

Bivalent RNA Interference to Increase Isoflavone Biosynthesis in Soybean (*Glycine max*)

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ABSTRACT

*In this work, a bivalent RNA interference (RNAi) plant-transformation vector was constructed to silence both the flavanone 3-hydroxylase (F3H) gene and the flavone synthase II (GmFNSII) gene in soybean (*Glycine max*). Two further unit RNAi vectors were constructed for each of these two genes. RNAi-mediated suppression of these genes effectively regulated flavone and isoflavone production in hairy roots that arose from soybean cotyledons transformed with *Agrobacterium rhizogenes* ATCC15834. Notably, the bivalent RNAi vector had a significantly higher effect for increasing isoflavone production compared with the two unit RNAi vectors. The study highlighted molecular methods that could be used to enhance isoflavone production in soybean and demonstrated the challenges associated with such metabolic engineering for the production of plant natural products.*

Key words: Bivalent RNA interference, Flavanone 3-hydroxylase, Flavone synthase II, Isoflavone biosynthesis, Soybean

INTRODUCTION

Flavonoids are secondary metabolites that are found throughout the plant kingdom and more than 10,000 flavonoids have been described to-date (Tahara 2007). All flavonoids are synthesized from two basic metabolites, malonyl-CoA and p-coumaroyl-CoA at a ratio of 3:1 to 15-carbon skeletons. The derived chalcone intermediates comprise two phenolic groups that are connected by an open three-carbon bridge. That linkage is part of an additional heterocyclic six-member ring that involves one of the phenolic groups on the adjacent ring. Based on the structure of their basic chalcone skeleton, various classes of flavonoids can be synthesized, including flavanones, isoflavones, flavanols, anthocyanins, flavonols and flavones (Kim et al. 2008; Fowler and Koffas 2009).

In nature, flavonoids are involved in many biological processes. Plant flavonoids are involved in defense responses to biotic and abiotic stresses. Anthocyanins are visible flavonoid pigments that give rise to the red and blue colors of certain ripe fruits and these pigments attract frugivores that can help disperse seeds (Wilson and Whelan 1990). Flavones are important for protecting against ultraviolet light, regulating auxin transport and controlling flower color (Harborne and Williams 2000). Isoflavones, the most abundant flavonoids in soybean (*Glycine max*), play diverse roles in plant-microbe interactions (Ferguson et al. 2010). For example, isoflavones function as preformed antibiotics, and they act as precursors for the pterocarpan phytoalexins (Ebel 1986; Rivera-Vargas et al. 1993). Isoflavones also function as signaling molecules for the induction of microbial genes involved in the infection and

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symbiosis between soybean and *Bradyrhizobium japonicum* (Dixon and Sumner 2003; Ferguson and Mathesius 2003). Furthermore, they have direct but complex effects on human nutrition and health; they can reduce cholesterol levels and prevent certain cancers (Weisshaar and Jenkinst 1998; Lee et al. 2005; Kim et al. 2006; Kim and Lee 2007). Isoflavones are especially important for preventing many hormone-dependent cancers and these compounds can improve women's health (Beecher 2003).

Metabolic engineering tools have opened up new possibilities for studying the regulation and accumulation of plant secondary metabolites and investigating their roles in plant defenses (Subramanian et al. 2005). Successes in enhancing the production capacities have come from the manipulation of blocks of genes that encode segments of pathways, over- or under-expression of genes controlling putative rate-limiting steps in pathways, and the expression of transcription factors that regulate entire metabolic pathways

(Wu and Chappell 2008). In soybean, the isoflavones daidzein I, genistein II and glycitein III are synthesized via the phenylpropanoid pathway and these compounds are stored in the vacuole as glucosyl- and malonyl-glucose conjugates (Graham 1991). Genistein II, dihydroflavonol, and apigenin are produced from the same naringenin substrate via three different branches (Fig. 1), which are mediated by isoflavone synthase, flavanone-3-hydroxylase, and flavone synthase II, respectively. Importantly, the genes encoding each of these enzymes have been identified (Steele et al. 1999; Zabala and Vodkin 2005; Jiang et al. 2010). Ralston et al. (2005) described the RNA interference (RNAi) of soybean isoflavone synthase genes using the soybean cotyledon system, and this led to enhanced plant susceptibility to *Phytophthora sojae* in hairy roots. Jiang et al. (2010) suggested that RNAi-mediated suppression of isoflavone synthase pathway genes could enhance isoflavone production in soybean.

