and CCW Induction Treatments

Soybean cultivars used in this study included Jack, Qinyangdadou (QY), Kefeng No.1 (KF), Nannong 1138-2 (1138-2), Wanxianbaidongdou (WX) and Nannong 99-10 (99-10). All cultivars were grown in growth chambers with a photoperiod of 16/8 h (day/night), and a temperature of 26 °C during the day and 24 °C at night. The different tissues of KF and 1138-2 were used to identify the tissue expression of *GmERF54*. The roots, stems and leaves of plants at the V4 stage, flowers of plants at the R2 stage [57], and pod walls and seeds of plants at 15 days after flowering were collected. CCW induction treatment was carried out after KF and 1138-2 plants grew to the V4 stage. Two third-instar CCW larvae were placed on the third leaf from the top with a white net bag, which was used to prevent the larvae running away. Then, after eating for 2 h, the larvae were removed to complete the induction. Plant leaves with or without induction (control) were sampled at 1 h, 6 h, 12 h, 24 h and 48 h after CCW attack for total RNA extraction. The leaves of QY at the V4 stage were collected for DNA and RNA extraction. The DNA and RNA of QY leaves were used to clone *GmERF54*. Jack seeds were used to generate transgenic hairy roots.

2.2. Cloning and Bioinformatics Analysis of GmERF54

Genomic DNA was extracted using a DNAsecure Plant Kit (TIANGEN, Beijing, China). Total RNA was extracted using an RNA extraction kit (RNAsimple Total RNAKit, TIANGEN), and a reverse transcriptase kit (DNAsecure Plant Kit, TIANGEN) was used to obtain the first-strand cDNA. The quality and concentration of DNA and RNA were detected by gel electrophoresis and spectrophotometer. The cDNA sequence of *GmERF54* was cloned from the leaf cDNA of QY by PCR using specific primers (primers in Table S1), which were designed by Primer 5.0 software according to the mRNA sequence of *Glyma.12G110400* on NCBI (https://www.ncbi.nlm.nih.gov/, accessed on 14 March 2018). The procedure for cDNA cloning was as follows: initial denaturation for 3 min at 94 °C, followed by 35 cycles each consisting of denaturation for 15 s at 94 °C, annealing for 15 s at 58 °C and extension for 90 s at 72 °C; the final cycle was extended for 5 min at 72 °C. The cDNA sequence of *GmERF54* was connected into an entry T vector using pClone007 Blunt Simple Vector Kit (TsingKe, Beijing, China, TSV-007BS); then, the recombinant T plasmid with *GmERF54* cDNA was named as T-GmERF54 and confirmed by sequencing. The DNA sequence of *GmERF54* was cloned from the leaf DNA of QY by PCR using the same specific primers. The PCR procedure for DNA sequence cloning was similar to that for cDNA sequence cloning, except that the extension time of 90 s in the cycles was changed to 3 min.

BioXM software (http://202.195.246.60/BioXM/, accessed on 19 March 2018) was used to analyze the gene sequence and protein properties. Protein domain prediction was performed on the website SMART (http://smart.embl-heidelberg.de/index2.cgi, accessed on 8 April 2018).

2.3. Gene Expression Analysis

Total RNA was extracted using the RNA extraction kit (RNAsimple Total RNAKit, TIANGEN), and the first-strand cDNA was obtained using the reverse transcriptase kit (DNAsecure Plant Kit, TIANGEN). Approximately 100 mg of plant tissue was used for RNA extraction. Quantitative real-time PCR (qRT-PCR) was conducted using fluorescence quantitative PCR analyzer ABI 7500 (Applied Biosystems, Carlsbad, CA, USA) with the Sequence Detection System (SDS) analysis software (version 1.4). The gene relative expression level was analyzed using the $2^{-\Delta\Delta C}{}_{T}$ method [58], and the soybean *tubulin* gene (*Glyma.03g124400*) was used as a control gene. The procedure for qRT-PCR was as follows: initial denaturation for 30 s at 95 °C, followed by 40 cycles each consisting of denaturation

for 10 s at 95 °C and extension for 30 s at 60 °C. The primers used in qRT-PCR are listed in Table S1.

2.4. Subcellular Localization

The coding sequence (with the stop codon removed) of *GmERF54* was cloned into the pFGC5941 vector, which had a green fluorescent protein (GFP) gene driven by the CaMV 35S promoter. Empty vector 35S:GFP and recombinant vector 35S:GmERF54-GFP were transformed into *Nicotiana benthamiana* leaves for transient expression, respectively. Laser confocal microscopy (Zeiss, Jena, Germany, LSM780) was used to detect GFP signals in tobacco leaves.

2.5. Transformation of Soybean Hairy Roots

The *GmERF54* coding sequence (CDS) of QY was cloned into the vector pMDC83 to construct the pMDC83-GmERF54 (GmERF54-OE) overexpression vector, which contained a double CaMV 35S promoter. A 503-bp incomplete CDS fragment of *GmERF54* was linked to vector pB7GWIWG2(II) to construct pBI-GmERF54-RNAi (GmERF54-RNAi) vector. GmERF54-OE, GmERF54-RNAi and their respective empty vectors, pMDC83 (Control-OE) and pB7GWIWG2(II) (Control-RNAi), were independently transformed into *Agrobacterium rhizogenes* strain K599 to transform soybean hairy roots. The cotyledons of soybean variety Jack were infected by the transformed K599 to generate transgenic hairy roots, as described previously [59]. The cotyledons were cultured on white medium containing carbenicillin disodium (500 μg/mL) and cefotaxime disodium (50 μg/mL). The medium was changed after 14 days. Four weeks later, DNA and RNA were extracted from hairy roots for positive identification and gene expression analysis. Primers are listed in Table S1.

