Cloning and Functional Analysis of the Soybean *GmRIQ2* Promoter

Binbin Zhang [1], Huayi Yin [1], Zhihui Sun [1], Xiaohui Song [1], Jing Deng [1], Qian Zhang [2,*] and Dongmei Li [1,*]

1   Key Laboratory of Soybean Biology in Chinese Education Ministry (Northeastern Key Laboratory of Soybean Biology and Genetics & Breeding in Chinese Ministry of Agriculture), Northeast Agricultural University, Harbin 150030, China; zhangbinbin@neau.edu.cn (B.Z.); yhy9762@163.com (H.Y.); Arashi1203SZH@163.com (Z.S.); kxysxh09@163.com (X.S.); 13199075975@163.com (J.D.)
2   Key Laboratory of Germplasm Enhancement and Physiology and Ecology of Food Crops in Cold Region, Ministry of Education, Northeast Agricultural University, Harbin 150030, China
*   Correspondence: zhq2016@neau.edu.cn (Q.Z.); ldm2010@neau.edu.cn (D.L.)

Abstract: The *RIQ* gene can optimize the structure of light-harvesting complex II by regulating the degree of granum stacking, and ultimately participates in plant light stress responses. The *GmRIQ2* gene contributes to plant photoprotection and may be involved in negative regulation of yield. To understand the regulatory mechanisms of *GmRIQ2* expression, we isolated the *GmRIQ2* promoter (*PGmRIQ2*) from the KenFeng 16 soybean genome. Sequence analysis showed that several cis-acting elements were involved in hormone responsiveness, seed-specific regulation, anaerobic induction and light responsiveness. *PGmRIQ2* was expressed in soybean roots. GUS activity was detected in *Arabidopsis thaliana* rosette leaves, stems and roots, indicating that *PGmRIQ2* tended to drive expression in rosette leaf vascular tissue and had spatiotemporal expression characteristics at the early growth stage. Three promoter deletion fragments of 450 bp, 284 bp and 194 bp were obtained by 5′ deletion. These three fragments all drove *GUS* gene expression in *Arabidopsis thaliana* rosette leaves, stems and roots. *GUS* gene expression decreased with deletion fragment truncation. In addition, the *PGmRIQ2* auxin (IAA), abscisic acid (ABA) and methyl jasmonate (MeJA) response elements were located at base pairs −1661~−450, −450~−284 and −284~−194, respectively, and the promoter was induced by IAA, ABA, MeJA and light.

Keywords: soybean; *GmRIQ2*; promoter; functional identification

1. Introduction

Regulation at the transcriptional level is the most critical link between the complex expression and multi-stage regulation of genes [1]. Promoters are contributors to transcriptional regulation and have several important characteristic sequences [2]. These sequences interact with transcription factors or the external environment to ensure accurate gene transcription and expression [2–4]. In addition, promoters indirectly control the initiation and degree of transcription, participating in the internal mechanism of functional gene expression regulation [5]. In plant genetic engineering, promoters are often used to regulate the expression of foreign genes in order to improve crop quality, increase crop yield, and enhance crop adaptability under adverse conditions [6]. Constitutive promoters have a wide range of applications in plant genetic engineering due to their highly efficient expression throughout the growth period in plants [7–9]. However, there are problems with the use constitutive promoters, such as metabolite accumulation and energy waste caused by excessive consumption, and the occurrence of gene silencing [10–12]. Therefore, researchers have focused on inducible promoters and tissue-specific promoters, hoping that they can replace constitutive promoters as regulators [3]. The former regulate the expression of foreign genes only in the presence of inducing conditions or factors and can be purposefully regulated according to desired outcomes [13,14]. The use of the latter can

effectively alleviate unnecessary waste caused by overexpression because expression occurs only during a specific period or in a specific location [14–16].

Soybeans are rich in high-quality protein and fat. They are mainly used in the food and industrial material industries [17]. However, soybean plants are often exposed to a complex and changeable environment during the growth process, which affects normal growth and development and, in severe cases, leads to reduced production [18,19]. The existing research on soybean yield and stress resistance has revealed good prospects for development through soybean breeding [20]. The results of this kind of research can enable not only mitigation of the damage to crops caused by environmental changes but also cultivation of high-quality and high-yielding soybean varieties. In this regard, promoter function analyses are beneficial for mastering the regulatory mechanism of gene expression and can provide new efficient and convenient methods for plant genetic engineering through breeding, especially for soybean.

RIQ is a gene discovered in 2016 that protects the normal growth and development of plants by quenching excess light energy, which may be negatively correlated with yield [4,21–23]. In our previous research, *GmRIQ2* (accession NO.: *Glyma.04G174400*) could enhance the tolerance of strong light stress and reduces photoinhibition in soybean [23]. However, there are few studies on the function or gene structure of the *RIQ* gene. In view of this scarcity, this study was performed to provide new ideas for exploration of the regulatory mechanism of the *RIQ2* gene via analysis of the function of the promoter of the *RIQ2* gene. Thus, the objectives of this study were to (1) verify the functions *PGmRIQ2* (*GmRIQ2* promoter) and determine the gulatory abilities of promoter of different deletion fragments, and (2) clarify the regulatory effects of different hormones on *PGmRIQ2*. This could provide a theoretical reference for research on the molecular function and mechanism of the *GmRIQ2* gene and encourage further in-depth exploration of the regulatory network of its internal functional elements.

2. Materials and Methods

2.1. Plant Materials and Growth Conditions

The soybean cultivars KenFeng 16 (KF16) and DongNong 50 (DN50) were used as plant materials and were provided by the Soybean Research Institute of Northeast Agricultural University (Harbin, China). In a previous study, the *RIQ2* gene was cloned from KF16, which is a main cultivar in Heilongjiang province, and the transformation efficiency of DN50 was higher than other cultivars; thus, these two cultivars were used as plant materials in this study. The soybean plants were grown in black soil: Vermiculite (1:1, both were purchased locally) under a photoperiod of 16/8 h light/dark at 23 °C in a greenhouse. At the same time, Columbia wild-type (WT) *Arabidopsis thaliana* seeds were raised on Murashige-Skoog Basal Salt Mixture medium supplemented with 4.43 g/L MS (Phyto Technology Laboratory, Shawnee Mission, KS, USA), 30 g/L sucrose and 8 g/L agar, adjusted to pH 5.8. The seedlings of *Arabidopsis thaliana* were grown in black soil: vermiculite (1:1) under a photoperiod of 16/8 h light/dark at 23 °C in a light incubator and were used for subsequent genetic transformation experiments.

2.2. Promoter Cloning and Sequence Analysis

An online tool of the Berkeley Drosophila Genome Project (BDGP) [24] was used to predict the transcription start site and core segment in the *GmRIQ2* gene promoter sequence, and the regulatory sequence of the *GmRIQ2* gene promoter was deduced with the online analysis website PlantCARE.

Polymerase chain reaction (PCR) was carried out using the primer pairs *PGmRIQ2-1661-F* and *PGmRIQ2-1661-R* (shown in Table 1) using PrimeSTAR Max with soybean KF16 genomic DNA as a template. The cycling conditions were as follows: 94 °C for 5 min; 35 cycles of 94 °C for 15 s, 55 °C for 15 s, and 72 °C for 90 s; and 72 °C for 10 min. Based on the site distribution of the *pCAMBIA3301* vector, DNAMAN software (version 6.0, Lynnon Biosoft Co., San Ramon, CA, USA) was used to analyze the restriction enzyme

cleavage sites within the promoter sequence of the *GmRIQ2* gene. Finally, the *Xbar*I and *Bgl*II restriction enzyme cleavage sites were selected. The PCR amplification product was purified, recovered and cloned into the pGM-T vector, and the positive ampicillin (Amp)-resistant clones were selected on LB plates containing Amp and sequenced. The full-length sequence of the promoter was named *PGmRIQ2*.

Table 1. List of primers used for PCR.

