Mol. Cells, Vol. 25, No. 2, pp. 247-252

Molecules and Cells

Accumulation of Flavonols in Response to Ultraviolet-B Irradiation in Soybean Is Related to Induction of Flavanone 3-β-Hydroxylase and Flavonol Synthase

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(Received August 10, 2007; Accepted October 9, 2007)

There are several branch points in the flavonoid synthesis pathway starting from chalcone. Among them, the hydroxylation of flavanone is a key step leading to flavonol and anthocyanin. The flavanone 3-β-hydroxylase (*GmF3H*) gene was cloned from soybean (*Glycine max* cultivar Sinpaldal) and shown to convert eriodictyol and naringenin into taxifolin and dihydrokaempferol, respectively. The major flavonoids in this soybean cultivar were found by LC-MS/MS to be kamepferol *O*-triglycosides and *O*-diglycosides. Expression of *GmF3H* and flavonol synthase (*GmFLS*) was induced by ultraviolet-B (UV-B) irradiation and their expression stimulated accumulation of kaempferol glycones. Thus, GmF3H and GmFLS appear to be key enzymes in the biosynthesis of the UV-protectant, kaempferol.

Keywords: Flavanone 3-β-Hydroxylase; Flavonoid; Flavonol Synthase; *Glycine max.*

Introduction

The plant phenylpropanoid pathway generates diverse compounds such as lignins, (iso)flavonoids, and anthocyanins. Members of a typical class of phenylpropanoids, flavonoids, have C6-C3-C6 skeletons. There are estimated to be more than 10,000 flavonoids (Tahara, 2007), and their biological functions in plants as well as in humans are as diverse as their structures. They have important roles in plant growth and development, and protect plants from environmental stresses such as UV, heat, cold, microorganism and pests (Dixon and Steele, 1999; Shirley, 1996). In humans they act as antioxidants and radical scavengers, and have estrogenic activity, growth stimulatory activity, and antimicrobial activity (Havsteen, 2002; Shim et al., 2007).

The biosynthetic pathway of flavonoids has been established by characterizing the genes involved (Winkel-Shirley, 2001); chalcone synthesized from malonyl-CoA and *p*-coumaroyl-CoA by chalcone synthase (CHS) is the starting material for flavonoid biosynthesis. Chalcone isomerase (CHI) eventually converts chalcone to flavanone, the precursor of diverse flavonoid-classes. Flavanone is a precursor of other classes of flavonoids such as isoflavones, flavanols, anthocyanins, flavonols, and flavones. A key step in the biosynthesis of flavonol and anthocyanin is the addition of a hydroxyl group to carbon 3 of flavanone catalyzed by flavanone 3-β-hydroxylase (F3H). F3H is one of the dioxygenases whose activity is dependent on oxoglutarate and Fe^{2+}. It catalyzes the reduction of oxygen to yield hydroxylated substrates in addition to succinate and CO_2 (Forkmann, 1991; Forkmann and Heller, 1999; Forkmann and Martens, 2001), and is a target enzyme for modifying the anthocyanin pathway in plants (Liu et al., 2002; Wiseman, 1998; Zabala and Vodkin, 2005).

Soybean is known to contain isoflavones such as diadzein and genistein as well as flavones (apigenin and luteolin) and flavonols (kaempferol and quercetin) (Boué et al., 2003; Graham, 1991). There have been several studies of isoflavone biosynthesis in soybean (Kim et al., 2005; Lozovaya et al., 2007; Yu et al., 2000; 2003). In addition, several soybean mutants affecting genes of anthocyanin or flavonol biosynthesis have been identified (Fasoula et al., 1995; Toda et al., 2002; Zabala and Vodkin, 2005). These mutants are affected in either dihy-

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Abbreviations: CHI, chalcone isomerase; CHS, chalcone synthase; DFR, dihydroflavonol 4-reductase; F3H, flavanone 3-β-hydroxylase; FLS, flavonol synthase.

droflavonol 4-reductase (DFR), CHS, flavonol synthase (FLS) or F3H. However, none of these soybean enzymes except FLS has been biochemical characterized (Takahshi et al., 2007). Purification of these enzymes is time-consuming and some of them are unstable.

