RESEARCH ARTICLE

Isolation and characterization of GmMYBJ3, an R2R3-MYB transcription factor that affects isoflavonoids biosynthesis in soybean

Mingzhu Zhao, Tianliang Wang, Ping Wu, Wenyun Guo, Liantai Su, Ying Wang, Yajing Liu, Fan Yan, Qingyu Wang*

Jilin Key Laboratory for Crop Genetic Engineering, College of Plant Science, Jilin University, Changchun, China

* qywang@jlu.edu.cn

Abstract

Isoflavonoids are secondary metabolites that play a variety of roles in plant-microbe interactions and plant defenses against abiotic stresses. Here we report a new MYB transcription factor (TF) gene, GmMYBJ3, that is involved in the isoflavonoids biosynthesis. The GmMYBJ3 gene is 1,002 bp long and encodes a protein of 333 amino acids. Amino acid sequence analysis showed that GmMYBJ3 is a typical R2R3 MYB TF. Yeast expression experiment demonstrated that GmMYBJ3 has its transcription activity in the nucleus and is transiently expressed in onion epidermal cells. The GmMYBJ3 gene was transformed into soybean and the expression activity of the GmMYBJ3 gene was significantly positively correlated with total isoflavonoid accumulation in soybean. Transient expression assays indicated that GmMYBJ3 can activate CHS8 expression. Furthermore, we analyzed the expressions of several genes known involved in the isoflavonoid biosynthesis, including CHS8, CHI1A, PAL1, IFS2 and F3H, in the GmMYBJ3 transgenic plants. The results showed that the expression levels of CHS8 and CHI1A were significantly increased in the transgenic plants compared to wild-type plants, but those of PAL1, IFS2 and F3H remained similar between the transgenic and wild-type plants. These results suggest that GmMYBJ3 participates in the isoflavonoid biosynthesis through regulation of CHS8 and CHI1A in soybean.

1. Introduction

Isoflavonoids are a group of plant natural secondary metabolites. Isoflavonoids are mainly found in the Papilionideae family, and are abundant in soybean, Glycine max (L.) Merr., and other legume species. They play a variety of roles in plant-microbe interactions, such as functioning as signal molecules for symbiosis between soybean and Bradyrhizobium japonica [1,2]. They also serve as phytoalexins in response to pathogen attacks [3,4] and abiotic stresses such as UV irradiation and drought [5–7]. Interest in these compounds has recently increased...
Isoflavonoids are synthesized through a branch of the phenylpropanoid pathway present in legumes. Chalcone establishes the first step in the branched pathway for the synthesis of flavonoids and isoflavonoids [10] and is generated in a reaction catalyzed by chalcone synthase (CHS). CHS is encoded by a single gene in *Arabidopsis thaliana*, but there are multiple copies in other plants, including petunia and soybean [11,12]. The soybean genome contains a gene family consisting of nine CHS gene members, designated from CHS1 to CHS9, with CHS1 having a duplicate [13]. Comparative gene expression analysis between soybean cultivars with contrasting seed isoflavonoid contents revealed a critical role of the CHS7 and CHS8 genes in isoflavonoid biosynthesis [14]. RNAi silencing of the CHS8 gene reduced the level of isoflavonoids in soybean hairy roots, providing a line of evidence that CHS8 is involved in regulation of isoflavonoid biosynthesis [15]. In the isoflavonoid biosynthesis, isoflavonoid synthase (IFS) is also necessary. The first key step of isoflavonoid biosynthesis is liquiritigenin and naringenin conversion to daidzein or genistein, which is catalyzed by IFS. Two IFS genes were identified in soybean, IFS1 and IFS2, and there are 14 amino acids that differ between their protein products. The expression of IFS1 in *A. thaliana* can induce the production of the isoflavone genistein in this non-legume plant [16].

The phenylpropanoid pathways are predominantly regulated at the transcriptional level by members of transcription factors, especially the MYB TFs [17–19]. Genetic markers linked to corresponding quantitative traits may be used to select for favorable alleles effectively, and was verified in some agronomic traits [20,21]. Therefore, we focus in this study on the MYB TFs that are mapped in the soybean isoflavonoid QTL. Even though interaction exists between MYB TFs and other factors to regulate several branches of the phenylpropanoid pathways, MYB proteins are believed to be the key components [18]. For example, *GmMYB176* affects isoflavonoid biosynthesis in soybean hairy roots through interaction with 14-3-3 protein in the nucleus [22]. The genes required for the synthesis of flavonoids and anthocyanins are induced by maize MYB C1 in tomato, except for F3H, F3’5H and CHI [23]. Introduction of CRC (a chimeric transcription factor of maize C1 and R) into soybean resulted in a significant increase in seed isoflavonoid levels [24]. *GmMYB39* down regulates the isoflavonoid biosynthesis in soybean [25]. MYB134 regulates proanthocyanidin synthesis in poplar [26]. Overexpression of *AtMYB12* in *Arabidopsis* displays a flavonol accumulation in transgenic lines [27].

In this study, we compared the isoflavonoid QTLs previously mapped [28–30] and found a common isoflavonoid QTL mapped with different populations in different environments. Only one R2R3 MYB TF, *GmMYBJ3*, was found in this common QTL, suggesting that it is likely a candidate gene for isoflavonoid biosynthesis. We associated the expression activity of *GmMYBJ3* with the isoflavonoid content in soybean. Subcellular localization assays in onion epidermal cells indicated that *GmMYBJ3* encodes a nucleus-localized protein and yeast hybrid analysis showed that it has transcriptional activity. Transient expression assays showed that *GmMYBJ3* could activate CHS8 expression. Overexpression of *GmMYBJ3* in the soybean transgenic plants up-regulates the expression level of CHS8 and CHI1A and increases the total isoflavonoid content.