Primer Name	Primer Sequence (From 5′ to 3′)	Tm (°C)
PGmRIQ2-1661-F	GCTCTAGA(*Xbar*I)CAGCACCAACCAAGCAGTTCTCTC	55
PGmRIQ2-1661-R	GAAGATCT(*Bgl*II)TACCATGAGGTTGCGCCTCAGAACCAGAGT	55
PGmRIQ2-450-F	GCTCTAGAGC(*Xbar*I)CAGCACCAACCAAGCAGTTCTCTCT	56
PGmRIQ2-450-R	GAAGATCTTC(*Bgl*II)TACCATCCTGTTTGGGATTCTTTTA	56
PGmRIQ2-284-F	GCTCTAGAGC(*Xbar*I)CAGCACCAACCAAGCAGT	55
PGmRIQ2-284-R	GAAGATCTTC(*Bgl*II)TACCATATTGGCGATTTGACCATG	55
PGmRIQ2-194-F	GCTCTAGAGC(*Xbar*I)CAGCACCAACCAAGCAGT	56
PGmRIQ2-194-R	GAAGATCTTC(*Bgl*II)TACCATGCTAGGTGCAGGGAAACG	56
Bar-F	GCGGTACCGGCAGGCTGAAG	55
Bar-R	CCGCAGGAACCGCAGGAGTG	55

2.3. Construction and Transformation of a Promoter Expression Vector

PGmRIQ2 was used to replace the CaMV35S promoter in the plant expression vector pCAMBIA3301. *PGmRIQ2* was connected to the β-glucuronidase (*GUS*) gene to form the *pCAMBIA3301GmRIQ2::GUS* plant expression vector. The vector construction is shown in Figure 1. The recombinant vector was transformed into *E. coli*, and the positive clones were identified by sequencing and stored. Then, the recombinant plasmids with the correct sequences were introduced into *Agrobacterium tumefaciens* strain EHA105 and *Agrobacterium rhizogenic* strain K599. To evaluate promoter activity and transformation efficiency, the expression of the reporter gene *GUS* gene was studied.
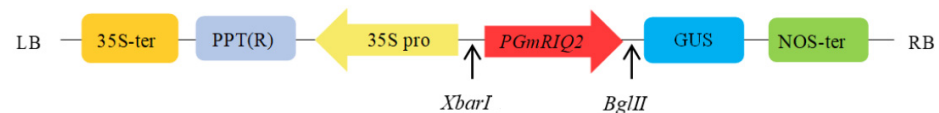

Figure 1. Schematic diagram of the *pCAMBIA3301PGmRIQ2::GUS* construct.

2.4. Construction and Transformation of the Expression Vector for Promoter Deletion Fragments

Based on the results of bioinformatics analysis of *PGmRIQ2* and 5′ end deletion analysis, a series of deletion fragments of *PGmRIQ2* were generated. Three deletion promoter fragments were ultimately constructed. The fragments amplified by PCR with the primer pairs *PGmRIQ2-450-F/PGmRIQ2-450-R*, *PGmRIQ2-284-F/PGmRIQ2-284-R*, and *PGmRIQ2-194-F/PGmRIQ2-194-R* (shown in Table 1) and the recombinant plasmid *pCAMBIA3301PGmRIQ2::GUS* as a template were named *PGmRIQ2-450* (450 bp), *PGmRIQ2-284* (284 bp) and *PGmRIQ2-194* (194 bp), respectively.

Similarly, the recombinant plasmids were introduced into *Agrobacterium tumefaciens* strain EHA105 after the amplified fragment was cloned into the plasmid *pCAMBIA3301* and determined to have the correct sequenced (Figure 2).

2.5. Cultivation and Identification of Transgenic Plants

To obtain hairy roots, Agrobacterium rhizogene was used to infect soybean cotyledon nodes. The completely and healthy soybean seeds (DN50) were selected and disinfected with 10% sodium hypochlorite (NaClO) solution for 15 h, and then rinsed with sterile water 3 times. The seeds were sown in germination medium (GM) (Table 2 and Figure 3), and cultured under light for 5–7 days. The soybean cotyledonary nodes were scratched on a sterile operating table and infected with Agrobacterium rhizogenes K599 for 30 min.

After that, the soybean cotyledon nodes were washed with sterile water 3–5 times, and the surfaces were wiped dry. The nodes were placed on solid co-culture medium (SCCM), cultured in the dark for 3 days, transferred to hairy root induction medium (RM), and cultured under light until hairy roots grew. DNA was extracted from the soybean roots by the cetyltrimethylammonium bromide (CTAB) method [25], and the primer pairs (*PGmRIQ2-1661-F* and *PGmRIQ2-1661-R*, *PGmRIQ2-450-F* and *PGmRIQ2-450-R*, *PGmRIQ2-284-F* and *PGmRIQ2-284-R*, *PGmRIQ2-194-F* and *PGmRIQ2-194-R*) were PCR-amplified as shown in Table 1 to detect positive roots. *pCAMBIA3301* was used as control. The cycling conditions were as follows: 94 °C for 5 min; 35 cycles of 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 30 s; and 72 °C for 10 min. The induced transformation efficiency of transgenic soybean roots was calculated as follows: hairy root transformation rate = number of hairy roots/number of explants × 100%; positive induction rate = number of positive roots/number of stained hairy roots × 100%.
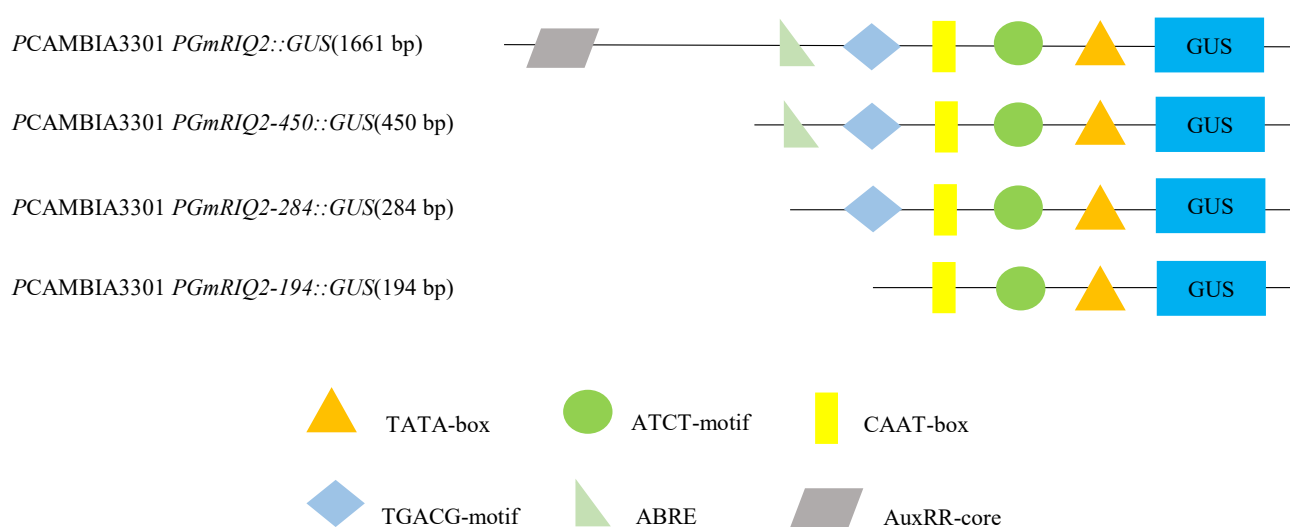

Figure 2. Construction of the recombinant expression vector of the *PGmRIQ2* deletion fragment.

Table 2. Media for soybean cotyledon node transformation [26,27].

Medium	PH	Composition
GM	5.8	B5: 3.21 g; sucrose: 20 g; agar: 7.5 g
SCCM	5.4	Murashige and Skoog (MS): 0.449 g; sucrose: 30 g; MES: 3.9 g; agar: 7.5 g; acetosyringone (As): 100 mg/L; DTT: 154 mg/L
RM	5.6	MS: 0.449 g; sucrose: 30 g; MES: 0.59 g; agar: 7.5 g; cephalosporins: 250 mg/L

The surfaces of *Arabidopsis thaliana* seeds were successively disinfected with 75% ethanol and 10% NaClO. The seeds were washed with sterile water, sown on 1/2 MS solid medium, incubated at 4 °C for 2–3 days, and then moved into a light incubator. After the seeds germinated, the seedlings were moved in a greenhouse with a photoperiod of 16/8 h light/dark at 23 °C. When flower buds appeared, the method of Clough et al. [28] was used. The opened flowers were cut, while the unopened bud leaves were completely soaked with transgenic *Agrobacterium tumefaciens* strain EHA105 for about 40 s. After the infestation was completed, the plants were treated in the dark for 24 h, and then transferred to a light incubator to continue to cultivate for 3–5 d under the original light conditions. The above steps were repeated 1–2 times until the seeds were harvested. Positive transgenic *Arabidopsis thaliana* was identified by phosphinothricin (PPT) resistance screening and PCR. PCR was carried out using the primer pairs *PGmRIQ2-450-F*/*PGmRIQ2-450-R*, *PGmRIQ2-*

