J. Plant Biol. (2010) 53:425–432
DOI 10.1007/s12374-010-9132-9

ORIGINAL RESEARCH

# Flavonoid Production Is Effectively Regulated by RNAi Interference of Two Flavone Synthase Genes from *Glycine max*

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Received: 3 August 2010 / Revised: 7 October 2010 / Accepted: 11 October 2010 / Published online: 3 November 2010


**Abstract** Flavonoids are a group of secondary metabolites found in many higher plants. The multiple roles of their flavone subclass include protection against UV damage, regulation of auxin transport, and modulation of flower color. In soybean (*Glycine max*), flavone synthase II (FNS II) is the key enzyme responsible for flavone biosynthesis. Two FNS II genes from soybean cultivar Hefeng 47 were cloned according to basic local alignment search tool (BLAST) contexts using flavone synthase sequences reported in other species. These were named GmFNSII-1 and GmFNSII-2. Sequence alignments showed that the cDNA of GmFNSII-1 was identical to that of CYP93B16, whereas GmFNSII-2 was clearly distinct. Functional assays in yeast (*Schizosaccharomyces pombe*) suggested that these two enzymes could convert (2S)-naringenin into apigenin. The two GmFNSII genes had similar tissue-specific expression patterns, but GmFNSII-2 was significantly expressed in the roots after treatment with 0.4 M glucose. This demonstrates that the gene plays an important role in the response to defense signals in soybean. RNA interference-mediated suppression of those GmFNSII genes effectively regulated flavone and isoflavone production in hairy roots that arose from soybean cotyledons transformed with *Agrobacterium rhizogenes* (ATCC15834). Our study also highlights some of the challenges associated with metabolic engineering of plant natural products.

**Keywords** Flavones · Flavone synthase II · Isoflavones · Soybean · Subcellular localization

**Abbreviations**

| | |
|---|---|
| BLAST | basic local alignment search tool |
| FNS II | flavone synthase II |
| GmFNSII | soybean flavone synthase II |
| MS medium | Murashige and Skoog medium |
| NADPH | reduced nicotinamide adenine dinucleotide phosphate |
| qRT-PCR | quantitative RT-PCR |

Flavonoids are well-known secondary metabolites in plants, and more than 10,000 compounds have already been reported (Tahara 2007). All flavonoids are synthesized from two basic metabolites—malonyl-CoA and p-coumaroyl-CoA—to 15-carbon skeletons at a 3:1 ratio. The derived chalcone intermediates comprise two phenolic groups 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. Derived from chalcone, various classes of flavonoids can be synthesized, including flavanones, isoflavones, flavanols, anthocyanins, flavonols, and flavones (Kim et al. 2008; Fowler and Koffas 2009).

Plant flavonoids have a wide array of physiological functions in defense responses to biotic and abiotic stresses. Many of these roles, including ultraviolet (UV) protection, regulation of auxin transport, and modulation of flower

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color, have been attributed to a subclass known as flavones (Harborne and Williams 2000). Isoflavones, the most abundant flavonoids in soybean (*Glycine max*), play diverse roles in plant–microbe interactions (Ferguson et al. 2010). Moreover, those compounds have direct but complex effects on human health, e.g., reducing cholesterol levels and preventing certain cancers (Weisshaar and Jenkinst 1998; Lee et al. 2005; Kim et al. 2006; Kim and Lee 2007). They are especially critical to the prevention of many hormone-dependent cancers and can aid in improving women's health (Beecher 2003).

In all higher plants, flavones are produced through a branch of the phenylpropanoid pathway. Their biosynthesis from flavanones involves the introduction of a double bond between C2 and C3 in the heterocyclic ring of the flavanone skeleton. Interestingly, two fundamentally different enzymatic systems—flavone synthase (FNS) I and II—are able to generate flavones directly from flavanones. Both enzymes can convert naringenin to apigenin and eriodoctyol to luteolin. Enzymatic studies in *Petroselinum crispum* have revealed that FNSI, a soluble 2-oxoglutarate- and $Fe^{2+}$-dependent dioxygenase, catalyzes this reaction (Britsch 1990). FNSI has been described mainly in members of Apiaceae and was only recently identified from monocotyledonous plants as well (Martens et al. 2001; Kim et al. 2008). In contrast, FNS II, a membrane-bound cytochrome P450 monooxygenase, is active in *Antirrhinum majus*, where it requires reduced nicotinamide adenine dinucleotide phosphate (NADPH) and molecular oxygen (Heller and Forkmann 1993). This enzyme occurs commonly among higher plants. All FNS II proteins belong to the plant cytochrome P450 subfamily CYP93B.