2.6. Forced Feeding Experiment of CCW

The hairy roots of soybean were used to conduct the forced feeding experiment of CCW using previously described methods [51,52]. Four-week-old fresh hairy roots containing GmERF54-OE, GmERF54-RNAi, Control-OE and Control-RNAi vectors were placed in plastic culture jars, respectively, and each culture jar had four third-instar larvae. Total weight of the four larvae was recorded before feeding, and 2, 4 and 6 days after feeding. The average larval weight was calculated. Samples in the plastic culture jar were replaced with fresh hairy roots at each weighing. No fewer than six replicates were set up in the experiment. Microsoft Excel 2021 was used for statistical analysis.

2.7. Transcriptional Activation Activity Analysis

The pGBKT7 (BD) vector with a GAL4 DNA-binding domain and the pGADT7 (AD) vector with a GAL4 activation domain were used to analyze the transcriptional activation activity of GmERF54. *GmERF54* (full-length CDS of *GmERF54*), *GmERF54*-N (N-terminal truncated 133 amino acid) and *GmERF54*-C (C-terminal truncated 130 amino acid) were cloned from vector T-GmERF54 and subcloned into vector pGBKT7 to construct BD-GmERF54, BD-GmERF54-N and BD-GmERF54-C bait vectors, respectively. The recombinant vectors were co-transformed into yeast cells with the AD-T vector. BD-53+AD-T and BD-lam+AD-T were used as positive and negative controls, respectively. Transformed yeast cells were cultured on quadruple-dropout media with X-α-Gal (40 μg/L) (-Leu/-Trp/-His/-Ade/X-α-Gal). X-α-Gal (5-bromo-4-chloro-3-indolyl-alpha-D-galactoside) was used to visualize the results.

2.8. Yeast Two-Hybrid (Y2H) Assay

The cDNA library of soybean leaf and root was used to identify the proteins interacting with GmERF54. In the library, cDNA was fused with the GAL4 activation domain on vector AD. Vector BD-GmERF54-C (loss of transcriptional activation activity) was co-transformed into Y2H Gold yeast strain with the cDNA library, and then, the transformed yeast cells were cultured on quadruple-dropout media with X-α-Gal (40 μg/L) (-Leu/-

Trp/-His/-Ade/X-α-Gal) for 10 days at 30 °C. Plasmids were extracted from yeast cells grown normally on quadruple-dropout medium and transformed into *E. coli* competent state. The transformed *E. coli* cells were grown on LB medium containing ampicillin (100 mg/mL), then recombinant AD vectors were screened out for sequencing (primers in Table S1). The sequences were analyzed by Nucleotide BLAST in the NCBI database (https://blast.ncbi.nlm.nih.gov/Blast.cgi, accessed on 20 June 2019). The BD-GmERF54-C vector was co-transformed into Y2H Gold strain with the screened recombinant AD plasmid to further test the positive interaction.

2.9. Bimolecular Fluorescence Complementation (BiFC) Assay

The CDS of *GmERF54* was cloned into the N-terminal of YFP of the SPYNE173 vector (YFP^N) to generate the GmERF54-YFP^N vector and the CDS of *GmPLT2* was cloned into the C-terminal of YFP of the 35S-SPYCE (M) vector (YFP^C) to generate GmPLT2-YFP^C. The constructs were introduced into *Agrobacterium tumefaciens* strain EHA105 and were then cotransformed into *Nicotiana benthamiana* leaves. The combinations of GmERF54-YFP^N and YFP^C and YFP^N and GmPLT2-YFP^C were used as negative controls. The yellow fluorescent protein (YFP) signal in tobacco leaves was observed by confocal microscopy (Leica TCS SP2, Mannheim, Germany). Primers are listed in Table S1.

2.10. Promoter Cloning and Analysis

Genomic DNA was extracted using a DNAsecure Plant Kit (TIANGEN). Specific primers (Table S1) were used to clone the promoter fragments of *GmERF54* from the leaf DNA of the insect-resistant cultivar WX and the insect-susceptible cultivar 99-10. The β-glucuronidase (GUS) reporter system was used to analyze gene promoter activity [60]. Two promoter sequences were cloned into vector pCAMBIA1381z containing the *GUS* reporter gene to obtain the recombinant vectors *GmERF54*$_{WX}$*:GUS* and *GmERF54*$_{99\text{-}10}$*:GUS*, respectively. If the promoters of *GmERF54* have activity, they can drive the reporter gene *GUS* to express β-glucuronidase which breaks down colorless chemical 5-bromo-4-chloro-3-indolyl-beta-D-glucuronide (X-Gluc) in GUS staining solution into a visualized blue product. Thus, sites with GUS activity are shown in blue. Recombinant vectors were transformed into *Agrobacterium rhizogenes* K599 strain, and then, the transformed strain infected cotyledons of soybean variety Jack to obtain hairy roots. The detailed procedure followed that in Section 2.5. Four weeks later, transgenic hairy roots were treated with methyl jasmonate (MeJA; 100 μM) solution for 30 min, 1 h and 3 h, and with indole-3-acetic acid (IAA; 20 μM) solution for 30 min, 1 h and 3 h at room temperature, respectively. In addition, the hairy roots containing recombinant vector *GmERF54*$_{WX}$*:GUS* and *GmERF54*$_{99\text{-}10}$*:GUS* were inoculated with CCW for 30 min and 1 h. Following these treatments, hairy roots were sampled and placed in GUS staining solution for 6 h at 37 °C in the dark. Then, they were decolorized by 75% ethanol continuously, and soaked for 5 min each time until they were colorless.