284-F/PGmRIQ2-284-R and *PGmRIQ2-194-F/PGmRIQ2-194-R* (shown in Table 1) with transgenic *Arabidopsis thaliana* leaf genomic DNA as a template.
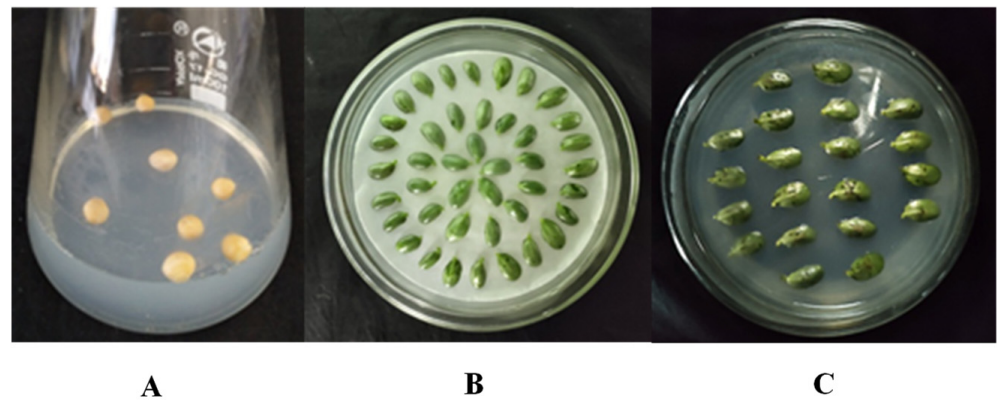

A   **B**   **C**

Figure 3. Process of hairy root formation induced by *Agrobacterium rhizogenes* K599 in soybean cotyledon nodes. (**A**) Germination. (**B**) Co-cultivation. (**C**) Hairy root induction.

2.6. Histochemical Staining

According to the method of Jefferson et al. [29], the plant tissue was stained. The washed tissue and GUS staining solution (0.1 M phosphate buffer pH 7.0, 0.5 M EDTA, 50 mM $K_3Fe(CN)_6$, 50 mM $K_4Fe(CN)_6$, Triton X-100, X-Gluc and ddH_2O) were added to an Erlenmeyer flask. The pressure switch was turned on, and a vacuum was established. The vacuum was maintained for approximately 1 h, after which the samples were incubated overnight (37 °C). After staining, the staining solution was poured out, and the samples were decolorized with 70% ethanol three times. After complete decolorization, a stereomicroscope was used to observe and photograph the samples.

2.7. Hormone and Stress Management of Transgenic Arabidopsis Thaliana

Transgenic third-generation *Arabidopsis thaliana* seeds were sown in MS medium, vernalized at 4 °C for 2–3 days, transplanted to mixed nutrient soil and cultured in a light incubator for 15 days. Two groups of transgenic *Arabidopsis thaliana* were prepared. One group was treated with medium containing 50 μM auxin (IAA), and the other group was treated with medium lacking IAA. After treatment for 0.5, 1, 2, 4, 6, 8, 12 and 24 h, the leaves of the plants were taken, and GUS activity was measured. The hormone in the above steps was replaced with 100 μM abscisic acid (ABA) or 100 μM methyl jasmonate (MeJA), and the procedure was repeated to obtain plant materials treated with different hormones.

Transgenic *Arabidopsis thaliana* seeds were sown in MS medium containing 5 mg/L PPT, vernalized at 4 °C for 2–3 days, and transferred to a light incubator with a light intensity of 120 μmol/m^2/s for 10 days. Two groups of transgenic *Arabidopsis thaliana* were prepared. One group was transferred to MS medium containing 250 mM mannitol for 2 days, and the other group was kept in the original MS medium for 2 days to measure GUS activity. The stress stimulus in the above steps was replaced by 200 mM NaCl or 300 μmol/m/s of light to obtain plant materials with different stress treatments.

2.8. GUS Fluorometric Quantitative Analysis

According to the method of Jefferson et al. [29], the GUS activity of the plant tissue was detected by fluorescence quantitative analysis. GUS extraction buffer (0.1 M phosphate buffer pH 7.0, 0.5 M EDTA, 10% SDS, Triton X-100, β-mercaptoethanol and ddH_2O) was used to extract GUS protein from the soybean leaves, which was stored at 4 °C until use. A fluorescence spectrophotometer was used to measure the absorbance at 595 nm, and the concentration of GUS protein was calculated. Then, the fluorescence spectrophotometer was set to an excitation wavelength of 365 nm, an emission wavelength of 455 nm, and a

slit width of 10 nm, and the fluorescence value was measured. Finally, the GUS activity was calculated. The determination was repeated three times for each sample.

2.9. Hormone Treatment of Soybean Plants

Soybean seedlings were grown in a greenhouse with a photoperiod of 16/8 h light/dark at 23 °C until the first fully expanded trifoliate leaves appeared, and were sprayed with medium containing 50 μM IAA. The leaves were taken at 0, 1, 3, 6, 9, 12 and 24 h after treatment; frozen with liquid nitrogen immediately; and stored at -80 °C until RNA extraction and fluorescence quantitative analysis. The hormone in the above steps was replaced with 100 μM ABA or 100 μM MeJA, and the procedure was repeated to obtain plant materials treated with different hormones.

All experiments were performed in triplicate. All data were analyzed using the general linear model procedure (GLM) in SPSS 17, IBM, New York, NY, USA. Comparisons were made using Duncan's Multiple Range test ($p < 0.05$). The data are shown as the mean $\pm$ SE of three independent replicates.

2.10. Total RNA Extraction and Quantitative Real-Time (qRT)-PCR Analysis

Total RNA from soybean leaves was extracted by the TRIzol method (Takara, Shiga, Japan) [30]. cDNA was synthesized according to the instructions of a reverse transcription kit with ReverTra Ace qPCR RT Master mix with gDNA Remover (Toybo Life Science Shanghai). qRT-PCR analysis was performed on a Chromo4 RT-PCR system (Bio-Rad, Hercules, CA, USA). The soybean *Actin4* gene (GenBank Accession No. AF049106) was used as the internal standard control, and the total RNA was reverse-transcribed into single-stranded cDNA using the primer pairs shown in Table 3. The qRT-PCR cycling conditions were as follows: 95 °C for 5 min and 40 cycles of 95 °C for 10 s, 60 °C for 20 s, and 72 °C for 30 s. All reactions were performed three times.

Table 3. Primers used for qRT-PCR.

Primer Name	Primer Sequence (From 5′ to 3′)
Actin4-S	GTGTCAGCCATACTGTCCCCATT
Actin4-A	GTTTCAAGCTCTTGCTCGTAATCA
GmRIQ2-S	GTCACATAAAAACACACCACGA
GmRIQ2-A	TGCTTGATATCTCAACTCAGCT

3. Results

3.1. Bioinformatics Analysis of the PGmRIQ2 Sequence

PGmRIQ2 was predicted with the BDGP online tool [24]. The results showed that there were four core promoter regions in the *PGmRIQ2* sequence, which were located from base pairs -103 to -153, -516 to -566, -843 to -893, and -983 to -1033. The predicted probabilities were 0.83, 0.85, 0.87 and 0.94. The predicted transcription initiation sites were T, A, A and A. The cis-acting elements of *PGmRIQ2* were predicted by using the PlantCARE online analysis website (Figure 4 and Table 4). It was found that the sequence contains a large number of basic promoter components, such as a TATA box and CAAT box. Also present were some cis-regulatory elements involved in abiotic stress responses, such as those involved in light responses (the ATCT-motif, Box II, the G-box, the GATA-motif and the TCT-motif); elements related to hormone responses (cis-acting elements involved in ABA responsiveness (ABA response elements, ABREs), a cis-acting regulatory element involved in IAA responsiveness (the AuxRR-core), and cis-acting regulatory elements involved in MeJA responsiveness (CDTCA- and TGACG-motifs)); a cis-acting regulatory element essential for anaerobic induction (the anaerobic response element, ARE); and a cis-acting regulatory element involved in seed-specific regulation (the RY-element).