### 2. Results

#### 2.1. Isolation and sequence analysis of *GmMYBJ3*

Two adjacent QTLs, GLY1 and qGm06, were previously mapped to Chromosome 6 that control soybean isoflavonoid content [31,32]. GLY1 were mapped to a region closely linked with SNP marker BARC-031337-07051 using a population consisting of 274 F5:8 RILs...
(recombining inbred lines) derived from Essex × Williams 82 and grown at three locations (Knoxville, TN; Harrisburg, IL; and Stuttgart, AR). The SNP of BARC-031337-07051 was physically mapped to the 16679945 position of the chromosome 6 (http://soybase.org/). The QTL qGm06 was mapped to an interval between BARC-063661-18416 and BARC-066175-19800 using a population consisting of 188 F7 RILs derived from Magellan × PI 437654 and grown at two locations (the University of Missouri Bradford Research and Extension Center, and the University of Missouri Delta Research Center). The SNP of BARC-063661-18416 was physically mapped to the 17230167 position and that of BARC-066175-19800 to the 18736894 position of Chromosome 6 (http://soybase.org/). Therefore, the region between BARC-031337-07051 and BARC-066175-19800, physically spanning from the 16679945 position to the 18736894 position, contains at least one of the two QTLs controlling soybean isoflavonoid content. Therefore, we searched this region carefully and found only one MYB transcription factor (TF) and the gene ID of this MYB TF is Glyma.06g193600 (Version: Glyma2.0). We designated this gene as GmMYBJ3. GmMYBJ3 has a full-length ORF of 1002 bp and was predicted to encode a protein of 333 amino acid residues with a calculated mass of 37.7 kDa and a pl of 5.81. The CDD v3.15 (https://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi) was used to search for the conserved domains located at amino termini and responsible for binding to DNA sequence. As shown in Fig 1, there are two MYB imperfect repeats, R2 and R3, which consist of 50 (13–63) and 48 (66–114) amino acids, respectively. Phylogenetic analysis (Fig 2) showed that GmMYBJ3 clustered with Arabidopsis AtMYB60 that regulates anthocyanin biosynthesis in lettuce [33]. Both anthocyanin and isoflavonoid are products of the phenylpropanoid metabolic pathway. Because GmMYBJ3 was found from the soybean isoflavonoid QTL region, it is homologous to AtMYB60, and its R2R3 participates in regulating the phenylpropanoid pathway, we hypothesized that GmMYBJ3 is involved in the transcriptional regulation of isoflavonoid biosynthesis.

2.2 Subcellular localization of the GmMYBJ3 protein

The GmMYBJ3 protein was localized in the nucleus by online prediction (http://www.csbio.sjtu.edu.cn/bioinf/plant/). To further confirm this localization result of the GmMYBJ3 protein, we deleted its stop codon and fused its ORF to the N terminus of GFP that is under control of the CaMV 35S promoter. The pBI121 constructs containing GmMYBJ3:GFP and containing GFP alone were transformed into onion epidermal cells, respectively, through Agrobacterium EHA105-mediated transient expression. The results showed that the GmMYBJ3:GFP fusion protein resided in the nucleus of the cells, which was consistent with the online predicted result, but the control construct containing GFP alone was visualized throughout the cells (Fig 3).

2.3 Transcriptional activation of GmMYBJ3 in vivo

The transcriptional activity of GmMYBJ3 was tested using the pGBKT7 vector that expresses proteins fused to the GAL4 DNA binding domain from the constitutive ADH1 promoter in the yeast GAL4 system (PT3248-5, Clontech, USA). The transcriptional activator protein would activate the HIS and lacZ reporter genes, thus allowing yeast to survive on the histidine-deficient medium and showing color in the β-galactosidase assay. The fusion plasmid pGBKTT7-GmMYBJ3 and the empty vector pGBKTT7 were transformed into yeast AH109 cells, respectively, and screened on the solid medium SD/-Trp so that the positive transformants will survive (Fig 4A and 4B). To further characterize the positive transformants, we streaked the transformants on the solid medium SD/-Trp/-His/-Ade plus 3-AT. The fusion plasmid cells survived on the SD/-Trp/-His/-Ade plus 3-AT plate, but the pGBKTT7 control plasmid cells did not. This result indicated that the fusion effectors were expressed and able to activate the
expression of the HIS reporter gene (Fig 4C). For the 5-bromo-4-chloro-3-indolyl \(\beta\)-D-galactopyranoside activation assay, strong blue signals were observed for the pGBKT7-\textit{GmMYBJ3} cells, reflecting that the \textit{lacZ} reporter gene was activated, whereas no colorful signal was detected for the pGBKT7 cells, indicating that no transcriptional activation occurred (Fig 4D).

### 2.4 \textit{GmMYBJ3} activates the transcription of \textit{CHS8} \textit{in vivo}

To find whether \textit{GmMYBJ3} activates \textit{CHS8} expression in soybean, we performed a transient expression experiment using tobacco leaves. A \textit{CHS8pro:GUS} fusion reporter construct, an
effector plasmid and a control plasmid were constructed (Fig 5A). The GUS activity of the 35Spro:GUS construct without the effector was higher than the GUS activity of the transformed CHS8pro:GUS construct in tobacco leaves. However, the co-transfection experiment with the effector and CHS8pro:GUS reporter constructs showed that the GUS activity was 1.26-fold higher than that of the 35Spro:GUS control plasmid and was 1.59-fold higher than that of the CHS8pro:GUS reporter plasmid. The GUS activity was significantly different between the CHS8pro:GUS construct and the CHS8pro:GUS construct co-transfected with the effector plasmid. From the results of the transient expression assay (Fig 5B), we noted that GmMYBJ3 highly likely trans-activated CHS8pro:GUS expression.

2.5 GmMYBJ3 is expressed in the tissues where isoflavonoids are synthesized in soybean

To confirm that GmMYBJ3 expresses in tissues where isoflavonoids accumulate, we investigated the expression of GmMYBJ3 in various tissues of soybean (Fig 6A) by quantitative RT-PCR and analyzed the correlation between its expression level and isoflavonoids content. The results showed that GmMYBJ3 expressed in all the tissues studied, but its expression level increased as embryos developed. Fig 6B shows the contents of isoflavonoids in tissues. Correlation analysis showed that the GmMYBJ3 expression level positively correlated with the total isoflavonoids accumulation in soybean (Pearson’s r = 0.674, P ≤ 0.05) (S1 Table). These results indicated that the expression activity of GmMYBJ3 was associated with the synthesis of isoflavonoids.

