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Soybean flower-specific R2R3-MYB transcription factor gene *GmMYB108* induces anthocyanin production in *Arabidopsis thaliana*

Ju Yeon Moon[1†], Saet Buyl Lee[2†], Yu Jeong Jeong[1], Gah-Hyun Lim[3], Gilok Shin[1], Man-Soo Choi[4], Jeong Ho Kim[5], Ki Hun Park[5], Jiyoung Lee[1], Jae Cheol Jeong[1] and Cha Young Kim[1*]

Abstract

R2R3-MYB transcription factors (TFs) are known to play a key role in regulating the expression of structural genes involved in plant flavonoid biosynthesis. However, the regulatory networks and related genes controlling isoflavonoid biosynthesis in soybean are poorly understood. We previously reported that ethephon application increases the production of isoflavonoids in soybean leaves. In this study, we attempted to identify a potential regulatory gene that positively controls isoflavonoid production in response to ethephon treatment in soybean (*Glycine max* L.). RNA sequencing (RNA-seq) revealed that ethephon application led to the upregulation of 22 genes, including the genes for R2R3-MYB TFs, related to isoflavonoid biosynthesis in soybean plants. Ethephon treatment highly induced the expression of *GmMYB108*, and its expression was exclusively enriched in flowers as determined using in silico and real-time quantitative PCR analyses. Furthermore, *GmMYB108* overexpression resulted in an intense accumulation of anthocyanins as well as total flavonoid production in the leaf tissues of transgenic *Arabidopsis* plants. In addition, *GmMYB108* overexpression increased the transcript levels of several genes involved in the biosynthesis of anthocyanins and their regulatory pathways in *Arabidopsis*. These results suggest that *GmMYB108* is a potential positive regulator of the biosynthesis of flavonoids and anthocyanins in soybean flowers.

Keywords *Glycine max*, Anthocyanin, Flavonoid, Isoflavonoid, Transcription factor, Ethephon, Flower

Introduction

Soybean (*Glycine max* (L.) Merrill) is one of the world's most important legumes, as it is a major source of edible vegetable oil and protein. Physiological and genetic studies have suggested that the health benefits of soybeans are related to secondary polyphenolic metabolites, mainly anthocyanins, isoflavones, and phenolic acids, which are subclasses of flavonoids in plants [1–3].

Isoflavonoid compounds, including isoflavones and anthocyanins, are among the most effective natural antioxidants. Anthocyanins impart pink-to-purple color to diverse plant fruits, flowers, and vegetative tissues, depending on the vacuolar pH and chemical modifications; they are also important pigments in soybean seeds [4–7]. Although numerous studies have been conducted on flower pigmentation in petunia, *Arabidopsis*, and

[†]Ju Yeon Moon and Saet Buyl Lee have authors contributed equally to this work.

*Correspondence:
Cha Young Kim
kimcy@kribb.re.kr
[1] Biological Resource Center, Korea Research Institute of Bioscience Biotechnology (KRIBB), Jeongeup 56212, Republic of Korea
[2] Department of Agricultural Biotechnology, National Institute of Agricultural Sciences (NIAS), Rural Development Administration (RDA), Jeonju 54874, Republic of Korea
[3] Department of Biological Sciences, Pusan National University, Busan 46241, Republic of Korea
[4] Department of Southern Area Crop Science, Crop Production Technology Research Division, National Institute of Crop Science (NICS), Rural Development Administration (RDA), Miryang 50424, Republic of Korea
[5] Division of Applied Life Science (BK21 Plus), Gyeongsang National University, Jinju 52828, Republic of Korea

maize [7, 8] related studies in soybean have been limited. Understanding the regulation of flavonoid biosynthesis is important for developing crops rich in these secondary metabolites.

The accumulation of isoflavones or anthocyanins in plants occurs via multi-step enzymatic reactions in association with their biosynthetic genes in the flavonoid branch of the phenylpropanoid pathway [9–14]. In general, phenylalanine is transformed into *p*-coumaroyl-CoA by the enzyme activities of phenylalanine ammonia-lyase (PAL), cinnamic acid 4-hydroxylase (C4H), and 4-coumarate:CoA ligase (4CL); *p*-Coumaroyl-CoA and malonyl-CoA are then converted to the naringenin chalcone [2′,4,4′,6-tetrahydroxychalcone (THC)] by chalcone synthase (CHS), which is the initial rate-limiting enzyme in the flavonoid biosynthesis pathway [15]. Chalcone isomerase (CHI) can convert THC or isoliquiritigenin into naringenin and liquiritigenin, which are flavanones that act as common substrates for flavones, isoflavones, and anthocyanins. Isoflavone synthase (IFS) directs flavanones to the isoflavone biosynthesis pathway. In soybean, abundant isoflavones, including genistein, glycitein, and daidzein, are synthesized by the enzymatic action of IFS and hydroxyisoflavanone dehydratase (HID; [14, 16]. Malonyltransferase converts isoflavones to their corresponding malonylisoflavones (malonylgenistein, malonyldaidzein, and malonylglycitein). On the contrary, flavanone 3-hydroxylase (F3H) is the enzyme that most commonly competes with IFS for flavanones, common precursors of anthocyanins, flavanols, and proanthocyanidins [17]. This enzyme converts its substrate to the corresponding dihydroflavonols, which are transformed into anthocyanins with the help of dihydroflavonol 4-reductase (DFR) and anthocyanidin synthase (ANS), which are glycosylated under the control of flavonoid glycosyltransferases (UGTs) [10].

The major transcriptional regulator of flavonoid biosynthesis in various model plant species is a ternary protein complex (MBW) consisting of MYB, a basic helix-loop-helix (bHLH), and WD40-repeat proteins [18–20]. Of the four MYB protein groups, R2R3-type MYBs are primarily involved in regulating flavonoid biosynthesis in several plant species, including *Arabidopsis* and apple (*Malus domestica*) [21–31]. In *Arabidopsis*, three R2R3-type MYB TFs (AtMYB11, AtMYB12, and AtMYB111) with differential spatial expression patterns regulate the expressions of diverse enzymes such as CHS, CHI, and F3H [9, 32, 33]. In *Arabidopsis*, four major R2R3-MYB TFs—AtMYB75 (production of anthocyanin pigment 1, PAP1), AtMYB90 (PAP2), AtMYB113, and AtMYB114—are known to regulate flavonoid biosynthesis at different developmental stages in *Arabidopsis* leaves [22, 34]. Moreover, *Arabidopsis* MYB proteins (PAP1 and PAP2), bHLHs (transparent testa 8, TT8; glabra 3, GL3; and enhancer of glabra 3, EGL3), and the WD40 protein (transparent testa glabra 1, TTG1) interact and activate anthocyanin synthesis in vegetative tissues [35]. The transcripts of two R2R3-MYB genes—*MdMYB3* and *MdMYB10*—are expressed at higher levels in apple cultivars with red skin than those without red skin [36, 37]. Overexpression of *MdMYB10* induces anthocyanin production in both heterologous (tobacco) and homologous systems, depending on the coexpression of two TF genes, *MdbHLH3* and *MdbHLH33* [36, 37]. The ectopic expression of *MdMYB3* in *Nicotiana tabacum* results in a greater accumulation of pigments and anthocyanins in the flowers than in the wild type and contributes to the activation of *CHI*, *CHS*, *ANS*, *UFGT*, and *DFR* in transgenic tobacco plants. These reports suggest that R2R3-type MdMYB TFs are responsible for regulating anthocyanin biosynthesis and flower development. To date, a wide range of flower colors has been identified, with purple and white being the most common colors of soybean flowers. However, the research on flower pigmentation of soybean has been limited [4, 38].