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1     GTAATCTACC ATATAAAAAA TTTAAACTAA AGCTAATCTA AACTTAATAC
51    GTTATGGTAC CTAAAATTAT TCCTAATCTA AAATTATTAA ATTACGGTAC
101   CTAAACCTAT ATAAAAAATT TCAACTAAAC TTAATCTATC ATATAAAATT
151   ATTCCTAATT TACCATATAA AAAATTATTC CTAATTTGTC ATATAAAAAA
                       ATCT-motif
201   TTTAAACTAA A ATCTAATCT A TCCTATAACA TTAAGAATTG AAACTAAACT
                                                       TCT-motif
251   ATACAAAATT ATTATATTAC ACTACCTAAA CTATACAAAA TTATTA TCTT
                    ARE
301   AC ACTACCTA AAACCA TTTT ATTATCAGAA TATAAATATT TATATAAATA
351   AAAACATACA AAAGATTCAA TATATAACTT ACAAATTTTT TACGAAAATG
       PGmRIQ2-1661-F        PGmRIQ2-450-F      PGmRIQ2-284-F   PGmRIQ2-194-F
401   GAAGAACACC AACAAGAA CA GCACCAACCA AGCAGT TCTC TC TTCACACA
451   ATATTTCACT CTCTCAGATA ACAAGTAAAC TCGAAAACGC AGGCAAACAA
501   TTGAAGGCCA AACACAGTTT ATAAACGAAA GAAAAAGCAC GAAGCGCCAA
                                                        PGmRIQ2-194-R
551   TGGCACCAGC GCCTTCAATA GGCACGAAGC GCCAATGGGA ATGG CGTTTC
                            TGACG-motif     CGTCA-motif
601   CCTGCACCTA GC TGCCTAGC CCTGCTGCTG CTGCT TGACG G GACAAGACA
                                                       PGmRIQ2-284-R
651   GGGTCAAAGC GCCAACACCA CTGGCGTTTT GCAATA CATG GTCAAATCGC
                                                          R Y element
701   CAAT CAGGTT AGCGCATCCC TCCAGCAGTC AACACTTGTC CGC CATGCAT
                                                   ABRE      G-box
751   GAAGTCGCCA GTTCTACTGG CGCGTCCCAT GCTG GGTACG TGCA AAAACA
801   GCACCCCATG GAAAATATTT CCAAAACAGC CCTAGTTTGA GAAATTCTTT
              PGmRIQ2-450-R
851   G TAAAAGAAT CCCAAACAGG GAAATTTGCC AAAGCATCGC TAGTCTTCTC
901   TGACACGATA ATGAGTTGAC AAAGACATAA TCCAGAAATT AAATAAACAT
                                                         AuxRR-core
951   CAAAACTAAT CTACCATAAT CCAATGGAAA ACAATAACTT AAATAT ATGG
1001  ACCA GGGAAA TTAGGAAAAC AATATATTAG ATATAGATAG AAAGAATTTA
1051  ACAGGAATGC AACTTGTACT TGTAAAAGAC CGTCTAACTA ACAAAACATG
1101  AAGGGTGAAG ACTACAAATT ATCAAAACAC CATAGAAGAG AGCCAAGGTA
1151  ACATGACAAA GTTCTCCAAA ATTCAAATAA CACATTTTTT AATGTGCCAG
1201  TCAAAAATAA AGAATTCAAT CTGTGGTTGA TAAGATGAGT TAAAATAAAT
1251  TATAACTAAG TTCTTCCAAA TAAACTTGGC ATTTTATTGA AGTCATTGAT
1301  GACATTTATT TAATTAATTT CCCAGGCATA GATATATTAA TTTAAAATAA
1351  AAAATTTATT TATATTAAAA TTATTAGTAA ATTCATCAAA ATGTTTTATT
1401  CAATAAAACA TTATTGCACA AATGTAAATC ATTAGTTACT TTATTAACTT
1451  TTATTAATAA TTGAACGAAA AATTTATAAA GTGATCTAAT TTAGATATGA
1501  CTAATGTAAA AAGGTTACTG AATAATTAAG GGAATAAATA ATATAATAAT
1551  TATTATTTAA TTATTTAATC ATAAATATAA GTAGATTTGT TAGTTATTAC
1601  TTTTAAAAAA TTAATTAATT AGCATAATAA AAACTTTTAT ATTCATAATG
                    ARE
1651  TATATCAATT AAACCA TATC TAAAATTACA CACTAACTAT AAAAAAAATA
1701  TATAATTTAG TCCAAACATT AGCATTTTTT TTGGCCAACT AAAAGCTTCA
1751  TTTTTTATTT CTGGATCCAT CCCTAAATTT TCTCAACCTC TAATTCATTT
1801  GTAGTATAAC AAAGTAAAAA GAGAGAGGAA TAAGCTGGAG AGAAAATATC
                       GATA-motif
1851  AGTTTCCAAT A AAGGATAAA G CTAAGACAA AAATGATTGG TGGAGATCAA
                       CGTCA-motif   TGACG-motif
1901  GCAACGAGAT CG CGTCA CAT AAAAACACAC CACGATTGTT ACTACTAATT
1951  TATATTTAGT CTTATTATTA TCACAGAAAA TGTCCTTTGT GTCTCTACCT
            Box II    G-box     ABRE                    PGmRIQ2-1661-R
2001  CTGCATCGTC CC ACGTG GCA GCTGAGTTGA GATATCAAGC AGAAGAAGC A
2051  CTCTGGTTCT GAGGCGCAAC CTC GTCCCAA CAACC
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Figure 4. Sequence analysis of the *GmRIQ2* promoter (*PGmRIQ2*). The explanations of the gene sequences marked by different colors and black boxes are shown in Table 4.

Table 4. Predicted positions and functions of cis-regulatory elements in the *GmRIQ2* promoter (*PGmRIQ2*).

Element	Core Sequence	Position	Number Present	Function
ABRE	ACGTG	785 (+), 2012 (−)	2	*Cis*-acting element involved in ABA responsiveness
ARE	AAACCA	311 (+), 1661 (+)	2	*Cis*-acting regulatory element essential for anaerobic induction
ATCT-motif	AATCTAATCC	210 (+)	1	Part of a conserved DNA module involved in light responsiveness
AuxRR-core	GGTCCAT	998 (−)	1	*Cis*-acting regulatory element involved in IAA responsiveness
Box II	CCACGTGGC	2011 (+)	1	Part of a light responsive element
CGTCA-motif	CGTCA	636 (−), 1913 (+)	2	*Cis*-acting regulatory element involved in MeJA responsiveness
G-box	CACGTG	787 (+), 2010 (−)	2	*Cis*-acting regulatory element involved in light responsiveness
GATA-motif	AAGGATAAGG	1862 (+)	1	Part of a light responsive element
RY-element	CATGCATG	744 (+)	1	*Cis*-acting regulatory element involved in seed-specific regulation
TCT-motif	TCTTAC	297 (+)	1	Part of a light responsive element
TGACG-motif	TGACG	636 (+), 1913(−)	2	*Cis*-acting regulatory element involved in MeJA responsiveness

3.2. GUS Gene Expression from PGmRIQ2 in Different Crops

The recombinant plant expression vector containing the 1661 bp full-length *GmRIQ2* promoter and the *GUS* gene and the *pCAMBIA3301* vector containing the *CaMV35S* promoter and the *GUS* gene were transferred into soybean and *Arabidopsis thaliana*, respectively. Histochemical staining was used to quickly determine whether *PGmRIQ2* drove *GUS* gene expression and to analyze the transformation efficiency. The promoter was confirmed to drive *GUS* gene expression.

After PCR amplification, the results showed that the target bands were 1661 bp and 403 bp in size, indicating that two positive transgenic roots were successfully obtained (Figure 5A). By histochemical staining (Figure 5B), it was found that the hairy roots of transgenic soybean were blue under the influence of the *PGmRIQ2* or *CaMV35S* promoter. The strong constitutive *CaMV35S* promoter resulted in a darker color than the *PGmRIQ2* promoter. Combined with the results of PCR amplification, the findings revealed that both the *pCAMBIA3301GmRIQ2::GUS* recombinant expression vector and the *pCAMBIA3301* empty vector were successfully transferred into soybean roots and could drive the expression of the *GUS* reporter gene. As shown in Table 5, the hairy root transformation rates of the two positive materials were 42.22% and 40%, respectively, while the positive induction rates of the two positive materials were 71.05% and 77.78%, respectively. The results showed that although the recombinant expression vector containing *PGmRIQ2* had a slightly higher hairy root conversion rate than the other vector, the positive induction rate was lower. However, there was no significant difference between the vectors.

Table 5. Comparison of induction and transformation of hairy roots by different recombinant expression vectors.