In soybean, genistein, one type of isoflavone, dihydroflavonol/anthocyanins, and apigenin are all produced with the same naringenin substrate via three different branches of the phenylpropanoid pathway (Fig. 1). Dihydroflavonol and genistein are mediated, respectively, by flavanone-3-hydroxylase (F3H) and isoflavone synthase, for which the encoding gene has been identified (Steele et al. 1999; Zabala and Vodkin 2005). Fliegmann et al. (2010) have described inducible activity for a flavone synthase, GmFNS II (CYP93B16), from cell cultures of soybean, by which naringenin is converted to apigenin.

Here, we report the cloning of two different flavone synthase II homologs from soybean—GmFNSII-1 and GmFNSII-2. We aligned their sequences to determine similarity and performed functional assays in yeast (*Schizosaccharomyces pombe*). A hairy root protocol was followed to analyze how silencing of the GmFNSII genes in transgenic roots might affect the production of flavones and isoflavones. Our objectives were to obtain new insight into the requirements for metabolic engineering methods that could enhance isoflavone production in soybean and to investigate the challenges associated with such engineering of plant natural products.

## Materials and Methods

### Cloning and Sequence Analysis of *GmFNSII-1* and *GmFNSII-2*

To obtain the full-length cDNAs for *GmFNSII-1* (GU568027) and *GmFNSII-2* (GU568028), we isolated total RNA with Trizol Reagent (Invitrogen) from the root tissues of field-grown soybean cultivar "Hefeng 47"seedlings. These were sampled after 24 h of treatment with 0.4 M glucose in an MS solution. First-strand cDNAs were cloned by RT-PCR with a PrimeScript™ 1st Strand cDNA Synthesis Kit (TaKaRa). They were then used as templates in PCR for full-length GmFNSII cDNA amplification with the following primers: GmFNSII-1F/R for *GmFNSII-1* and GmFNSII-2F/R for *GmFNSII-2* (Table 1). The corresponding PCR products were cloned into the pMD18-T vector (TaKaRa) and sequenced.

A homology search was carried out using the BLAST program, and multiple sequence alignments were performed with Clustal W and Genedoc software. Relationships between protein sequences and motifs were predicted by the Hits database. A phylogenetic tree was then constructed with MEGA2 by the neighbor-joining method, and bootstrap values were generated by 1,000 repeats.

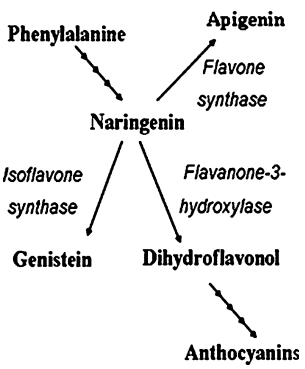



**Fig. 1** Partial diagram of phenylpropanoid pathway, including intermediates and enzymes involved in flavone synthesis, as well as some branch pathways. *Dotted arrows* represent multiple steps

**Table 1** Primers used in these experiments

| Primer | Primer sequence (5′-3′) |
|---|---|
| GmFNSII-1F | ATGATTTCTGAGTCCCTCTTGTTAG |
| GmFNSII-1R | CTACATTTGACGAAAAGGAGTGGGA |
| GmFNSII-2F | ATGATATCTGAGTCCCTCTTGTTAG |
| GmFNSII-2R | CTACACTTGAGGAAAAGAGGTGGGA |
| qRT_FNS-1F | AGGTCCAGTTAACTCGGAACCAT |
| qRT_FNS-1R | GCTTCACACACTAATTGCGTGT |
| qRT_FNS-2F | AGGTCCAGTTAACTCGGAACCAT |
| qRT_FNS-2R | GCTTCACACACTAATCGCTTGT |

### *In vitro* Yeast Assays for Enzyme Activity

The coding regions of *GmFNSII-1* and *GmFNSII-2*, starting from the start codon (ATG) and ending at the stop codon, were cloned into the pESP-2 yeast (*S. pombe*) expression vector (Stratagene) under the control of an *nmt1* promoter at multi-cloning sites. Those constructs, as well as the pESP-2 vector (as our control), were transformed into competent yeast cells. The protein content of each microsome preparation was assayed per the Bradford protein microassay (Bio-Rad). We also performed an *in vitro* microsomal enzyme assay according to the protocols of Yu et al. (2000).

Aliquots of the above extracts were analyzed on an Agilent 1100 series HPLC system, using a Venusil MP-C18 column (2.1×150 mm, 5 μm; Agela Technologies, Inc.). Samples were diluted in methanol and then separated with an 18-min linear gradient of 20% methanol–80% 10 mM ammonium acetate (pH 5.6) going up to 100% methanol. The flow rate was 0.1 mL $min^{-1}$. Elution of metabolites was monitored by a photodiode array. Retention times and UV spectra were compared with those of authentic standards when available (Ralston et al. 2005).