Furthermore, we previously reported that the treatment with ethylene or ethephon (an ethylene biosynthetic *precursor)* induced high levels of isoflavonoid production, including daidzein, genistein, malonyldaidzin, and malonylgenistin in soybean leaves [39]. Therefore, to fully understand the regulatory networks and associated genes that control isoflavonoid biosynthesis in response to ethylene or ethephon treatment in soybean plants, it is first important to identify a key regulatory gene responsible for the biosynthesis of isoflavonoids in soybean.

Materials and methods

Plant materials, growth conditions, and elicitor treatment

The seeds of soybean (*G. max* (L.) Merrill, Daewon) were obtained from the National Institute of Crop Science, Miryang, Republic of Korea, and were cultivated in a controlled plant chamber at 23 ± 2 °C with 16 h light/8 h dark cycles, and 75% relative humidity. For ethephon treatment, soybean plants were cultivated for approximately 4 weeks up to the V4 growth stage. Four-week-old soybean plants were treated with 200 ppm ethephon as described previously [39]. Entire leaves were collected for further analysis at 0, 6, 12, and 24 h post-treatment with ethephon. For gene expression analysis in soybean tissues, soybean plants were cultivated for approximately 11 weeks up to the R6 growth stage. Total RNA was extracted from the shoots, roots, and leaves of 13-day-old soybean plants (VC stage); mature leaves and flowers of 7 week-old soybean plants (R2 stage); and developing pod

coats and seeds from plants at 25 days after flowering (R6 stage).

The seeds of *Arabidopsis thaliana* ecotype Columbia (Col-0) were surface-sterilized and grown vertically on half-strength Murashige and Skoog (MS) agar medium under long-day growth conditions (16 h light/8 h dark cycles) at 22 ± 2 °C as described previously [40]. Healthy seedlings were transplanted to soil on trays for further experiments.

Transcriptome (RNA-Seq) analysis of ethephon-treated soybean plants

The total RNA was isolated from soybean leaves treated with ethephon for 24 h as previously described [41, 42]. The quantity and quality of total RNA were determined using the DeNovix DS-11 spectrophotometer (DeNovix Technologies, Wilmington, DE, USA) and an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). An RNA-Seq library was constructed and sequenced on the Illumina HiSeq 2000 instrument at Theragen Etex Bio Institute (Suwon, South Korea). The quality of raw reads was evaluated using FastQC (v.0.11.9); the reads were filtered by eliminating poor-quality reads with Q < 20 from the raw reads. Clean reads with an average Q values of 20 were processed using the Tuxedo protocol [43]. Reads of each sample were aligned to the soybean reference genome sequence Wm82 genome browser (m82.a1.v1.1). Transcript expression value was quantified as fragments per kilobase of transcript sequence per million reads mapped (FPKM) and estimated fold change (FC). Differential gene expression analyses between the untreated (group 1) and ethephon-treated (group 2) soybean samples were performed using Cufflinks [44].

Transcripts with expression FC > 0 and FC ≤ 0 (cut-off set at $P < 0.05$) were considered significantly upregulated and downregulated genes, respectively, and are shown in the Scatter plot (Theragen Etex Bio Institute).

Generation of *GmMYB*-overexpressing transgenic *Arabidopsis plants*

The entire coding sequences of *GmMYB* genes were amplified from the soybean cDNA templates with the specific primers listed in Additional file 1: Table S1. The amplified coding regions of *GmMYB* genes were introduced to the NdeI/SpeI or BamHI/SpeI sites of the modified pGreenII0229 binary vector with a Flag tag under the control of a duplicated cauliflower mosaic virus (CaMV) *35S* promoter, resulting in *d35S*-Flag::GmMYBs, and the *d35S*-Flag::mYFP construct was used as a control [45]. Transgenic *Arabidopsis* plants were obtained using the *Agrobacterium*–mediated floral dip method [46]. The transgenic *Arabidopsis* plants were screened on 1/2 Murashige and Skoog (MS) basal medium with 50 mg·L^{-1} kanamycin. T1 generation plants were obtained from three batches of transformed *Arabidopsis* plants (T_0 lines).

RNA isolation, cDNA synthesis, and real-time quantitative RT-PCR (qRT-PCR)

Total RNA extraction, cDNA synthesis, and qRT-PCR were conducted as previously described [41, 42]. For qRT-PCR analysis, gene-specific primers from soybean and *Arabidopsis* plants are used (Additional file 1: Table S1). The qRT-PCR reaction conditions were as follows: first denaturation at 95 °C/15 min; 40 cycles of 95 °C/20 s, primer-specific annealing 55 °C/20 s, and extension 72 °C/20 s; and dissociation for a melting curve with 65 – 95 °C in 0.5 °C increment/5 s. Data were normalized against the expression levels of soybean *Actin11* and *Arabidopsis PP2A*, used as reference controls.

Quantification of anthocyanins, total flavonoids, and total phenolic compounds

The content of anthocyanins, total flavonoids, and total phenolic compounds was determined using the methods described previously [40–42]. The total anthocyanins were measured spectrophotometrically at wavelength 530 nm and 657 nm as described previously [40] The total flavonoid content and total phenolic content were determined at 510 nm and 725 nm with UV–Vis spectrophotometer, respectively, and calculated as catechin (CAT) and chlorogenic acid (CGA) equivalents using the methods described previously [42]. The isoflavone content was quantified according to the protocol described previously [39]. Briefly, collected soybean leaves (0.1 g of dry weight (DW)) were used for isoflavone extraction in 3 mL of 80% (v/v) ethanol. Isoflavone content was determined using the Agilent HPLC 1260 Series with the ZORBAX Bonus-RP (150 mm × 4.6 mm, 5 μm) column (Agilent Technologies). Each isoflavone was identified and quantified based on the respective standard. Measurement of total flavonoid content in methanol extracts of *Arabidopsis* leaves was performed as described previously [47] with slight modifications. The harvested leaves (1 g of fresh weight) were extracted in 3 mL of 80% (v/v) methanol. The sample solution was prepared by adding and incubating with 10% (w/v) $AlCl_3$ and 1 M NaOH in that order, followed by the addition of water. The absorption of extracts was measured immediately at 510 nm using a UV – visible spectrophotometer. Total phenolic content of the methanolic extracts was estimated using the Folin – Ciocalteu method with minor modifications [41]. The methanol extract was analyzed at 725 nm on a UV – visible spectrophotometer.