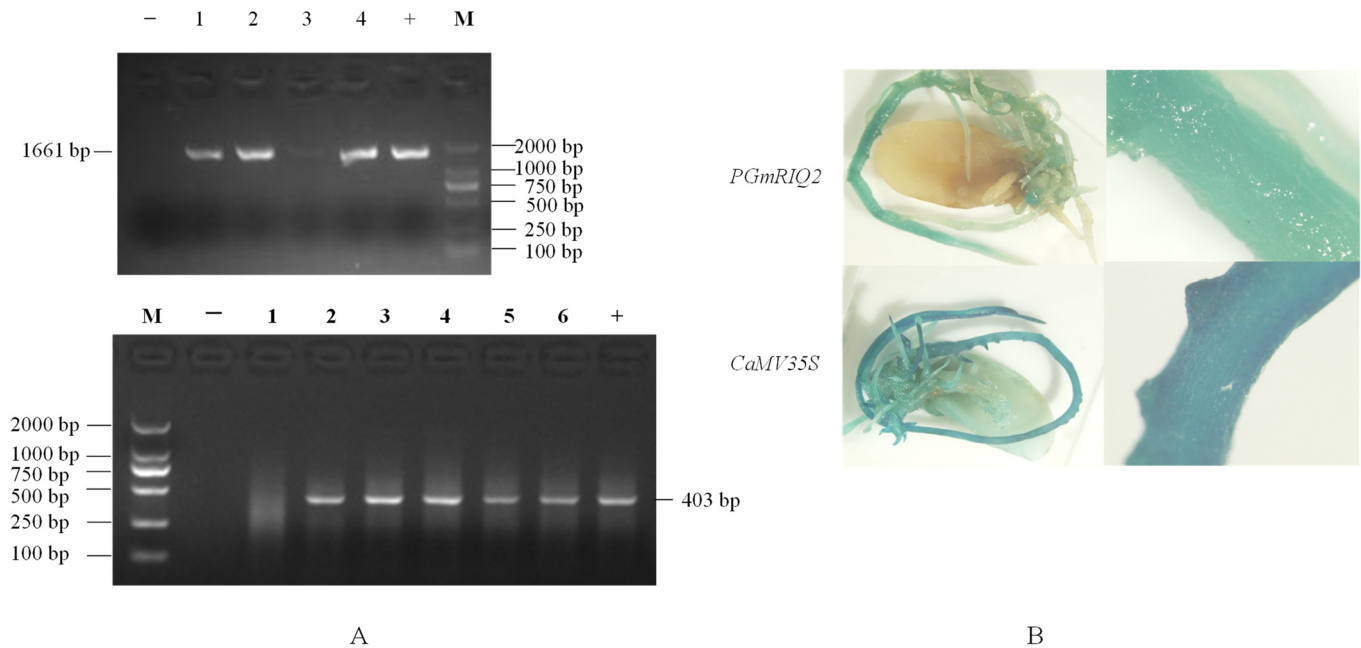| *Agrobacterium rhizogenes* Type	Number of Explants	Number of Hairy Roots	Positive Number	Conversion Rate	Positive Induction Rate
pCAMBIA3301PGmRIQ2::GUS	90	38	27	42.22%	71.05%
pCAMBIA3301::GUS	90	36	28	40%	77.78%

Figure 5. PCR detection of transgenic soybean root tissue and GUS histochemical staining of soybean root tissue. (**A**) PCR detection of transgenic soybean root tissue. 1–6: different transgenic roots GUS *pCAMBIA3301GmRIQ2::GUS* PCR amplification bands; +: positive control; −: negative control; M: DNA Marker (DL2000). (**B**) Transgenic *pCAMBIA3301PGmRIQ2::GUS* and *pCAMBIA3301* soybean roots.

PCR amplification and PPT resistance screening were used to confirm the successfully produced transgenic *Arabidopsis thaliana* plants (Figure 6A,B). Histochemical staining showed that the *GUS* gene was expressed in *Arabidopsis thaliana* under the influence of the two promoters (Figure 6C). Although the *PGmRIQ2* resulted in a slightly lower degree of staining (lighter staining, Figure 6C(5–11)) than the *CaMV35S* promoter (Figure 6C(1–4)), the blue color was obviously deeper and the expression level was obviously higher in the vascular tissue of rosette leaves than in the rest of the leaf tissue (Figure 6C(9–11)). This indicated that the *GUS* gene was efficiently expressed in the vascular tissues of rosette leaves. Subsequently, we found that the *GUS* gene expression level increased and that the expression area expanded with extension of the *Arabidopsis thaliana* growth period under the influence of the *PGmRIQ2* promoter; thus, it had obvious spatiotemporal expression characteristics. In the early stage of growth, *GUS* was expressed only in the rosette leaves. It was gradually expressed in the whole plant, but it remained dominantly expressed in the vascular tissues of the rosette leaves.

3.3. GUS Gene Expression Driven by PGmRIQ2 Deletion Mutants in Arabidopsis Thaliana

Based on the bioinformatics analysis of *PGmRIQ2* combined with the predicted positions and functions of the cis-acting elements in the sequence, *PGmRIQ2* was truncated by the 5′ end deletion method, and three promoter deletion fragments were constructed. The deletion promoters were named *PGmRIQ2-450* (450 bp), *PGmRIQ2-284* (284 bp) and *PGmRIQ2-194* (194 bp). To further study the ability of the *PGmRIQ2* deletion mutant to drive the inducible activity of the *GUS* gene, the promoter deletion fragment-GUS constructs were transferred to *Arabidopsis thaliana* through an *Agrobacterium tumefaciens*-mediated inflorescence soaking method. The positive *Arabidopsis thaliana* plants were confirmed by PCR amplification and PPT screening (Figure 7A,B), and then the expression of the *GUS* gene was observed by histochemical staining (Figure 7C). We clearly observed that transgenic *Arabidopsis thaliana* was stained blue under the action of *PGmRIQ2-450*, *PGmRIQ2-284* or

PGmRIQ2-194, consistent with the expression pattern of the full-length promoter; however, the expression levels were different. With truncation of the promoter, the expression level of the *GUS* gene decreased. However, the differences in *GUS* gene expression were more obvious in rosette leaves (Figure 7C(2,6,10)) and roots (Figure 7C(4,8,12)) than in stems (Figure 7C(3,7,11)). The *GUS* gene was obviously preferentially expressed in the vascular tissue of rosette leaves. The expression levels in roots were lower than those in rosette leaves and stems. These results showed that the promoter deletion fragments of different lengths were all able to drive *GUS* expression, but the resulting expression level gradually decreased with fragment shortening. *GUS* was most highly expressed in rosette leaves, followed by stems, while it was most weakly expressed in roots. It was dominantly expressed in vascular tissue.

3.4. Functional Analysis of the GmRIQ2 Full-Length and Deletion Fragment Promoters

Transgenic *Arabidopsis thaliana* plants with *GmRIQ2* promoters of different lengths were treated with hormones and stress. GUS activity was determined by GUS fluorescence quantitative analysis. Transgenic *Arabidopsis thaliana* plants with full-length *PGmRIQ2* were treated separately with 50 μM IAA, 100 μM ABA and 100 μM MeJA (Figure 8). GUS activity first increased and then decreased with prolonged treatment. It peaked at 6 h, 6 h and 4 h after the respective treatments, reaching levels significantly higher than those in the control plants. These results suggest that these three hormones can induce *GUS* gene expression and are inducers of *PGmRIQ2* and that the optimal response times for the hormones at the tested concentrations are 6 h, 6 h and 4 h, respectively.

Similarly, transgenic *Arabidopsis thaliana* plants with *PGmRIQ2* deletion mutants were treated separately with 50 μM IAA, 100 μM ABA and 100 μM MeJA, and samples were taken at the optimal response time for GUS fluorescence quantitative analysis. The results showed that GUS activity was significantly upregulated only in transgenic *Arabidopsis thaliana* with *PGmRIQ2* after IAA induction (Figure 9A). The responses of the other plants to IAA were significantly reduced, and there were no significant differences between these plants and the control plants. These preliminary data suggest that the IAA-responsive regulatory element is located between base pair -1661 and base pair -450, consistent with the predicted position of the GGTCCAT element. After ABA induction, GUS activity was significantly increased in transgenic *Arabidopsis thaliana* containing *PGmRIQ2* and *PGmRIQ2-450* (Figure 9B). However, the responses of the other two materials to ABA were not markedly different from that of the control, indicating that the promoter segment with the missing response element had a weakened ability to elicit a response to ABA. These preliminary data suggest that the regulatory element for ABA is between base pair -450 and base pair -284, consistent with the predicted position of the ACGTG element. After treatment with MeJA, GUS activity was upregulated in transgenic *Arabidopsis thaliana* containing the *PGmRIQ2*, *PGmRIQ2-450* and *PGmRIQ2-284* promoters (Figure 9C). However, GUS activity in transgenic *Arabidopsis thaliana* with *PGmRIQ2-194* was significantly decreased, which indicated that the ability of the promoter fragment without the MeJA response element to elicit a response was weakened. These preliminary data suggest that the regulatory element for MeJA is between base pair -284 and base pair -194, consistent with the predicted position of the TGACG element.

