Production of the Isoflavones Genistein and Daidzein in Non-Legume Dicot and Monocot Tissues

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Metabolic engineering for production of isoflavones in non-legume plants may provide the health benefits of these phytoestrogens from consumption of more widely used grains. In legumes, isoflavones function in both the symbiotic relationship with rhizobial bacteria and the plant defense response. Expression of a soybean isoflavone synthase (IFS) gene in Arabidopsis plants was previously shown to result in the synthesis and accumulation of the isoflavone genistein in leaf and stem tissue (Jung et al., 2000). Here we further investigate the ability of the heterologous IFS enzyme to interact with the endogenous phenylpropanoid pathway, which provides the substrate for IFS, and produces genistein in several plant tissue systems. In tobacco (*Nicotiana tabacum*) floral tissue that synthesizes anthocyanins, genistein production was increased relative to leaves. Induction of the flavonoid/anthocyanin branch of the phenylpropanoid pathway through UV-B treatment also enhanced genistein production in Arabidopsis. In a monocot cell system, introduced expression of a transcription factor regulating genes of the anthocyanin pathway was effective in conferring the ability to produce genistein in the presence of the IFS gene. Introduction of a third gene, chalcone reductase, provided the ability to synthesize an additional substrate of IFS resulting in production of the isoflavone daidzein in this system. The genistein produced in tobacco, Arabidopsis, and maize (*Zea mays*) cells was present in conjugated forms, indicating that endogenous enzymes were capable of recognizing genistein as a substrate. This study provides insight into requirements for metabolic engineering for isoflavone production in non-legume dicot and monocot tissues.

Isoflavones form a group of distinct secondary metabolites produced predominately in leguminous plants. Natural roles for isoflavones are in both positive and negative plant-microbial interactions. One role is in establishing the symbiotic relationship between the plant and rhizobial bacteria for the formation of nitrogen-fixing root nodules, where isoflavones act as chemoattractants for the rhizobial bacteria and as inducers of *nod* gene expression (Van Rhijn and Vanderleyden, 1995; Pueppke, 1996). Another major role is during the disease resistance response, where the synthesis of isoflavones is induced to provide defense compounds. The isoflavone daidzein is the precursor to the major phytoalexins including medicarpin and glyceollins, which are produced in alfalfa and soybean, respectively (Blount et al., 1992; Graham, 1995). The isoflavone genistein has antifungal activity (Rivera-Vargas et al., 1993), is the precursor to the phytoalexin kievitone made by *Phaseolus vulgaris* (Garcia-Arenal et al., 1978), and is involved in the pathway leading to the glyceollin response of soybean cells (Graham, 1998; Graham and Graham, 2000). There is also evidence for a role for isoflavones in human health as a dietary component (for review, see Davis et al., 1999; Messina, 1999). Thus control of isoflavone synthesis would have multiple applications. Due to complexities in regulation of interrelated biochemical pathways, metabolic engineering to affect the isoflavone biosynthetic capacity of a target plant tissue presents a challenge.

Isoflavones are synthesized as part of the phenylpropanoid pathway (Fig. 1). The phenylpropanoid pathway has multiple branches common to legumes as well as non-legumes, which provide numerous compounds including lignins, anthocyanins, and certain classes of phytoalexins that have roles in normal development, as well as serving as protectants to many environmental stresses (Dixon et al., 1995). Sets of genes encoding enzymes of the pathway are developmentally and tissue-specifically regulated, and may be induced by environmental stresses such as nutrient deficiency, prolonged cold, pathogen attack, and exposure to UV light (Dixon and Paiva, 1995). It has been shown that different transcription factors of the Myb gene family (Jin and Martin, 1999) can control the expression of different sets of genes in the pathway. Overexpression of snapdragon Myb308 in tobacco (*Nicotiana tabacum*) reduces expression of some pathway genes, including cinnamate 4-hydroxylase (C4H), 4-coumaroyl-CoA ligase (4CL), and cinnamyl alcohol dehydrogenase (CAD), causing a reduction in synthesis of lignins and phenolic compounds (Tamagnone et al., 1998). On the other hand, the maize (*Zea mays*) Myb C1, in conjunction with the maize Myc factor R, activates a set of genes leading to an enhancement in the biosynthesis and accumulation of anthocyanin (Grotewold et al., 1998; Bruce et al., 2000, and refs. therein). These genes include phenylalanine ammonia lyase (PAL), chalcone synthase