Flavonols protect plants from ultraviolet B (UV-B) irradiation. In soybean, the UV-B resistant cultivar contains more flavonol than the susceptible cultivar (Reed et al., 1992; Sullivan and Teramura, 1990). There has been no report concerning the relationship between the induction of genes involved in flavonol biosynthesis and the accumulation of flavonols. For this reason we analyzed the major flavonols in soybean by LC/MS/MS and observed the changes of these flavonols upon UV-irradiation. In addition, we showed that genes involved in flavonol biosynthesis such as *GmF3H* and *GmDFR* were induced by UV-irradiation.

Materials and Methods

Chemicals Flavonoids were purchased from Indofine Chemicals (Hillsborough, USA) or Sigma (Sigma-Aldrich, USA). Solvents for HPLC were from Merck (USA).

Cloning of *GmF3H* Total RNA from whole two-week-old UV-B-treated soybean (*Glycine max* Sinpaldal) plants was isolated with a plant total RNA isolation kit (Qiagen, USA), and cDNA was synthesized using Omniscript reverse transcriptase (Qiagen, USA) with oligo dT as primer. Polymerase chain reactions (PCR) were performed with HotStart Taq Polymerase (Qiagen) under the following conditions: 40 cycles of 1 min denaturation at 94°C, 1 min annealing at 55°C, and 1 min amplification at 72°C. Primers were 5′-AGACAACAATGGCACCAACA-3′ as forward primer and 5′-ACTAAAAACACCAAGGGGGC-3′ as reverse primer, both designed based on the sequence in GenBank access number AY669324. The PCR product was subcloned into pGEMT- Easy (Promega, USA) and sequenced.

Expression of *GmFLS* encoding flavonol synthase (Takahashi et al., 2007) and *GmDFR* encoding dihydroflavonol 4-reductase (Fasoula, 1995) in response to UV-B irradiation was investigated as described in Kim et al. (2004). Primers used were: 5′-GAGCAACCAGGCATCACAAC-3′ and 5′-GGTCGCCAATG-TGAATAACG-3′ based on the *GmFLS* sequence (GenBank accession number AB246668), and 5′-AGTGACGTTGAGTTT-TGCCG-3′ and 5′-CTGGCCTTACAAGCCACTCA-3′ for *Gm DFR* (from GenBank accession number AF167556).

Expression of *GmF3H* in *E. coli* The open reading frame of *GmF3H* was amplified with Pfu DNA polymerase (Intron Biotech, Korea) and was inserted into pGEX 5X-3 (Amersham Biotech, USA). The construct was transformed into *E. coli* BL21 (DE3), and a transformant was grown in LB medium containing 50 μg/ml ampicillin to an absorbance at 600 nm of 0.7. At this point, isopropyl-beta-D-thiogalactopyranoside (IPTG) was added at a final concentration of 0.1 mM and the culture was grown for five more hours at 30°C. The cells were harvested and washed with PBS buffer (10 mM NaH_2PO_4, 150 mM NaCl, pH 7.0), resuspended in the same buffer, and lysed by sonication. The expressed protein was purified with a GSTrap affinity column (Amersham Biotech, USA).

Preparation of 2*S*-naringenin and 2*S*-eriodictyol The 2*S*-naringenin was prepared by HPLC (Varian, USA) using a Chiralpack AD-RH column (4.6 mm × 150 mm, Daicel Chemical Industries). An isocratic elution system in CH_3CN/H_2O buffer (3:7, pH 2.0) was used at a flow rate of 0.4 ml/min over a 1 hr period (Miyahisa et al., 2005). 2*S*-eriodictyol was prepared using a Chiralpak OJ-RH column (4.6 mm × 150 mm, 5 μm particle size, Chiral Technologies Inc., USA). The mobile phase consisted of acetonitrile, water and phosphoric acid (20:80:0.04, v/v/v). Separation was carried out isocratically at a flow rate of 0.4 ml/min, with ultraviolet (UV) detection at 288 nm (Jaime et al., 2007).