Transgenic *Arabidopsis thaliana* with *PGmRIQ2* promoter fragments of different lengths were treated with 250 mM mannitol, 200 mM NaCl and 300 μmol/m^2/s light. Quantitative analysis of GUS fluorescence showed that the GUS activity of all materials did not change significantly after treatment with 250 mM mannitol or 200 mM NaCl, and the regulatory elements related to drought and high-salt responses were not predicted to exist in the *PGmRIQ2* sequence (Figure 10A,B). Therefore, NaCl was not an inducer of *PGmRIQ2*. However, in our previous studies, the *GmRIQ2* gene was induced by drought, suggesting that there might be elements related to drought induction in other regulatory regions of the promoter. After 300 μmol/m^2/s light treatment, the GUS activity of all materials was significantly increased (Figure 10C). Promoter element prediction showed that there were

multiple light response regulatory elements in the *PGmRIQ2* sequence. Given the results and previous research by our group, we conclude that the *GmRIQ2* gene is induced by light and that light is the inducer of *PGmRIQ2*.
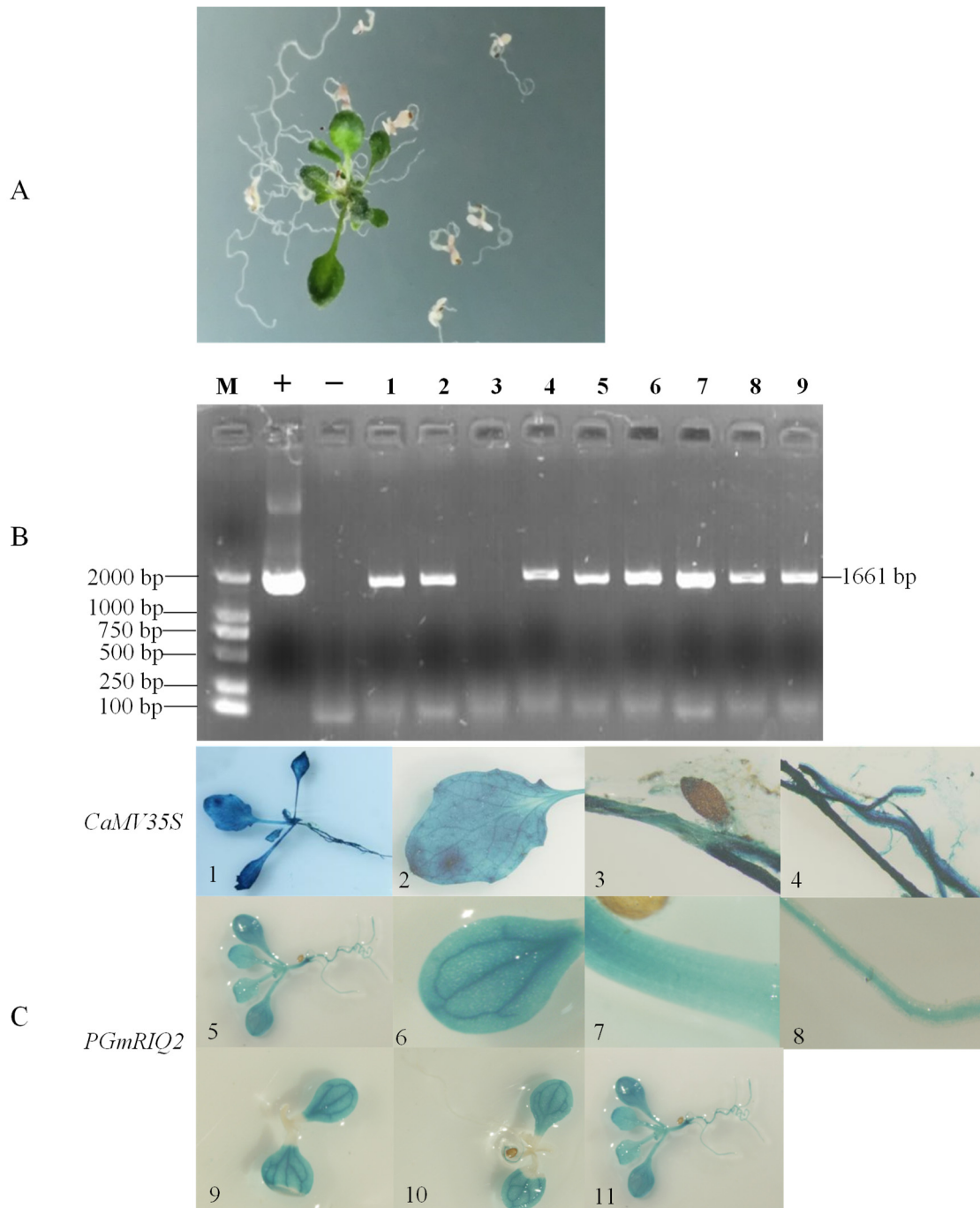

Figure 6. Detection of transgenic *Arabidopsis* with the full-length promoter of the *GmRIQ2* gene and GUS staining. (**A**) 5 mg/L PPT resistance screening. (**B**), PCR detection. M: DNA marker (DL2000); +: positive control; −: negative control; 1–9: *pCAMBIA3301GmRIQ2::GUS* PCR amplification bands. (**C**), GUS staining results. 1–4: expression of the *GUS* gene in the whole plant, rosette leaves, stems and roots of transgenic *pCAMBIA3301 Arabidopsis thaliana*; 5–8: expression of the *GUS* gene in the whole plant, rosette leaves, stems and roots of transgenic *pCAMBIA3301PGmRIQ2::GUS Arabidopsis thaliana*; 9–11: expression of the *GUS* gene at 4, 6 and 10 days after germination of transgenic *pCAMBIA3301PGmRIQ2::GUS Arabidopsis thaliana*.
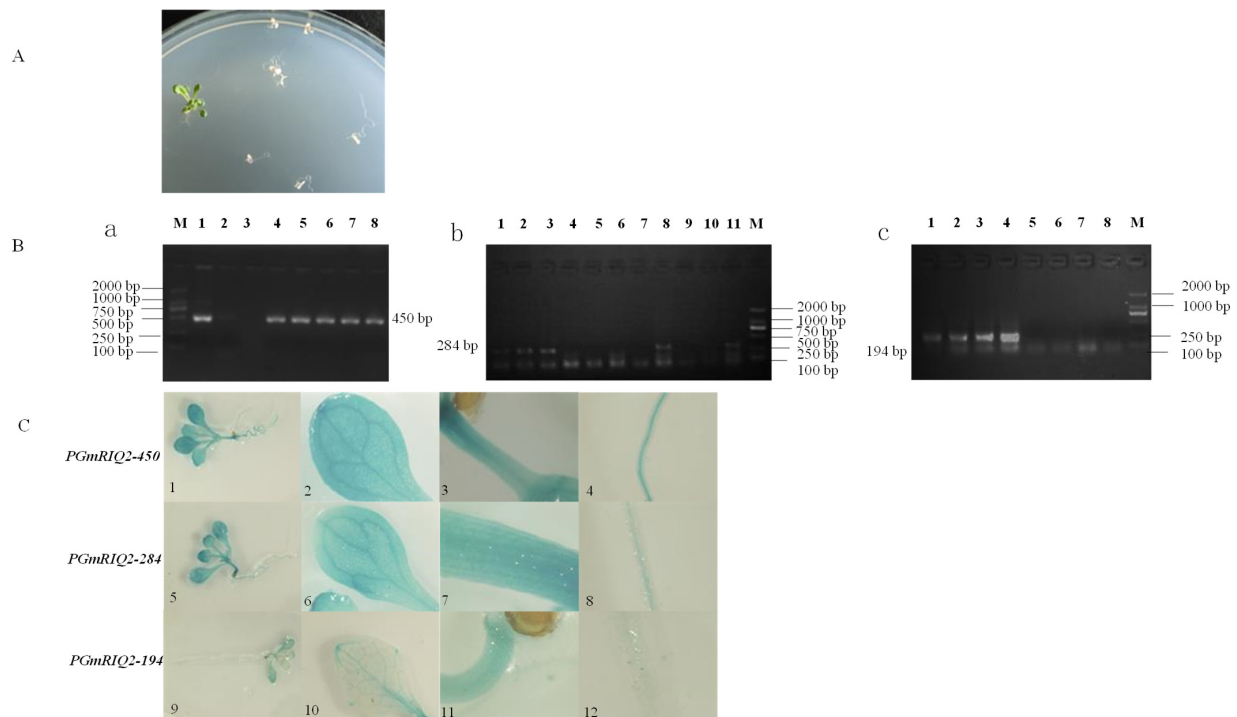
Figure 7. Detection of transgenic *Arabidopsis* with the *GmRIQ2* gene deletion fragment promoter and GUS staining. (**A**) PPT resistance screening. (**B**) a and c: 1–8 PCR amplification bands with annealing temperature of 56 °C; M: DNA marker (DL2000); b: 1–11 PCR amplification bands with annealing temperature of 56 °C. (**C**) GUS staining results. 1–4: expression of the *GUS* gene in the whole plant, rosette leaves, stems and roots of transgenic *pCAMBIA3301PGmRIQ2-450::GUS*; 5–8: expression of the *GUS* gene in the whole plant, rosette leaves, stems and roots of *pCAMBIA3301PGmRIQ2-284: GUS*; 9–12: expression of the *GUS* gene in the whole plant, rosette leaves, stems and roots of *pCAMBIA3301PGmRIQ2-194::GUS Arabidopsis thaliana*.
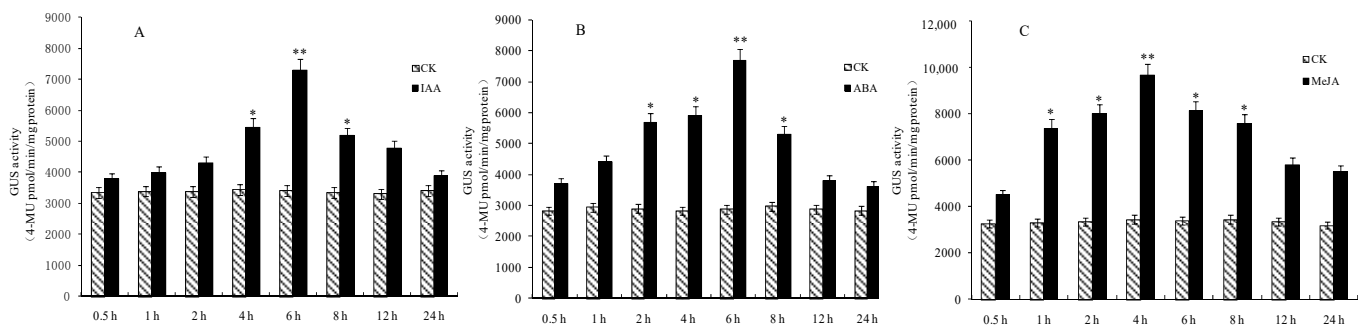
Figure 8. Determination of GUS activity in *Arabidopsis thaliana* with the full-length promoter of the *GmRIQ2* gene transformed by hormone treatment. (**A**) IAA: 50 μM; (**B**) ABA and (**C**) MeJA: 100 μM. The data are shown as the mean ± SE of three independent replicates. * and ** indicate significant difference at 0.05 and 0.01 levels, respectively.