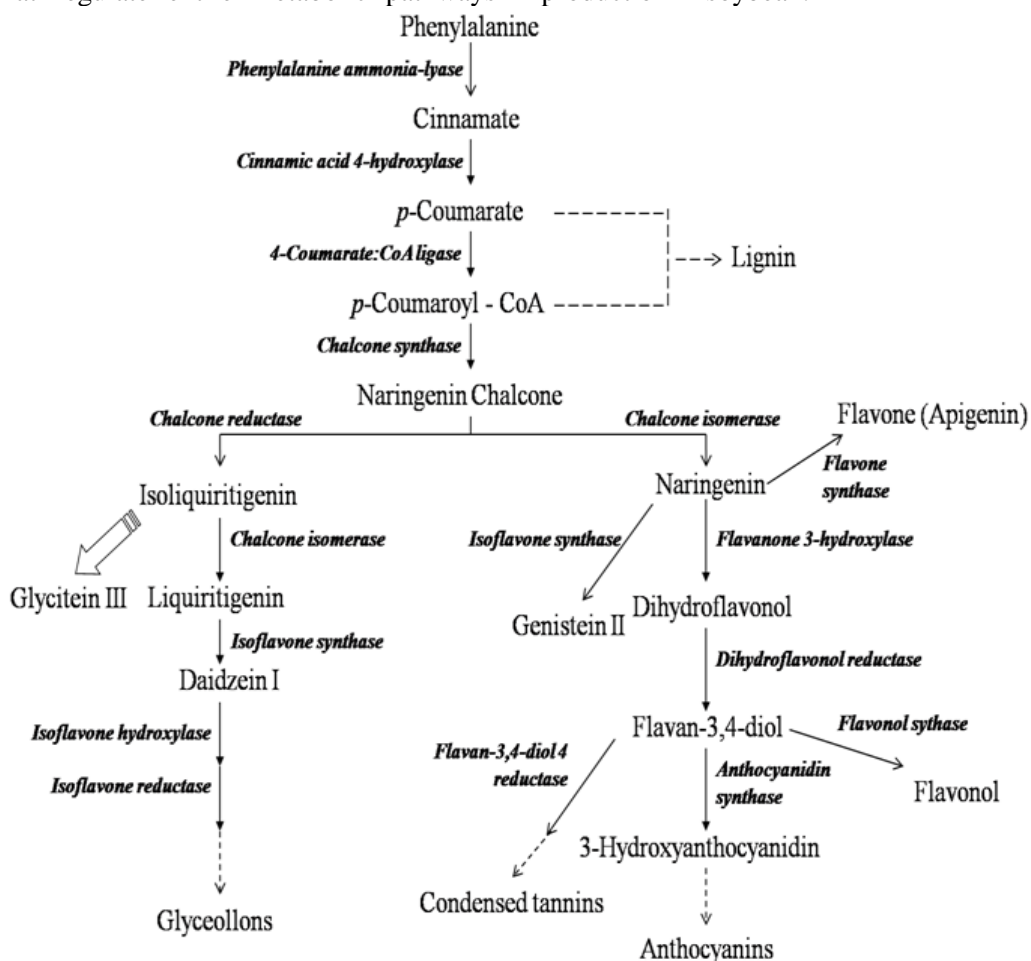


Figure 1 - Partial diagram of phenylpropanoid pathway, including intermediates and enzymes involved in flavonoids synthesis and some branch pathways. Dotted arrows indicate multiple steps.

In this present study, two unit RNAi plant-transformation vectors were constructed to silence the flavanone 3-hydroxylase gene (*F3H*) and the flavone synthase II gene (*GmFNSII*) in soybean. In addition, a bivalent RNAi vector was constructed for both of these genes.