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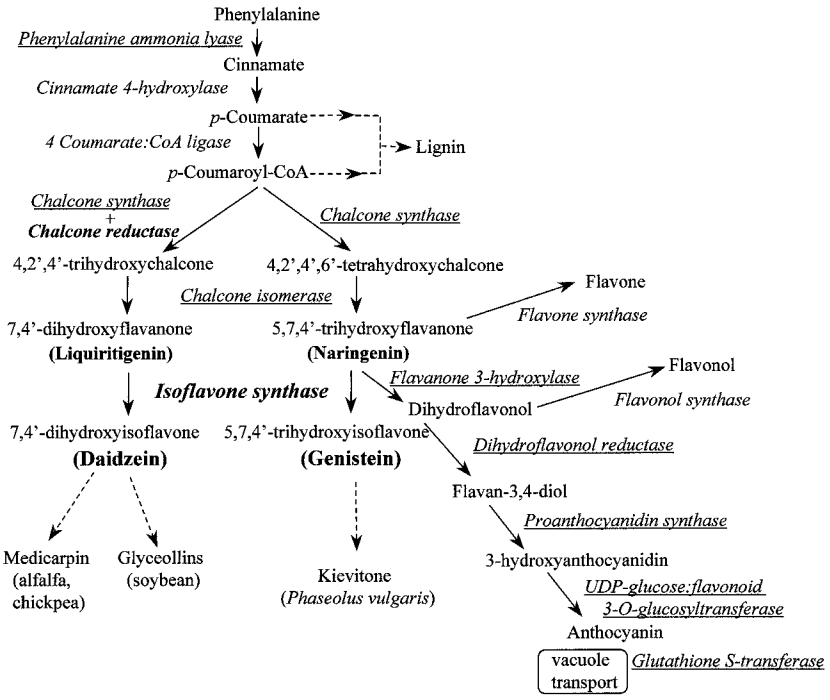

Figure 1. A partial diagram of the phenylpropanoid pathway showing intermediates and enzymes involved in isoflavone synthesis, as well as some branch pathways. The enzymes in bold are encoded by genes expressed as transgenes in this study. Genes encoding the underlined enzymes have been shown to be activated in maize by C1 and R. Dotted arrows represent multiple steps. Enzymes are indicated in italics.

(*c2*), chalcone isomerase (*chi*), flavanone 3-hydroxylase (*f3h*), flavanone/dihydroflavanol reductase (*a1*), proanthocyanidin synthase (*a2*), UDP-Glc:flavonoid 3-*O*-glucosyltransferase (*bz1*), and a glutathione *S*-transferase (*bz2*).

The substrate for producing the isoflavone genistein is an intermediate in the branch of the phenylpropanoid pathway that leads to synthesis of the flavonoids, which include flavanones, flavones, flavonols, proanthocyanidins, and anthocyanins. This substrate, naringenin, is the product of chalcone synthase and chalcone isomerase, enzymes that are common to most plants (Fig. 1). The chalcone reductase (CHR) activity of legumes is necessary to produce the second substrate for isoflavone synthesis, liquiritigenin, which is converted to daidzein (Fig. 1). Legumes and few other species have a unique enzymatic function that carries out a 2,3 migration of the B-ring of naringenin or liquiritigenin, resulting in the production of the isoflavones. This key enzyme that redirects phenylpropanoid pathway intermediates from flavonoids to isoflavonoids is the cytochrome P450 monooxygenase, isoflavone synthase (IFS). We, along with two other groups, recently have independently reported the identification of the long-sought IFS gene through expression screening of candidate expressed sequence tags (ESTs; Akashi et al., 1999; Steele et al., 1999; Jung et al., 2000). Cloning of the IFS gene provides an unprecedented opportunity for metabolic engineering to influence isoflavone synthesis in both legumes and non-legumes. Since in legumes the levels of isoflavones are genetically and environmentally influenced (Eldridge and Kwolek, 1983; Tsukamoto et al., 1995), those levels may be enhanced and stabilized through the introduction of an IFS transgene, potentially leading to improved disease resistance or nutritional qualities. Cereal crops, which do not naturally produce isoflavones, are major food staples for both humans and livestock. Synthesis of isoflavones in these crops such as maize, wheat, or rice may enhance their nutritional value.

We reported previously that the expression of soybean IFS in Arabidopsis resulted in the production of genistein in this plant that does not naturally synthesize isoflavones (Jung et al., 2000). Here we demonstrate that: (a) production of genistein in tobacco plants expressing soybean IFS is dependent on properties of individual tissues, (b) the level of genistein produced in IFS-transformed Arabidopsis plants is UV-B stress-regulated, and (c) a transcription factor is required in addition to IFS for synthesis of genistein in maize black mexican sweet (BMS) cells. All of these effects are related to a correlation between the level of genistein synthesized and the general activity of the phenylpropanoid pathway. We find that the genistein produced in these novel environments, the Arabidopsis, tobacco, and BMS tissues, is not in the aglycone form, but is found as conjugates. In addition to genistein, we direct the novel production of daidzein in BMS cell lines using an additional transgene, soybean CHR. This work demonstrates both the potential for and the complexities of metabolic engineering of isoflavone levels in non-legumes.

