

# Identification of novel MYB transcription factors involved in the isoflavone biosynthetic pathway by using the combination screening system with agroinfiltration and hairy root transformation

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**Abstract** Soybean isoflavones are functionally important secondary metabolites that are mainly accumulated in seeds. Their biosynthetic processes are regulated coordinately at the transcriptional level; however, screening systems for key transcription factors (TFs) are limited. Here we developed a combination screening system comprising a simple agroinfiltration assay and a robust hairy root transformation assay. First, we screened for candidate MYB TFs that could activate the promoters of the chalcone synthase (CHS) gene *GmCHS8* and the isoflavone synthase (IFS) genes *GmIFS1* and *GmIFS2* in the isoflavone biosynthetic pathway. In the agroinfiltration assay, we co-transformed a *LjUbi* (*Lotus japonicus polyubiquitin* gene) promoter-fused MYB gene with target promoter-fused GUS ( $\beta$ -glucuronidase) gene constructs, and identified three genes (*GmMYB102*, *GmMYB280*, and *GmMYB502*) as candidate regulators of isoflavone biosynthesis. We then evaluated the functional regulatory role of identified three MYB genes in isoflavone biosynthesis using hairy roots transformation assay in soybean for the accumulation of isoflavones. Three candidate MYB genes showed an increased accumulation of total isoflavones in hairy root transgenic lines. Accumulation of total isoflavones in the three MYB-overexpressing lines was approximately 2- to 4-folds more than that in the vector control, confirming their possible role to regulate isoflavone biosynthesis. However, the significant accumulation of authentic *GmCHS8*, *GmIFS1*, and *GmIFS2* transcripts could not be observed except for the *GmMYB502*-overexpressing line. Therefore, the analysis of isoflavone accumulation in transgenic hairy root was effective for evaluation of transactivation activity of MYB TFs for isoflavone biosynthetic genes. Our results demonstrate a simple and robust system that can potentially identify the function of orphan TFs in diverse plant metabolic pathways.

**Key words:** agroinfiltration, co-transformation, hairy root, isoflavone, soybean, transcription factor.

## Introduction

Isoflavones are polyphenolic compounds synthesized by leguminous plants and are distinctly abundant in soybean seeds. Soybean seeds are known to be an excellent source of isoflavones in the human diet. Soy isoflavones are considered to have many human health benefits, e.g. the risk reduction of hormone-dependent cancers (Dixon 2004; Peterson and Barnes 1991), post-menopausal symptoms (Nestel et al. 1999), cardiovascular disease (Messina 1999), and osteoporosis

(Potter et al. 1998). In plants, isoflavones play an important role in growth, development, and survival. They regulate symbiosis between leguminous plants and rhizobia by inducing the expression of nodulation genes (Ferguson and Mathesius 2003; Kosslak et al. 1987; Novák et al. 2004). Furthermore, isoflavones induce phytoalexin production, which is vital in plant-microbe interactions during pathogen attack (Aoki et al. 2000; Lygin et al. 2013; Yoneyama et al. 2016).

In soybean, isoflavones are coordinately synthesized by multiple biosynthetic enzymes via a legume-specific

Abbreviations: CHI, chalcone isomerase; CHR, chalcone reductase; CHS, chalcone synthase; DAP, days after pollination; GUS,  $\beta$ -glucuronidase; IFS, isoflavone synthase; *LjUbi*, *Lotus japonicus polyubiquitin* gene; qRT-PCR, quantitative reverse transcription-polymerase chain reaction; TF, transcription factor.

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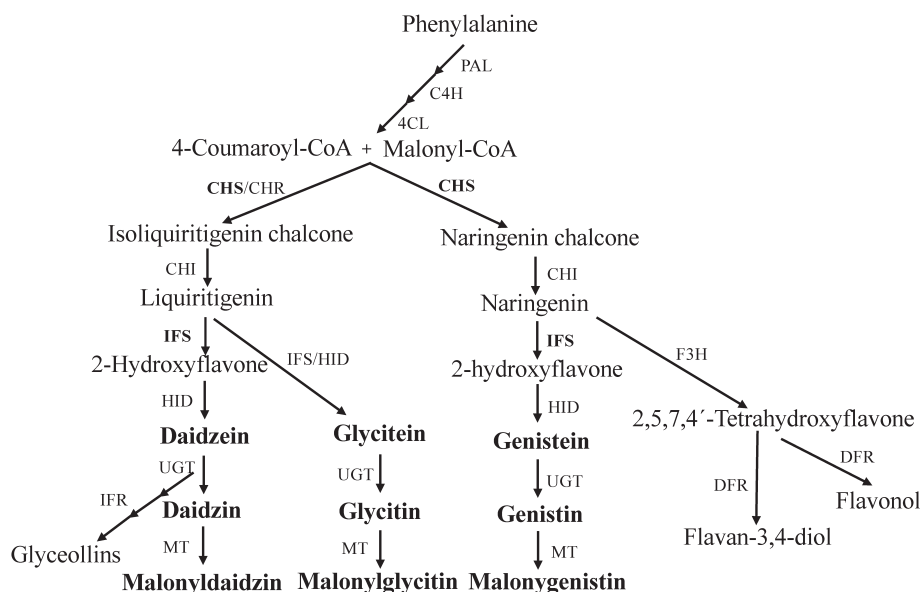


Figure 1. Schematic representation of the isoflavone biosynthetic pathway of soybean. Arrows represent individual enzymatic steps. Abbreviations: 4CL, 4-coumarate-CoA ligase; C4H, cinnamic acid 4-hydroxylase; CHI, chalcone isomerase; CHR, chalcone reductase; CHS, chalcone synthase; DFR, dihydroflavonol 4-reductase; F3H, flavone 3-hydroxylase; HID, 2-hydroxyisoflavone dehydratase; IFR, isoflavone reductase; IFS, isoflavone synthase; MT, isoflavone 7-O-glucoside 6-O-malonyltransferase; PAL, phenylalanine ammonia lyase; and UGT, uridine diphosphate glycosyltransferase. The major isoflavone biosynthetic enzymes and isoflavones are represented as a bold character.