MATERIALS AND METHODS

Cloning full-length cDNAs of *GmFNSII* and *F3H*

GmFNSII and *F3H* cDNA sequences were identified from the SoyBase *G. max* genomic database (<http://soybase.org/SequenceIntro.php>). To obtain the full-length cDNAs for *GmFNSII-2* (GenBank: GU568028) and *F3H* (GenBank: AF198451), total RNA was isolated from the leaf tissues of field-grown soybean cultivar "Hefeng 47" seedlings using Trizol Reagent (Invitrogen). First-strand cDNAs were cloned by reverse-transcriptase polymerase chain reaction (RT-PCR) using the PrimeScript™ 1st Strand cDNA Synthesis Kit (TaKaRa). These cDNAs were used as templates in the PCR for full-length *GmFNSII* and *F3H* cDNA amplification using the following primers: GmFNSII-F/R for *GmFNSII* and F3H-F/R for *F3H* (Table 1). The corresponding PCR products were cloned into the pMD18-T vector (TaKaRa) and then sequenced.

Table 1 - Primers used in this study.

Primer name	Primer sequence (5'-3')
GmFNSII-F	ATGATATCTGAGTCCCTCTTGTAG
GmFNSII-R	CTACACTTGAGGAAAAGAGGTGGGA
F3H-F	CTTGAAGCATTGCATTCTGCTATTT
F3H-R	TAAGGTATACATAGATGAGCCAGCT
BAR-F	TCGAGTCAAATCTCGGTGACGGGCA
BAR-R	CTCGAGTCTACCATGAGCCCAGAAC
Actin-F	AACTTCGTGTTGTTTCCGAGGAG
Actin-R	CACCAGAGTCCAATAACAATACCAG
qRT_FNS-F	AGGTCCAGTAACTCGGAACCAT
qRT_FNS-R	GCTTCACACACTAATCGCTTGT
qRT_F3H-F	ATTCATTGTCTCCAGCCATCT
qRT_F3H-R	TTACTTTGTCGCTGTATTCTCA

Construction of bivalent RNAi vectors

An RNAi vector was constructed for silencing the *GmFNSII* transcripts. A 322-bp coding region located between 897–1218 of the *GmFNSII* cDNA sequence was selected and amplified by the PCR using pMD18–*GmFNSII* as the template. The forward (5'-CTG(ATCGAT/GGTACC)

AACTCGGAACCATGTCAAATC-3') and reverse (5'-CCGG(TCTAGA/CTCGAG)GTTTACGCAAACCTATTGAACCT-3') primer sequences contained two different sets of restriction sites (Table 2). The PCR products were cloned into the pHANNIBAL vector in opposing orientations on either side of a PDK intron to create an invert repeat driven by the cauliflower mosaic virus (CaMV) 35S promoter (Wesley et al. 2001). Then, this *GmFNSII* RNAi construct was cloned into the pCAMBIA3300 plant expression vector (<http://www.cambia.org/daisy/cambia/home.html>), which included the 35S: *BAR* fragment and was digested with *Sac I/Pst I*. The newly construct was named p3300-GmFNSIII.

An RNAi vector was constructed for silencing the *F3H* transcripts. A 303-bp coding region located between 694-997 of the *F3H* cDNA sequence was selected and amplified by the PCR using pMD18-F3H as the template. The forward (5'-CTG(ATCGAT/GGTACC)TGACCTCACTCTTGGCCT-3') and reverse (5'-CCGG(TCTAGA/CTCGAG)TCTCCTTCTCTTATCTTCAGAG-3') primer sequences contained two different sets of restriction sites (Table 2). The PCR products were cloned into the pHANNIBAL vector and then this *F3H* RNAi construct was cloned into pCAMBIA3300 as described above and named p3300-F3Hi.

Table 2 - Restriction sites used in this study.