RESULTS

Production of Genistein in Tobacco Expressing IFS Is Related to Tissue-Specific Activity of the Phenylpropanoid Pathway

We previously reported production of the isoflavone genistein in Arabidopsis plants expressing a soybean IFS gene regulated by the cauliflower mosaic virus (CaMV) 35S promoter (35S/P-IFS; Jung et al., 2000). Genistein was shown to be present in hydrolyzed extracts prepared from the combined leaves and stems of Arabidopsis transformants. To assess the production of genistein in tobacco plants, we transformed *N. tabacum* cv SR1 with a binary vector containing the same 35S/P-IFS gene (pOY204). The presence of the transgene in 18 individual transformants was confirmed by PCR (data not shown). Extracts were prepared from leaf tissue of all 18 independent tobacco transformants and analyzed by HPLC. Genistein was not detected in the hydrolyzed tobacco leaf extracts using the same procedure that had clearly shown the accumulation of genistein in the Arabidopsis transformants.

To determine whether poor expression of the IFS transgene was the reason for the lack of genistein synthesis in tobacco leaves, the presence of IFS mRNA was assessed by northern-blot analysis. Figure 2A (lanes 3 and 5) shows that a strong band hybridizes to the IFS probe in the leaf RNA samples from two representative tobacco transformants indicating that the IFS transgene is effectively transcribed. Presence of the IFS protein was assessed in leaves of two transgenic tobacco lines using an antibody raised to a synthetic peptide derived from the amino acid sequence of the IFS protein. Because the cytochrome P450 IFS is a membrane bound enzyme, microsomes were prepared from the transformed tobacco leaves for western-blot analysis. The IFS antibody did detect a protein of the expected size in the tobacco leaf microsomes (Fig. 2B), which comigrates with the IFS protein expressed in yeast microsomes (data not shown). These results show that the IFS transgene is expressed and the protein does accumulate in the tobacco leaves.

To assess activity of the IFS protein found in the transgenic tobacco leaves, an in vitro enzyme activity assay was performed. The tobacco leaf microsomes were incubated in vitro with the naringenin substrate for IFS and NADPH cofactor. This reaction produced a small amount of genistein as detected by HPLC. The genistein product was increased by addition to the reaction of yeast microsomes prepared from a strain expressing a plant cytochrome P450 reductase. These additional microsomes could enhance the re-