Anthocyanin content was quantified according to the protocol described previously [40].

Subcellular localization of GmMYB108::mYFP

The coding region of *GmMYB108* was translationally fused to mYFP at the C-terminus and inserted into the pGreenII0229 binary vector aforementioned, resulting in *d35S*-GmMYB108::mYFP; *d35S*-Flag::mYFP was used as a control. The recombinant constructs were used in an *Agrobacterium*-mediated transient expression assay with *N. benthamiana* as described previously [48]. For confocal microscopy observation, *N. benthamiana* leaves were examined 24 h after agro-infiltration using laser scanning confocal microscopy (FV1000; Olympus, Tokyo, Japan). YFP fluorescence was excited at 514 nm and detected at 524–550 nm. Images were performed with an ImageJ software.

Results

Identification of R2R3-type MYB TF genes expressed in response to ethephon application in soybean plants

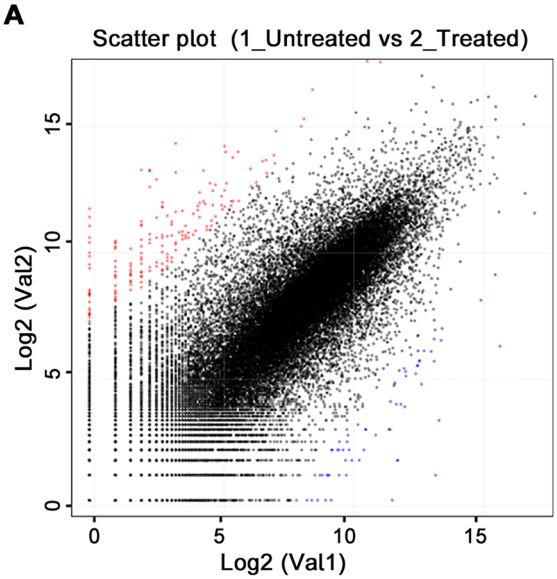
Ethephon-induced increase (24 h to 96 h) in the production of dietary isoflavones (daidzin, genistin, malonyldaidzin, and malonylgenistin) has been reported in soybean leaves [39]. In addition, the expression levels of isoflavonoid biosynthesis genes, including *CHS, CHI, IFS, HID, IF7GT,* and *IFMaT* were highly induced in the 24 h ethephon-treated soybean leaves. Ethephon treatment also increased the early accumulation of isoflavones in soybean leaves over time; isoflavone quantification in the leaves was greatly increased from 6 to 48 h after ethephon application (Additional file 1: Table S2). Therefore, to identify the key regulatory MYB TF genes that control the isoflavonoid biosynthesis in response to ethephon in soybean plants, transcriptome analysis was performed to quantify the differences in mRNA expression between untreated and 24 h ethephon-treated soybean plants (Theragen Bio, https://www.theragenbio.com). A total of 42,167 of 54,174 reads of mRNAs were normalized whose expressions were increased by one-fold or more ($\log_2$ expression ratio > 0-FC) or reduced by ≤ 0-fold in the ethephon-treated soybean leaves: 8,718 mRNAs were upregulated and 33,449 mRNAs were downregulated (Fig. 1A and Additional file 2: Table S3). The number of reads from the untreated (group 1) and ethephon-treated soybean leaves (group 2) was 10,211 and 10,183, respectively, and 9,472 reads overlapped (Fig. 1A and Additional file 1: Fig. S1). A total of 301 gene out of 42,167 reads were mapped to each characterized gene (Fig. 1B and Additional file 2: Table S3). Of these, 224 transcripts were upregulated and 77 were downregulated in group 2 in comparison with those in group 1 (Fig. 1B). Moreover, 51 transcripts were newly induced only in group 2. The expression of 15 transcripts was inhibited by ethephon application, and they were therefore present only in group 1. Among the induced transcripts, several *MYB* TF genes were identified together with isoflavonoid biosynthesis-related genes, such as soybean *CHS*, *IFS*, and isoflavone 7-O-uridine diphosphate glycosyltransferase (*IF7GT*), across the transcriptome data, and the accessions were modified according to a new version of the database (https://www.uniprot.org, https://ensembl.gramene.org; Fig. 1C).

As MYB proteins are among the largest plant TF families and have key regulatory functions in the biosynthesis of compounds derived from phenylpropanoids in plants, including soybeans, six R2R3-type MYB TF genes (*GmMYB12a*, *GmMYB75*, *GmMYB108*, *GmMYB84*, *GmMYB140*, and *GmMYB156*) were further examined using qRT-PCR analysis [19, 28, 49]. Our study confirmed increased transcript levels of all six *GmMYB* genes upon ethephon application at the early time points (6 h, 12 h, and 24 h), compared with the levels in the untreated soybean plants (Fig. 2). Overall, the transcript levels of the six *GmMYB* genes increased in response to ethephon treatment; of note, *GmMYB108* was induced more than 100-fold by ethephon treatment compared with that in the control.

GmMYB108 is exclusively expressed in soybean flowers

To explore the temporal and spatial expression for the six *GmMYB* genes in soybean plants, gene expression profiles were searched using the online website soybean eFP Browser (https://bar.utoronto.ca/eplant_soybean) [50]. Interestingly, *GmMYB108* (*Glyma.20g117000*) was predicted to be exclusively expressed in the flowers, whereas *GmMYB12a* (*Glyma.03g221700*) showed high expression in the roots, and *GmMYB140* (*Glyma.09g167900*) and *GmMYB156* (*Glyma.19g260900*) displayed higher expression in the leaves than in the flowers (Additional file 1: Fig. S2). Although *GmMYB75* (*Glyma.14g066200*) and *GmMYB84* (*Glyma.05g234600*) were expected to be predominantly expressed in floral organs, they were also expressed at lower levels in the roots and leaves. Among them, to confirm the results obtained from the soybean eFP Browser, we further investigated the expression patterns of *GmMYB108* in soybean tissues or organs at different developmental stages using qRT-PCR of RNA samples from the shoots, leaves, and roots at VC stage; mature leaves and flowers of 7 week-old soybean plants (R2 stage); and developing pod coats and seeds from plants at 25 days after flowering (R6 stage). Consistent with the results of digital gene expression analysis (Additional file 1: Fig. S2), *GmMYB108* expression was higher in soybean flowers than in other tissues, such as the shoots, leaves, roots, or seeds (Fig. 3). These results