Name of Restriction sites	Sequence of restriction site (5'-3')
<i>Cla</i> I	AT CGAT
<i>Xba</i> I	T CTAGA
<i>Kpn</i> I	GGTAC C
<i>Xho</i> I	C TCGAG
<i>Sac</i> I	GAGCT C
<i>Pst</i> I	CTGCA G
<i>Pvu</i> I	CGAT CG

A bivalent RNAi vector was constructed for silencing the *GmFNSII* and *F3H* transcripts. The *F3H* RNAi construct in pHANNIBAL and p3300-GmFNSIII were double digested using *Pst I* and *Pvu I* restriction sites and then the *F3H* RNAi construct was cloned into p3300-GmFNSIII; this was named p3300-GmFNSIII-F3Hi. The newly constructed p3300-F3Hi, p3300-GmFNSIII and p3300-GmFNSIII-F3Hi vectors were inserted into *A. rhizogenes* ATCC15834 for the subsequent transformation of the soybean cotyledons. The pCAMBIA3300 vector served as a control.

pHANNIBAL, pCAMBIA3300 and *A. rhizogenes* were kindly gifted by Dr Tang (Fudan-SJTU-Nottingham Plant Biotechnology R&D Center).

Soybean cotyledon transformation and southern blotting

Wild-type and transformed strains of *A. rhizogenes* were maintained by culturing on yeast extract peptone (YEP) agar. For the transformed strains, all the media contained 50 µg mL⁻¹ kanamycin. Cultures for plant inoculation were grown overnight in 10 mL of YEP broth at 28°C. The cells were pelleted by centrifugation at 5,000 ×g and 4°C for 10 min. Cell pellets were drained briefly and gently re-suspended in quarter-strength Murashige aninSkoog medium to a final optical density of 0.5 at 600 nm before the cotyledon tissues were inoculated.

The cotyledon transformations were carried out as follows. Soybean cultivar “Hefeng 47” seeds that had been stored in a cold room were surface-sterilized in 70% ethanol for 1 min and then in 100 mL of 15% Clorox for 30 min, with occasional agitation. Afterward, ten seeds were placed into each Petri dish that contained germination medium (Murashige-Skoog medium with 0.4 mg L⁻¹ 6-Benzyladenine). Seeds were germinated for 5-7 d at 26°C under a 16-h photoperiod. Then, cotyledons showing no surface blemishes were sampled at 0.3 cm away from the petiole end by making small, circular (0.4 cm diameter) incisions. The cut surfaces of six cotyledons were placed on to 9-cm sterile filter paper in each plate, and this formed a wet surface on which the cotyledons were exposed to 20 µL of *A. rhizogenes* suspension. Plates were wrapped in Parafilm and kept in a Percival incubator at 22°C operating a 12-h photoperiod (Subramanian et al. 2005).

After transformation for three weeks, genomic DNA (30-60 µg) of hairy roots containing the wild-type and transformed vectors were cleaved with *Pst* I and digested DNA was then fractionated on a 1.0% agarose gel, denatured, and transferred onto nylon positively charged membranes (Osmonics). Southern blotting followed the manufacturer's instructions (Amersham). The DNA probe (approximately 600 bp), including the *BAR* coding region in pCAMBIA3300 vector, which was detected using the primers BAR-F and BAR-S (Table 1), was labeled with alkaline phosphatase and the membrane exposed to film

with CDPStarTM chemiluminescent detection reagents (Amersham, RPN3690). Hybridization and stringency washes were performed at 55°C.

Quantitative RT-PCR analysis of *GmFNSII* and *F3H*

Total RNA was isolated separately from each of fifteen transgenic hairy roots that resulted from using the wild-type and transformed vectors. Samples from the hairy roots were treated with Trizol (Invitrogen) and then DNase I to remove any contaminating genomic DNA. First-strand cDNAs were cloned by RT-PCR with a PrimeScriptTM 1st Strand cDNA Synthesis Kit (TaKaRa). Quantitative real-time (qRT)-PCR was performed with SYBR-Green and a 7300 Real-Time PCR System (Applied Biosystems). Initial transcript concentrations were estimated according to the comparative threshold cycle method (Bovy et al. 2002). Transcript abundance of the soybean *Actin* gene (V00450) in each RNA preparation was used as the internal standard. The PCR primers used were Actin-F and Actin-R for *Actin*, qRT_FNS-F and qRT_FNS-R for *GmFNSII*, and qRT_F3H-F and qRT_F3H-R for *F3H* (Table 1).