GmF3H enzyme assay and analysis of reaction products The reaction mixture contained 1 mM ascorbate, 2 mg catalase (bovine, Sigma, USA), 100 μM $FeSO_4$, 160 μM 2-oxoglutaric acid, 10 mM Tris/HCl (pH 8.0), 40 μM of flavonoid substrate and 12.5 μg of purified recombinant GmF3H. The mixture was incubated at 37°C for 1 h, then extracted with ethyl acetate and evaporated by vacuum. When enzyme kinetics were calculated based on Lineweaver-Burke plots, concentrations of eriodictyol from 10 to 100 μM were used. The reaction products were analyzed by high performance liquid chromatography as described in Kim et al. (2006).

The LC-MS/MS instruments were a Varian 212 HPLC photodiode array detector (Varain355) (Varian Inc., USA), and a Varian 500-MS ion trap spectrometer. A Chromsep HPLC column (150 × 2 mm) was used at a flow rate of 0.2 ml/min. The mobile phase consisted of A (0.1% formic acid in water) and B (0.1% formic acid in acetonitrile). The gradient increased linearly from 0% to 10% B in 2 min, to 40% B in 10 min, and 70% in 20 min. The system was operated with MS workstation software (version 6.9, Varian, Inc.). Mass spectra were acquired simultaneously using an electrospray ionization (ESI) source in positive and negative ionization mode at 600 V. The spray needle voltage was set at 5 kV, capillary voltage was 80 V, and capillary temperature 220°C. The sheath gas pressure was set at 35psi and the auxiliary gas pressure at 10psi. Full scan mass spectra were recorded for the range of *m/z* 50-2000.

Extraction of flavonoids from *Glycine max* Soybean plants were exposed to UV-B for 2 h as described in Kim et al. (2004) and kept in normal light for 23 h. Flavonoids were then extracted. Flavonoids were also extracted control soybean plants. We performed this experiment twice.

Total phenolic compounds were isolated from soybean leaves by grinding them to a fine power in liquid nitrogen with pestle and mortar, and extracted with 80% methanol. The extract was

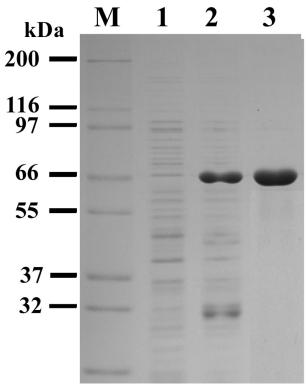


Fig. 1. Expression and purification of recombinant GmF3H protein. Lane M, protein size marker; lane 1, *E.coli* lysate before induction; lane 2, *E. coli* lysate after induction; lane 3, purified recombinant GmF3H.

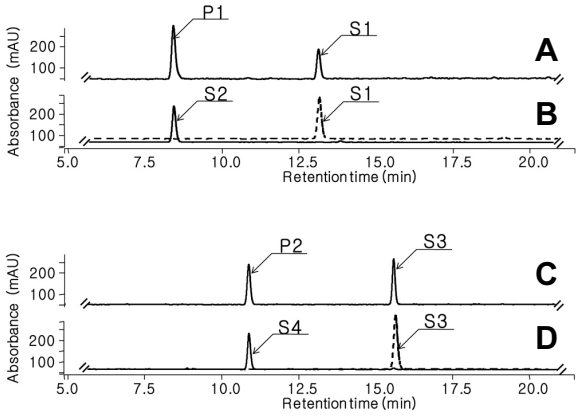


Fig. 2. Analysis of GmF3H reaction products by high performance liquid chromatography. **A.** Reaction products of 2*S* eriodictyol with GmF3H. P1, reaction product; S1, substrate (2*S*-eriodictyol). **B.** Authentic compounds. S1, 2*S*-eriodictyol; S2, taxifolin. **C.** Reaction products of 2*S*-naringein. P2, reaction product; S3, substrate (2*S*-naringenin). **D.** Authentic compounds. S3, 2*S*-naringenin; S4, dihyrokaempferol.

filtered through a 0.45 μm PTFE membrane (Whatman, UK). The resulting fraction was evaporated and redissolved in water. After eliminating undissolved material by centrifugation, the sample was purified with a C-18 Sep-pak cartridge (Waters, USA). The resulting samples were analyzed by HPLC and LC/MS/MS.