branch of the phenylpropanoid pathway (Figure 1). This branch includes many steps; several biosynthetic genes such as those encoding CHS (chalcone synthase), CHI (chalcone isomerase), IFS (isoflavone synthase), HID (2-hydroxyisoflavone dehydratase), UGT (uridine diphosphate glycosyltransferase), and MT (isoflavone 7-O-glucoside 6-O-malonyltransferase) are involved in the core branch for isoflavone biosynthesis (Akashi et al. 1999; Dastmalchi et al. 2015; Jung et al. 2000; Latunde-Dada et al. 2001; Steele et al. 1999). The regulatory mechanism of isoflavone biosynthesis through the phenylpropanoid pathway has been extensively studied; the transcriptional levels of isoflavone biosynthetic genes are directly regulated by the ectopic expression of plant transcription factors (TFs) such as MYB, bHLH (basic Helix Loop Helix), bZIP (basic leucine Zipper Protein), WRKY, MADS-box, and WD40 (Grotewold et al. 2000; Nesi et al. 2001; Ramsay and Glover 2005; Yi et al. 2010; Yu et al. 2003).

Through advances in high-throughput sequencing technologies, the annotated genome information from many economically important crops including soybean has become available (Lam et al. 2010; Schmutz et al. 2008). However, functional information on TFs regulating important biosynthetic pathways is still limited because it is difficult to assume the precise function of huge numbers of TFs based on amino acid sequence similarity alone.

In common plant genomes, ~7% of total transcripts encode TFs. Some of these could be potential tools for manipulation of the phenylpropanoid pathway

and improving the levels of isoflavone biosynthesis and accumulation in legume species (Broun 2004). In soybean, 5,671 genes encode putative TFs; MYBs comprise the largest family, representing 14% of the total TFs (Aoyagi et al. 2014). Manipulating MYB TFs endogenous to legumes could alter the isoflavone biosynthesis by changing the transcriptional activities of the biosynthetic genes.

Using co-expression profiling analysis, one group has recently identified the target genes of orphan TFs (Du et al. 2018; Ye et al. 2017). Others have identified potential TFs related to a specific biosynthesis pathway, lignin biosynthesis in *Arabidopsis*, by assaying transient expression of target promoters and confirming the results in stable transgenic lines (Newman et al. 2004; Zhou et al. 2009). Nevertheless, it is still not possible to screen TFs involved in a specific biosynthetic pathway in economically important crops in a simple, rapid and reliable way. This is because establishing stable transformants carrying promoter-reporter systems requires regeneration and analysis of numerous lines, which is labor-intensive and time consuming. Therefore, here we developed a method to evaluate the function of orphan TFs by combining two different approaches, a general but preliminary agroinfiltration assay using *Nicotiana benthamiana* (a close relative of tobacco) (Berger et al. 2007; Kapila et al. 1997; Yang et al. 2000), and a robust hairy root transformation assay in soybean (Kereszt et al. 2007; Ron et al. 2014).

In this study, we succeeded in identifying novel soybean MYB TF candidates that induced the

transcription of members of the isoflavone biosynthetic pathway, CHS and IFS, in the transient expression assay system. We evaluated the capacity of these candidates, *GmMYB102*, *GmMYB280*, and *GmMYB502*, to activate the isoflavone biosynthetic pathway by using the soybean hairy root transformation system.

## Materials and methods

### Plant materials

Mature seeds and developing seeds at 21, 28, 35, 42, 49, 56, 63, and 70 days after pollination (DAP) were collected randomly from a field-grown soybean [*Glycine max* (L.) Merr.] cv. Fukuyutaka at Saga University. The pod wall and seed coat were separated from the developing seed and quickly frozen with liquid nitrogen and stored at  $-80^{\circ}\text{C}$  for further analysis.

### Construction of plasmids

The open reading frames of MYB genes were amplified from the cDNA synthesized from developing seeds of c.v. Fukuyutaka by using KOD FX Neo DNA polymerase (TOYOBO, Osaka, Japan). The amplified cDNA fragments were introduced into the entry vector pENTER/D-TOPO (Invitrogen, CA, USA). To prepare the effector constructs, the MYB cDNA fragments were transferred from the pENTER clones to the destination binary vector pUB-GW-GFP by using Gateway technology (Maekawa *et al.* 2008); the open reading frame fragment of the GUS ( $\beta$ -glucuronidase) reporter gene (Jefferson *et al.* 1986) gene obtained from pCambia1391z was also cloned into pUB-GW-GFP as a positive control. Next, to construct the reporter plasmids, the amplified upstream DNA fragments of CHS8, IFS1, and IFS2 derived from cv. Fukuyutaka were cloned into separate pCambia1391z vectors. We confirmed that all of these constructs (Supplementary Figure S1) had the correct nucleotide sequences. Finally, these constructs were introduced into *Agrobacterium tumefaciens* strain EHA105 by the freeze-thaw method (Holsters *et al.* 1978) and their glycerol stocks were stored at  $-80^{\circ}\text{C}$  until use. The primer sequences are listed in Supplementary Table S1.