### Quantitative RT-PCR Analysis of *GmFNSII-1* and *GmFNSII-2*

Total RNA was separately isolated from the roots, stems, leaves, flowers, immature seeds, and immature siliques of field-grown "Hefeng 47". Samples from those plants were treated with Trizol (Invitrogen) and then DNase I to remove contaminating genomic DNA. First-strand cDNAs were cloned by RT-PCR with a PrimeScript™ 1st Strand cDNA Synthesis Kit (TaKaRa). 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 an internal standard. PCR primers included qRT_FNS-1F and qRT_FNS-1R for *GmFNSII-1*, and qRT_FNS-2F and qRT_FNS-2R for *GmFNSII-2* (Table 1).

For glucose treatment, seeds of "Hefeng 47" were placed in pots in a controlled climate chamber (16-h photoperiod, 25°C), and the resultant seedlings were excavated when their first pairs of true leaves were fully expanded (about 10 d). The seedlings were then placed in an MS solution supplemented with 0.4 M glucose. Root tissues were collected after 24 h of treatment. Total RNA was isolated and digested with DNase I. Transcript levels were analyzed using real-time RT-PCR as described above.

### Construction of GmFNSII RNAi Vectors

An RNAi vector was constructed for silencing the GmFNSII transcripts. A 322-bp coding region that was >96% identical between *GmFNSII-1* and *GmFNSII-2* was selected and amplified by PCR using pMD18—GmFNSII-2 as the template. The forward primer sequence 5′-CTG (ATCGAT/GGTACC)AACTCGGAACCATGTCAAATC-3′, and the reverse primer sequence 5′-CCGG(TCTAGA/CTCGAG) GTTTACGCAAACTATTGAACCT-3′ contained two different sets of restriction sites. PCR products were cloned into the pHANNIBAL vector in opposite orientations on either side of a PDK intron to create an invert repeat driven by the CaMV35S promoter (Wesley et al. 2001). This GmFNSII RNAi construct was then cloned into plant expression vector p1304+PBI121, which was obtained by adding the 35S:GUS fragment from pBI121 into pCambia1304. The newly constructed pCAMGUS–GmFNSII–RNAi vector was inserted into *Agrobacterium rhizogenes* strain ATCC15834 for transformation of our soybean cotyledons. Vector p1304+PBI121 served as a control. pHANNIBAL, p1304+PBI121, and *A. rhizogenes* were all the kind gifts of Dr. Tang (Fudan-SJTU-Nottingham Plant Biotechnology R&D Center).

### Soybean Cotyledon Transformation

Wild-type and transformed strains of *A. rhizogenes* were maintained by culturing on yeast extract peptone (YEP) agar. For the transformed strains, all media contained 50 μg $mL^{-1}$ kanamycin. Cultures for plant inoculation were grown overnight in 10 mL of YEP broth at 28°C. Cells were then spun down for 10 min at 5,000 rpm in a tabletop centrifuge at 4°C. The pellets were drained briefly and then gently re-suspended in a quarter-strength MS medium to a final $OD_{600}$ of approximately 0.5 before the cotyledon tissues were inoculated.

Soybean 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 shaking. Afterward, ten seeds each were placed in Petri dishes containing a germination medium (MS basal medium with 0.4 mg $L^{-1}$ 6-BA). The cotyledon transformation was carried out as follows. Seeds were germinated for 5 to 7 d at 26°C, under a 16-h photoperiod. Cotyledons that had no surface blemishes were then sampled at 0.3 cm away from the petiole end by making small, roughly circular incisions (0.4 cm diam.). The cut surfaces of six cotyledons each were placed in plates for suspension with 20 μL of *A. rhizogenes*. Plates were then wrapped in Parafilm and kept in a Percival Incubator at 22°C under a 12-h photoperiod (Subramanian et al. 2005).

### Histochemical Analysis of *GUS* Expression

After transformation for 3 weeks, the hairy roots that resulted from using the pCAMGUS-GmFNSII-RNAi vector were immersed in a GUS-staining solution that comprised 0.05% 5-bromo-4-chloro-indolyl-β-D-glucuronide and a 100 mM sodium phosphate buffer (pH 7.0) that contained 10 mM EDTA, 0.1% Triton, and 0.5 mM $K_4Fe(CN)_6 6H_2O$. Tissues were allowed to stain for 24 h at 37°C (Jefferson et al. 1987). To stop this process, the roots were removed and washed with 70% ethanol. GUS-staining patterns were visualized with a dissecting microscope (Olympus SZX12).