Results and Discussion

Cloning and characterization of Gm*F3H* We searched for an F3H in the soybean genome index of the Institute of Genome Research (TIGR), retrieved one and named the gene *GmF3H*. A BLAST search revealed that GmF3H had 78% amino acid sequence identity to *Petunia hybrida* F3H (Britsch et al., 1986). A full length cDNA of *GmF3H* was cloned by RT-PCR; the open reading frame consisted of 1128-bp encoding a 42.7-kDa protein. GmF3H contained the conserved iron and 2-oxoglutarate binding sites: his at 77, his at 219, asp at 221 and his at 277 for iron binding; arg at 287 and ser at 289 for 2-oxoglutarate binding.

Although GmF3H had strong homology to other F3Hs, it seemed necessary to verify its function *in vitro*. Therefore *GmF3H* was subcloned into the *E. coli* expression vector pGEX 5X-2 fused with the glutathione *S*-transferase gene to facilitate purification of the expressed protein. Most of the expressed GmF3H was soluble. We examined the purity of the recombinant protein by SDS-PAGE. As shown in Fig. 1, its molecular weight is about 68-kDa, consistent with the sum of the molecular weights of GmF3H, 42.7 kDa and glutathione-*S*-transferase, 26-kDa.

We used two flavanones, eriodictyol and naringenin, as possible substrates for the GmF3H. HPLC analysis of the reaction products obtained with each substrate revealed a new peak differing in retention time from the substrate. After prolonged incubation the substrates were still present and no new peak was produced. This indicates that GmF3H uses only one of the two enantiomers of flavanones and that the reaction products were not further converted into either kaempferol or quercetin. It is known that F3H uses the *S*-enantiomer much better than the *R*-form (Britsch et al., 1986). Since the enantiomers of naringenin and eriodictyol can be separated with a chiral column (Jaime et al., 2007; Miyahisa et al., 2005) we were able to determine which configurations acted as substrates. Incubation of 2*S*-narigenin or 2*S*-eriodictyol with the recombinant GmF3H gave rise to the new peaks whereas incubation of 2*R*-narigenin and 2*R*-eriodictyo did not. This indicates that GmF3H uses only the 2*S*-forms. The 2*S*-naringein reaction product had a retention time at 11.3 min (Fig. 2), the same as dihydrokaempferol, and the 2*S*-eriodictyol reaction product had a retention time of 8.5 min, like taxifolin. Tandem mass spectrometry analysis confirmed that the reaction products were dihydrokaempferol and taxifolin, respectively (Fig. 3). We tested whether GmF3H contains an FLS since the FLS converts eriodictyol into taxifolin which is eventually transformed into quercetin. The reaction products obtained after prolonged incubation of eriodictyol with GmF3H consisted only of taxifolin, indicating that GmF3H does not metabolize taxifolin further. Taken together, these results suggest that GmF3H encodes a flavanone 3-β-hydroxylase.

The Km and Vmax for eriodictyol were found to be 87 μM and 102.4 μkat/mg protein, resepctively. GmF3H uses 2*S*-eriodictyol (100%) more efficiently than 2*S*-naringenin (90%). Two methoxy eriodictyol, homoeriodictyol (3′-methoxy eriodictyol) and hesperetin (4′-methoxy eriodictyol) also served as substrates (Table 1).