### Agroinfiltration assay

Agroinfiltration was performed as described previously (Yang *et al.* 2000) with some modifications. Briefly, EHA105 glycerol stock was used to inoculate 4 ml of YEP medium [1% (v/v) tryptophan, 0.5% (v/v) yeast extract, 0.5% (w/v) NaCl, pH 7] supplemented with kanamycin ( $50\text{ mg ml}^{-1}$ ). *Agrobacteria* were grown overnight at  $28^{\circ}\text{C}$  with agitation (200 rpm). Then 2 ml of the cultured *agrobacteria* were used to inoculate 25 ml of the YEP medium supplemented with kanamycin and grown for a further 8 h. *Agrobacterial* cells were harvested by centrifugation for 10 min at  $2400\times g$ . The pellet was suspended in 10 mM MES (2-[*N*-Morpholino]ethanesulfonic acid)-KOH buffer (pH 5.6) containing 10 mM  $\text{MgCl}_2$  and adjusted to a final bacterial concentration of optical density at 600 nm. Finally, freshly prepared  $100\text{ }\mu\text{M}$  acetosyringone (Sigma-

Aldrich, St. Louis, MO, USA) was added to the *agrobacterial* suspension, which was kept at room temperature for 1 h before agroinfiltration to enhance the transient expression efficiency. *Nicotiana benthamiana* plants were grown in a plant growth chamber under 16 h light/8 h dark period at  $25^{\circ}\text{C}$  and 60–70% relative humidity. Four weeks after the plants were placed in the chamber, healthy and green plant leaves were used for the transient assay. The prepared *agrobacterial* suspension was infiltrated into the underside of the top 3 or 4 leaves by using a blunt-tipped 5 ml plastic syringe (Terumo Corp., Tokyo, Japan) with gentle pressure. The container in which the agroinfiltrated plants were housed was covered with clear plastic wrap until sample collection to avoid severe drying conditions.

### Protein extraction and fluorometric GUS assay

The infiltrated leaves were collected 3 days after infection and homogenized with 650  $\mu\text{l}$  of GUS extraction buffer [50 mM  $\text{NaH}_2\text{PO}_4$  (pH 7.0), 0.1% sarcosyl (*N*-lauroylsarcosine sodium salt), 10 mM EDTA (pH 8.0), 0.1% Triton X-100, 10 mM  $\beta$ -mercaptoethanol, and 20% methanol]. The total soluble protein content was estimated with the Bradford assay (Bradford 1976) and GUS activity was quantified by a highly sensitive fluorometric assay (Jefferson 1987). Repeated measurements of GUS activities with MUG (4-methylumbelliferyl- $\beta$ -D-glucuronide hydrate; Sigma-Aldrich) as a substrate were taken in triplicate over a kinetic reaction period (0, 15, and 30 min) at  $37^{\circ}\text{C}$ . Finally, the reactions were stopped by the addition of 0.2 M  $\text{Na}_2\text{CO}_3$  and fluorescence was measured with the excitation wavelength of 356 nm and the emission wavelength of 448 nm using a fluorescence spectrometer (Infinite 200 Pro, Tecan, Männedorf, Switzerland).

### Isoflavone extraction and high performance liquid chromatography analysis

For extraction of isoflavones from developing seeds, each sample was freeze-dried and ground into a fine powder with a Multi-beads Shocker (Yasui Kikai, Osaka, Japan). Precisely 100 mg of fine powder was extracted with 1 ml of 0.1% acetic acid (v/v) in 70% ethanol (v/v), vortexed, and sonicated for 30 min. The samples were centrifuged at  $4^{\circ}\text{C}$ ,  $14,000\times g$  for 1 min. The extraction procedure was then repeated twice and the supernatant fractions were combined and filtered through a  $0.45\text{ }\mu\text{m}$  filter. The extracts were analyzed by high performance liquid chromatography (HPLC) on a Hydrosphere C18 column (50 mm length  $\times$  4.6 mm internal diameter; YMC Co. Ltd., Kyoto, Japan) at  $35^{\circ}\text{C}$ . The samples were separated with a linear gradient of solvent A (0.3% acetic acid) and solvent B (0.3% acetic acid and 0.5% acetonitrile) at a flow rate of  $1.5\text{ ml min}^{-1}$ . The isoflavones were detected by measuring absorbance at 254 nm, and isoflavone peaks were confirmed by comparison with the retention time of nine authentic isoflavone standards (genistin, daidzin, glycitin, genistein, daidzein, glycitein, malonylgenistin, malonyldaidzin, and malonylglycitin; Wako, Osaka, Japan). For extraction of isoflavones from soybean

transgenic hairy roots, we measured the fresh weight of the roots, froze them in liquid nitrogen, and then ground them into a fine powder by using the Multi-beads Shocker. The powders were used as initial materials for isoflavone extraction. Subsequently, extraction and analysis were performed as above.

### Soybean hairy root assay

Soybean transgenic hairy roots were developed according to the method of Chen et al. (2018) by using soybean cv. Toyoshirome with some modifications. To generate the hairy roots, the effector constructs and the empty vector pUB-GW-GFP as described above were transformed into the *Agrobacterium rhizogenes* strain LBA1334. Independent positive transgenic hairy root lines were selected by visualization of strong GFP (green fluorescent protein) signals, and were then used for measurement of isoflavones content and gene expression analysis.

### Quantitative reverse transcription-polymerase chain reaction analysis

Total RNA was isolated from developing seeds and transgenic hairy roots of soybean (fresh weight, 50–100 mg) by using an RNeasy Plant Mini Kit (QIAGEN, Hilden, Germany). The first-strand cDNA was synthesized from 1 µg of total RNA with a ReverTra Ace kit (TOYOBO), and oligo(dT)20 primer. cDNA (10 ng) was used as the template for quantitative reverse transcription-polymerase chain reaction (qRT-PCR) analysis with rTaq enzyme and EvaGreen dye (Biotium, Inc., Fremont, CA, USA) and fluorescence intensity was monitored. The qRT-PCR analysis was carried out in 96-well plates using the LightCycler 96 System (Roche Diagnostics K.K., Tokyo, Japan) in a final volume of 10 µl per well. Each reaction was performed using the following conditions: pre-incubation at 95°C for 5 min, 40 cycles at 95°C for 15 s, 60°C for 15 s, 72°C for 20 s, and melting analysis at 95°C for 10 s, 65°C for 1 min followed by final extension at 97°C. Two housekeeping genes, *GmACTIN* (*Glyma.19G147900*, for developing seeds) and *GmELFa* (*Glyma.19G052400.1*, for hairy roots), were used in each PCR plate for normalization of transcript levels. Data were analyzed with the LightCycler 96 SW 1.1 software package (Roche Diagnostics K.K) and transcript levels were calculated by following the guidelines in Livak and Schmittgen (2001). The gene-specific primer sequences for qRT-PCR are listed in Supplementary Table S2.