3. Results

3.1. GmERF54 Shows Differential Expression in Insect-Resistant and Insect-Sensitive Soybeans

The insect-resistant soybean WX and insect-sensitive soybean 99-10 at the time of the highest induced resistance, were taken to analyze the transcriptional profiles of CCW induction by RNA-seq sequencing technology [56]. A soybean ERF transcription factor gene *Glyma.12G110400* (*GmERF54*) was screened out from the transcriptional profile data. The *GmERF54* expression level was significantly down-regulated in WX (LOG2 (RK_RPKM/RCK_RPKM) = −12.8, FDR = 2.35×10^{-4}) after insect induction, while it was significantly up-regulated in 99-10 (LOG2 (SK_RPKM/SCK_RPKM) = 11.7, FDR = 2.21×10^{-2}). Here, we used another relatively resistant cultivar (KF) and susceptible cultivar (1138-2) to verify the expression pattern of this gene in response to the CCW induction. It was found that the relative expression level of *GmERF54* in leaves of KF and 1138-2 cultivars was consistently down-regulated from 1 to 48 h after CCW induction,

compared with that of the control (Figure 1). One hour after insect induction, the *GmERF54* expression in KF decreased significantly, although it also decreased in 1138-2, but not significantly. These results indicate that *GmERF54* responds to the CCW attack, and may be involved in the defense response to CCW in soybean.

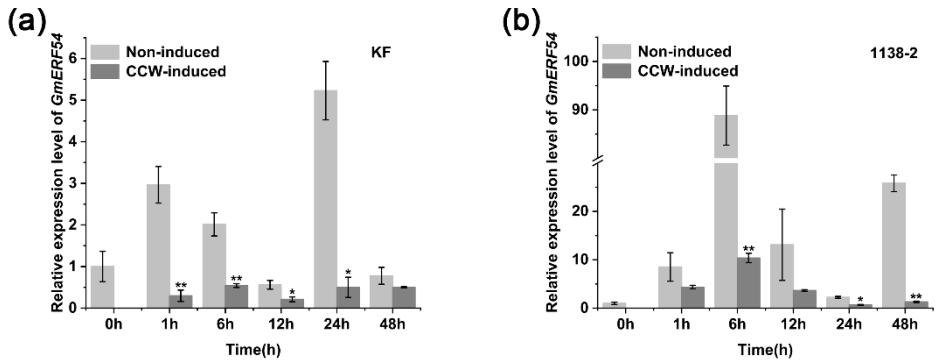

Figure 1. Relative expression levels of *GmERF54* in non-induced and CCW-induced leaves of soybean plants at the V4 stage (n = 3). (**a**) CCW-induced expression analysis of *GmERF54* in KF (the insect-resistant accession). (**b**) CCW-induced expression analysis of *GmERF54* in 1138-2 (the insect-susceptible accession). The qRT-PCR results were normalized to the *tubulin* control gene and relative to the expression level of non-induced plant leaves at 0 h. A two-tailed t-test was used to detect the statistical significance. *: $p < 0.05$; **: $p < 0.01$. Error bars denote ± SD.

GmERF54 was cloned from QY leaf cDNA and DNA, respectively. The cDNA and DNA sequences of *GmERF54* were the same by sequencing and gel electrophoresis (Figure 2a), indicating that the gene had no intron. The full-length coding sequence of *GmERF54* is 1221 bp, and it encodes 406 amino acids. Its protein contains an AP2 conserved domain (Figure 2b). The homologous gene of *GmERF54* in *Arabidopsis* is *AtERF53* (*At2g20880*), and the similarity of their protein sequences is 37.41% (Figure S1). The overexpression of *AtERF53* in *Arabidopsis rglg1rglg2* double mutant enhanced the drought [61] and heat stress [62] tolerance of the mutant. Thus, *GmERF54* may also be involved in the response to stress in plants. The qRT-PCR analysis showed that *GmERF54* was expressed in all tissues of both KF and 1138-2, and the expression level of *GmERF54* was different in insect-resistant and insect-susceptible soybean cultivars (Figure 2c).

3.2. GmERF54 Localized in the Nucleus

The recombinant expression vector pFGC5941-GmERF54 (35S:GmERF54-GFP) was transiently transformed into tobacco (*N. benthamiana*) leaves, and laser confocal microscopy was used to detect the GFP signals. The GFP signals showed that the 35S:GmERF54-GFP fusion protein was distributed in the nucleus of tobacco cells (Figure 3). This indicated that *GmERF54* probably functions in the nucleus.

3.3. GmERF54 May Negatively Regulate Soybean Resistance to CCW

The soybean hairy root system was used to preliminarily identify *GmERF54*. GmERF54-OE and GmERF54-RNAi were constructed to promote and inhibit the expression of the *GmERF54* gene in soybean hairy roots, respectively. The two vectors and their corresponding empty vectors were transformed into the soybean hairy roots, and approximately 20 dishes of transgenic hairy roots were obtained for each vector (Figure 4a). Transgenic hairy roots were examined by PCR (Figure 4b) and qRT-PCR. The expression level of *GmERF54* was significantly increased in the gene-overexpressed hairy roots compared with the Control-OE, and significantly decreased in the gene-interfered hairy roots compared with the Control-RNAi (Figure 4c).