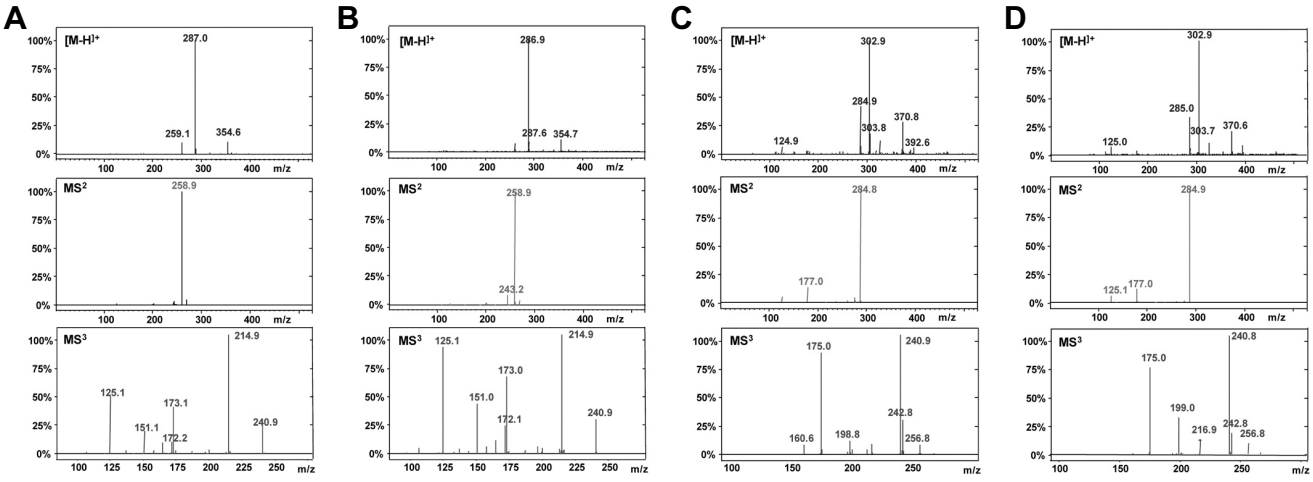
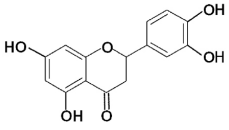
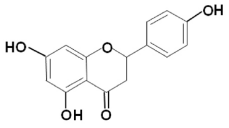
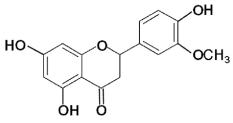
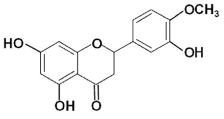


Fig. 3. Identification of reaction products of GmF3H by tandem mass spectrometry. **A.** reaction product of 2*S*-naringenin. **B.** authentic dihydrokaempferol. **C.** reaction product of 2*S*-eriodictyol. **D.** authentic taxifolin.

Table 1. Substrate specificity of GmF3H.

Substrate	Structure	Relative activity (%)
Eriodictyol		100
Naringenin		90
Homoeriodictyol		16
Hesperetin		56

UV-B irradiation induces expression of *GmF3H* and *GmFLS*, and results in the accumulation of flavonols

There are no reports of dihydrokaempferol or taxifolin in soybean. These compounds are substrates for two enzymes, FLS and DFR (Shirley, 2001). FLS converts dihydrokaempferol into kaempferol, and taxifolin into quercetin, both of which are known as UV-protectants, and DFR channels these substrates into the biosynthesis of anthocyanin. We investigated the expression of *GmF3H*, *GmFLS*, and *GmDFR* upon UV-B irradiation by RT-PCR. Total RNA was isolated 0, 2, 8, 12, 20 h after UV-B irradiation (Fig. 4). Expression of *GmF3H* was not detected in the absence of UV-B irradiation whereas *GmFLS* and *GmDFR* were expressed. However, all three genes were maximally expressed two hour after UV-B irradiation. Overall expression of *GmF3H* was the highest followed by *GmFLS* and *GmDFR*.

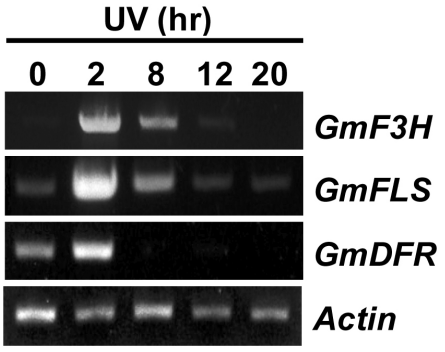


Fig. 4. Expression of *GmF3H*, *GmFLS*, and *GmDFR* upon UV-B irradiation Total RNA was isolated at 0, 2, 8, 20 h after UV-B irradiation from whole soybean plants (Kim et al, 2004). RT-PCR was conducted with the soybean actin gene as a control. PCR was conducted for 33 cycles for *GmF3H*, 35 cycles for *GmFLS*, and 38 cycles for *GmDFR* .