### Statistical analysis

We calculated the normalized value (zero mean and variance 1) for each gene expression data and for the total amount of isoflavone molecules (assigned as HPLC data in the figure) during seed development. The package “heatmap.2” was used for the analysis of hierarchical clustering and heat map construction with default parameters. Statistical test for complementation test was conducted with single-factor ANOVA, Tukey post hoc test,  $p < 0.05$ . All analyses were performed in R software (<https://www.r-project.org/>).

## Results

### Transactivation analysis of 12 orphan MYB TFs by agroinfiltration assay

We selected three isoflavone biosynthetic genes, *GmCHS8*, *GmIFS1*, and *GmIFS2* as major up-regulated target genes in the isoflavone biosynthetic pathway, because the expression of these three genes also showed a higher correlation with isoflavone accumulation in developing soybean seeds (Dhaubhadel et al. 2003, 2007; Yi et al. 2010). We also selected 12 candidate MYB TF genes whose expression highly correlated with *GmIFS1* expression by using annotation data and RNA-seq Atlas data in the soybean genome database (<https://soybase.org/>).

Hereafter, to evaluate the capacity of the 12 orphan R2-R3 type MYB TFs to transactivate the promoters of *GmCHS8*, *GmIFS1*, and *GmIFS2*, we designed a agroinfiltration assay into the leaves of a foreign species, *N. benthamiana*, via agroinfiltration. This assay system consisted of a reporter plasmid containing a *GUS* gene fused with the target gene (*GmCHS8*, *GmIFS1*, or *GmIFS2*) promoter, and an effector plasmid containing one of the candidate MYB genes or the positive control *GmMYB176* gene (Yi et al. 2010) fused with the strong housekeeping *L. japonicus* polyubiquitin (*LjUbq1*) promoter (Maekawa et al. 2008).

Out of 13 co-transformations of MYB TFs with *GmCHS8* reporter, 7 MYB TFs showed significantly higher GUS activities at  $p < 0.01$  compared with the reporter alone (Figure 2A and Table 1): i.e., *GmMYB4* (2.27-fold [MYB plus reporter/reporter alone]), *GmMYB77* (2.28-fold), *GmMYB102* (2.76-fold), *GmMYB280* (1.82-fold), *GmMYB502* (2.26-fold), *GmMYB522* (2.09-fold), and *GmMYB176* (2.18-fold). In addition, 3 MYB TFs showed significantly higher GUS activities at  $p < 0.05$  compared with the reporter alone: i.e., *GmMYB128* (1.82-fold), and *GmMYB250* (1.63-fold) (Figure 2A and Table 1). In contrast, *GmMYB202* significantly suppressed GUS activity compared with the reporter alone (0.24-fold,  $p < 0.05$ ) (Figure 2A and Table 1).

When *GmIFS1* was used as the reporter, co-transformation of seven MYB TFs showed significantly higher GUS activities at  $p < 0.01$  than transformation with reporter alone (Figure 2B and Table 1): i.e., *GmMYB4* (1.85-fold), *GmMYB77* (1.98-fold), *GmMYB102* (1.65-fold), *GmMYB250* (1.58-fold), *GmMYB280* (1.72-fold), *GmMYB502* (1.70-fold), and *GmMYB176* (1.77-fold). In contrast, co-transformation with *GmMYB202* showed 0.52-fold suppression in GUS activity at  $p < 0.05$  compared with reporter alone (Figure 2B and Table 1).

In co-transformations with *GmIFS2* reporter, higher GUS activity was observed for six MYB TFs compared