2.6 Overexpression of GmMYBJ3 enhances isoflavonoid content in transgenic soybean

To demonstrate the function of GmMYBJ3 in isoflavonoid biosynthesis, the overexpression of GmMYBJ3 was performed using cv. Jilin 35 as the host plant through the Agrobacterium-
mediated transformation of soybean embryonic tips (unpublished). The T₀ transgenic plants were selected on the medium containing Basta. Southern blots were prepared from the leaves of independent T₂ plants and hybridized to confirm the transgenic plants. The results showed that GmMYBJ3 was transformed into Jilin 35 cultivar (Fig 7A). Quantitative RT-PCR with the positive T₂ transgenic plants showed that the expression level of GmMYBJ3 was significantly increased in the transgenic plants compared to the WT control (Fig 7B) and the isoflavonoids content was measured by HPLC (Fig 7C). HPLC analysis showed that the glycitin, genistin and total isoflavonoid contents were significantly increased in the positive transgenic plants compared to the WT control, but the contents of daidzin, genistein and daidzein largely remained unchanged.

2.7 Expression of the genes known to be involved in the isoflavonoid biosynthesis in the seeds of transgenic plants

The enzymes, PAL1 (phenylalanine ammonia-lyase), CHS8 (chalcone synthase), IFS2 (isoflavone synthase) and CHI1A (chalcone isomerase), are known to catalyze the biosynthesis of isoflavonoids in soybean seeds [14,15,35], and F3H (flavanone-3-hydroxylase) is a competitive enzyme for anthocyanin synthesis in a branched fashion. Therefore, we further analyzed the expressions of the genes encoding these enzymes in the T₂ transgenic plants to infer the role of
The results showed that the CHS8 and CHI1A genes were significantly up-regulated in the seeds of the T2 transgenic plants. However, the expression levels of PAL1, IFS2 and F3H underwent little changes compared to the WT plants (Fig 8). These changes suggested that GmMYBJ3 may be a regulator of the isoflavonoid biosynthesis pathway in soybean seeds.

Fig 4. Transcription activation analysis of the GmMYBJ3 protein. (A and B) Yeasts containing the pGBK vector only and the pGBK-GmMYBJ3 construct screened on the solid medium SD/-Trp. (C) Yeasts containing the pGBK vector only and the pGBK-GmMYBJ3 construct grown on the solid medium SD/-Trp/-His/-Ade with 10 mM 3-AT. (D) 5-bromo-4-chloro-3-indoly/β-D-galactopyranoside activation detection of transformed yeast thalli. The red dashed lines in C and D represent the regions where the yeasts containing the pGBK vector only were streaked.
Fig 5. Transcriptional activation analysis of *GmMYBJ3* in tobacco leaves. (A) Schematic diagrams of the reporter, effector and control plasmid constructs used for the transient expression assays. (B) Transcriptional activation analyzed by GUS activity assay. The data are presented as the averages of three independent assays ± SD. ** represents significant difference at $P \leq 0.01$ between the CHS8pro:GUS construct and the CHS8pro:GUS construct co-transfected with the effector plasmid. +,– represent whether the reporter and/or effector plasmids were co-transfected or not.

https://doi.org/10.1371/journal.pone.0179990.g005
Fig 6. *GmMYBJ3* expression and isoflavonoids accumulation. (A) Expressions of *GmMYBJ3* in different tissues of soybean cv. Williams 82 determined by quantitative RT-PCR. The tissues are roots (R), stem (S), leaf (L), flower (F), pod wall (PW), and embryos collected at 20 days after pollination (E20), 30 days after pollination (E30), 40 days after pollination (E40) and 50 days after pollination (E50). (B) Total isoflavonoid content analysis in these tissues by HPLC. In both (A) and (B), the data are the mean ± SD of samples in three independent plants, with three technical replicates for each plant.

https://doi.org/10.1371/journal.pone.0179990.g006
Fig 7. Genetic transformation and analysis of GmMYBJ3 in soybean. (A) Southern blot analysis of T2 transgenic plants using the bar gene fragment as a probe. The DNAs of transgenic plants were digested with Hind III. The bands present in the transgenic lines, but undetected in the wild type were used to define the positive transgenic plants. P, pCB35SR1R2-GFP-GmM YBJ3 vector DNA used as a positive control; WT, wild type; OE, GmMYBJ3 overexpression lines, with 1–6 representing different transgenic lines. (B) Quantitative
3. Discussion

The MYB TFs, especially the group of R2R3-MYB, are known to be involved in the regulation of the phenylpropanoid metabolic pathway [36]. In this study, we have identified a MYB transcription factor from soybean, designated as \textit{GmMYBJ3}, and characterized it in its functions in the phenylpropanoid metabolic pathway. Analysis of the \textit{GmMYBJ3} gene indicates that it is a typical R2R3-MYB with conserved R2 and R3 motif, and gene phylogenetic analysis shows that it clusters with \textit{GmMYBJ2}, and is also homologous to \textit{AtMYB60}. Overexpression of \textit{GmMYBJ2} enhances stress tolerance in \textit{Arabidopsis thaliana} [37]. \textit{AtMYB60} regulates stomatal movements, plant drought tolerance [38] and anthocyanin biosynthesis in lettuce [33]. Pleiotropy is very common in MYB TF [39], and \textit{GmMYBJ3} may play different roles in different physiological and biochemical processes. Because \textit{GmMYBJ3} was identified from a genomic region containing a soybean isoflavonoid QTL, we speculate that \textit{GmMYBJ3} may be involved in regulating the isoflavonoid biosynthesis. The sub-localization of \textit{GmMYBJ3} reveals that it is a nuclear localization protein, which is in agreement with its role as a transcription factor. Most R2R3 MYB TFs are presumed to be transcriptional activators with activation domains (ADs) in the C-terminal region [40], but it is known that the N-termini of MYB TFs are conserved. One yeast hybrid analysis has confirmed that the \textit{GmMYBJ3} gene has a transcription activation domain, even though further tests in the plant system remain.