GmF3H and *GmFLS* are directly involved in flavonol biosynthesis, and studies by others have shown that the UV-tolerance of soybean is correlated with its flavonol content (Reed et al., 1992; Sullivan and Teramura, 1990). The major flavonoids in soybean leaves are kaempferol and quercetin glycosides (Stafford, 1990). In order to see whether the induction of *GmF3H* and *GmFLS* is accompanied by the accumulation of flavonols, we examined flavonoid content by HPLC (Fig. 5). Several peaks in the characteristic UV-visible spectra of flavonoids were identified as kaempferol derivatives. To further characterize the individual peaks, we obtained MS and MS-MS spectra (Ta-

Table 2. Annotation of flavonoids peaks from HPLC profiles shown in Fig. 2.

Peak No.	Rt (min.)	λ_{max} (nm)	$[M+H]^+$	Main fragments	Compound identification
1	24.3	264, 346	757	594.9, 448.9, 432.6, 287	Kaempferol *O*-hexoside, *O*-hexosyl-rhamnoside I
2	24.8	264, 346	757	594.9, 449,432.9, 287	Kaempferol *O*-hexoside, *O*-hexosyl-rhamnoside II
3	26.0	264, 346	741	595.1, 449.1, 432.9 287	Kaempferol *O*-rhamnoisde, *O*-hexosyl-rhamnoside I
4	26.3	264, 346	741	595.1, 449.1, 432.9 287	Kaempferol *O*-rhamnoside, *O*-hexosyl-rhamnoside II
5	30.9	264, 346	594	448.8, 432.8, 287	Kaempferol *O*-hexosyl-rhamnoside I
6	32.2	264, 346	594	448.8, 432.8, 287	Kaempferol *O*-hexosyl-rhamnoside II

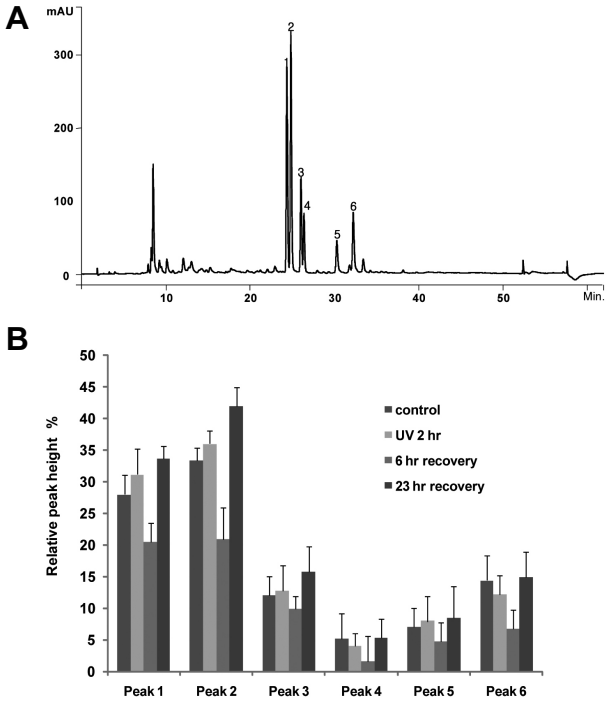


Fig. 5. A. HPLC elution profile of flavonoids from *Glycine max* leaves. HPLC chromatograms were monitored at 340 nm and the peak numbers correspond to the MS analysis in Table 2. **B.** changes in kaempferol derivatives upon UV-irradiation. Soybean plants were exposed to UV-B for 2 h, and transferred to normal light for 6 h and 23 h. Flavonoids were extracted straight after UV-irradiation and 6 h and 23 h later. Non-irradiated plants were used as controls.