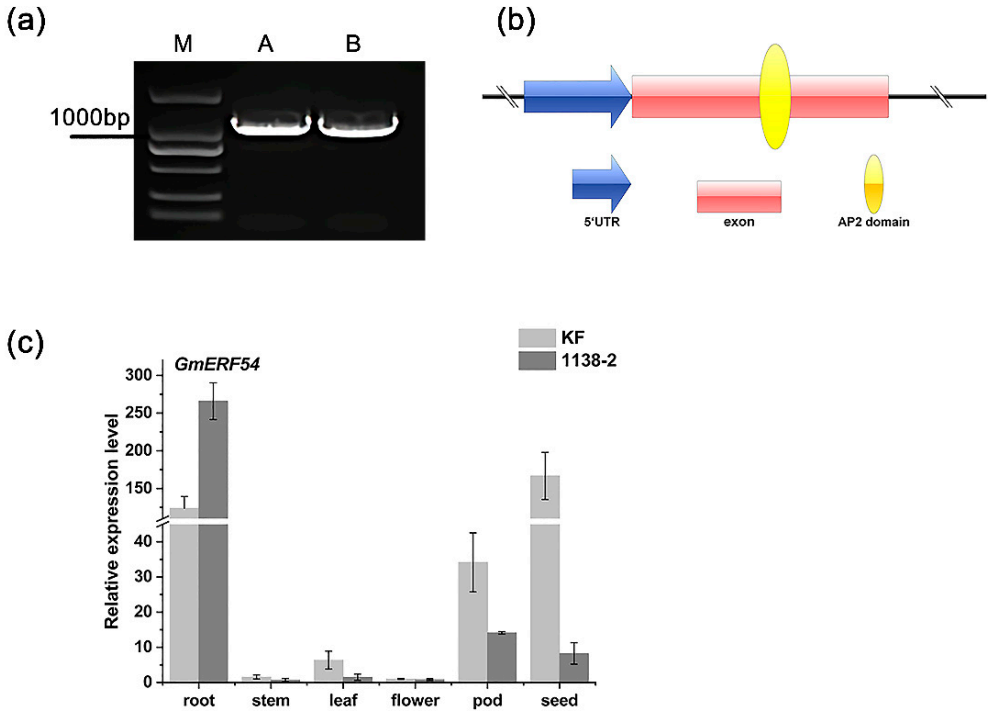

Figure 2. Sequence and expression analysis of *GmERF54*. (**a**) Agarose electrophoresis of *GmERF54* PCR products. M: DNA molecular marker (2000 bp); A: *GmERF54* product amplified in cDNA; B: *GmERF54* product amplified in DNA. (**b**) Schematic diagram of the *GmERF54* gene structure. UTR, untranslated region. (**c**) Expression analysis of *GmERF54* in different soybean tissues of KF and 1138–2 (n = 3). Roots, stems and leaves of plants at the V4 stage, flowers of plants at the R2 stage, and pod skins and seeds of plants at 15 days after flowering were used for tissue expression analysis. According to the $2^{-\Delta\Delta C}{}_{T}$ method [58], the relative expression levels were normalized to the housekeeping gene *Gmtubulin* in the same tissue at the same time, and were relative to the expression in flowers of KF (expression value in KF flowers = 1). Error bars denote ± SD.

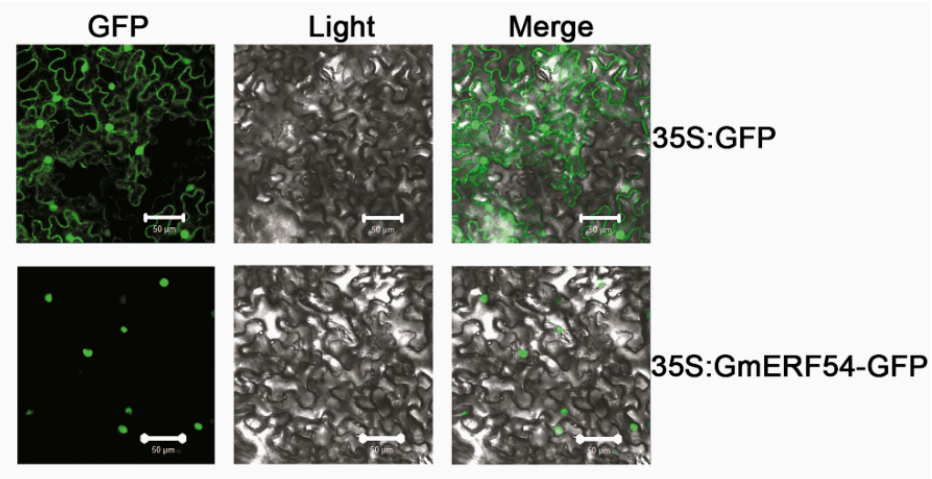

Figure 3. Subcellular localization of GmERF54 in tobacco mesophyll cells. GFP represents GFP fluorescence, light represents the bright field, and merge represents the combination of GFP fluorescence and the bright field. Scale bars: 50 μm.