Analysis of gene expressions during soybean embryo development revealed the crucial roles of the \textit{CHS7} and \textit{CHS8} genes in isoflavonoid biosynthesis [14]. Further studies of the \textit{CHS7} and \textit{CHS8} promoters have indicated that \textit{CHS8} is involved in isoflavonoid biosynthesis during seed development [15]. In the transient expression experiments of this study using tobacco leaves, the co-transfection of the \textit{CHS8} gene with the effector construct demonstrated that the \textit{CHS8pro:GUS} construct was activated by \textit{GmMYBJ3}, and the GUS activity of the co-transfected was 1.59-fold higher than that of the \textit{CHS8pro:GUS} construct. In previous studies, \textit{GmMYBJ176} was found to trans-activate the \textit{CHS8pro:GUS} expression by 4.7-fold in \textit{Arabidopsis} leaf protoplasts compared to the \textit{CHS8pro:GUS} and the level of endogenous \textit{CHS8} transcripts was increased by 169-fold in soybean protoplast within 48 h [41]; and \textit{GmMYBJ12B2} trans-activated \textit{CHS8pro:GUS} expression by 2-fold in soybean calli [42]. In this study, although \textit{GmMYBJ3} activated a lower expression level of \textit{CHS8} than \textit{GmMYBJ176} and \textit{GmMYBJ12B2}, the co-transfection results indicate that \textit{CHS8} is one of the target genes for \textit{GmMYBJ3}.

This study has also shown that \textit{GmMYBJ3} expresses in the tissues where isoflavonoids are synthesized in soybean. The expression of \textit{GmMYBJ3} is most active in embryos and increases gradually as they develop, even it also expresses in other tissues including root, stem, leaf and floret. The correlation between \textit{GmMYBJ3} expression level and total isoflavonoids accumulation further demonstrates that \textit{GmMYBJ3} is involved in the isoflavonoid biosynthesis in soybean. Moreover, the roles of \textit{GmMYBJ3} in regulating isoflavonoid biosynthesis have been further confirmed by the overexpression analysis in the transgenic plants. The results of significantly increased total seed isoflavonoid content and individual isoflavones, glycitin and genistin, but not for daidzin, genistein and daizein in the transgenic plants compared to the wild
type provide a strong line of evidence in supporting the roles of GmMYBJ3 in isoflavonoid bio-
synthesis. Because levels of total seed isoflavonoids were increased only by 1.5-fold in the soy-
bean transgenic plants, which is only about half of those in the soybean seeds of the maize
chimeric CRC gene transgenic plants [24], the function of GmMYBJ3 is likely weaker than the
maize CRC in regulating the phenylpropanoid pathway. Furthermore, C1 is a R2R3 MYB TF
involved in anthocyanin accumulation in maize and must interact with R (bHLH TF) to be
effective [43], whereas AtMYB11, AtMYB12 and AtMYB111 all can regulate flavonol synthesis
by themselves [27,44]. Further study is required to determine whether GmMYBJ3 needs to
interact with other protein factors to enhance its function.

Previous study showed that overexpression of LjMYB14 up-regulated expression of PAL,
C4H, 4CL, IFS and IFR genes in Lotus [34], and silencing GmMYB176 down-regulated the
expression level of CHS8 and reduced the isoflavonoid content in soybean hairy roots [41]. We
speculate that GmMYBJ3 may affect soybean isoflavonoid biosynthesis through regulation of
the structural genes for enzymes in the biosynthesis pathway. Of the twelve members in the

Fig 8. Quantitative RT-PCR analysis of the key genes, PAL1, CHS8, IFS2, CHI1A and F3H, involved in the isoflavonoids
synthesis in the seeds of positive T2 transgenic lines. WT, wild type; OE, independent GmMYBJ3 overexpression lines. The
expression data of transgenic and wild-type lines represent the mean ± SD of three independent plants, with each plant having three
technical replicates. * and ** represent significant differences at P ≤ 0.05 and 0.01, respectively, between the transgenic lines and WT.

https://doi.org/10.1371/journal.pone.0179990.g008
CHI family, CHI1A, PAL1 and IFS2 are involved in seed isoflavonoid biosynthesis [35], and CHS8 plays a crucial role in isoflavonoid biosynthesis [14,15]. Our analysis of the PAL1, IFS2, CHS8 and CHI1A expression in the seeds of wild-type and transgenic plants indicates that GmMYBJ3 does not have effect on PAL1 and IFS2, but does have effect on CHS8 and CHI1A. This result suggests that GmMYBJ3 is able to activate the expression of CHS8. These results all indicate that GmMYBJ3 participates in isoflavonoid biosynthesis by up-regulating the expression levels of CHS8 and CHI1A in soybean seeds.

In conclusion, we have isolated a novel R2R3 MYB TF, GmMYBJ3, from soybean. GmMYBJ3 encodes a nucleus-localized protein and has a transcription activation domain. GmMYBJ3 activates the expression of CHS8, thus enabling to enhance the total and individual isoflavone contents, especially glycitin and genistin, in soybean seeds by up-regulating the genes encoding the enzymes involved in the isoflavonoid biosynthesis pathway.

4. Materials and methods

4.1 Plants materials

Soybean (Glycine max L.) cv. Williams 82 was used for expression analysis of GmMYBJ3 in various soybean tissues. The cultivar was planted in the Crop Experimental Station of Jilin University near Changchun, China. Roots, stems and leaves were sampled between the ternate leaf stage and before flowering; flowers were sampled at the flowering stage; embryos were tagged on the first day of pollination and then collected on the 20th, 30th, 40th and 50th day after pollination and pod wall was separated from the 50th day embryo. All of the tissues were collected randomly from three independent plants, quickly frozen in liquid nitrogen and then stored at −80˚C.