ble 2). The first two peaks were identified as kaempferol *O*-hexoside and *O*-hexosyl-rhamnosides. The difference of their retention times is due to the different positions of glycosylation. Peaks 3 and 4 were identified as kaempferol *O*-rhanmoside, *O*-hexosyl-rhamnosides, and peaks 5 and 6 as kaempferol *O*-hexosyl-rhamosides, both of which are likely to be substrates for the synthesis of the other four kaempferol trigycosides. Surprisingly, all the major peaks in the HPLC turned out to be flavonol kaempferol derivatives (Fig. 5A; Table 2) Based on these results, we monitored the changes in kaempferol derivatives using HPLC. Soybean plants were treated with UV-B for 2 h and total flavonoids were extracted from their leaves after 0, 6, and 23 h. Total flavonoids did not change immediately after UV-treatment whereas the content of flavonoids fell by about 35% after 6 h. However, after 23 h it had increased by about 20% (Fig. 5B). Four peaks, 1, 2, 3, and 5 appeared to increase significantly by 23 h post treatment. We infer that UV-irradiation increases expression of *GmF3H*, which may have a role in the accumulation of flavonol-*O*-glycosides.

F3H has been a target for treatments to manipulate flavonoids such as anthocyanins and isoflavonoids in crops. Our results show that F3H is also one of enzymes that protects plants from UV-irradiation by generating the UV-protectant, kaempferol.

Acknowledgments This work was supported by a grant from the Biogreen 21 Program, Rural Development Administration, Republic of Korea and partially by grant KRF-2006-005-J03401 and the second Brain Korea 21 (Ministry of Education).

References

Boué, S.M., Carter-Wienthes, C.H., Shih, B.Y., and Cleveland, T.E. (2003). Identification of flavone aglycones and glycosides in soybean pods by liquid chromatography-tandem mass spectrometry. J. Chromatogr. A *991*, 61–68.

Britsch, L., and Grisebach, H. (1986). Purification and characterization of (2*S*)-flavanone 3-hydroxylase from *Petunia hybrida*. Eur. J. Biochem. *156*, 569–577.

Dixon, R.A., and Steele, C.L. (1999). Flavonoids and isoflavonoids-a gold mine for metabolic engineering. Trends. Plant Sci. *4*, 394–400.

Fasoula, D.A., Stephens, P.A., Nickell, C.D., and Vodkin, L.O. (1995). Cosegregation of purple-throat flower color with DNA polymorphism in soybean. Crop Sci. *35*, 1028–1031.

Forkmann, G. (1991). Flavonoids as flower pigments: the formation of the natural spectrum and its extension by genetic engineering. Plant Breed. *106*, 1–26.

Forkmann, G., and Heller, W. (1999). *Biosynthesis of flavonoids*. In Comprehensive Natural Products Chemistry, (Amsterdam, Nederland; Elsevier), pp. 713–748.

Forkmann, G., and Martens, S. (2001). Metabolic engineering and applications of flavonoids. Curr. Opin. Biotechnol. *12*, 155–160.

Havsteen, B.H. (2002). The biochemistry and medical significance of the flavonoids. Pharmacol. Ther. *96*, 67–202.

Graham, T.L. (1991). Flavonoid and isoflavonoid distribution in developing soybean seedling tissues and in seed and root exudates. Plant Physiol. *95*, 594–603.

Miyahisa, I., Kaneko, M., Funa, N., Kawasaki, H., Kojima, H., Ohnishi, Y., and Horinouchi, S. (2005). Efficient production of (2*S*)-flavanones by *Escherichia coli* containing an artificial biosynthetic gene cluster. Appl. Microbiol. Biotechnol. *68*, 498–504

Kim, B.G., Ko, J.H., Hur, H.-G., and Ahn, J.-H. (2004). Classification and expression analysis of cytochrome P450 genes from soybean. Agric. Chem. Biotechnol. *47*, 173–177.

Kim, H.K., Jang, Y.H., Baek, I.S., Lee, J.H., Park, M.J., Chung, Y.S., Chung, J.I., and Kim, J.K. (2005). Polymorphism and expression of isoflavone synthase genes from soybean cultivars. Mol. Cells *19*, 67–73.

Kim, B.G., Lee, Y., Hur, H.-G., Lim, Y., and Ahn, J.-H. (2006). Flavonoid 3′-*O*-Methyltransferase from rice: cDNA cloning, characterization and functional expression, phytochemistry *67*, 387–394.