Flavonoid analysis by high-performance liquid chromatography (HPLC)

To evaluate flavonoid composition, 100 mg of transgenic hairy roots were ground in liquid N₂ and extracted for 2-h with 4 mL of 80% methanol using ultrasonic treatment (100 W). For HPLC, aliquots of each extract were analyzed at 260 nm on an Agilent 1100 series HPLC system using a Venusil MP-C18 column (2.1×150 mm, 5 µm, Agela Technologies, Inc.). Samples were separated using a linear gradient of 15–85% methyl alcohol in HPLC-grade water for 30 min at a flow rate of 1 mL min⁻¹. Retention times and UV spectra were compared with authentic standards where available, and the quantity of each compound was calculated by comparison with standard curves prepared using the authentic compounds (Sigma-Aldrich, St. Louis, MO). The quantities of the isoflavone aglycones, glucose-conjugates (daidzin, genistin and glycitin) and malonyl-glucose conjugates were determined for each individual isoflavone and summed to give the total amounts for daidzein I, genistein II and glycitein III.

RESULTS

Construction of the binary RNAi vectors

To determine the roles of *GmFNSII* and *F3H* in flavonoid production and in the accumulation of isoflavone in particular, two unit RNAi vectors and one bivalent RNAi vector were constructed for silencing the *F3H* and *GmFNSII* genes in soybean. A 322-bp coding region of *GmFNSII* cDNA and a

303-bp coding region of *F3H* cDNA were amplified and cloned into the pCAMBIA3300 RNAi binary vector containing a *BAR* selectable marker (Fig. 2). After verification by restriction enzyme digestion and sequencing, the newly constructed p3300-GmFNSIII, p3300-F3Hi and p3300-GmFNSIII-F3Hi vectors were transformed successfully into soybean cotyledons by *A. rhizogenes* to obtain the hairy roots.

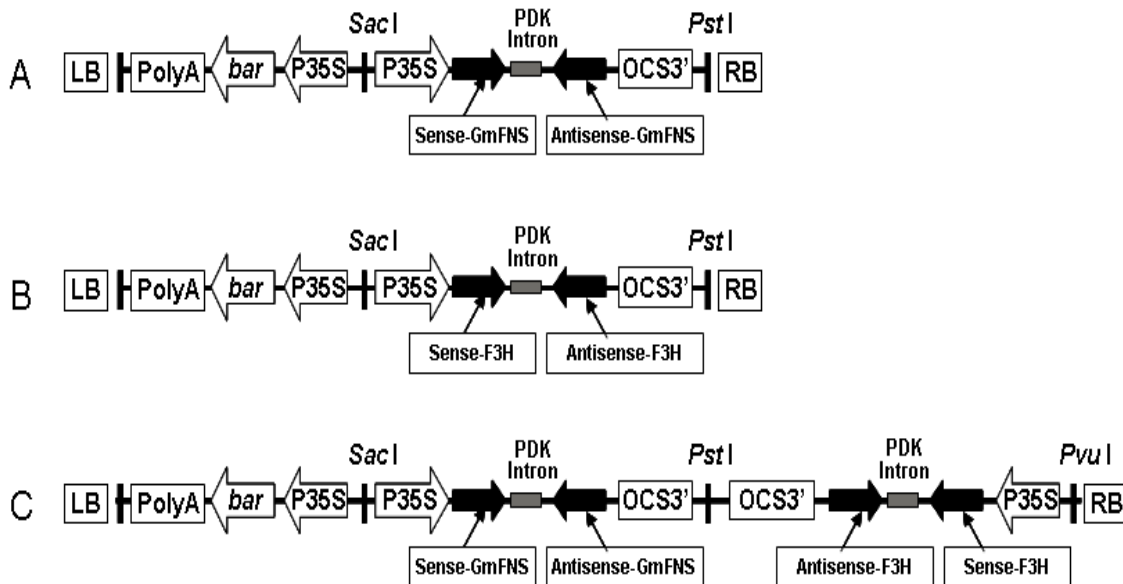


Figure 2 - T-DNA region of RNAi vectors. A: p3300-GmFNSIII, B: p3300-F3Hi, C: p3300-GmFNSIII-F3Hi.