### Flavonoid Analysis by HPLC

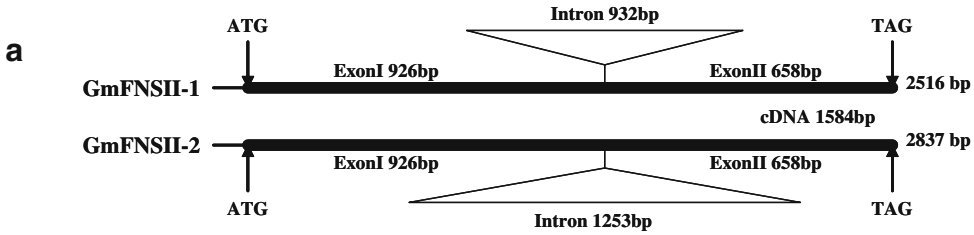
To evaluate their flavonoid composition, 100 mg of transgenic roots were ground under liquid $N_2$ and extracted for 2 h by ultrasound (100 W) with 400 mL of 80% methanol. For HPLC, aliquots of those extracts were analyzed at 236 nM on an Agilent 1100 series HPLC system, using a Venusil MP-C18 column. Samples were separated by a linear gradient of 0% to 55% acetonitrile in HPLC-grade water (pH 3.0 adjusted with phosphoric acid) for 30 min, at a flow rate of 0.2 mL $min^{-1}$.

## Results

### Two Putative GmFNS II Genes Were Obtained from the Soybean Genome

Using a BLAST search of the SoyBase *G. max* genomic database (http://soybase.org/SequenceIntro.php), we determined that our selected sequences were most closely aligned with *GeFNSII* (51%) from *Glycyrrhiza echinata* (CYP93B1; Akashi et al. 1999) and *MtFNSII* (53%) from *Medicago truncatula* (CYP93B12; Li et al. 2007). Among the resulting matches with complete open reading frames (ORFs), the two assemble contigs with greatest homology were selected as putative FNSII full-length genomic sequences. For further characterization, they were amplified from "Hefeng 47" and designated as *GmFNSII-1* and *GmFNSII-2*.

The ORF regions between *GmFNSII-1* and GmFNSII (CYP93B16, FJ767774) shared over 99% identity at the




b

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GmFNSII-1     1 MISESLLLVFLIVFISASLLKLLFVRENKPKAHLKNPPSPPAIPIIGHLHLLKPLIHHSFRDLSLRYGPLLSLRIGSVKF
GmFNSII-2     1 MISESLLLVFLIVFISASLLKLLFVRKNKPKAHLKYPPSPPAIPIIGHLHLLKPLIHHSFRDLCLRYGPLLSLRIGSVKF

GmFNSII-1    81 IVASTPSLAQEFLKTNELTYSSRKMNMAINMVTYHNATFAFAPYDTYWKFMKKLSTTELLGNKTLGHFLPIRTREVHDII
GmFNSII-2    81 IVASTPSLAKEFLKTNELTYSSRKMNMAINTVTYHNATFAFAPYDTYWKFMKKLSTTELLGNKTLGHFLPIRTQEVHDFI

GmFNSII-1   161 QFLFHKSKAQESVNLTEALLSLSNNVISQMMLSIKSSGTDSQAEQARTLVREVTQIFGEFNVSDFLGFCKNLDLQGFRKR
GmFNSII-2   161 QILFHKSKAQESVNLTEALLRLSNNVISRMMLSIKSSGTDSQAEQARALVREVTRIFGEFNVSDFLGFCKNMDLQSFRKR

GmFNSII-1   241 ALDIHKRYDALLEKIISDREELRRKSKVDGCEDGDDEKVKDFLDILLDVAEQKECEVQLTRNHVKSLILDYFTAATDTTA
GmFNSII-2   241 ALDIHKRYDALLEKIISDREELRRKSKEEGCEDGGDEKVKDFLDILLDVSEQKECEVQLTRNHVKSLILDYFTAATDTTA

GmFNSII-1   321 ISVEWTIAELFNNPKVLKKAQEEVDRVTGNTQLVCEADIPNLPYIHAIIKETMRLHPPIPMIMRKGIEDCVVNGNMIPKG
GmFNSII-2   321 ISVEWTIAELFNNPKVLKKAQEEVEKVTGNKRLVCEADISNLPYIHAIIKETMRLHPPIPMITRKGIEDCVVNGNMIPKG

GmFNSII-1   401 SIVCVNIWAMGRDPNIWKNPLEFKPERFLEGEGSAIDTKGHHFELLPFGSGRRGCPGMPLAMRELPTIIGALIQCFEWKM
GmFNSII-2   401 SIVCVNIWAMGRDPNIWKNPLEFMPERFLEGEGSAIDTKGHHFELLPFGSGRRGCPGMPLAMRELPTFIGALILCFEWKM