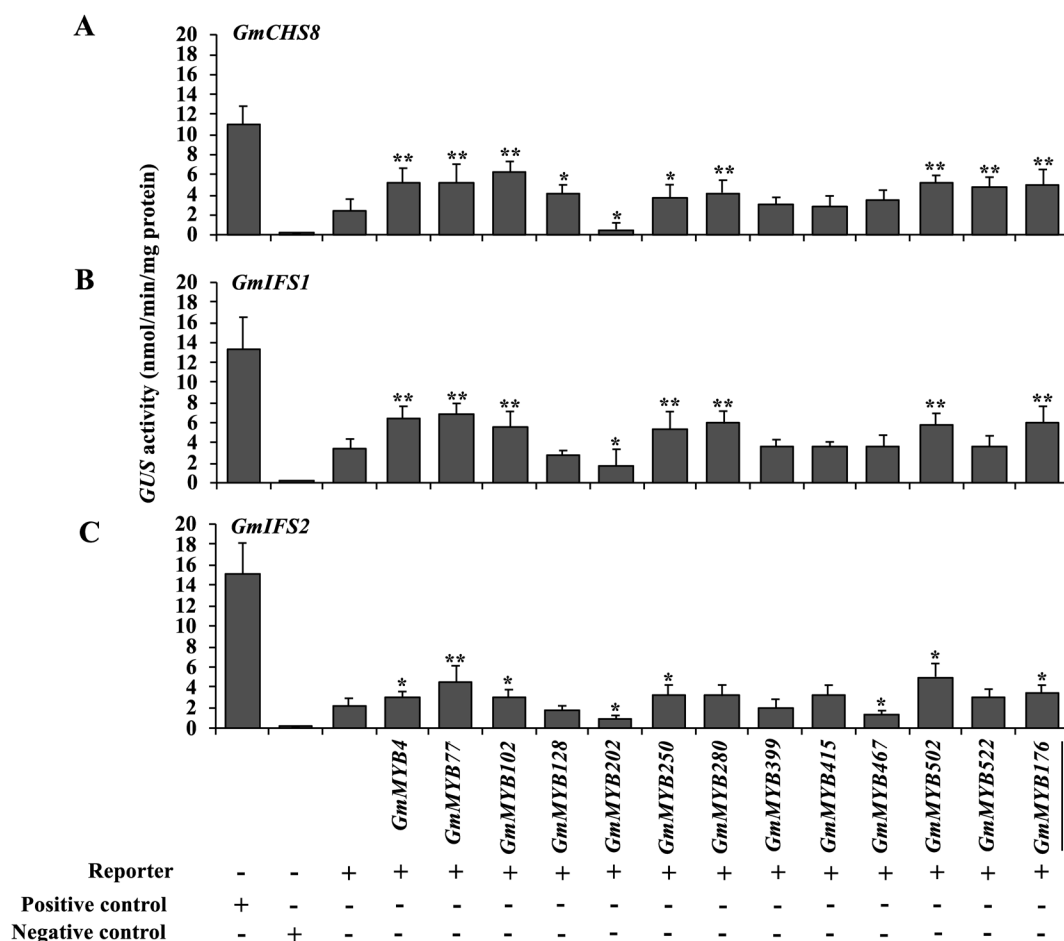


Figure 2. Comparison of the transcriptional activity of soybean MYB TFs in an agroinfiltration assay. The underlined effector construct was used as a positive control MYB TF. The data are presented as means and standard deviations of eight biological replicates. \* $p < 0.05$ , \*\* $p < 0.01$  versus reporter only (Student's *t*-test).

Table 1. Transactivation of multiple target promoters by transient expression of MYB transcription factors.

Name	MYB type	Number of amino acid residues	<i>GmCHS8</i>	<i>GmIFS1</i>	<i>GmIFS2</i>
GmMYB4	R2R3	406	++	++	+
GmMYB77	R2R3	322	++	++	++
GmMYB102	R2R3	275	++	++	++
GmMYB128	R2R3	287	+	+/-	+/-
GmMYB202	R2R3	377	-	-	-
GmMYB250	R2R3	330	+	++	+
GmMYB280	R2R3	252	+	++	+/-
GmMYB399	R2R3	452	+/-	+/-	+/-
GmMYB415	R2R3	289	+/-	+/-	+/-
GmMYB467	R2R3	320	+	+/-	-
GmMYB502	R2R3	284	++	++	+
GmMYB522	R2R3	373	++	+/-	+/-
GmMYB176	R1	285	++	++	+

MYB types are assigned according to Du et al. (2012), Aoyagi et al. (2014) and soybean genome information from <http://phytozome.jgi.doe.gov/>. ++,  $\geq 2$ -fold activation; +,  $\geq 1.5$ -fold activation; -,  $\geq 2$ -fold suppression and +/-,  $\geq 1.5$ -fold suppression of GUS activity compared to the reporter construct only. +/-, no change in GUS activity.

with transformation with reporter alone (Figure 2C). The highest increase in GUS activity was obtained with GmMYB77 (2.08-fold,  $p < 0.01$ ) (Figure 2C and

Table 1). Five other MYB TFs, GmMYB4 (1.36-fold), GmMYB102 (1.36-fold), GmMYB250 (1.50-fold), GmMYB502 (2.09-fold) and GmMYB176 (1.58-fold)



also showed significantly increased GUS activity (all  $p < 0.05$ ; Figure 2C and Table 1). GUS activities were significantly suppressed ( $p < 0.05$ ) by co-transformation with GmMYB202 (0.41-fold) and GmMYB467 (0.84-fold) (Figure 2C and Table 1).

In our co-transformation assay, six MYB TFs, GmMYB4, GmMYB77, GmMYB102, GmMYB250, GmMYB502, and GmMYB176, showed increased GUS activities for all three reporter constructs of isoflavone biosynthetic enzyme genes (i.e., *GmCHS8*, *GmIFS1*, and *GmIFS2*) (Figure 2), and one MYB TF, GmMYB202, suppressed GUS activity for all three reporter constructs (Figure 2). Thus, in this assay, the multiple target promoters of isoflavone biosynthetic pathway genes were differentially activated or suppressed by various MYB TFs.

These results suggest that this agroinfiltration assay with many orphan TFs and combinations of different promoter-GUS constructs may accurately identify functional TFs for target genes on the specific and coordinately regulated biosynthetic pathway.

#### Correlations between the expression of MYB genes and isoflavone biosynthetic enzyme genes in developing soybean seed

To investigate correlations between total isoflavone accumulation and expression of MYB genes, isoflavone biosynthetic enzyme genes in developing soybean seed, HPLC and qRT-PCR analysis were performed. Isoflavones gradually accumulated during seed development, becoming very abundant in the late maturation stages (63 to 70 DAP) (Figure 3). Total isoflavone content and the expression levels of 12 candidates, *GmMYB176* (positive control gene, which is known to activate *GmCHS8* reporter; Yi et al. 2010), and 20 genes encoding isoflavone biosynthetic enzymes across various seed development stages, and assessed the relationships between these values by hierarchical clustering (Figure 4). The accumulation patterns of isoflavones closely correlated with the expression patterns of seven isoflavone biosynthetic enzyme genes encoding CHI, chalcone reductase (CHR), CHS, and IFS, i.e., *GmCHI1A* ( $r = 0.87$ ), *GmCHR* (0.95), *GmCHS1* (0.89), *GmCHS7* (0.87), *GmCHS8* (0.85), *GmIFS1* (0.88), and *GmIFS2* (0.88); and 3 MYB genes, i.e., *GmMYB102* (0.84), *GmMYB280* (0.74), and *GmMYB502* (0.90). The transcript levels of these genes gradually increased during seed development, with a dramatic increase at the late development stage (Figure 4; 70 DAP). The clustering results strongly suggest that the expression of *GmCHS8*, *GmIFS1*, and *GmIFS2* is coordinately regulated in the soybean isoflavone biosynthetic pathway in developing seed, and that GmMYB102, GmMYB280, and GmMYB502 are putative candidates for transcriptional activators of these biosynthetic genes.