A



B

Group 1 (Val1)	Group 2 (Val2)	Genes		
		Sum	Up (Group 2 only)	Down (Group 1 only)
Untreated	Treated	301	224 (51)	77 (15)

cutoff : p-value < 0.05

C

No	Accession	Name	Val1	Val2
1	Glyma.09g075200	CHS6	0.88	318.21
2	Glyma.01g228700	CHS7	64.79	10251.5
3	Glyma.11g011500	CHS8	2.05	106.9
4	Glyma.20g241700	CHI	7	988.44
5	Glyma.02g042500	CHIA1	6.13	1672.65
6	Glyma.10g292200	CHI1B2	4.38	691.21
7	Glyma.20g241600	CHI1B1	0.96	7.25
8	Glyma.07g202300	IFS1	2.72	34.51
9	Glyma.13g242000	IFS2	268.81	40493.3
10	Glyma.16g175600	IF7GT	9.4	14.78
11	Glyma.16g175900	RhGT1	1.74	3.63
12	Glyma.13g056100	IF7MAT	5.17	83.87
13	Glyma.03g221700	MYB12a	0.03	0.54
14	Glyma.19g218800	MYB12b	0.03	0.03
15	Glyma.14g067500	MYB75	2.32	4.71
16	Glyma.02g254200	MYB83	3.79	6.48
17	Glyma.05g234600	MYB84	4.38	1720.44
18	Glyma.20g117000	MYB108	0	94.42
19	Glyma.10g048500	MYB118	6.13	1552.59
20	Glyma.09g167900	MYB140	25.39	6653.31
21	Glyma.19g260900	MYB156	32.4	19165
22	Glyma.08g042100	MYB184	1.75	553.66

cutoff : p-value < 0.1

Fig. 1 Transcriptome analysis of soybean plants treated with ethephon. **A** Scatter plot of the transcript profiles of soybean plants. Each point on the plot represents the expression data of a single gene in ethephon-treated soybean plants compared with those in the untreated plants. X-axis: log ratio of expression for the ethephon-treated soybean plants (group 2); Y-axis: log ratio of expression for the untreated soybean plants (group 1). Red and blue dots denote genes that were significantly upregulated and downregulated in group 2 compared with those in group 1 plants, respectively. $P < 0.05$. **B** Table representing the counts of transcripts with altered levels after ethephon treatment in **A**. **C** List of isoflavone biosynthesis-related genes selected in this study. The transcript levels of genes were compared between the untreated control (Val1) and the ethephon-treated samples (Val2)

suggest that *GmMYB108* is exclusively expressed in flowers. Thus, the flower-specific gene expression pattern indicates that *GmMYB108* may regulate flavonoid biosynthesis in soybean flowers.

Overexpression of *GmMYB108* promotes anthocyanin accumulation in *Arabidopsis* plants

Because many R2R3-type MYB proteins have been identified as major transcriptional regulators of flavonoid biosynthesis in various model plant species [18–20], we further investigated six R2R3-type MYB genes (*GmMYBs*) selected from the transcriptome analysis results shown in Fig. 1. To identify the key *GmMYB* genes involved in the regulation of flavonoid biosynthesis in soybean plants, we used *Arabidopsis* plants as a model system to overexpress the six *GmMYB* genes *in planta*. T_1 generation plants were obtained from three batches of transgenic *Arabidopsis* plants (T_0 lines). Interestingly, the *GmMYB108*-overexpressing transgenic *Arabidopsis* lines (*GmMYB108-OE*) from the T_1 and T_2 generations displayed high accumulation of intense purple pigment in rosette leaves with a curved shape, especially on the underside, and exhibited more severe growth defects, such as smaller leaf and shorter stems than the control plants (Fig. 4A and Additional file 1: Fig. S3). However, the other five lines, *GmMYB12a-OE*, *GmMYB75-OE*, *GmMYB84-OE*, *GmMYB140-OE*, and *GmMYB156-OE* exhibited no prominent phenotypes (data not shown); therefore, we focused on the *GmMYB108-OE* line for further analysis. Three independent lines (#1, #7, and #9) with different phenotypic intensities were selected from the *GmMYB108-OE* T_1 generation. The strongest phenotypic effects were observed in line #7, in which purple pigment accumulation and growth defects were more pronounced than those in the other lines (#1 and #9) and the control. Unfortunately, most *GmMYB108-OE* transgenic plants failed to develop into mature plants, eventually died, and were lethal without seed formation. As it is well known that anthocyanin is responsible for the blue-to-black coloration of various plant parts, we investigated whether the pigmentation induced by *GmMYB108* overexpression led to anthocyanin accumulation in the

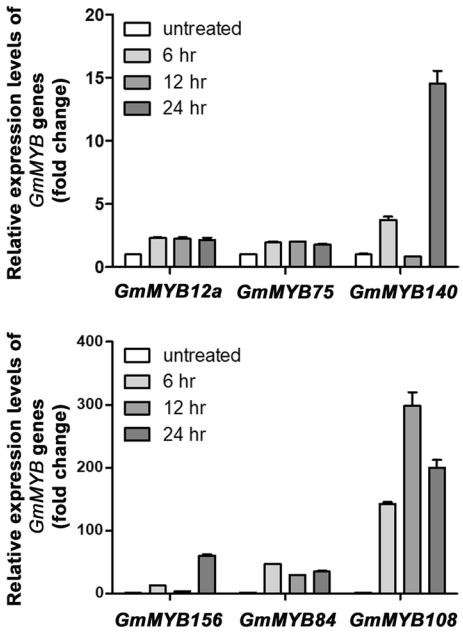


Fig. 2 Relative transcript levels of *GmMYBs* in soybean plants in response to ethephon treatment. Fold change in *GmMYB* expression in ethephon-treated soybean leaves at 6, 12, and 24 h relative to that in the untreated plants. Data were normalized against *GmActin11* expression and Log2 FC (LogFC) values were generated for the qPCR samples by comparing the expression of the genes in the ethephon-treated soybean plants to the untreated plants using the $2^{-\Delta\Delta Ct}$ method. Data are represented as mean ± standard deviation (SD) of three independent experiments