GmFNSII-1   481 LGSQGEILDHGRSLISMDERPGLTAPRANDLIGIPVARLNPTPFRQM
GmFNSII-2   481 FGSQGEILDHGKSLINMDERPGLTAPRANDLIGIPVARLNPTSFPQV
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**Fig. 2** Schematic representation of GmFNSII-1 and GmFNSII-2 genes and alignment of two derived amino acid sequences. **a** *Top* represents genomic sequence of *GmFNSII-1*; *bottom* is for *GmFNSII-2*. Intron locations are indicated, with sizes in base pairs. Full lengths of cDNAs are given under *thick solid lines* for each gene, and entire length of each sequence is shown to right of each *thick solid line*. **b** Alignment of two derived amino acid sequences from *GmFNSII-1* and *GmFNSII-2*. Identical amino acids are shaded in *black*; conserved residues, in *gray*. *Unshaded* amino acids represent differences between sequences for each gene. Amino acids are numbered at *left*

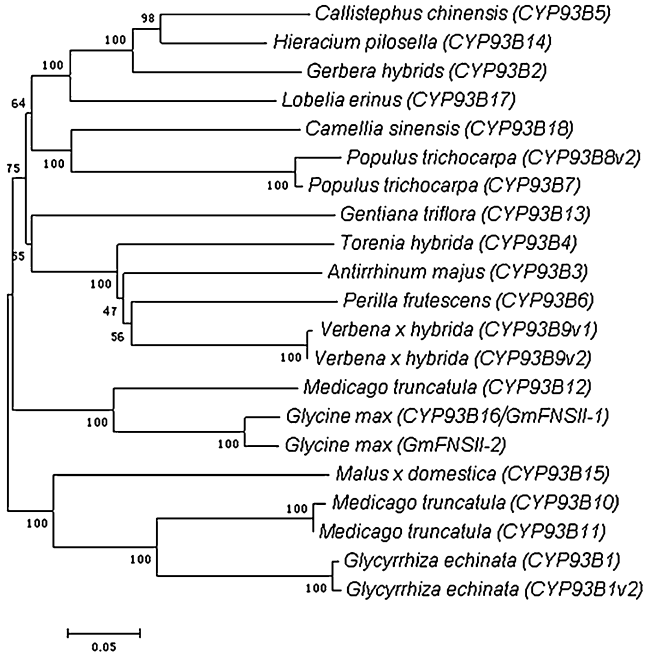


**Fig. 3** Phylogenetic comparison of deduced amino acid sequences for select FNSII-like genes from higher plants. FNS IIs were grouped into families based on sequence homology and substrate specificity. Their sequences and corresponding GenBank Accession Numbers are: CYP93B1 (*G. echinata*, AB001380); CYP93B1v2 (*G. echinata*, AB022733); CYP93B2 (*Gerbera hybrida*, AF156976); CYP93B3 (*A. majus*, AB028151); CYP93B4 (*Torrentia hybrida*, AB028152); CYP93B5 (*Callistephus chinensis*, AF188612); CYP93B6 (*Perilla frutescens* var. crispa, AB045592); CYP93B7 (*Populus trichocarpa*, XM_002334800); CYP93B8v2 (*P. trichocarpa*, XM_002328514); CYP93B9v1 (*Verbena* x *hybrida*, AB234903); CYP93B9v2 (*V.* x *hybrida*, AB234910); CYP93B10 (*M. truncatula*, DQ354373); CYP93B11 (*M. truncatula*, AC146789); CYP93B12 (*M. truncatula*, DQ335809); CYP93B13 (*Gentiana triflora*, AB193314); CYP93B14 (*Hieracium pilosella*, EU561012); CYP93B15 (*Malus* x *domestica*, EG631280); CYP93B17 (*Lobelia erinus*, AB221081); and CYP93B18 (*Camellia sinensis*, FJ169499). Of these sequences, *GmFNSII-1* and *GmFNSII-2* were most homologous to *MtFNSII* (CYP93B12), with 76% and 77% identity, respectively