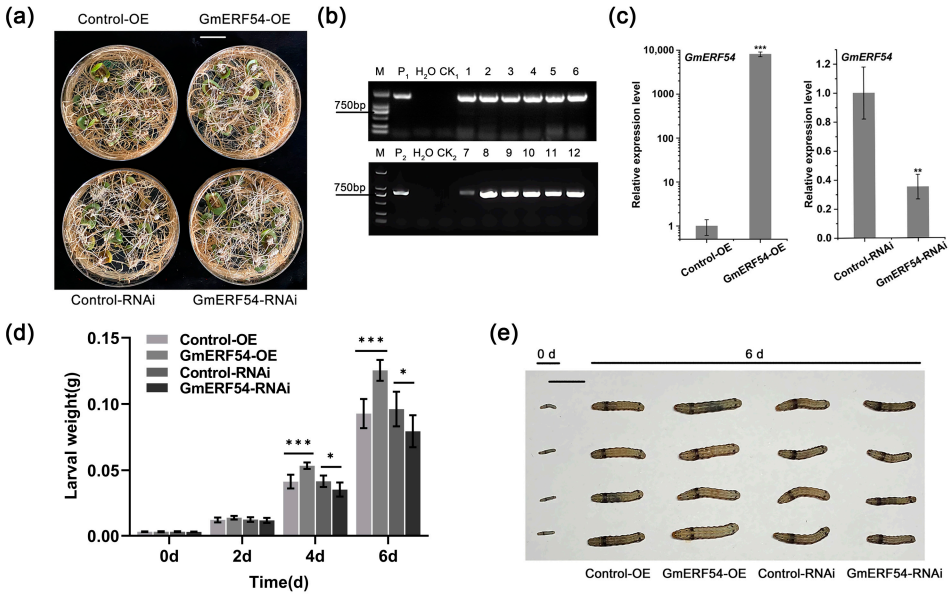

Figure 4. *GmERF54* transgenic soybean hairy roots and evaluation of CCW resistance. (**a**) *GmERF54* transgenic hairy roots. Scale bar = 2 cm. (**b**) Identification of transgenic soybean hairy roots at the DNA level. M: DNA Marker DL 2000; P_1: pMDC83-GmERF54 plasmid; P_2: pB7GWIWG2(II)-GmERF54 plasmid; H_2O: blank control; CK_1: soybean hairy roots containing vector pMDC83; CK_2: soybean hairy roots containing vector pB7GWIWG2(II); 1~6: soybean hairy roots containing pMDC83-GmERF54 plasmid; 7~12: soybean hairy roots containing pB7GWIWG2(II)-GmERF54 plasmid. (**c**) Relative expression level of the *GmERF54* gene in transgenic hairy roots. The qRT-PCR results were relative to the expression level of the Control-OE lines and Control-RNAi lines, respectively, and normalized to the *tubulin* control gene. $n = 3$. (**d**) Average larval weight of CCW feeding on *GmERF54* transgenic hairy roots after 0, 2, 4 and 6 days of feeding. $n \geq 6$. (**e**) Representative CCW larvae fed on transgenic hairy roots at days 0 and 6. Scale bar = 1 cm. Control-OE: the empty vector pMDC83; GmERF54-OE: over-expression of *GmERF54*; Control-RNAi: the empty vector pB7GWIWG2(II); GmERF54-RNAi: RNA interference of *GmERF54*. A two-tailed *t*-test was used to detect statistical significance. *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$. Error bars denote ± SD.

Then, transgenic hairy roots were used to feed CCW larvae. Following feeding for 4 and 6 days, the average weight of the CCW larvae feeding on GmERF54-OE hairy roots was significantly higher than that of the control, whereas the larval weight after feeding on GmERF54-RNAi hairy roots was significantly lower than that of the Control-RNAi (Figure 4d, e). We repeated the experiment twice and obtained similar results (Figure S2). These results show that *GmERF54* may negatively regulate soybean resistance to CCW.

3.4. GmERF54 Has Transcriptional Activation Activity and Interacts with GmPLT2

To understand how *GmERF54* regulates soybean insect resistance, Y2H assay was performed to screen proteins that may interact with GmERF54. Firstly, the Y2H system was used to verify whether GmERF54 has transcriptional activation activity. The results show that the co-expression of vector BD-GmERF54 and vector pGADT7-T (AD-T) in yeast resulted in the expression of the reporter gene (encoding α-galactosidase), which indicated that GmERF54 had transcriptional activation activity. The full length of GmERF54 was truncated, and its transcriptional activation domain was finally confirmed to exist in the C-terminal 130 amino acid region (Figure 5a).

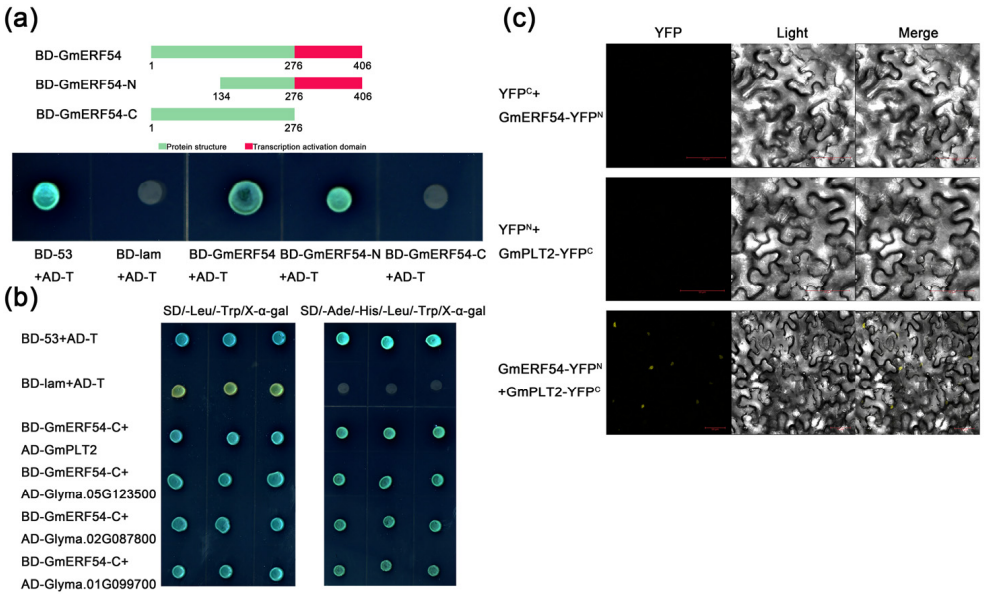

Figure 5. GmERF54 has transcriptional activation activity and interacts with GmPLT2. (**a**) Identification of GmERF54 transcription activation domain. BD-53 represents pGBKT7-53, BD-lam represents pGBKT7-lam, AD-T represents pGADT7-T, BD-GmERF54 represents pGBKT7-GmERF54, BD-GmERF54-C represents pGBKT7-GmERF54-C (C-terminal truncated 130 amino acid) and BD-GmERF54-N represents pGBKT7-GmERF54-N (N-terminal truncated 133 amino acid). (**b**) Yeast two-hybrid assay of the four proteins and GmERF54. The fact that yeast cells grew and turned blue on quadruple-dropout media (-Leu/-Trp/-His/-Ade/X-α-gal) is evidence of interaction. AD-GmPLT2 represents pGADT7-GmPLT2, AD-Glyma.05G123500 represents pGADT7-Glyma.05G123500, AD-Glyma.02G087800 represents pGADT7-Glyma.02G087800 and AD-Glyma.01G099700 represents pGADT7-Glyma.01G099700. SD/-Trp/-Leu/X-α-Gal represents double-dropout medium supplemented with X-α-Gal, and SD/-Trp/-Leu/-His/-Ade/X-α-Gal represents quadruple-dropout medium supplemented with X-α-Gal. (**c**) BiFC assay showed interaction between GmERF54 and GmPLT2 in tobacco cells. GmERF54 was fused with the N-terminal of YFP, and GmPLT2 was fused with the C-terminal of YFP. Scale bars: 50 μm.