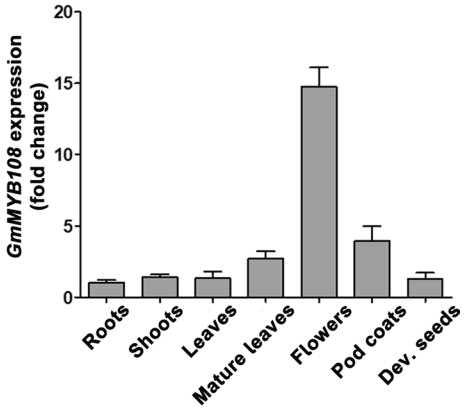


Fig. 3 Expression analysis of *GmMYB108* in soybean tissues. qRT-PCR analysis of *GmMYB108* in vegetative flowers, seeds, and pod coats at different developmental stages of soybean plants. Roots, shoots, and leaves were collected at the VC stage; mature leaves and flowers were harvested from 7 week-old plants (R2 stage); and developing pod coats and seeds from plants at 25 days after flowering (R6 stage). *GmActin11* was used as a reference gene. Data (mean ± SD) are representative of 3 technical and 3 biological replicates

transgenic *Arabidopsis* plants. As shown in Fig. 3B, optical microscopy was performed on the rosette leaves of the T_1 generation lines (#1 and #9), and the reddish color of the cytoplasmic sites of epidermal cells suggested higher anthocyanin accumulation in the transgenic *Arabidopsis* plants. To further validate the effect of the upregulated *GmMYB108*, we measured the content of secondary metabolites, including anthocyanin and flavonoid content, in *GmMYB108-OE* transgenic *Arabidopsis* plants (T_2 generations of *GmMYB108-OE* #1). The total flavonoid, phenolic, and anthocyanin content increased by 2.9-, 1.8-, and 5.3-fold in the *GmMYB108-OE Arabidopsis* plants, respectively, compared with those in the controls (Fig. 4C). A significant increase in anthocyanin content demonstrated the effects of *GmMYB108* overexpression on anthocyanin and flavonoid biosynthesis in *GmMYB108-OE* transgenic *Arabidopsis* plants.

Overexpression of *GmMYB108* alters the expression of several genes involved in anthocyanin biosynthesis and their regulation in *Arabidopsis* plants

To determine how *GmMYB108* regulates anthocyanin accumulation, the expression of specific genes involved in anthocyanin biosynthesis and their regulation were investigated using qRT-PCR analysis of *GmMYB108-OE* lines. qRT-PCR was performed using the T_2 generation lines (OE #1–1 and OE #1–18) and a control plant harboring EV (Additional file 1: Fig. S3). Despite the different expression levels between the lines, the expression of flavonoid and anthocyanin biosynthetic genes (*CHS*, *CHI*, *F3H*, *DFR*, *ANS*, and *UF3GT*) increased, together with the upregulation of *GmMYB108* expression in *GmMYB108-OE* lines (#1–1 and #1–18; Fig. 5A). Interestingly, the different expression levels between the two T_2 lines (#1–1 and #1–18) likely corresponded to the phenotypic analysis results, with greater anthocyanin accumulation in *GmMYB108-OE* line OE #1–1 and less in OE #1–18, regardless of the expression levels of *GmMYB108* in the two transgenic lines (Fig. 5B). Moreover, the expression of three R2R3-MYB TF genes, *MYB11*, *MYB12*, and *MYB111*, increased in the *GmMYB108-OE* lines compared with the EV control. These three genes are known to activate early flavonoid/anthocyanin biosynthetic genes, such as *CHS*, *CHI*, and *F3H* but not late genes [33, 51]. In contrast, the expression of late flavonoid and anthocyanin biosynthetic genes, including *DFR*, is transcriptionally regulated by the MBW complex. As shown in Fig. 5B, the expression of *MBW* (*MYB/bHLH/WD40*) genes, including *MYB75* (*PAP1*), *MYB90* (*PAP2*), *MYB113*, *MYB114*, *TT8*, *GL3*, and *EGL3*, was upregulated in *GmMYB108-OE* lines. Additionally, *GmMYB108* overexpression downregulated the R3-MYB TF gene *MYB-like 2* (*MYBL2*; Fig. 5B), which is a negative

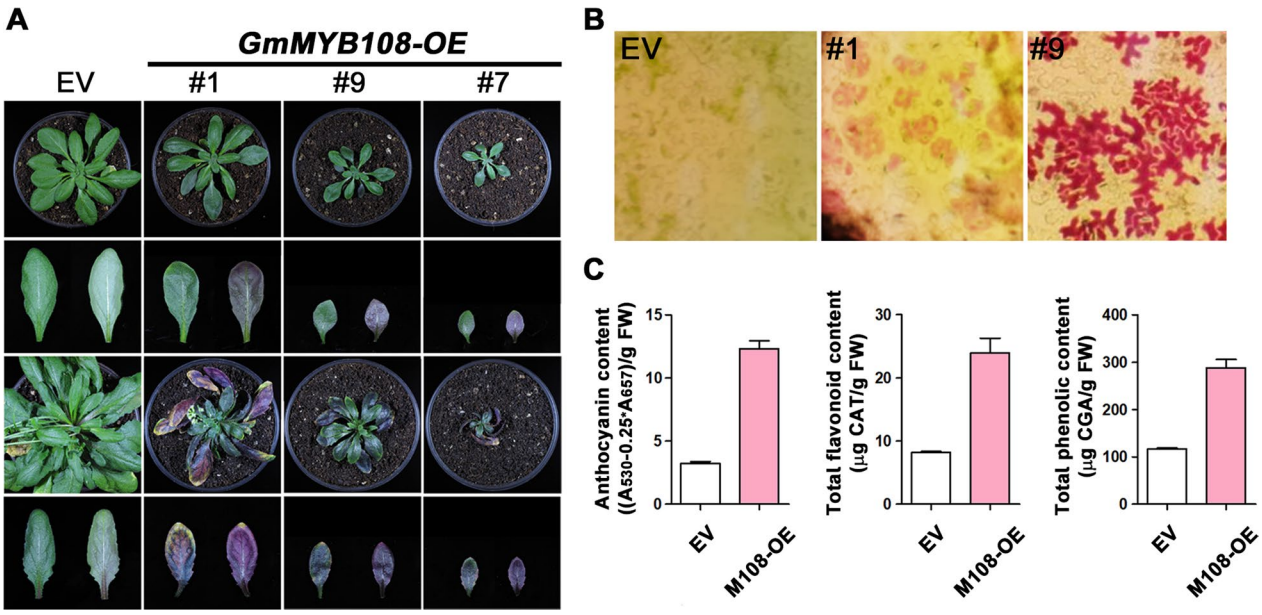


Fig. 4 Phenotypes of *GmMYB108-OE* transgenic *Arabidopsis* plants. **A** Rosette-leaf morphology in 38-day-old (two upper panels) and 2 month-old (two lower panels) *Arabidopsis* plants. **B** Dissecting microscopy of the epidermis of the *GmMYB108-OE* transgenic *Arabidopsis* rosette leaves. The red mosaic cells were abundant in the anthocyanin-accumulating cells of *GmMYB108-OE* transgenic *Arabidopsis* plants (#1 and #9), but the red color was almost absent in the control (EV). **C** Content of secondary metabolites of anthocyanins, total flavonoids, and total phenolic compounds in *GmMYB108-OE* transgenic *Arabidopsis* plants (T_1 generation). The analyses were performed by spectrophotometric quantification. Total flavonoid content are expressed as micrograms of CAT equivalent per gram of FW (μg of CAT/g FW) and total phenolic content are expressed as micrograms of CGA equivalent per gram of FW (μg of CGA/g FW). The values are expressed as mean ± SD of two technical repeats

regulator of anthocyanin biosynthesis in *Arabidopsis* [52]. These results suggest that *GmMYB108* overexpression activates the expression of anthocyanin biosynthetic genes in transgenic *Arabidopsis* plants, leading to increased accumulation of the anthocyanin pigment.