Cultivar Jilin 35 was used as the host plant for genetic transformation and overexpression analysis of GmMYBJ3 in soybean. The T2 transgenic plants generated and wild-type Jilin 35 were grown in the transgenic controlled field, Jilin University. The tissues for expression analysis of the genes were collected from the transgenic plants as described above.

4.2 Isolation and sequence analysis of the GmMYBJ3 gene

Isoflavonoid content QTL analysis allowed identification of a MYB TF (Glyma.06g193600) in the QTL region. Therefore, it was used an inquiry to search the soybean genome data (http://soybase.org/). A cDNA clone (GenBank accession number: KU664645) that is highly homologous to plant MYB TFs was identified and this cDNA clone was designated as GmMYBJ3. To reproduce the cDNA of the gene, total RNA was extracted from 50 d embryos of Williams 82 and reverse transcription polymerase chain reaction (RT-PCR) was performed using the primer pair, 5'-GAGCCCAAAGGGATCAAA-3' (forward) and 5'-CGACCTCAAGTCCGCTAC-3' (reverse) designed based on the cDNA sequence. The RT-PCR reaction contained 18.2 μL H2O, 1 μL of the first strand of cDNA, 1 μL (10 μM) of each primer, 2.5 μL of 10x PCR reaction buffer, 2 μL of dNTP mix and 0.3 μL of ExTa DNA polymerase (Takara, China). The PCR reaction program was 95˚C for 4 min, followed by 30 cycles (94˚C for 40 s, 58˚C for 40 s and 72˚C for 80 s) and 72˚C for 10 min at the final extension. The amplified fragments were cloned into the pMD-18T vector (Takara, China) and sequenced for confirmation. Phylogenetic analysis of the GmMYBJ3 gene with foreign genes was performed using MEGA 7 with the Neighbor-Joining (NJ) algorithm [45].

4.3 Subcellular localization assay

The coding region of GmMYBJ3 lacking a stop codon was ligated to the N-terminus of GFP to construct the pBI121-GmMYBJ3-GFP construct under the control of the cauliflower mosaic
virus 35S (CaMV 35S) promoter in the pBI121 vector. This construct was then introduced into the Agrobacterium tumefaciens Strain EHA105 and used for subcellular localization analysis with transient transformation in onion epidermal cells. The Agrobacterium culture was prepared as described by Yang et al. [46]. The onion cells were dipped in prepared Agrobacterium solution for 30 min and then plated on Murashige and Skoog (MS) medium and incubated at 25°C in the dark for 16–48 h. Hoechst No.33342 was used to stain the nuclei of the onion cells and the transformed onion cells were observed using a confocal microscope (Olympus, Japan).

4.4 Transactivation analysis in the yeast GAL4 system

The GmMYBJ3 coding sequence was cloned into the GAL4 DNA-BD binding domain in the pGBK7 vector. The transactivation assay (PT3024-1) was performed as described by its manufacturer (Clontech, USA). The pGBK7-GmMYBJ3 plasmid was transformed into the yeast strain AH109 by the lithium acetate-mediated method [47], and the transformants were screened on SD/-Trp at 28°C for 2 d. Then, the transformants from SD/-Trp were streaked onto the solid medium SD/-Trp/-His/-Ade plus 3-AT. The plates were incubated for 3 d until they were used for a β-galactosidase assay. For the β-galactosidase assay, the transformant cells were imprinted onto Whatman filter paper and lysed by freezing with liquid nitrogen for 10 s and then thawing at room temperature. This process was repeated 3 times to completely lyse the cells. The filter was incubated in 2.5 ml of Z buffer containing 0.8 mg of 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside supplemented with 21.51 g L⁻¹ Na₂HPO₄·12 H₂O, 6.22 g L⁻¹ NaH₂PO₄·H₂O, 0.75 g L⁻¹ KCl, and 2.46 g L⁻¹ MgSO₄·7H₂O at 30°C for 30 min to 8 h. The color reaction was monitored.

4.5 Transient expression

To construct the reporter plasmid, the 1577-bp CHS8 promoter (F: 5’-AGCTGAGCAAGTATACCAACC-3’; R: 5’-GAGGTTGAAATGAAGGTCG-3’) was amplified by PCR and cloned into the pCAMBIA1301 plasmid to replace the CaMV 35S promoter. To construct the effector plasmid, the full coding region of GmMYBJ3 was cloned into the pCB35SR1R2-GFP plasmid that was constructed as below and under the control of the CaMV 35S promoter. The reporter and the effector plasmids were introduced into the Agrobacterium EHA105 strain for further analysis. Agrobacterium-mediated transient expression was performed on the leaves of 4-week-old tobacco seedlings (Nicotiana tabacum cv. NC89) [46]. GUS activity analysis was performed after approximately 48 h [48]. All transfection experiments and GUS activity analysis were performed at least three times.

4.6 Quantitative real-time RT-PCR analysis

Total RNA was extracted from the soybean tissues sampled above using an RNAprep pure Plant Kit (Tiangen, China) and cDNA was synthesized using MMLV reverse transcriptase (Takara, China). Quantitative real-time RT-PCR analysis was performed with Applied Biosystems 7500 (Applied Biosystems, USA) using SYBR Premix Ex Taq (Takara, China). Each reaction contained 10 μL of SYBR Green I, 2 μL of cDNA samples, 0.4 μL of ROX Reference Dye II and 0.4 μL of 10 μM gene-specific primers for a final volume of 20 μL. The reaction was performed at 95°C for 30 s, followed by 40 cycles of 95°C for 5 s, 60°C for 34 s and 72°C for 30 s. Three biological replicates, with three technical replicates per biological replicate, were applied for the experiment. The soybean β-tubulin gene (GenBank accession No. GMU12286) was used as an internal control. The gene-specific primers used for real-time quantitative RT-PCR and their accession numbers are listed in Table 1. The data were analyzed using the comparative C_T method based on C_T values [49].
4.7 Isoflavonoid analysis by high-performance liquid chromatography (HPLC)

Approximately 0.5 g of dry soybean seeds was ground, mixed with 10 mL of 80% methanol in distilled water followed by sonication for 20 min, and subsequently incubated at 80˚C for 14 hours. The mixture was filtered through a 0.45-μm filter and transferred to 5-mL HPLC vials. Aliquots of this filtrate (20 μL) were utilized for HPLC analysis.