BD-GmERF54-C (C-terminal truncated 130 amino acid) without self-activation activity was used as a yeast bait vector to screen the interacting proteins. Nine putative interacting proteins were screened from the leaf cDNA library (Supplemental Table S2). According to the functional annotation of these genes, they are mostly involved in photosynthesis, respiration and other pathways related to energy accumulation and consumption. Four candidate interacting proteins were screened from the soybean root cDNA library: Glyma.02G087800, Glyma.01G099700, Glyma.05G123500 and Glyma.12G056300 (Supplemental Table S3). As shown in Figure 5b, the interactions between GmERF54 and these four proteins were preliminarily verified by one-to-one Y2H assay. The interaction between GmERF54 and Glyma.12G056300 (GmPLT2) in the nuclei of tobacco mesophyll cells was confirmed by BiFC assay (Figure 5c). The YFP signal was detected in the combination of GmERF54-YFP^N with GmPLT2-YFP^C. These results indicate the possible interaction between GmERF54 and GmPLT2.

3.5. The Sequence and Activity of GmERF54 Promoter Are Different in Insect-Resistant and Insect-Susceptible Cultivars

The promoter (1910 bp upstream of the CDS) of *GmERF54* from insect-resistant cultivar WX and insect-sensitive cultivar 99-10 was cloned. The promoter elements were predicted through the PlantCARE (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/, accessed on 26 August 2019) website, and the results show that the *GmERF54* promoter contained multiple putative elements, including hormone-responsive cis-acting elements,

light responsive elements and adversity responsive elements. This suggested that *GmERF54* may deal with various stresses by responding to different hormones. The comparison between the *GmERF54* promoter sequences of WX and 99-10 revealed that there were many differences between the two sequences, and the promoter of *GmERF54* in WX had four putative additional elements: AT-rich element, Box 4, ERE and L-box cis-response elements (Tables S4 and S5).

Soybean transgenic hairy roots of $GmERF54_{WX}$*:GUS* and $GmERF54_{99\text{-}10}$*:GUS* were used to access the activity of the *GmERF54* promoter. As shown in Figure 6, both *GmERF54* promoters from WX and 99-10 had activity and responded to CCW induction. Following 1 h of CCW treatment, the *GmERF54* promoter activity decreased, compared with that after 30 min of CCW treatment. The activity of the *GmERF54* promoter from 99-10 was higher than that from WX (Figure 6). These results indicate that the expression of *GmERF54* appeared to be down-regulated in both the insect-resistant accession (WX) and insect-susceptible accession (99-10) after 1 h of CCW attack.

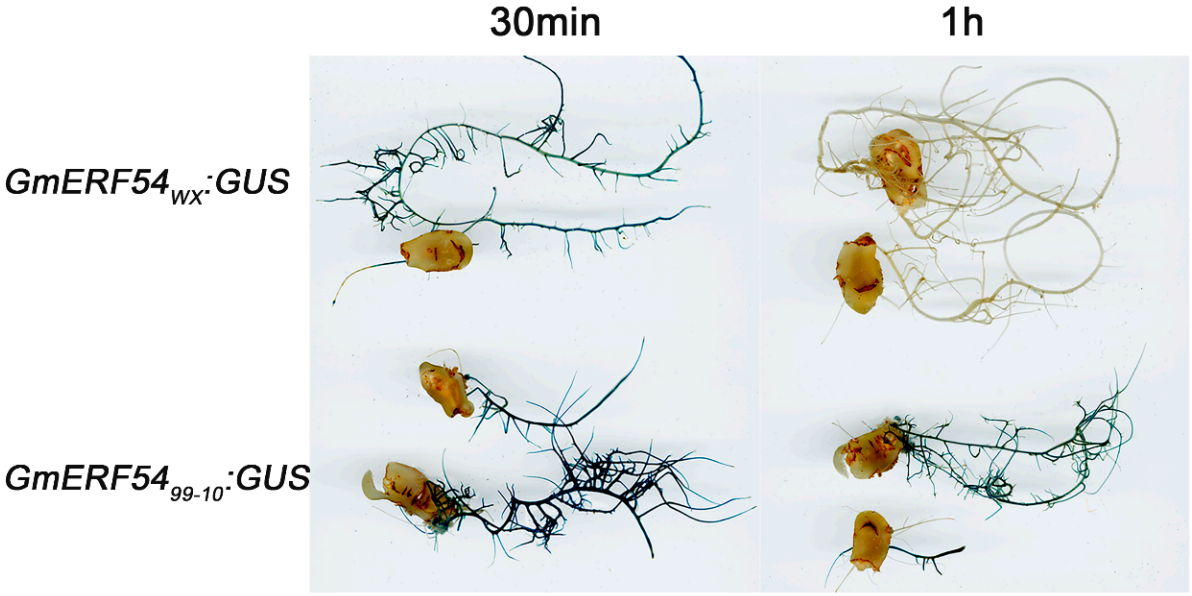

Figure 6. GUS staining of $GmERF54_{WX}$*:GUS* and $GmERF54_{99\text{-}10}$*:GUS* transgenic hairy roots with exposure to CCW attack. The $GmERF54_{WX}$*:GUS* and $GmERF54_{99\text{-}10}$*:GUS* recombinant vectors represent the promoter fragments from WX (insect-resistant cultivar) and 99-10 (insect-susceptible cultivar), respectively.

3.6. GmERF54 of Insect-Resistant and Insect-Susceptible Cultivars Responds Differently to Jasmonic Acid and Auxin

Jasmonic acid and its derivatives play a key role in inducing plant defense against a wide range of herbivorous insects [63]. The *GmERF54* promoter sequence contained putative jasmonic acid response elements, and the protein GmPLT2 that interacted with GmERF54 in yeast was associated with the auxin pathway, so *GmERF54* may be regulated by the two phytohormones. We conducted a GUS staining assay to study the response of *GmERF54* promoters from WX and 99-10 to MeJA and IAA treatment.