GmMYB108 is a nuclear protein

To verify the subcellular localization of GmMYB108, constructs harboring *d35S*-GmMYB108::mYFP or *d35S*-Flag::mYFP as control were transiently transformed into the leaf epidermal cells of *N. benthamiana* plants via *Agrobacterium*-mediated expression. As expected, mYFP fluorescence was ubiquitously distributed in the nucleus and cytoplasm, whereas fluorescence from GmMYB108-mYFP was detected exclusively in the nucleus of *N. benthamiana* epidermal cells, indicating that GmMYB108 is a nuclear protein (Fig. 6).

Discussion

Although a variety of MYB TF genes involved in the flavonoid biosynthetic pathway have been identified and characterized in various plant species, including *Arabidopsis*, *Medicago*, and soybean, the regulatory mechanisms of the structural genes involved in flavonoid biosynthesis have remained elusive. We previously reported that ethephon treatment strongly induced the production of total isoflavone content by transcriptional activation of the isoflavonoid biosynthesis genes in soybean leaves. The levels of total flavonoids and total phenolics were also increased by ethephon treatment [39]. Thus, to better understand the regulatory mechanisms of isoflavonoid biosynthesis in soybean, it is necessary to find out the upstream regulatory MYB TF genes that control the isoflavonoid biosynthesis by upregulating downstream isoflavonoid biosynthesis genes in soybean plants in response to ethephon.

Herein, we provide novel insights into the function of the soybean R2R3-MYB gene *GmMYB108* in flavonoid/anthocyanin biosynthesis. To identify the key regulatory R2R3-MYB gene responsible for the isoflavonoid biosynthesis, we employed ethephon as an inducer of isoflavonoid production in soybean plants and identified six R2R3-type MYB genes, as well as multiple genes encoding isoflavonoid-related enzymes, owing to their increased transcriptional levels (Figs. 1, 2). The upregulation of the six *GmMYB* genes in response to ethephon treatment suggests that these genes could be responsible for the accumulation of dietary isoflavones in soybean leaves by positively regulating isoflavone biosynthesis. In particular, *GmMYB108* exhibited more than 100-fold upregulation within 6 h of ethephon application—a pronounced increase over the fold change of the other five