3.5. Gene Expression of GmRIQ2 in Hormone-Treated Soybean

Soybean leaves were treated with hormones, and RNA was extracted from the leaves at different time points for fluorescence quantitative PCR analysis. The results showed that under the 50 μM IAA or 100 μM ABA treatment, the expression level of the *GmRIQ2* gene increased slightly at first and then decreased significantly with increasing treatment time (Figure 11A,B). As shown in Figure 11C, under treatment with 100 μM MeJA, the expression level of the *GmRIQ2* gene gradually decreased with increasing treatment time. This finding

indicated that the expression level of the *GmRIQ2* gene was negatively correlated with the treatment time. We conclude that the expression of the *GmRIQ2* gene is negatively regulated by IAA, ABA and MeJA.
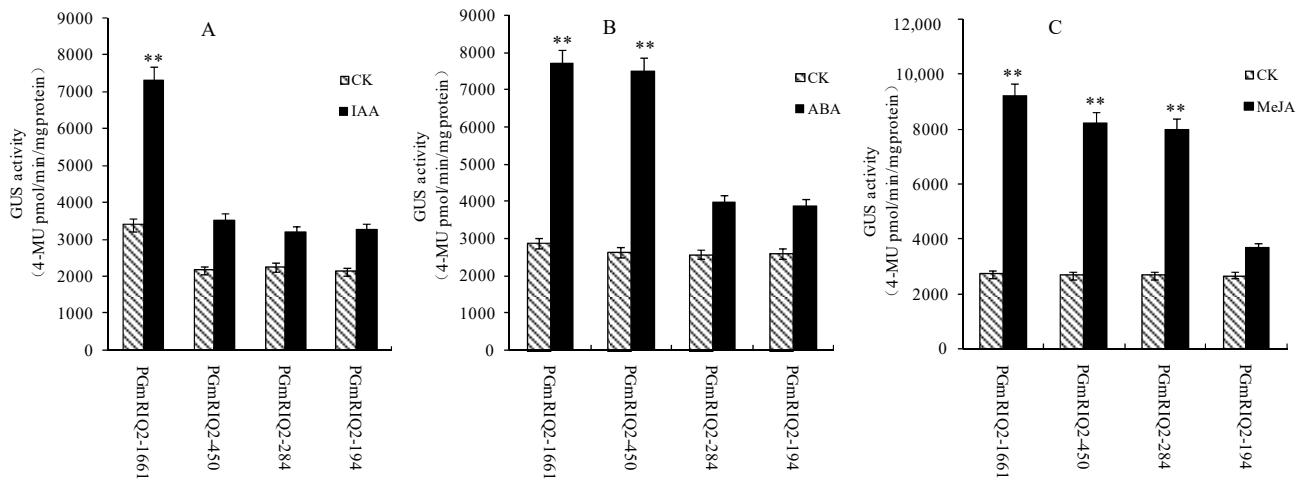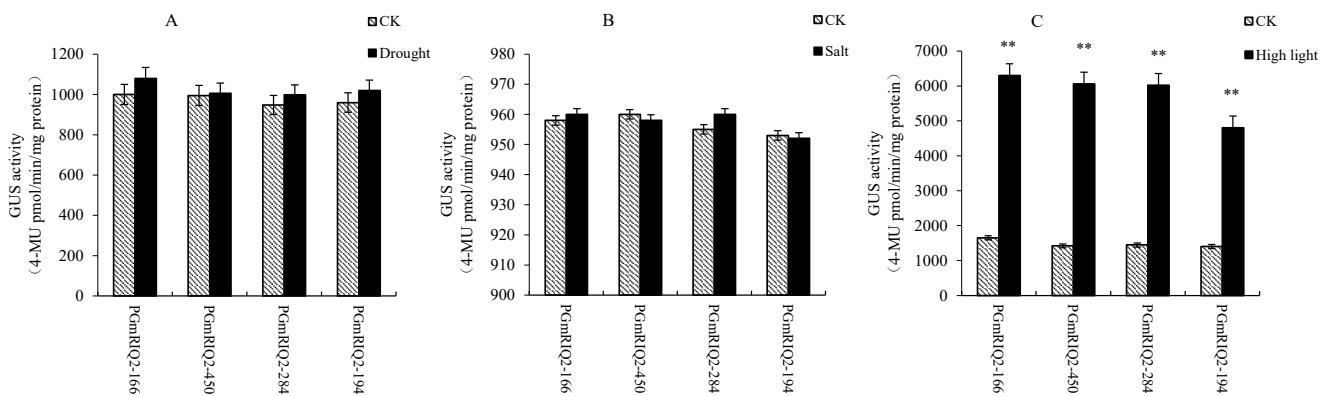

Figure 9. Hormone treatment after transformation of the *GmRIQ2* gene deletion fragment promoter in *Arabidopsis GUS*. (**A**) IAA: 50 μM; (**B**) ABA and (**C**) MeJA: 100 μM. The data are shown as the mean ± SE of three independent replicates. ** indicate significant difference at 0.01 levels.

Figure 10. Determination of GUS activity of the transformed *GmRIQ2* promoter (*PGmRIQ2*) in *Arabidopsis* under abiotic stress. (**A**) Drought: 250 mM mannitol; (**B**) salt: 200 mM NaCl; (**C**) high light: 300 μmol/m^2/s. The data are shown as the mean ± SE of three independent replicates. ** indicate significant difference at 0.01 levels.
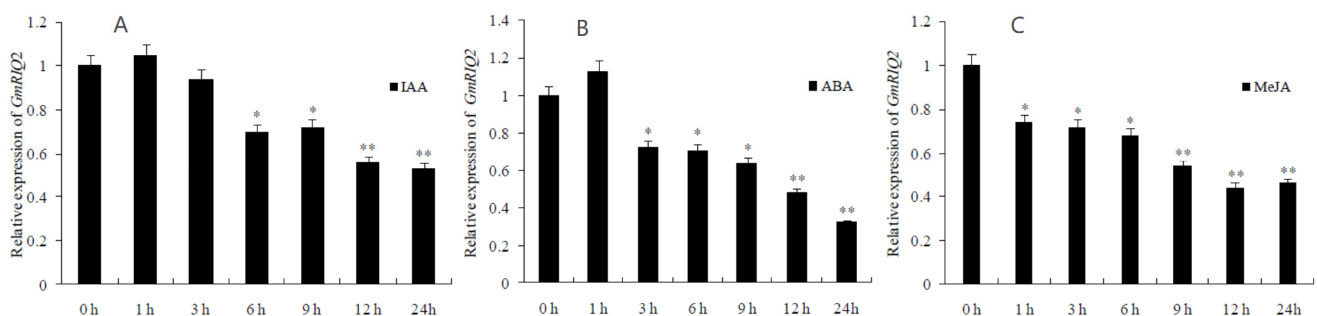

Figure 11. Effects of hormones on the expression levels of *GmRIQ2* in soybean KF16. (**A**) IAA: 50 μM; (**B**) ABA and (**C**) MeJA: 100 μM. The data are shown as the mean ± SE of three independent replicates. * and ** indicate significant difference at 0.05 and 0.01 levels, respectively.