The $GmERF54_{WX}$*:GUS* and $GmERF54_{99\text{-}10}$*:GUS* recombinant vectors described above were transformed into soybean hairy roots and treated with exogenous MeJA (100 μM) and IAA (20 μM) solutions for 30 min, 1 h and 3 h, respectively. The activity of the *GmERF54* promoters of both cultivars increased after 1 h of MeJA treatment and decreased after 3 h of MeJA treatment. Following treatment with MeJA for 1 h, the *GmERF54* promoter activity of 99-10 was lower than that of WX (Figure 7a). Following 1 h of treatment with IAA solution, the *GmERF54* promoter showed enhanced activity, which remained high after 3 h of treatment. In contrast to MeJA treatment, the *GmERF54* promoter activity of 99-10 was

higher than that of WX after 1 h treatment with IAA solution (Figure 7b). In conclusion, MeJA and IAA were involved in inducing the expression of *GmERF54*, but the *GmERF54* promoters in WX and 99-10 showed different responses to exogenous MeJA and IAA.

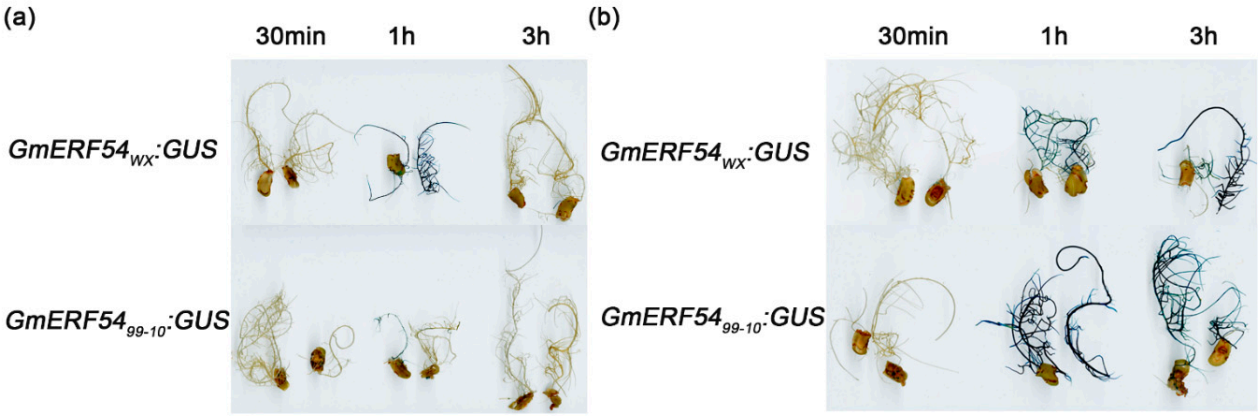

Figure 7. The expression of *GmERF54* is regulated by MeJA and IAA. (**a**) *GmERF54*$_{WX}$*:GUS* and *GmERF54*$_{99\text{-}10}$*:GUS* transgenic hairy roots were treated with MeJA (100 μM) for 30 min, 1 h and 3 h. (**b**) *GmERF54*$_{WX}$*:GUS* and *GmERF54*$_{99\text{-}10}$*:GUS* transgenic hairy roots were treated with IAA (20 μM) for 30 min, 1 h and 3 h. The *GmERF54*$_{WX}$*:GUS* and *GmERF54*$_{99\text{-}10}$*:GUS* recombinant vectors represent the promoter fragments from WX (insect-resistant cultivar) and 99-10 (insect-susceptible cultivar), respectively.

4. Discussion

AP2/ERF transcriptional factors play an important role in plant resistance to a series of stresses, such as mechanical injury, drought, high salinity and pathogen infection [64–66]. In this study, soybean *GmERF54* gene was cloned based on the previous transcriptome data, and its function was studied by various molecular and genetic engineering methods.

4.1. GmERF54 May Negatively Regulate Soybean Resistance to CCW

The AP2/ERF superfamily is one of the major transcription factor families in plants and participates in plant growth and development, as well as stress responses. In plants, studies on *AP2/ERF* genes mainly focus on the responses to abiotic stress [47,67,68] and pathogenic bacteria [69,70]. A few AP2/ERF TFs have been found to be involved in the regulation of insect resistance. For example, the *ERF-E2* gene in tomato negatively modulates the attraction to plant parasitic nematodes (PPNs) by altering the composition of root secretions, thereby reducing PPN damage to tomato. Knockdown of its homologous gene *ERF-E3* does not show a similar phenotype [71]. Another tomato gene, *JARE4*, positively regulates resistance to CCW by responding to JA and regulating the expression of steroidal alkaloid (SGA) biosynthesis genes, which leads to the accumulation of anti-nutritional metabolites [20]. The overexpression of the *Arabidopsis RAP2.6* gene increases the number of callose deposits at the feeding sites and enhances the resistance to beet cyst nematodes [72]. *OsERF3* has been reported to positively regulate the biosynthesis of JA, salicylic acid and ethylene, and improves the resistance of rice to striped stem borer (SSB) [73]. These functional insect resistance genes all positively regulate plant insect resistance. In this paper, the down-regulated expression of *GmERF54* in soybean hairy roots increased resistance to CCW, while the overexpression of *GmERF54* decreased resistance to CCW (Figure 4). These results suggest that *GmERF54* responds to CCW attack and may negatively regulate the resistance of soybean to CCW. According to the analysis of the *AP2/ERF* gene family in *Arabidopsis*, rice, soybean and potato, *GmERF54* belongs to subgroup A-6, which is distinct from that of the four genes mentioned above [46,74–76]. This may be the reason that *GmERF54* was different from the four genes in the regulation of insect resistance. Similarly, *GmCDPK17* and *GmCDPK38* from different subgroups of soybean *CDPK* genes have opposite effects on soybean resistance to CCW [77,78].