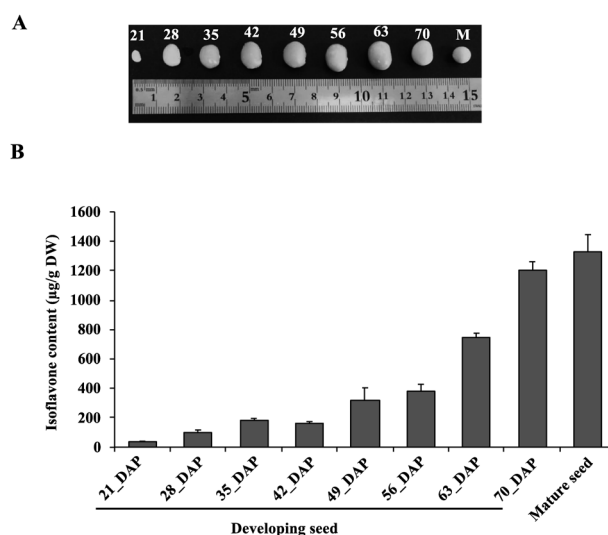


Figure 3. Morphology and total isoflavone content in developing soybean (cv. Fukuyutaka) seeds. Developing seed samples were collected at one week intervals during the period 21–70 days after pollination (DAP; A) and were used together with the mature seed sample for HPLC analysis of isoflavones (B). The data are presented as means and standard deviations of three technical replicates. DW, dry weight.

#### Effects of ectopic expression of MYB TFs in soybean hairy root on isoflavones and transcripts of the isoflavone biosynthetic genes

We chose three MYBs, GmMYB102, GmMYB280, and GmMYB502, for soybean hairy root transformation based on their high correlation ( $r = 0.84$ , 0.74 and 0.90, respectively) with isoflavone accumulation in developing soybean seeds (Figure 4) and their transactivation capacity for three major isoflavone biosynthetic genes in the co-transformation assay (Figure 2). To evaluate the functional potentiality of these MYBs, we developed soybean hairy root lines, each of which overexpressed one of the above MYB genes (GmMYB102-OE, GmMYB280-OE, and GmMYB502-OE lines, respectively). By qRT-PCR analysis of the hairy root lines, we confirmed that the transcript levels of all three genes were clearly increased compared with the vector control line (Figure 5A): GmMYB102-OE ( $>1,500$ -fold,  $p < 0.001$ ), GmMYB280-OE ( $>1,300$ -fold,  $p < 0.001$ ), and GmMYB502-OE ( $>30$ -fold,  $p < 0.001$ ). We also detected an increase in total isoflavone (daidzin, glycitin, malonyldaidzin, and malonylgenistin) content in the hairy root lines GmMYB102-OE (2.6-fold,  $p < 0.05$ ) and GmMYB502-OE (3.8-fold, at  $p < 0.01$ ) compared with the control (Figure 5B); a substantial increase was also observed in the line GmMYB280-OE (2.3-fold), but this was not statistically significant. The levels of two major isoflavones (malonyldaidzin and malonylgenistin) were 2-fold those in the control in all three hairy root lines (Figure 5B). These results suggest that at least three (GmMYB102, GmMYB280, and GmMYB502)

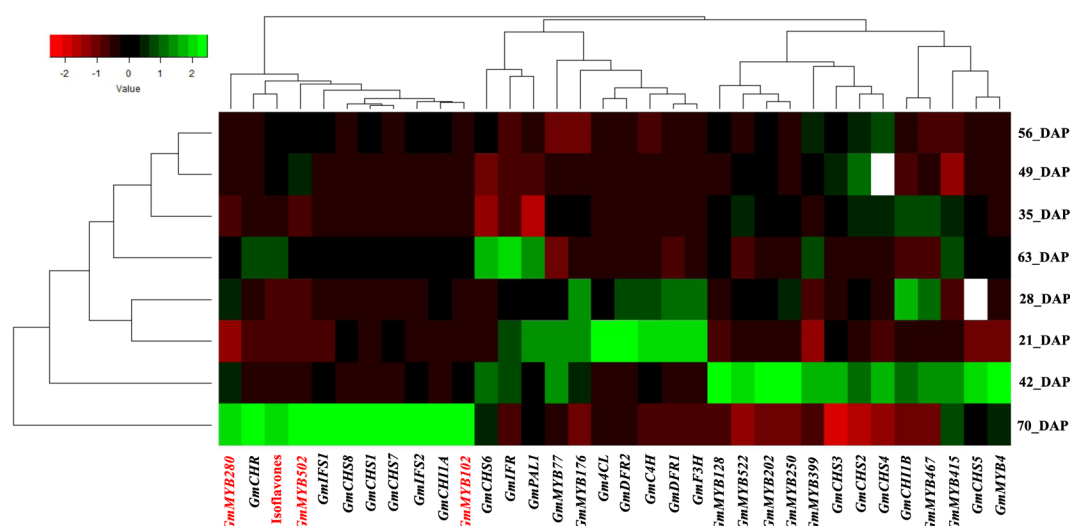


Figure 4. Hierarchical clustering and heat map analysis of gene expression profiles and the accumulation of isoflavones in developing soybean seeds. The columns represent the expression profiles of individual MYB TFs, isoflavone biosynthetic genes, and isoflavone content in developing seeds. The rows represent seed developmental stages. The white-color box in the heat map indicated that out of three replicates the expression of gene could not be detected in all the replication in qRT-PCR. The highly correlated three MYB TFs, GmMYB102, GmMYB280, GmMYB502 with isoflavones content in developing seeds are indicated by red characters. The analysis was performed in R software.

of the MYB TFs functionally identified by the soybean hairy root transformation assay could act as activators of isoflavone biosynthetic genes and increase the accumulation of target isoflavones.