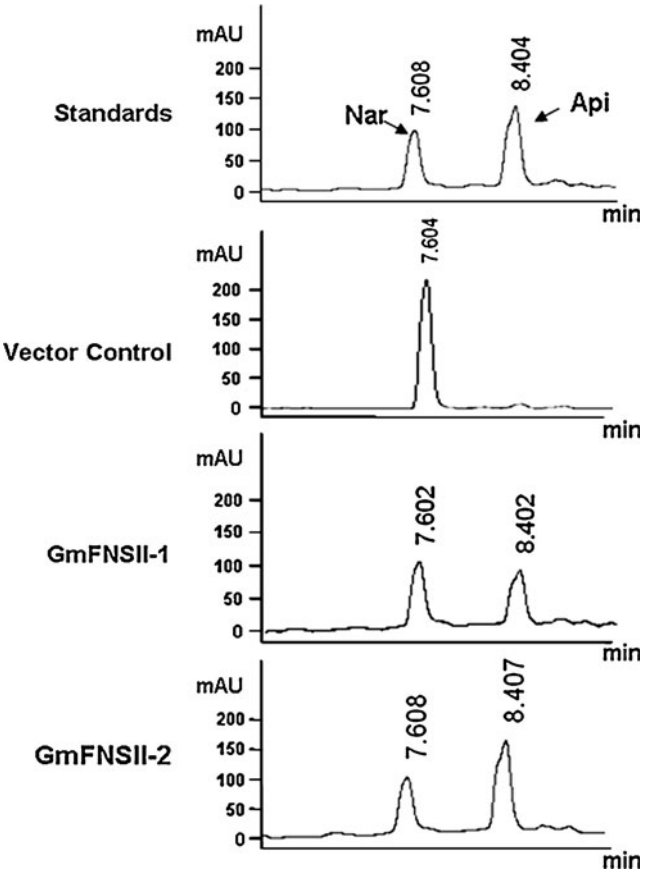


**Fig. 4** Reversed-phase HPLC profiles of extracts from yeast cells expressing *GmFNSII-1* or *GmFNSII-2*. Partial HPLC chromatograms show conversion of apigenin (*Api*) from naringenin (*Nar*). Yeast cells expressing those genes produced an apigenin (*Api*) peak not seen in vector-control reactions

nucleic acid level and over 99% identity at the amino acid level, differing by only one amino acid. Therefore, we considered them to be the same gene, which we refer to here as *GmFNSII-1*. *GmFNSII-1* (GU575289) and *GmFNSII-2* (GU575290) are 2,516 and 2,837 bp-long, respectively (Fig. 2a). The ORF regions between them share 95% identity at the nucleic acid level and 93% identity at the amino acid level (Fig. 2b). Both genes have one intron at the same location, i.e., position 927 from the start codon (ATG). By contrast, their intron sequence structures are highly divergent. As determined by alignments against their cDNA sequences, *GmFNSII-1* has one putative 932-bp-long intron while that of *GmFNSII-2* is 1,253 bp-long (Fig. 2a).

Full-length cDNAs of *GmFNSII-1* (GU568027) and *GmFNSII-2* (GU568028) were cloned by RT-PCR from "Hefeng 47" seedlings. Sequence analysis of their cDNAs revealed respective identities of 62% and 61% with *GeFNSII* (AB001380), and 76% and 77% identities with *MtFNSII* (DQ335809). The deduced amino acid sequences from both also share 50% identity with *GeFNSII* and 64% identity with *MtFNSII*. Based on an InterProScan Sequence Search, both GmFNSII genes show functional regions for cytochrome P450s. Their deduced amino acid sequences are also aligned with other members of the CYP93B family (Fig. 3). Of these, *GmFNSII-1* and *GmFNSII-2* are most homologous to *MtFNSII* (CYP93B12, DQ335809).

## Yeast Expression Assays Showed that GmFNSII-1 and GmFNSII-2 Are Functional Flavone Synthase

Functional assays of FNS II activity were performed by testing the ability of yeast cells expressing GmFNSII to convert (2S)-naringenin into apigenin. Vector pESP-2, conjugated into *GmFNSII-1* and *GmFNSII-2*, was transformed as a negative control into yeast cells. Those

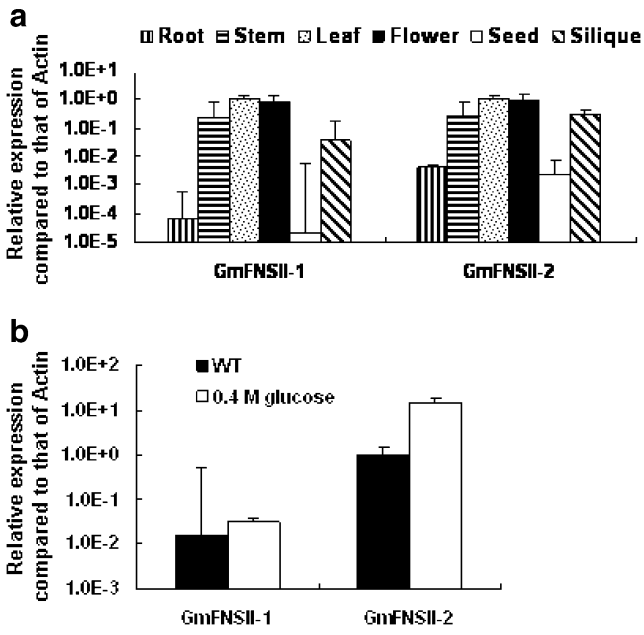


**Fig. 5** Real-time RT-PCR analyses of *GmFNSII-1* and *GmFNSII-2* expression patterns in different untreated tissues (**a**) and in roots after treatment with 0.4 M glucose (**b**). Transcript levels were estimated using relative Ct value method and normalized to that of soybean *Actin*. *Error bars* indicate range of possible values based on SD of replicate Ct values. Data are representative of three independent repeats

expressing *GmFNSII-1* and *GmFNSII-2* also converted naringenin directly into apigenin. In contrast, control yeast cells carrying empty vectors did not produce any apigenin (Fig. 4). This direct mode of conversion suggests that both GmFNSII-1 and GmFNSII-2 are indeed functional flavone synthase.