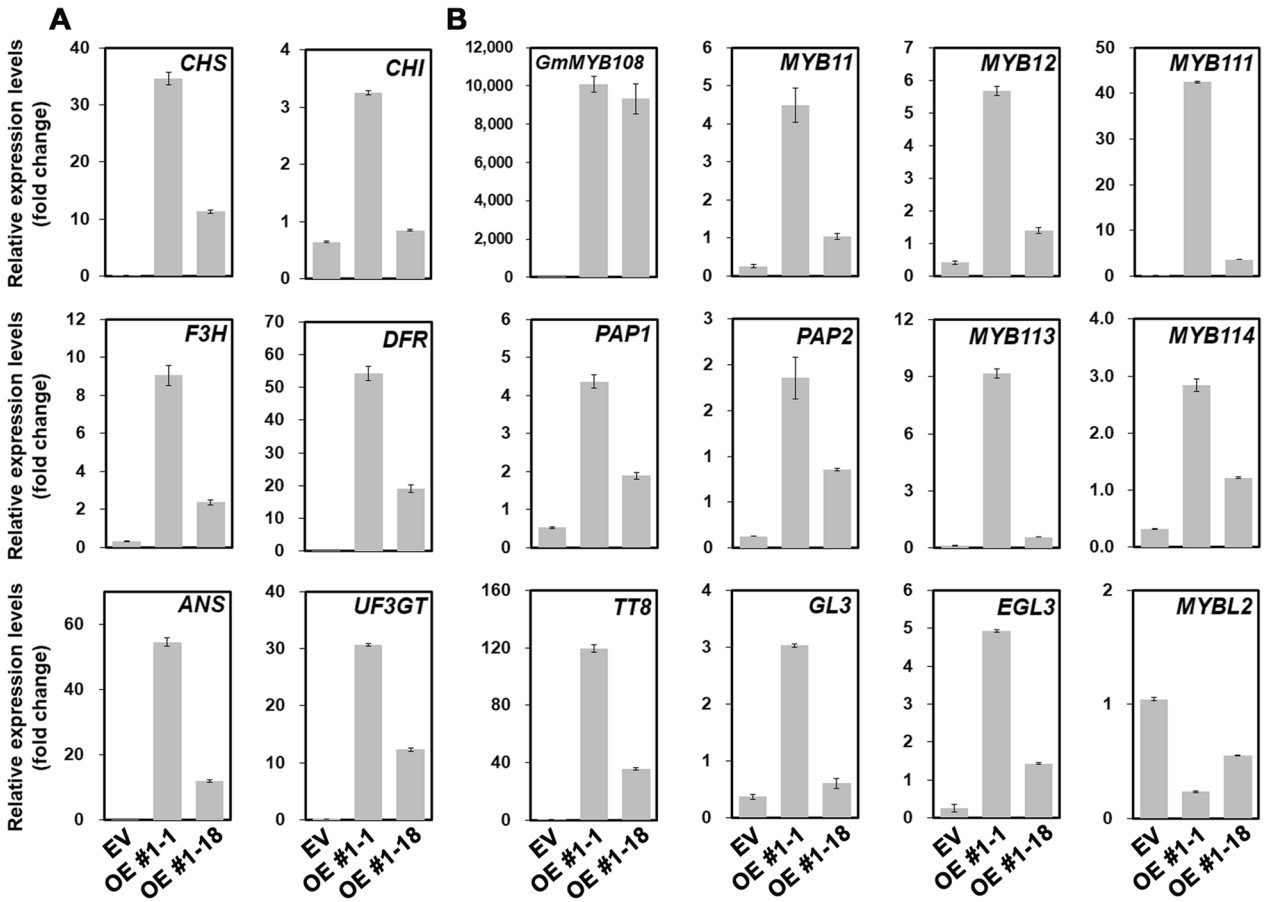


Fig. 5 Expression analysis of anthocyanin biosynthesis and regulatory genes in *GmMYB108-OE* transgenic *Arabidopsis* plants. The effect of *GmMYB108* overexpression on endogenous biosynthetic and regulatory genes was investigated using qRT-PCR analysis in *GmMYB108-OE* transgenic *Arabidopsis* plants (T_2 generation) (Additional file 1: Fig. S3). The relative expression levels (fold change) are depicted as mean ± SD of three biological replicates in triplicate. The protein phosphatase 2 regulatory subunit (*PP2A*) gene (At1g69960) was used as an endogenous reference to normalize gene expression. (A) Anthocyanin biosynthesis genes: chalcone synthase (*CHS*), chalcone isomerase (*CHI*), flavanone 3-hydroxylase (*F3H*), dihydroflavonol-4-reductase (*DFR*), anthocyanidin synthase (*ANS*), and UDP-glucose:flavonoid 3-*O*-glucosyltransferase (*UF3GT*). **B** *GmMYB108,* Anthocyanin-regulatory genes: *MYB11*, *MYB12*, *MYB111,* production of anthocyanin pigment 1 (*PAP1*), *PAP2*, *MYB113*, *MYB114*, transparent testa 8 (*TT8*), glabra 3 (*GL3*), enhancer of glabra 3 (*EGL3*), and MYB-like 2 (*MYBL2*)

GmMYB genes. Moreover, digital expression and qRT-PCR analyses suggested that *GmMYB108* was enriched in flowers compared with that in other reproductive organs, such as pod coats or seeds (Additional file 1: Figs. S2, S3). It is well known that the function of a gene is tightly linked to when and where it is expressed. It has been speculated that *GmMYB108* is intimately associated with isoflavonoid biosynthesis in soybean flowers, based on the enhanced expression of *GmMYB108* caused by ethephon treatment and flower-specific expression.

Ethylene or ethephon has been widely used in numerous studies, demonstrating its ability to stimulate various plant responses, including fruit ripening, abscission, flower induction, leaf senescence, and stem elongation [53]. Ethylene is considered to be a multifunctional plant hormone that regulates both growth and senescence [54, 55]. It is well known that ethylene can cause premature senescence symptoms such as leaf yellowing and abscission. In the ethephon treatment experiment on soybean leaves, we also observed that the application of ethylene or ethephon to soybean leaves stimulated senescence (leaf yellowing) over time. As *GmMYB108* was strongly induced by ethephon treatment, it will be very interesting to investigate whether the expression level of *GmMYB108* is increased during leaf yellowing in soybean plants. This will be an indication of whether *GmMYB108* expression is a factor in leaf senescence.

R2R3-type MYB TFs are known to be involved in the flavonoid biosynthesis pathways of different plant species [24, 33]. A previous study demonstrated that the soybean

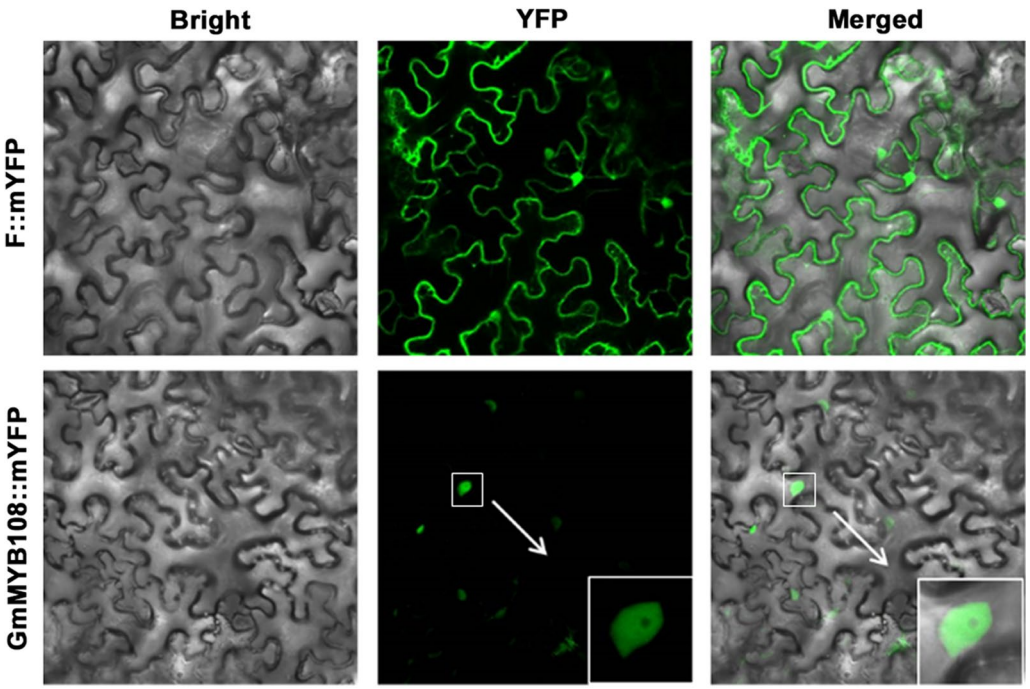


Fig. 6 Nuclear localization of GmMYB108 protein in *N. benthamiana* leaf epidermal cells. GmMYB108-mYFP represents leaves transformed transiently with the *d35S*-Flag: GmMYB108: mYFP vector construct, and the empty vector *d35S*-Flag::mYFP was used as a negative control (F::mYFP). Bright, bright field; YFP, YFP fluorescence (excitation peak at 514 nm and emission peak at 527 nm); Merged, combination. The bottom right-hand panels (white arrows) in the GFP and merged column represent ×3 magnification of the boxes showing the nucleus

R2R3-type MYB GmMYB29 regulates isoflavone biosynthesis by inducing the expression of *CHS8* and *IFS2* genes in soybean [56]. Furthermore, the study showed that *GmMYB29* overexpression in transgenic soybean hairy roots led to increased levels of isoflavonoids. As shown in our transcriptome analysis (Fig. 1C), ethylene treatment strongly upregulated the expression of several structural genes, such as *GmCHS6, GmCHS7, GmCHS8, GmCHIs, GmIFS1, GmIFS2, GmIF7GT,* and *GmIF7MAT*, involved in the isoflavone biosynthetic pathway. Thus, it is very important to elucidate whether the *GmMYB108* TF can regulate isoflavonoid biosynthesis by activating the structural genes involved in the isoflavone biosynthetic pathway. Further studies are needed to investigate whether the *GmMYB108* TF could bind to the promoters of isoflavone biosynthesis genes (*GmCHS7, GmCHI,* and *GmIFS2*, etc.) and activate their transcription. It will also be necessary to study the isoflavonoid content by overexpression of the *GmMYB108* gene in soybean plants and to examine the downstream gene expression.