For HPLC, a Phenomenex C18 column (Shimadzu LC-20A, Japan, 250 mm × 4.6 mm, 5.0 μm) and binary gradient elution were used with solvent A (methanol, chromatography purity) and solvent B (distilled water). The ratio of solvent A was 15%–45% at 0–20 min, 45%–60% at 20–30 min, 60%–80% at 30–35 min, 80%–90% at 35–40 min, and 90%–15% at 40–45 min at a flow rate of 1 mL min⁻¹. The temperature of the column was maintained at 40˚C and the detector wavelength was set at 254 nm. Isoflavones were identified by comparison with authentic standards of daidzein, genistein, daidzin, genistin and glycitin (Sigma, St. Louis, MO, USA), and quantification of the isoflavones was carried out by reference to an external standard.

4.8 Plasmid construction and plant transformation

The GATEWAY cloning technology was used to construct the plant expression vector [50]. First, the GmMYBJ3 coding region was cloned into the entry vector pDONR221 (Invitrogen, USA) via a BP recombinant reaction. Second, the fragment in the entry vector was transferred to the destination vector, pCB35SR1R2-GFP, based on the LR recombinant reaction. The pCB35SR1R2-GFP-GmMYBJ3 construct was heat shocked into A. tumefaciens EHA105 and then transformed into embryonic tips of soybean cv. Jilin 35. The transformed soybean embryonic tips were grown at 25˚C/22˚C with a light/dark cycle of 16 h light and 8 h darkness and a relative humidity of 60%. Transgenic T₀ and T₁ plants were cultured in a phytotron at 28˚C/22˚C with a light/dark cycle of 16 h light and 8 h darkness and a relative humidity of 60%.

4.9 Southern blot analysis

Southern blot analysis was performed to detect transgenic plants. DNA was extracted from the young leaves of T₂ transgenic and wild-type plants (Jilin 35) using the CTAB method [51].
Approximately 10 μg of genomic DNA was digested with Hind III (Takara, China). The digests were separated on a 0.8% agarose gel and blotted onto a Hybond-N+ nylon membrane (Roche Applied Science, Germany). The 501-bp PCR fragments (amplified with primer 5'-ACCCACGTCATGCCAGTT-3' and 5'-CTAGGGGGATCTACCATG-3') containing the bar gene (phosphinothricin acetyltransferase gene) coding region was labeled with Digoxigenin (DIG)-high prime and used as a probe for hybridization. Probe labeling, prehybridization, hybridization, membrane washing and signal detection were performed according to the manufacturer’s instruction (Roche Applied Science).

4.10 Statistical analysis
Statistical analysis was completed using the SPSS 21.0 program. Differences were defined at the two-tailed significance level of $P \leq 0.05$.

Supporting information
S1 Table. The correlation between the GmMYBJ3 expression level and total isoflavonoid content. * represent significant ($P \leq 0.05$) correlation at the level of 2-tailed.

Acknowledgments
Thanks are given to Dr. Meiping Zhang, Jilin Agricultural University, for critically reading and editing this manuscript.

Author Contributions
Data curation: MZ YW.
Formal analysis: MZ.
Investigation: MZ WG YL.
Resources: PW.
Supervision: QW.
Validation: MZ TW.
Visualization: LS.
Writing – original draft: MZ FY.
Writing – review & editing: MZ FY.

References
1. Pueppke SG. The genetic and biochemical basis for nodulation of legumes by rhizobia. Crit Rev Bio-technol. 1996; 16(1):1–51. http://dx.doi.org/10.3109/07388559609146599 PMID: 8935908
2. Ferguson BJ, Mathesius U. Signaling interactions during nodule development. Journal of Plant Growth Regulation. 2003; 22(1):47–72. http://dx.doi.org/10.1007/s00344-003-0032-9
3. Aoki T, Akashi T, Ayabe S-I. Flavonoids of leguminous plants: structure, biological activity, and biosynthesis. J Plant Res. 2000; 113(4):475–88. http://dx.doi.org/10.1007/PL00013958
4. Dixon RA, Achinnine L, Kota P, Liu CJ, Reddy M, Wang L. The phenylpropanoid pathway and plant defence—a genomics perspective. Mol Plant Pathol. 2002; 3(5):371–90. http://dx.doi.org/10.1046/j.1364-3703.2002.00131.x PMID: 12056934
5. Dixon RA, Paiva NL. Stress-induced phenylpropanoid metabolism. The plant cell. 1995; 7(7):1085. http://dx.doi.org/10.1105/tpc.7.7.1085 PMID: 12242399

6. Gutierrez-Gonzalez JJ, Gutlikonda SK, Tran L-SP, Aldrich DL, Zhong R, Yu O, et al. Differential expression of isoflavone biosynthetic genes in soybean during water deficits. Plant and cell physiology. 2010; 51(6):936–48. http://dx.doi.org/10.1038/pcc.2009.86 PMID: 20430761

7. Caldwell CR, Britz SJ, Mirecki RM. Effect of temperature, elevated carbon dioxide, and drought during seed development on the isoflavone content of dwarf soybean [Glycine max (L.) Merrill] grown in controlled environments. J Agric Food Chem. 2005; 53(4):1125–9. http://dx.doi.org/10.1021/jf0355351 PMID: 15713029

8. Messina MJ. Legumes and soybeans: overview of their nutritional profiles and health effects. The American journal of clinical nutrition. 1999; 70(3):439s–50s. PMID: 10479216

9. Rochfort S, Panozzo J. Phytochemicals for health, the role of pulses. J Agric Food Chem. 2007; 55 (20):7981–94. http://dx.doi.org/10.1021/jf071704w PMID: 17784726