4. Discussion

4.1. Application of the GUS Gene in Promoter Activity Analysis

The *GUS* gene is a commonly used reporter gene. Histochemical staining can be used for rapid and easy localization, and enzyme activity detection can be used for simple and sensitive quantitative analysis [31–34]. To study the function of *PGmRIQ2* in heterologous plants, *GUS* was used as a reporter gene. GUS histochemical staining showed that *PGmRIQ2* drove GUS activity in rosette leaves, stems and roots of transgenic *Arabidopsis thaliana*. The GUS enzyme activity test showed that *PGmRIQ2* was regulated by IAA, ABA, MeJA and light, but it did not respond to drought or high-salt stress. Previously, our research group transformed the *GmRIQ2* gene into soybean cotyledon nodes through *Agrobacterium tumefaciens*-mediated transformation to obtain transgenic overexpressing soybean lines. PEG-simulated drought treatment showed that overexpression of the *GmRIQ2* gene improved soybean drought resistance [23]. We speculate that although the predicted *PGmRIQ2* sequence does not contain regulatory elements responding to drought, related elements in other regulatory regions of the promoter may exist. This possibility needs to be further explored.

4.2. Construction of Promoter Deletion Fragments by the 5′ End Deletion Method

Stable expression is widely used for plant genetic transformation due to its ability to produce genetically stable offspring and reliable experimental results [35,36]. Use of the 5′ end deletion method to construct a series of promoter deletion fragments of different lengths enables analysis of the localization and function of regulatory elements [37]. The distribution and basic functions of the elements in the *PGmRIQ2* sequence were explored to provide a molecular basis for functional research on the *GmRIQ2* gene. In this study, the 5′ end deletion method and histochemical staining were used to verify that all three promoter deletion fragments had transcriptional activity and that the expression patterns were consistent with that of the full-length promoter. The deletion promoter with a length of only 194 bp still drove gene expression. After hormone and stress treatment, the GUS enzyme activity test showed that the IAA, ABA, and MeJA response elements were located between base pairs −1661~−450, −450~−284 bp and −284~−194 bp, respectively. The promoters were induced by light signals, consistent with the initial predicted element distribution. We preliminarily speculate that *PGmRIQ2* may be an inducible promoter. High-salt treatment did not significantly affect the enzyme activity of transgenic *Arabidopsis thaliana*, which indicates that the *PGmRIQ2* sequence does not contain elements that respond to high-salt stress, consistent with the predictions. However, whether the *GmRIQ2* gene is induced by high-salt stress needs to be explored in depth.

4.3. Sequence Analysis of PGmRIQ2

According to the results of bioinformatics prediction, the promoter of the *GmRIQ2* gene contains several basic promoter elements, suggesting that the promoter has a basic initiating function. In addition, a large number of regulatory elements are present. The effects of changing light intensity indicate that *PGmRIQ2* is induced by light signals, consistent with the finding that the predicted promoter sequence contains multiple light response elements. Therefore, light is likely to participate in the expression of the *GmRIQ2* gene as an important regulatory signal, which is consistent with the conclusions of previous studies showing that the *GmRIQ2* gene is regulated in response to light signals. Previous studies have also shown that the upstream sequence of the Phvul.009G190100 gene, which is homologous to the *Arabidopsis thaliana AtRIQ2* gene, contains several *cis*-acting elements involved in the light response. It is speculated that its activity may be regulated by light signals, and this characteristic may be closely related to the localization of the gene product in chloroplasts [22]. In the current study, the hormone treatment experiment also indicated that the promoter is induced by IAA, ABA, and MeJA. This finding is consistent with the prediction that the promoter sequence contains one AuxRR core, two ABREs, two CGTCA-motifs, and two TGACG-motif response elements. It is speculated that the

GmRIQ2 gene may also be induced and regulated by hormones. Previous studies have shown that the BnMicEmUp gene, which is homologous to the *Arabidopsis thaliana AtRIQ2* gene, is regulated by ABA. ABA signalling may activate gene expression during embryonic development. This provides a reliable basis for the induction of *GmRIQ2* gene expression by hormones [21]. These results are helpful for comprehensively understanding the molecular mechanisms and pathways of gene regulation and provide references for functional analysis of the *GmRIQ2* gene.

4.4. GmRIQ2 Gene Promoter Expression of Vascular Tissue Specificity

The constituent promoter is highly expressed due to its different decisiveness during the growth period of the plant. Thus, the application of constituent promoter in the plant gene engineering is very wide [8]. However, there is also a waste of metabolic accumulation and energy waste caused by excessive consumption, resulting in damage to the balance and genetic silence [10,12]. Therefore, researchers have focused on the inducible promoter and tissue-specific promoters, which were expected to replace the constitutive promoters. The inducible promoter regulates exogenous gene expression only in certain conditions or with certain factors and can be used to adjust according to people's expectations [3]. The tissue-specific promoters effectively alleviate excessive expressions of unnecessary waste by expressing in a specific period or place [13].

Li found that the transgenic *Arabidopsis thaliana* containing the lily MIR171a promoter drives *GUS* gene expression in the early stage of growth, but gradually does not express the *GUS* gene with the seedlings development. This promoter showed significant spatial and space specificity [38]. Bai et al. found that *GmbZIP33* promoter of soybean was a typical tissue-specific promoter, which was significantly expressed in the vascular tissue of the transgenic Arabidopsis, but its activity was weaker than the *CAMV35S* promoter [39]. The results of this study show that the activity of the soybean *PGmRIQ2* is slightly weaker than that of the *CaMV35S* strong promoter. The vascular tissue specificity of *PGmRIQ2* is not obvious in the transgenic soybean root system. However, there is obvious vascular tissue specificity in the vascular tissues of the rosette leaves, stems and roots of the transgenic *Arabidopsis thaliana*, especially the rosette leaf vascular tissues. And there is spatial and space specificity in the early growth of transgenic *Arabidopsis thaliana*. Promoter deletion fragments also show obvious advantages in vascular tissue expression, and their expression pattern is consistent with the full-length promoter. After analysis, no elements related to the promoter sequence and specific expression were found. This may be a phenomenon caused by the expression of the promoter in a heterologous plant, and it may also be affected by external environmental factors [40].

In this study, the three promoter deletion fragments were cloned based on the distribution of hormone-related elements. In subsequent studies, the three promoter deletions can be rescinded in conjunction with the new prediction method and converted to the plant to perform functional analysis. At present, there are several specific promoters that have been identified, which are generally used in anti-disease and anti-seepage genetic engineering [41]. However, the actual promoters applied are limited due to their lower expression efficiency and the restriction of heterologous expression [42]. Therefore, *PGmRIQ2* will provide a useful research direction and application prospects for the specific promoter of the vascular tissue.

5. Conclusions

In this research, the deletion method of 5′ end and tissue staining were used to verify that the three deletion promoter fragments of *PGmRIQ2* (*PGmRIQ2-450*, *PGmRIQ2-284* and *PGmRIQ2-194*) all have transcriptional activity, and the expression pattern is consistent with the full-length promoter of *PGmRIQ2*. Furthermore, these deletion promoter fragments all have the function of driving the expression of the reporter gene. After hormone or stress treatment, the GUS enzyme activity test confirmed that the response elements of IAA, ABA and MeJA are located between −1661~−450 bp, −450~−284 bp and −284~−194 bp,

respectively. At the same time, the *PGmRIQ2* is induced by light signals, and the *PGmRIQ2* has the characteristics of an inducible promoter. Our study provides an important reference for research on the molecular function and mechanism of the *GmRIQ2* gene and for further in-depth exploration of the regulatory network of its internal functional elements.

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