## Discussion

Biosynthesis and accumulation of isoflavones and related molecules require the coordinated activation or suppression of multiple genes encoding enzymes in the isoflavone biosynthetic pathway (Sablowski et al. 1994). In leguminous plants, MYB TFs transcriptionally regulate isoflavone biosynthetic genes and affect the isoflavone levels without disturbing other branches of the phenylpropanoid pathway (Yi et al. 2010; Yu et al. 2003). The R2R3-type MYB TFs consists of the largest family in plants (Dubos et al. 2010) and they play a function as transcriptional activator or repressor in diverse aspects (Dubos et al. 2010; Prouse et al. 2012). To date, R2R3-MYB TFs have been identified from several plant species, such as *Arabidopsis* (Park et al. 2008), maize (Fornalé et al. 2010; Sonbol et al. 2009), *Ginkgo* (Xu et al. 2014), grape berry (Huang et al. 2014), *Chrysanthemum* (Zhu et al. 2013) and strawberry (Paolocci et al. 2011), which act as transcriptional regulator of several biosynthetic genes involved in the phenylpropanoid biosynthetic pathway. Here, we evaluated 12 R2R3-type MYB TFs as candidates for involvement in the isoflavone biosynthetic pathway of soybean by conducting a transient co-transformation assay and a stable hairy root transformation assay.

The co-transformation assay is known to be useful for screening both positive and candidate negative regulators from orphan TFs. However, there was a need to develop

an easy and cost-effective transient multi-reporter system to reduce the false-positive detection rate.

In the transient expression assay, we analyzed the functional regulatory roles of the 12 candidate MYB TFs by using a multiple reporter system with *GmCHS8*, *GmIFS1*, and *GmIFS2* promoters. *CHS* and *IFS* play a critical role in the isoflavone biosynthetic pathway of legumes and are coordinately regulated by MYB TFs (Akada and Dube 1995). Most of the MYB TFs coordinately activated or suppressed the GUS activities from multiple reporters. Out of the 12 MYB TFs, 5 MYB TFs, GmMYB4, GmMYB77, GmMYB102, GmMYB250, and GmMYB502, showed significant transcriptional activation of all targets tested (Figure 2).

Yi et al. (2010) reported that transient expression of *GmMYB176* showed huge potential to activate the transcription of a single reporter, *GmCHS8*, in *Arabidopsis* leaf protoplasts; and RNAi silencing of *GmMYB176* in soybean hairy roots resulted in reduced levels of isoflavones. In transient assays with a single reporter gene, many MYB TFs have been detected as candidates for regulators of isoflavone biosynthesis, and some of them have been confirmed to affect isoflavone accumulation in soybean (Han et al. 2017; Li et al. 2013; Yi et al. 2010). In contrast, our multiple reporter system based on the *GmCHS8*, *GmIFS1*, and *GmIFS2* coordinately regulated genes in the isoflavone biosynthetic pathway, could detect MYB TFs that affected multiple metabolic reactions in this pathway; therefore, regulating these MYBs might be particularly useful for improving isoflavone accumulation.

Additionally, we used an R1-type MYB TF, GmMYB176, as a positive control to test the reliability

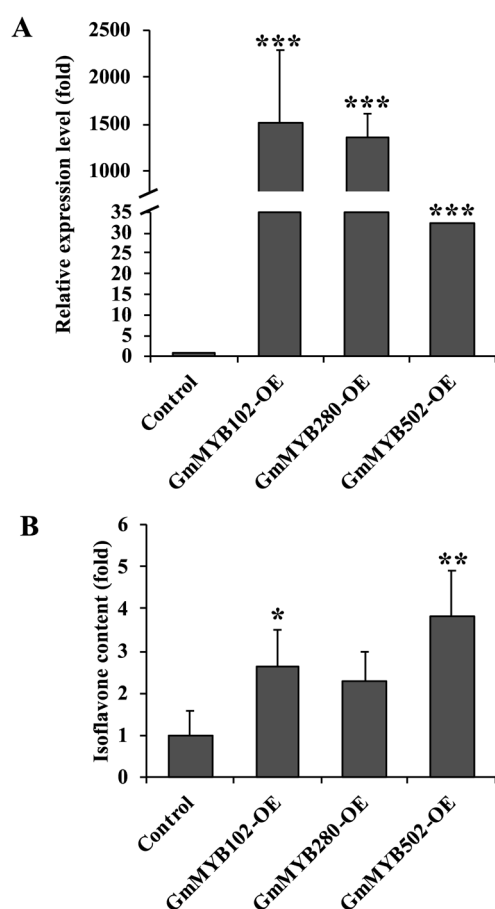


Figure 5. Isoflavone accumulation in soybean MYB-overexpressing hairy root lines. Relative expression levels of MYB genes were measured in the roots of the MYB-overexpressed (OS) hairy root lines and normalized with *GmELF1* (A). The data are presented as means  $\pm$  standard deviations of eight biological replicates. Effects of the overexpressed MYB genes on isoflavone content in the transgenic hairy roots were compared to the control (B). The data are presented as means  $\pm$  standard deviations of three independent hairy root lines. \* $p < 0.05$ , \*\* $p < 0.01$  versus an empty vector line, Student's *t*-test.

of our transient assay system. We confirmed that GmMYB176 could activate *GmCHS8* reporter as previously reported (Yi et al. 2010), and we showed that it also coordinately activated both *GmIFS1* and *GmIFS2* reporters (Figure 2). In a recent study, combined analysis of transcripts and isoflavone contents in soybean hairy roots expressing *GmMYB176*-RNAi or overexpressing *GmMYB176* demonstrated that GmMYB176 regulates multiple steps in isoflavone biosynthesis and affects isoflavone accumulation (Vadivel et al. 2019). These results show that our co-transformation system has great potential for evaluating the effects of orphan TFs on multiple target genes in complex metabolic pathways like isoflavone biosynthesis.