10. Caldwell CR, Britz SJ, Mirecki RM. Effect of temperature, elevated carbon dioxide, and drought during seed development on the isoflavone content of dwarf soybean [Glycine max (L.) Merrill] grown in controlled environments. J Agric Food Chem. 2005; 53(4):1125–9. http://dx.doi.org/10.1021/jf0355351 PMID: 15713029

11. Koes RE, Spelt CE, van den Elzen PJ, Mol JN. Cloning and molecular characteriz ation of the chalcone synthase multigene family of Petunia hybrida . Gene. 1989; 81(2):245– 57. https://do i.org/10.1016 /0378-1119( 89)90185- 6 PMID: 2806915

12. Todd JJ, Vodkin LO. Duplicat ions that suppress and deletio ns that restore expression from a chalcone synthase multigene family. The Plant Cell. 1996; 8(4):687–9 9. https://dx.doi.o rg/10.1105/tpc .8.4.687 PMID: 12239396

13. Tuteja JH, Vodkin LO. Structural features of the endogenous silencing and target loci in the soybean genome. Crop Sci. 2008; 48(Supplement_1):S -49–S-68. http://dx.doi.org/10.21 35/cropsc i2007.10.21 35cpg

14. Dhaubhadel S, Gijzen M, Moy P, Farhangkhoei M. Transcrip tome analysis reveals a critical role of CHS7 and CHS8 genes for isoflavonoid synthesis in soybean seeds. Plant Physiol. 2007; 143(1):326– 38. http://dx.doi.org/10.1104/pp.106.086306 PMID: 17098860

15. Yi J, Derynck MR, Chen L, Dhaubhadel S. Differential expression of CHS7 and CHS8 genes in soybean. Planta. 2010; 231(3):741–53. http://dx.doi.org/10.1007/s00042-009-0179-z PMID: 20016991

16. Jung W, Yu O, Lau S-MC, O’Keefe DP, Odell J, Fader G, et al. Identification and expression of isoflavone synthase, the key enzyme for biosynthesis of isoflavones in legumes. Nat Biotechnol. 2000; 18 (2):208–12. http://dx.doi.org/10.1038/72671 PMID: 10657130

17. Marles MS, Ray H, Gruber MY. New perspectives on proanthocy anidin biochemistr y and molecular reg- ulation. Phytochemistry. 2003; 64(2):367–83. http://dx.doi.org/10.1016/S0031-942 2(03)0037 7-7 PMID: 12943753

18. Stracke R, Werber M, Weisshaar B. The R2R3-M YB gene family in Arabidopsis thaliana. Curr Opin Plant Biol. 2001; 4(5):447–56. http://dx.doi.org/10.1016/S1369-5266(00)00199-0 PMID: 11597504

19. Weisshaar B, Jenkins GI. Phenylpropanoid biosynthesis and its regulation. Curr Opin Plant Biol. 1998; 1(3):251–7. http://dx.doi.org/10.1016/S1369-5266(98)00113-1 PMID: 9666590

20. Bommert P, Nagasawa NS, Jackson D. Quantitative variation in maize kernel row number is controlled by the FASCIATE D EAR2 locus. Nat Genet. 2013; 45(3):334–7. http://dx.doi.org/10.1038/ng.2534 PMID: 23377180

21. Huang X, Qian Q, Liu Z, Sun H, He S, Luo D, et al. Natural variation at the DEP1 locus enhances grain yield in rice. Nat Genet. 2009; 41(4):494–7. http://dx.doi.org/10.1038/ng.352 PMID: 19305410

22. Vadivel AKA, Sukumaran A, Li X, Dhaubhadel S. Soybean isoflavono ids: role of GmMYB176 interac- tome and 14-3-3 proteins. Phytochem Rev. 2016; 15(3):391–403. http://dx.doi.org/10.1007/s11101- 015-9431-3

23. Gómez-Maldonado J, Avila C, Torre F, Cañas R, Cánovas FM, Campbell MM. Functional interactions between a glutamine synthetase promoter and MYB proteins. The Plant Journal. 2004; 39(4):513–26. http://dx.doi.org/10.1111/j.1365-313X.2004.02153.x PMID: 15272871

24. Yu O, Shi J, Hession AO, Maxwell CA, McGonigle B, Odell JT. Metabolic engineering to increase isoflavone biosynthesis in soybean seed. Phytochemistry. 2003; 63(7):753–63. http://dx.doi.org/10.1016/S0031-9422(03)00345-5 PMID: 12877915

25. Liu X, Yuan L, Xu L, Xu Z, Huang Y, He X, et al. Over-expression of GmMYB39 leads to an inhibition of the isoflavonoid biosynthesis in soybean (Glycine max. L). Plant biotechnoloy reports. 2013; 7 (4):445–55. http://dx.doi.org/10.1007/s11101-013-0283-2

26. Meliway RD, Tran LT, Prouse MB, Campbell MM, Constabel CP. The wound-, pathogen-, and ultravio- let B-responsive MYB134 gene encodes an R2R3 MYB transcription factor that regulates
proanthocyanidin synthesis in poplar. Plant Physiol. 2009; 150(2):924–41. http://dx.doi.org/10.1104/pp.109.139071 PMID: 19395405

27. Mehrtens F, Kranz H, Bednarek P, Weisshaar B. The Arabidopsis transcription factor MYB12 is a flavon-specific regulator of phenylpropanoid biosynthesis. Plant Physiol. 2005; 138(2):1083–96. http://dx.doi.org/10.1104/pp.104.058032 PMID: 15923334

28. Primomo VS, Poya S, Ablett GR, Jackson C-J, Gijzen M, Rajcan I. Mapping QTL for individual and total isoflavone content in soybean seeds. Crop Sci. 2005; 45(6):2454–64. http://dx.doi.org/10.2135/cropsci2004.0672

29. Zeng G, Li D, Han Y, Teng W, Wang J, Qiu L, et al. Identification of QTL underlying isoflavone contents in soybean seeds among multiple environments. Theor Appl Genet. 2009; 118(8):1455–63. http://dx.doi.org/10.1007/s00122-009-0994-5 PMID: 19266178

30. Kassem MA, Meksem K, Iqbal M, Njiti V, Banz W, Winters T, et al. Definition of soybean genomic regions that control seed phytoestrogen amounts. BioMed Research International. 2004; 2004(1):52–60. http://dx.doi.org/10.1155/S1110724304304018 PMID: 15123888

31. Smallwood CJ. Detection of quantitative trait loci for marker-assisted selection of soybean isoflavone genistein. Master’s Thesis, University of Tennessee, 2012.