The RNAi strategy down-regulated *GmFNSII* and *F3H* expression in soybean

After transformation for three weeks, hairy roots containing the wild-type and transformed vectors were identified by southern blotting and transformed lines carrying the *BAR* gene were selected (Fig. 3). To investigate the effect of the new constructions, *GmFNSII* and *F3H* expression levels were analyzed by qRT-PCR. Total RNA was extracted from the transgenic hairy roots and reverse-transcribed to cDNAs. As expected, *GmFNSII* and *F3H* RNAi transgenic hairy roots showed significant reductions (to <10%) in *GmFNSII* and *F3H* expression (Fig. 4). At the same time, in the unit RNAi transgenic hairy roots, expression of *GmFNSII* in *F3H* RNAi transgenic hairy roots and expression of *F3H* in *GmFNSII* RNAi roots showed significant addition, especially for *GmFNSII* (about a two-fold increase compared with the control).

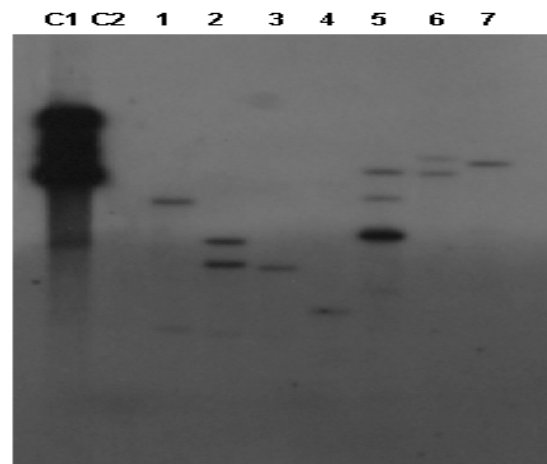


Figure 3 - Southern blotting analysis of transformed hairy roots. C1: Plasmid DNA of pCAMBIA3300, C2: DNA from non-transformed plant root, 1-5: DNA from hairy roots.

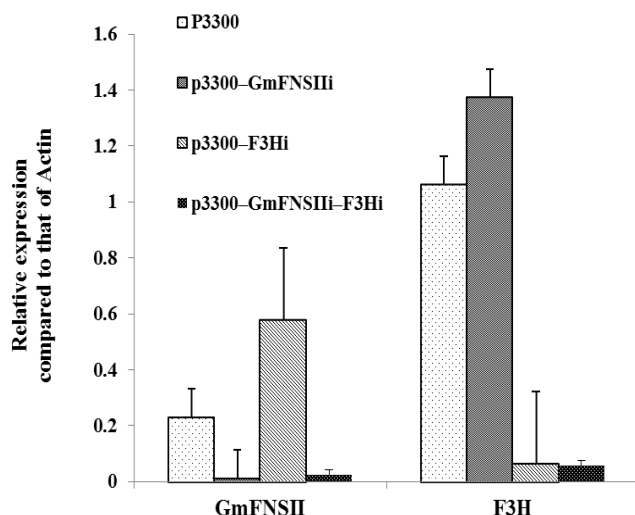


Figure 4 - RNAi-mediated silencing. qRT-PCR analysis showed significant regulation of *GmFNSII* and *F3H* expression in transgenic hairy roots.

Silencing of *GmFNSII* and *F3H* caused high levels of isoflavone accumulation

Single hairy roots of independent positive transformation events were analyzed by HPLC to determine the effects of silencing on the production profiles of root flavones and isoflavones. Mean levels of individual isoflavones and total isoflavone levels varied between the 15 individual transgenic hairy roots. The isoflavone component profile of a control transformant (transformed with just the pCAMBIA3300 vector) showed that daidzein I was the most abundant of the isoflavones in hairy roots (Fig. 5). Genistein II was generally the next abundant component, while glycitein III was found at lowest levels.