GmMYB202 significantly suppressed GUS activities of all tested target reporters. One possible explanation of this result is that there are diverse functional roles of MYB TFs and some negatively regulate transcription. Several R2R3-type MYB TFs that negatively regulate

target gene transcription have been reported. GmMYB39 negatively regulated the transcription of *GmCHS8* reporter in a co-transfection assay and reduced the total isoflavone level in soybean hairy roots (Liu et al. 2013). In a dual luciferase assay system, GmMYB100 suppressed the transcript levels of both *GmCHS7* and *GmCHI* reporters and decreased levels of isoflavones (Yan et al. 2015). Therefore, agroinfiltration assays might be detectable both positive and negative candidate regulators from orphan TFs.

Furthermore, we demonstrated that three of these MYB genes, *GmMYB102*, *GmMYB280*, and *GmMYB502* and seven isoflavone biosynthetic genes, *GmCHS1*, *GmCHS7*, *GmCHS8*, *GmCHR*, *GmCHI1A*, *GmIFS1*, and *GmIFS2* were highly transcribed at the late stage (70 DAP) of seed development, i.e., the stage with the highest isoflavone accumulation (Figure 4). It has been reported that the expression patterns of the major isoflavone biosynthetic genes *CHS7*, *CHS8*, *IFS1*, and *IFS2* correlate with the isoflavone accumulation pattern in the late stage of soybean seed development (Dhaubhadel et al. 2003, 2007). The results of the transient assay and the expression profile analysis suggested that GmMYB102, GmMYB280, and GmMYB502 are likely seed-specific activators of the isoflavone biosynthetic pathway.

MYB TFs can regulate isoflavone biosynthetic genes through binding to the target DNA binding sites of the upstream promoter region (Solano et al. 1995). We tried to find the common DNA-binding motifs in each promoter for *GmCHS8*, *GmIFS1*, or *GmIFS2* by searching the PLACE database ([www.dna.affrc.go.jp/PLACE/](http://www.dna.affrc.go.jp/PLACE/)). This program predicted three type1 MYBCORE elements (CNGTTR) located in the  $-388$  bp,  $-453$  bp, and  $-1005$  bp regions of the *GmCHS8* promoter (1662 bp), four MYBCORE elements located in the  $-387$  bp,  $-1328$  bp,  $-1354$  bp, and  $-1567$  bp regions of the *GmIFS1* promoter (1740 bp), and a single MYBCORE element located in the  $-1245$  bp region of the *GmIFS2* promoter (1547 bp). MYBCORE elements consist of two sub-groups (type1 and type2); in a previous report, *Arabidopsis* R2R3-MYBs were shown to display high binding affinity to the type1 MYBCORE elements in a yeast one-hybrid system (Kelemen et al. 2015). Therefore, type1 MYBCORE elements could be involved in the coordinate expression of *GmCHS8*, *GmIFS1*, and *GmIFS2* genes via specific MYB TFs in soybean.

Finally, we confirmed activation of isoflavone biosynthesis in the roots of all the soybean hairy root lines overexpressing the three putative MYB TFs, i.e., GmMYB102-OE, GmMYB280-OE, and GmMYB502-OE. In these hairy roots, ectopically expressed MYB TFs may induce the expression of multiple endogenous genes in the isoflavone biosynthetic pathway, which in turn may enhance isoflavone accumulation. We also analyzed the transcripts of several isoflavone biosynthetic



enzyme genes in the above lines. The GmMYB502-OE hairy root line displayed substantial overexpression of genes encoding isoflavone biosynthetic enzymes, but the other two overexpressing lines showed only limited effects on the induction of these genes (Supplementary Figure S2). Recently, by conducting transcriptome analysis of *GmMYB176*-overexpressing hairy roots, Vadivel *et al.* (2019) identified several differentially expressed isoflavone biosynthetic gene families; among them, 16 were down-regulated and 6 were upregulated, suggesting dual roles for GmMYB176 as a positive and negative regulator of isoflavone biosynthetic genes. Possible explanations of some irregular gene expressions in our hairy root experiments may be that some other unidentified endogenous factor could be involved in the regulation of biosynthetic pathway gene expression and/or some other metabolic pathways of glyceollins etc. (Lygin *et al.* 2013) are also affected by overexpressed MYB TFs. However, we consider that the change of accumulation patterns of isoflavones in transgenic hairy roots reflects the activation or suppression of their biosynthetic pathways by MYB TFs.

Novel mutant resource development with reverse genetics and genome editing technologies instead of transgenic technology is a current trend for improving various crops, but the development of a simple and efficient method to identify the key regulator TF in a specific biosynthetic pathway as the target is still desired. In recent years, *Agrobacterium*-mediated transient assays have become more widely used and are considered to be an excellent substitute to stable transformation due to their easy operation and high transformation efficiency (Tsuda *et al.* 2012; Wroblewski *et al.* 2005). In comparison, the functional analysis of plant TFs by *Agrobacterium*-mediated stable transformation is time-consuming and typically requires several months to obtain a dozen T1 transgenic plants for further analysis (Li *et al.* 2009; Yang *et al.* 2000). Our system could provide a more practical and easy way to analyze the function of orphan TFs by combining a highly efficient and reproducible transient agroinfiltration assay with a reliable hairy root transformation assay.

Therefore, we propose this system as a rapid, simple alternative to stable transformation for transcriptional activity evaluation of orphan TFs that regulate multiple target genes in a specific biosynthetic pathway in common plant species.

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