Further studies have shown that *GmERF54* responds differently to CCW induction in insect-resistant cultivar and insect-susceptible cultivar (Figure 1). The expression of the *GmERF54* gene decreased from 1 to 48 h after CCW induction in both the insect-resistant cultivar (KF) and insect-susceptible cultivar (1138-2), but the expression decreased to a significant level in KF 1 h after CCW induction (Figure 1). It was speculated that *GmERF54* in KF may respond to CCW induction earlier or faster than that in 1138-2. Similarly, the *Gbve1* gene in resistant cotton has a faster and stronger response to *Verticillium dahliae* inoculation than in *Verticillium*-susceptible upland cotton [79].

The jasmonate (JA) signaling pathway is a conserved core pathway in herbivore-induced responses [80]. JA and its derivatives have been proven to be involved in stress resistance and the regulation of growth and development [81]. In this study, *GmERF54* responded to MeJA treatment (Figure 7). This suggests that *GmERF54* is a JA response gene and may be involved in the JA pathway. *AP2/ERF* genes improve the resistance to biotic stress mainly by regulating the synthesis pathway of defense-related metabolites and the expression of resistance genes. *Arabidopsis ERF114* regulates the expression of phenylalanine ammonia-lyase1 (*PAL1*), accumulates lignin, and enhances the resistance to *Pseudomonas syringae* pv. *tomato* (Pst) DC3000 [82]. Lignin has also been reported to be associated with insect resistance in plants [83]. Defense genes *PDF1.1/1.2a/1.2b/1.2c/1.3/1.5* (PLANT DEFENSIN) were up-regulated in the ERF- or ERF6-overexpressed *Arabidopsis* [84]. *Arabidopsis ERF96* can directly bind to GCC elements of *PDF1.2a* and affect the expression of *PDF1.2a* [85]. *PDFs* are jasmonate-responsive defense genes and play a role in plant resistance to insects [86]. These results suggest that *GmERF54* might regulate soybean insect resistance via the JA mediation pathway.

4.2. GmERF54 May Be Involved in Insect-Injury-Induced Plant Regeneration

Insect resistance mechanisms include non-preference, antibiosis and tolerance [87]. Tolerance is the ability of plants to regenerate and/or reproduce after herbivore attack [14]. The role of ERF transcription factors in the regulation of plant regeneration after injury has been studied. The AP2/ERF transcription factor *ERF115* has been shown to regulate root stem cell regeneration after injury [88,89]. In *Arabidopsis*, *ERF109* expression is induced by injury and JA, and it promotes auxin accumulation at the wound site after injury, leading to root regeneration [90]. Another *AP2/ERF* gene, *RAP2.6L*, was reported to promote calli regeneration [91]. The *Arabidopsis* gene *AtERF111* is strongly induced after injury, and the overexpression of *AtERF111* increases the number and length of root hair in transgenic plants, thus confirming the role of *AtERF111* in acting as a stress signal and participating in wound repair [92]. In this study, the Y2H and BiFC assay results show that GmERF54 may interact with GmPLT2 (Figure 5), an AP2-like ethylene-responsive transcription factor PLETHORA (PLT). The *PLT* genes downstream of the auxin pathway have been reported to regulate cell division capacity around the injured tissues of plants [93]. The PLETHORA regulators *PLT1* and *PLT2* are involved in establishing the stem cell niche [94,95]. In addition, the PLT-CUC2 (CUP-SHAPED COTYLEDON2) module promotes vascular regeneration under injury through local auxin biosynthesis [96]. Plant organ regeneration usually involves the activation of mitosis. Another protein interacting with GmERF54 in yeast, Glyma.05g123500 (Figure 5), is predicted to be a p34cdc2 protein kinase. Such kinases have been reported to be necessary for plant mitosis [97,98]. These results indicate that *GmERF54* may participate in the regeneration response after CCW attack.

Damaged plants increase their regeneration and reproduction ability by regulating the balance between growth and resistance through the JA and auxin signaling pathways [99]. In this study, it was found that *GmERF54* can respond to the induction of MeJA and IAA (Figure 7), indicating that *GmERF54* may be involved in the JA and auxin pathways, thus regulating plant defense and regeneration responses after injury. The hypothesis that *GmERF54* regulates the regeneration needs to be verified in the future.

5. Conclusions

In conclusion, an ERF gene *GmERF54* was cloned and identified. The expression patterns of *GmERF54* differ between insect-resistant and insect-susceptible soybean cultivars. The down-regulated expression of *GmERF54* increased the resistance of transgenic soybean hairy roots to CCW, while *GmERF54*-overexpressed transgenic hairy roots exhibited decreased resistance to CCW. GmERF54 had transcriptional activation activity and interacted with AP2/ERF GmPLT2. The *GmERF54* promoters of insect-resistant and insect-susceptible cultivars were differently induced by CCW attack and both MeJA and IAA treatments. This study reveals the role of ERF TF in soybean resistance to CCW and contributes to the breeding of insect resistance in soybean.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/agronomy13020596/s1. Table S1: Primer pairs used in this paper; Table S2: GmERF54 interaction proteins in soybean leaf cDNA library; Table S3: GmERF54 interaction proteins in soybean root cDNA library; Table S4: Motif in *GmERF54* promoter of Wanxianbaidongdou; Table S5: Motif in *GmERF54* promoter of Nannong 99-10. Figure S1: Protein sequence alignment of GmERF54 and AtERF53; Figure S2: *GmERF54* transgenic soybean hairy roots and evaluation of CCW resistance.

Author Contributions: Conceptualization, L.C., R.Q., D.Y. and H.W.; validation, L.C., R.Q. and X.L. (Xiao Li); formal analysis, L.C., R.Q. and X.L. (Xiao Li); investigation, L.C., R.Q. and X.L. (Xinyu Liu); writing—original draft preparation, L.C.; writing—review and editing, L.C., D.Y. and H.W.; visualization, L.C. and R.Q.; supervision, D.Y. and H.W.; project administration, D.Y. and H.W. All authors have read and agreed to the published version of the manuscript.

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