Since genistein II, dihydroflavonol and apigenin are all produced from the same naringenin substrate, silencing any of the encoding genes should lead to increased accumulation of the other two compounds. As expected, >90% of the *GmFNSII* RNAi roots had significantly lower levels of apigenin (the major flavone) compared with the control roots. Some of these transgenic roots had undetectable levels of apigenin, indicating effective silencing. At the same time, genistein was found at markedly higher levels. The complete opposite result was observed in the *F3H* RNAi roots, which contained significantly greater levels of apigenin and genistein (Fig. 5). Daidzein I, genistein II and glycitein III levels were summed

to give the total isoflavone levels. *GmFNSII* RNAi roots and *F3H* RNAi roots had significantly higher levels of total isoflavones (1.3- and 1.9-fold, respectively). The bivalent RNAi vector was significantly more effective for increasing isoflavone production in the roots than the two unit RNAi vectors and total isoflavone levels were two-fold higher than the control roots.

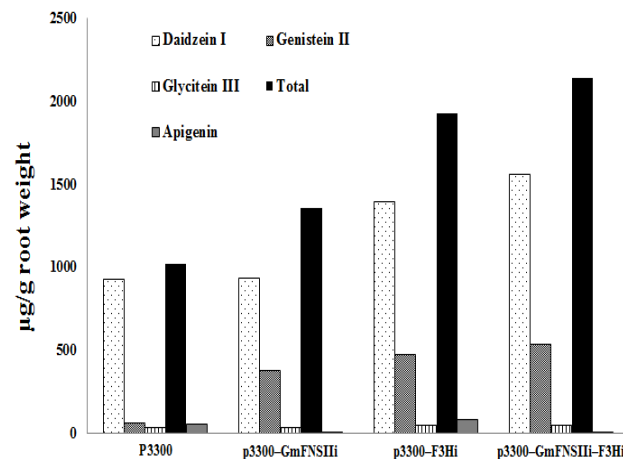


Figure 5 - HPLC analysis of flavonoid content in transformed hairy roots. Mean levels of individual isoflavones and total isoflavone levels varied between 15 individual transgenic hairy roots. Daidzein I, genistein II and glycitein III levels were summed to give the total isoflavone level.

DISCUSSION

Flavonoids are major secondary plant metabolites that mediate diverse biological functions in essential physiological processes and exert significant ecological impacts (Fowler and Koffas 2009). Many flavonoids are used as nutrients, colorants, flavors, fragrances and medicines (Newman and Cragg 2007, Wu and Chappell 2008). Metabolic engineering of the plants for the production of natural products can help alleviate the demands for limited natural resources. Successes in enhancing isoflavone biosynthesis production in soybean include the manipulation of the blocks of genes that encode segments of pathways, up- and down-regulation of putative rate-limiting steps in pathways, and the expression of transcription factors that regulate entire metabolic pathways (Yu et al. 2003; Subramanian et al. 2005; Lozovaya et al. 2007; Jiang et al. 2010).

In this present study, RNAi-mediated suppression of *GmFNSII* or/and *F3H* in the hairy roots of soybean that arose from cotyledons transformed with *A. rhizogenes* was tested to define the roles of these genes in the accumulation of flavones and isoflavones. qRT-PCR data showed significant reductions of *GmFNSII* and *F3H* expression in transgenic hairy roots, which confirmed that the new constructions were very effective in down-regulating the two target genes. These RNAi constructions were expressed under the control of the constitutive CaMV 35S promoter, and therefore it was expected that the transgene effect would not be restricted to the hairy roots but would also influence the flavonoid pathway in other parts of the soybean.

HPLC analysis showed that down-regulation of *F3H* had a greater effect for increasing the total isoflavone production compared with down-regulation of *GmFNSII*. One possible reason for this was the low expression activation of *GmFNSII*. The expression levels of *GmFNSII* were only approximately one-fifth of the expression of *F3H* in the vector-control hairy roots. However, since both the genes showed tissue-specific expression patterns and differential transcript levels in all the tissues (Ralston et al. 2005; Jiang et al. 2010), it was not possible to speculate whether the results would be repeated if different tissues were tested in transgenic plants.

CONCLUSION

This is the first time that a bivalent RNAi plant-transformation vector has been constructed for silencing both the flavanone 3-hydroxylase gene and the flavone synthase II gene in soybean. This study showed that the bivalent RNAi roots were significantly more effective for increasing isoflavone production than the two unit RNAi roots. The attempt to further enhance isoflavone accumulation in soybean reinforced the knowledge on the relationships between different genes and highlighted an effective combination of different approaches in one method.

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