Despite extensive studies on the important roles of R2R3-MYB TFs in developmental events, such as the regulation of seed germination, specification of cell fate, and other processes, including flavonoid biosynthesis, relatively few *MYB* genes in soybean plants have been studied for their roles in reproductive development [22–25, 28, 30, 31, 57]. Yang et al. identified the soybean R2R3-MYB TF gene *GmMYB181* with an exclusive expression pattern in soybean flower tissues. In addition, *GmMYB181-OE* transgenic *Arabidopsis* plants showed altered expression of over 3000 genes involved in the development of floral organs and seeds/fruits, suggesting a potential role for *GmMYB181* in the development of reproductive organs [58]. Considering that *GmMYB108* exhibited exclusive expression pattern in flowers, we suggest the possibility that *GmMYB108* plays a crucial role in the regulation of various physiological, developmental and biochemical processes in flowers, including the flower development and the biosynthesis of flavonoids/anthocyanins. However, there is currently a lack of data regarding the role of *GmMYBs* in flavonoid/anthocyanin accumulation in soybean flowers. Previous studies have demonstrated that six independent loci (*W1*, *W2*, *W3*, *W4*, *Wm*, and *Wp*) play crucial roles in regulating flower pigmentation in soybean plants. Among them, the *W2* locus has been shown to encode an MYB TF that regulates the vacuolar acidification of flower petals [59, 60]. Furthermore, Takahashi et al. suggested that silencing of *GmMYB-G20-1* changes the flower color from purple to grey/blue and that *GmMYB-G20-1* may correspond to the *W2* gene [60]. Maloney et al. reported the effect of flavonols on the development of tomato

pollen. The mutation of the tomato *F3H* gene resulted in reduced flavonoid content in plants and was associated with impaired pollen tube growth and germination [61]. Therefore, it will be interesting to find out the exact role of *GmMYB108* in soybean flowers by mutating the *GmMYB108* gene using the CRISPR/Cas9 gene editing system.

GmMYB108 markedly upregulated the expression of multiple structural genes involved in flavonoid and anthocyanin biosynthesis pathways, such as *CHS*, *CHI*, *F3H*, *DFR*, and *ANS*, and downregulated *MYBL2*, a negative regulator of the anthocyanin biosynthesis pathway. The transcript levels were proportional to the phenotypes of transgenic *Arabidopsis* plants (T_2; Fig. 5). These results suggest that *GmMYB108* positively affects multiple steps in the anthocyanin biosynthesis pathway. Four major R2R3-MYB TFs–AtMYB75 (PAP1), AtMYB90 (PAP2), AtMYB113, and AtMYB114–are known to regulate flavonoid biosynthesis at different developmental stages in *Arabidopsis* leaves. GmMYB108 shared a few similarities with the four R2R3-MYB TFs, except for the R2R3 domains (Additional file 1: Fig. S4B), indicating that GmMYB108 differentially affects the flavonoid biosynthetic pathway. The phylogenetic analysis with GmMYB108 revealed the two homologous R2R3-type MYBs in *Arabidopsis*: AtMYB116 (74% identity of amino acids) and AtMYB62 (52% identity of amino acids; Additional file 1: Fig. S4). AtMYB62 and AtMYB116 are members of subgroup 20 (there are 23 subgroups of the R2R3-type MYB family in *Arabidopsis*), which is related to cluster 38 of the soybean R2R3-type MYB superfamily (47 clusters), whereas GmMYB108 presents ambiguity among different phylogenetic trees [49]. Interestingly, *AtMYB62* and *AtMYB116* are highly expressed in flowers and petals during the early stages of flowering (*Arabidopsis* Atlas eFP Browser, bar.utoronto.ca/efp/cgi-bin/efpWeb.cgi), similar to the preferential expression of *GmMYB108* in flower tissues [62]. *AtMYB62*-overexpressing plants also showed anthocyanin accumulation, similar to the *GmMYB108-OE* transgenic *Arabidopsis* plants [63].

In this study, we identified a new R2R3-type MYB TF gene *GmMYB108* in soybean as a potential regulator of flavonoid and anthocyanin accumulation. *GmMYB108* was predicted to be involved in isoflavonoid biosynthesis in soybean plants. The in silico and qRT-PCR analyses revealed the exclusive expression of *GmMYB108* in soybean flower tissues. The ectopic overexpression of *GmMYB108* increased the accumulation of anthocyanins and flavonoids in the leaf tissues of transgenic *Arabidopsis* plants and upregulated multiple genes involved in anthocyanin biosynthesis and its regulatory pathways in *Arabidopsis*. However, to determine the exact function of *GmMYB108* in soybean, especially in the floral organs of soybean plants, and to further study how it regulates the accumulation of flavonoids, anthocyanins, and isoflavones, *GmMYB108* must be expressed in homologous soybean plants. Therefore, we are currently attempting to generate *GmMYB108*-overexpressing transgenic soybean plants.

Abbreviations

ANS	Anthocyanidin synthase
CHI	Chalcone isomerase
CHS	Chalcone synthase
C4H	Cinnamic acid 4-hydroxylase
4CL	4-Coumarate:CoA ligase
DFR	Dihydroflavonol-4-reductase
EGL3	Enhancer of glabra 3
GL3	Glabra 3
HID	Hydroxyisoflavanone dehydratase
IFS	Isoflavone synthase
MYBL2	MYB-like 2
PAP1	Production of anthocyanin pigment 1
PAL	Phenylalanine ammonia-lyase
THC	2′,4,4′,6′-Tetrahydroxychalcone
TT8	Transparent testa 8
TTG1	Transparent testa glabra 1
UGT	UDP-glycosyltransferase
UF3GT	UDP-glucose:flavonoid 3-O-glucosyltransferase
PAL	Phenylalanine ammonia-lyase

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s13765-024-00877-8.

Additional file 1. Fig. S1. Venn diagrams of the differentially expressed genes (DEGS) in soybean plants after 24 h of ethephon treatment. **Fig. S2.** Digital gene expression of GmMYB genes in various tissues of soybean plants. **Fig. S3.** Phenotypes of GmMYB108-OE transgenic Arabidopsis plants. **Fig. S4.** Sequence analyses of GmMYB108. **Table S1.** Primer pairs used for gene constructs and qRT-PCR in this study. **Table S2.** Quantitative analysis of dietary isoflavones in ethephon-treated soybean leaves.

Additional file 2. Table S3. RNA-Seq FPKM in ethephon-untreated and ethephon-treated soybean plants.

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Author contributions
CYK conceived and supervised this study. CYK, SBL, GS, and JYM designed the experiments. YJJ and SBL prepared transgenic plants. SBL, GL, JHK, and MSC grew soybean plants and performed sampling. JHK and GL performed ethephon treatment and sampling. JYM, SBL, and GL performed the RNA extraction and qRT-PCR experiments. KHP, JL, and JCJ analyzed the data. CYK and JYM wrote the manuscript. KHP, JL, and JCJ commented on the manuscript. All authors read and approved the final manuscript.

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Availability of data and materials
The datasets generated and analyzed during the current study are available from the corresponding author on reasonable request.

Declarations

Ethics approval and consent to participate
Not applicable.

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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