Liu, C.-J., Blount, J.W., Steele, C.L., and Dixon, R.A. (2002). Bottlenecks for metabolic engineering of isoflavone glycoconjugates in *Arabidopsis*. Proc. Natl. Acad. Sci. USA *99*, 14578–14583.

Lozovaya, V.V., Anatoliy, V., Lygin, A.V., Zernova, O.V., Ulanov, A.V., Li, S., Hartman, G.L., and Widholm, J. (2007). Modification of phenolic metabolism in soybean hairy roots through down regulation of chalcone synthase or isoflavone synthase. Planta *225*, 665–679.

Reed, H.E., Teramura, A.H., and Kenworthy, W.J. (1992). Ancestral U.S. soybean cultivars characterized for tolerance to ultraviolet-B radiation. Crop Sci. *32*, 1214–1219.

Shirley, B.W. (1996). Flavonoid biosynthesis: new functions for an old pathway. Trends Plant Sci. *1*, 377–382.

Shim, H.Y., Park, J.H., Paik, H.D., Nah, S.Y., Kim, D.S., and Han, Y.S. (2007). Acacetin-induced apoptosis of human breast cancer MCF-7 cells involves caspase cascade, mitochondria-mediated death signaling and SAPK/JNK1/2-c-Jun activation. Mol. Cells *24*, 95–104.

Stafford, H.A. (1990). Flavonoid Metabolism (Florida: CRC Press).

Sullivan, J.H., and Teramura, A.H. (1990). Field study of the interaction between solar ultraviolet-B irradiation and drought on photosynthesis and growth in soybean. Plant Physiol. *92*, 141–146.

Tahara, S. (2007). A journey of twenty-five years through the ecological biochemistry of flavonoids. Biosci. Biotechnol. Biochem. *71*, 1387–1404.

Takahashi, R., Githiri, S.M., Hatayama, K., Dubouzet, E.G., Shimada, N., Aoki, T., Ayabe, S.-I., Iwashina, T., Toda, K., and Matsumura, H. (2007). A single-base deletion in soybean flavonol synthase gene is associated with magenta flower color. Plant Mol. Biol. *23*, 125–135.

Toda, K., Yang, D., Yamanaka, N., Watanabe, S., Harada, K., and Takahashi, R. (2002). A single-base deletion in soybean flavonoid 3′-hydroxylase gene is associated with gray pubescence color. Plant Mol. Biol. *50*, 187–196.

Winkel-Shirley, B.W. (2001). Flavonoid biosynthesis. A colorful model for genetics, biochemistry, cell biology, and biotechnology. Plant Physiol. *126*, 485–493.

Wiseman, E., Hartmann, U., Sagasser, M., Baumann, E., Palme, K., Hahlbrock, K., Saedler, H., and Weisshaar, B. (1998). Knock-out mutants from an *En-1* mutagenized *Arabidopsis thaliana* population generate phenylpropanoid biosynthesis phenotype. Proc. Natl. Acad. Sci. USA *95*, 12432–12437.

Yáñez, J.A., Miranda, N.D., Remsberg, C.M., Ohgami, Y., and Davies, N.M. (2007). Stereospecific high-performance liquid chromatographic analysis of eriodictyol in urine. J. Pharm. Biomed. Anal. *43*, 255–262.

Yu, O., Jung, W., Shi, J., Croes, R.A., Fader, G.M., McGonigle, B., and Odell, J.T. (2000). Production of the isoflavones genistein and daidzein in non-legume dicot and monocot tissues. Plant Physiol. *124*, 781–793.

Yu, O., Shi, J., Hession, A.O., Maxwell, C.A., McGonigle, B., and Odell, J.T. (2003). Metabolic engineering to increase isoflavone biosynthesis in soybean seed. Phytochemistry *63*, 753–763.

Zabala, G., and Vodkin, L.O. (2005). The *wp* mutation of *Glycine max* carries a gene-fragment-rich transoson of the CACTA superfamily. Plant Cell *17*, 2619–2632.