### Tissue-Specific Expression Patterns of *GmFNSII-1* and *GmFNSII-2*

To investigate the biological functions of *GmFNSII-1* and *GmFNSII-2*, their expression patterns were analyzed by real-time quantitative RT-PCR. Total RNA was extracted from the roots, stems, leaves, flowers, immature seeds, and immature siliques and reverse-transcribed to cDNAs. Both genes were expressed in all tissues (Fig. 5a). However, transcript levels were lower in the roots and immature seeds than in other tissues and were in fact hardly detectable for *GmFNSII-1* in either roots or seeds.

We tested the effects of osmotic stress by treating seedlings with 0.4 M glucose for 24 h. In root samples, transcript levels were not significantly altered for *GmFNSII-1* while those of *GmFNSII-2*, with normally low expression, were sharply up-regulated (about 15-fold over the control) after 1 d of exposure (Fig. 5b).

### Silencing of GmFNSII Effectively Regulated Flavonoid Production

To determine the role of GmFNSII in flavonoid production, and in the accumulations of flavone and isoflavone in particular, we silenced *GmFNSII-1* and *GmFNSII-2* simultaneously in hairy roots. A 322-bp coding region with >96% identity between *GmFNSII-1* and *GmFNSII-2* was amplified and cloned into an RNAi binary vector containing a *GUS* selectable marker (Fig. 6a). This pCAMGUS–GmFNSII–RNAi construct had been transformed into soybean cotyledons by *A. rhizogenes* to obtain hairy roots.

Our histochemical analysis of *GUS* expression (Fig. 6b) revealed an abundance of transgenic roots. As expected, GmFNSII RNAi transgenic roots showed a significant reduction (to <10%) in GmFNSII expression, especially for *GmFNSII-2* (Fig. 6c). The effects of silencing on production profiles for root flavones and isoflavones were assayed by HPLC. Over 90% of the GmFNSII RNAi roots had significantly lower levels of apigenin, the major flavone, compared with the vector-control roots. Some of these transgenic roots had undetectable levels of apigenin, indicating effective silencing. At the same time, genistein, a major isoflavone in soybean roots, was obviously increased (Fig. 6d).

## Discussion

Flavonoids are major plant secondary metabolites that mediate diverse biological functions in essential physiological processes and exert significant ecological impacts (Fowler and Koffas 2009). Fliegmann et al. (2010) have described the inducible activity of flavone synthase in cell cultures from soybean, where naringenin is converted to apigenin. That gene has been classified as *GmFNS II* (CYP93B16). Here, we found two genes that encode flavone synthase from “Hefeng 47” soybean. Sequence alignment showed that *GmFNSII-1* cDNA was the same as for CYP93B16 whereas that of *GmFNSII-2* was clearly distinct. Analyses of molecular evolution, as well as measurements of enzyme function, strongly suggest that GmFNSII represents functional flavanone-2-hydroxylases that catalyze the conversion of (2S)-naringenin to apigenin.

Soybean FNSII activity was first reported in cell suspension cultures under osmotic stress. Such conditions can induce the production of a microsomal NADPH-dependent flavone synthase (flavone synthase II), which catalyzes the conversion of (2S)-naringenin to apigenin (Kochs et al. 1987). Our results from real-time quantitative RT-PCR demonstrated that GmFNSII genes are expressed to varying degrees in all tissues from field-grown “Hefeng 47”. Other elicitors, such as drought stress, may also induce