32. Gutierrez-Gonzalez JJ, Vuong TD, Zhong R, Yu O, Lee J-D, Shannon G, et al. Major locus and other novel additive and epistatic loci involved in modulation of isoflavone concentration in soybeans. Theor Appl Genet. 2011; 123(8):1375–85. http://dx.doi.org/10.1007/s00122-011-1673-x PMID: 21850478

33. Park J-S, Kim J-B, Cho K-J, Cheon C-I, Welham T, Hiraka H, et al. Transcription factors of Lotus: regulation of isoflavonoid biosynthesis requires coordinated changes in transcription factor activity. Plant Physiol. 2012; 159(2):531–47. http://dx.doi.org/10.1104/pp.112.194753 PMID: 22529285

34. Dastmalchi M, Dhaubhadel S. Soybean chalcone isomerase: evolution of the fold, and the differential expression and localization of the gene family. Planta. 2015; 241(2):507–23. http://dx.doi.org/10.1007/s00425-014-2200-5 PMID: 25385351

35. Vom Endt D, Kijne JW, Memelink J. Transcripton factors controlling plant secondary metabolism: what regulates the regulators? Phytochemistry. 2002; 61(2):107–14. http://dx.doi.org/10.1016/S0031-9422(02)00185-1 PMID: 12169302

36. Su L-T, Wang Y, Liu D-Q, Li X-W, Zhai Y, Sun X, et al. The soybean gene, GmMYB J2. Acta Physiologica Plantarum. 2015; 37(7):1–12. http://dx.doi.org/10.1007/s11738-015-1889-5

37. Cominelli E, Galbiati M, Vavasseur A, Conti L, Sala T, Vuylsteke M, et al. A guard-cell-specific MYB transcription factor regulates stomatal movements and plant drought tolerance. Curr Biol. 2005; 15(13):1196–200. http://dx.doi.org/10.1016/j.cub.2005.05.048 PMID: 16005291

38. Shirley BW, Kubasek WL, Storz G, Bruggemann E, Koornneef M, Ausubel FM, et al. Analysis of Arabidopsis mutants deficient in flavonoid biosynthesis. The Plant Journal. 1995; 8(5):659–71. http://dx.doi.org/10.1046/j.1365-313X.1995.008050659.x PMID: 8282787

39. Weston K. Myb proteins in life, death and differentiation.Curr Opin Genet Dev. 1998; 8(1):76–81. http://dx.doi.org/10.1016/S0959-437X(98)80065-8 PMID: 9529609

40. Yi J, Derynick MR, Li X, Telmer P, Marsolais F, Dhaubhadel S. A single-repeat MYB transcription factor, GmMYB176, regulates CHS8 gene expression and affects isoflavonoid biosynthesis in soybean. The Plant Journal. 2010; 62(6):1019–34. http://dx.doi.org/10.1111/j.1365-313X.2010.04214.x PMID: 20345602

41. Li X-W, Li J-W, Zhai Y, Zhao Y, Zhao X, Zhang H-J, et al. A R2R3-MYB transcription factor, GmMYB12B2, affects the expression levels of flavonoid biosynthesis genes encoding key enzymes in transgenic Arabidopsis plants. Gene. 2013; 532(1):72–9. http://dx.doi.org/10.1016/j.gene.2013.09.015 PMID: 24060295

42. Grotewold E, Chamberlin M, Snook M, Siame B, Butler L, Swenson J, et al. Engineering secondary metabolism in maize cells by ectopic expression of transcription factors. The Plant Cell. 1998; 10(5):721–40. http://dx.doi.org/10.1105/tpc.10.5.721 PMID: 9596332

43. Stracke R, Ishihara H, Huel G, Barsch A, Mehrtens F, Niehaus K, et al. Differential regulation of closely related R2R3-MYB transcription factors controls flavonol accumulation in different parts of the Arabidopsis thaliana seedling. The Plant Journal. 2007; 50(4):660–77. http://dx.doi.org/10.1111/j.1365-313X.2007.03078.x PMID: 17419845
45. Kumar S, Stecher G, Tamura K. MEGA7: Molecular Evolutionary Genetics Analysis version 7.0 for bigger datasets. Mol Biol Evol. 2016: msw054. http://dx.doi.org/10.1093/molbev/msw054 PMID: 27004904

46. Yang Y, Li R, Qi M. In vivo analysis of plant promoters and transcription factors by agroinfiltration of tobacco leaves. The Plant Journal. 2000; 22(6):543–51. http://dx.doi.org/10.1046/j.1365-313x.2000.00760.x PMID: 10886774

47. Gietz D, St Jean A, Woods RA, Schiestl RH. Improved method for high efficiency transformation of intact yeast cells. Nucleic Acids Res. 1992; 20(6):1425. PMID: 1561104

48. Jefferson RA. Assaying chimeric genes in plants: the GUS gene fusion system. Plant molecular biology reporter. 1987; 5(4):387–405. http://dx.doi.org/10.1007/BF02667740

49. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2−ΔΔCT method. methods. 2001; 25(4):402–8. http://dx.doi.org/10.1006/meth.2001.1262 PMID: 11846609

50. Karimi M, Inzé D, Depicker A. GATEWAY™ vectors for Agrobacterium-mediated plant transformation. Trends Plant Sci. 2002; 7(5):193–5. http://dx.doi.org/10.1016/j.plasms.2015.06.003 PMID: 11992820

51. Porebski S, Bailey LG, Baum BR. Modification of a CTAB DNA extraction protocol for plants containing high polysaccharide and polyphenol components. Plant molecular biology reporter. 1997; 15(1):8–15. http://dx.doi.org/10.1007/BF02772108