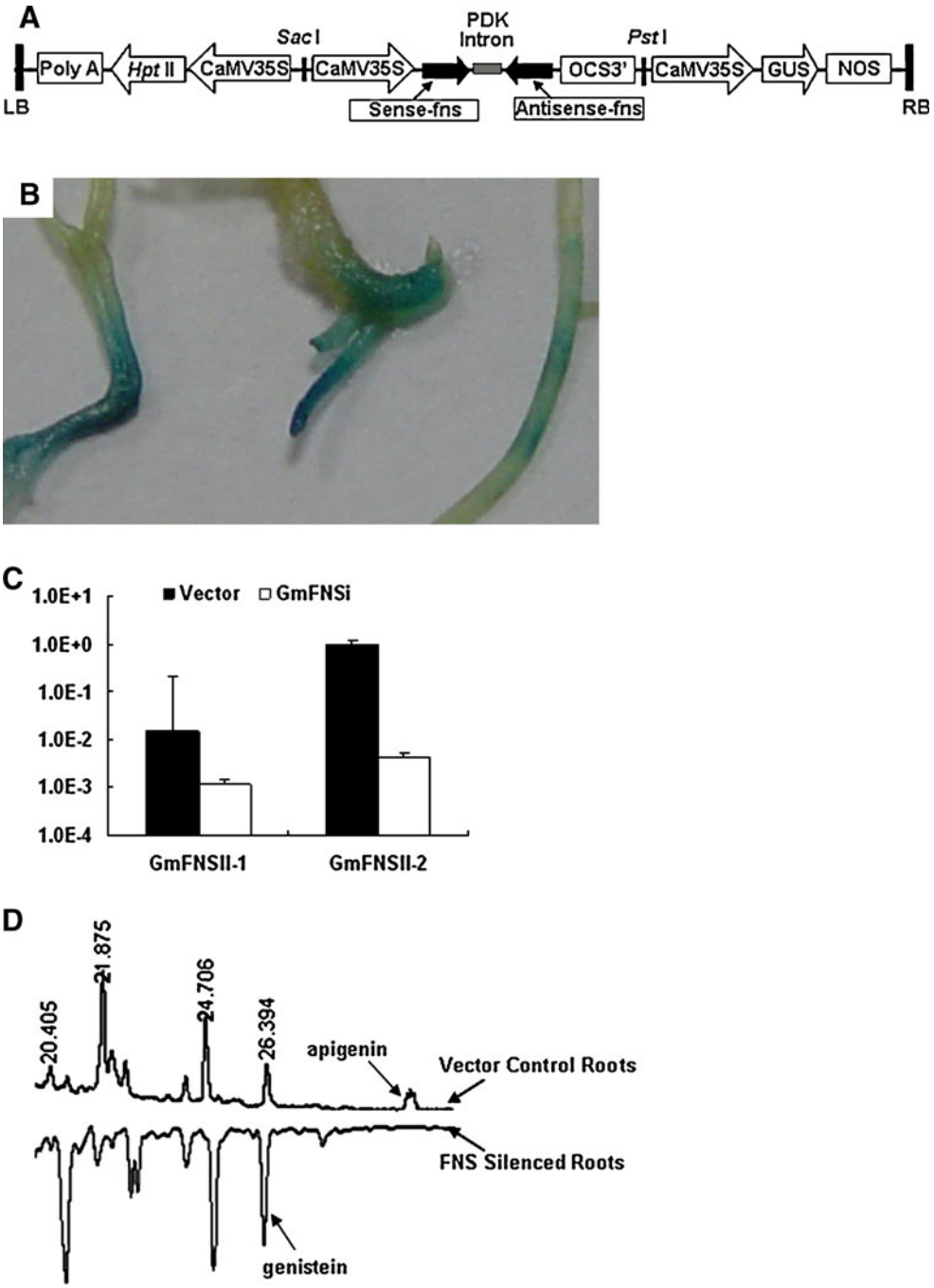


**Fig. 6** **a** T-DNA region of pCAMGUS–GmFNSII-RNAi vector. **b** GUS-staining of transgenic roots from RNAi-mediated silencing of GmFNSII genes. **c** RNAi-mediated silencing; real-time RT-PCR analysis showed significant reduction in expression. **d** RNAi-mediated GmFNSII-silencing reduced apigenin levels in hairy roots

GmFNSII production under field conditions. We obtained very similar results when we treated entire soybean seedlings with 0.4 mM glucose. However, when plants were exposed to osmotic stress, *GmFNSII-2*, with normally low expression, was highly induced in the roots. One possible reason is that activation of enzymes within the biosynthetic pathway is coordinated by defense signals (e.g., from 0.4 M glucose treatment) (Ralston et al. 2005). Enzymes that form a complex are coordinately regulated to enable proper channeling of metabolites. Further investigation is necessary to understand whether this phenomenon is also associated with GmFNSII-functioning in response to a defense signal in soybean.

To further define the role of GmFNSII in the accumulation of flavones and isoflavones, we introduced an RNAi construct that targeted both *GmFNSII-1* and *GmFNSII-2* in hairy roots that had developed from transformed soybean cotyledons. Silencing of the mRNA led to significantly reduced apigenin levels but also an obvious increase in genistein. This suggests that, in soybean, flavones and genistein are produced through different branches of the phenylpropanoid pathway that utilize the same substrate

(Fig. 1). Such evidence also implies that a metabolic engineering strategy can be effectively targeted to enhance levels of isoflavones in that crop (Yu et al. 2003).

**Acknowledgements** This work was financed by the National Natural Science Fund (31071453), the National Fund on "Breeding of New Cultivars" (2009ZX08004-009B) and the Shanghai Horticulture Key Discipline Construction Fund (B209). The English editing services by Priscilla Light and Theresa Herman are also acknowledged.

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