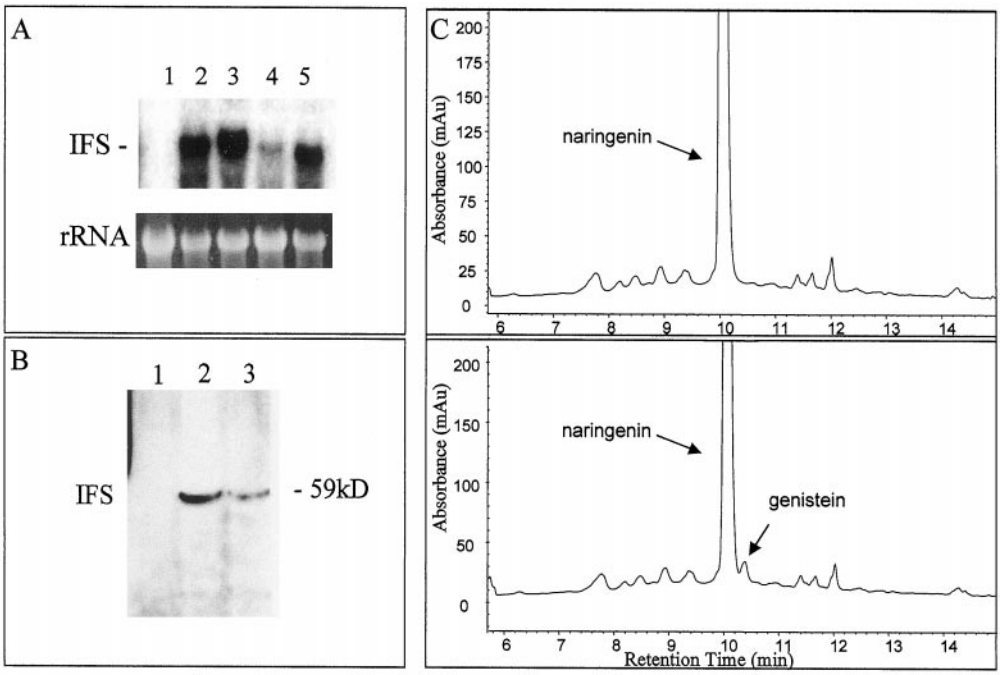

Figure 2. Expression of IFS mRNA, protein, and enzyme activity in tobacco leaves. A, Northern blot of RNA from IFS transgenic tobacco plants. Samples contain approximately 20 μg of total RNA. Lane 1, Wild-type tobacco leaves; lane 2, transgenic number 16 petals; lane 3, transgenic number 16 leaves; lane 4, transgenic number 3 petals; lane 5, transgenic number 3 leaves. The band that hybridizes to the IFS probe is labeled. The same lanes with ethidium bromide-stained rRNA control bands are shown below. B, Western blot of microsomal proteins from IFS transgenic tobacco leaves. Lane 1, Wild-type tobacco leaves; lane 2, transgenic number 3 leaves; lane 3, transgenic number 17 leaves. The band that reacts with the IFS antiserum is labeled. C, Chromatograms at 260 nm of products of the tobacco leaf microsome IFS assay samples from wild type (top) and IFS transformant number 3 (bottom). The naringenin and genistein peaks are labeled.

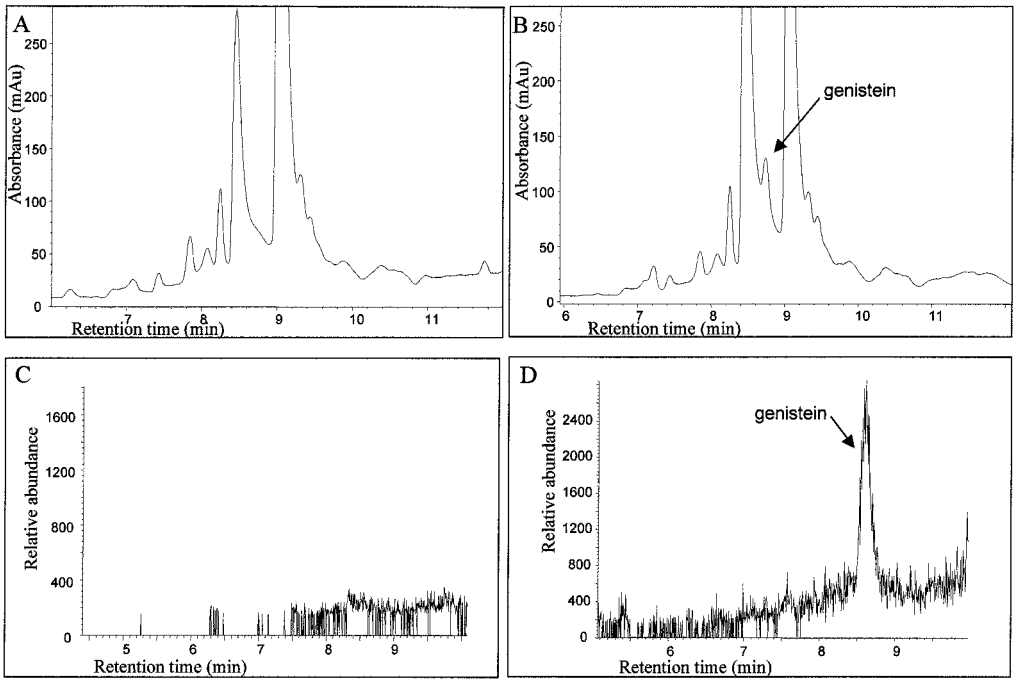

Figure 3. Genistein accumulation in tobacco petal extracts. A and B, HPLC assay of extracts from wild-type (A) and IFS transgenic tobacco petals (B) showing A_{260}. The arrow indicates the peak with retention time and spectrum corresponding to genistein. C and D, GC-MS assay of extracts from wild-type (C) and IFS transgenic tobacco petals (D) showing selected ion monitoring at 414 *m/z*.

action since, in general, cytochrome P450 reductases are required to transfer electrons from NADPH to cytochromes P450 (Bolwell et al., 1994). Figure 2C shows that genistein is produced in the in vitro assay, indicating that active IFS enzyme is present in the tobacco leaves. Therefore expression of the IFS gene and enzyme activity should provide the potential for isoflavone synthesis in tobacco leaves.

Availability of the naringenin substrate for IFS may be a factor limiting the synthesis of genistein in tobacco leaves. In tobacco flowers where the dark pink flower color indicates the accumulation of anthocyanin, the flavonoid branch of the phenylpropanoid pathway that produces the naringenin intermediate is active. Northern-blot analysis of RNA prepared from flower petals of two independent tobacco transformants showed that IFS mRNA is present, although the level of IFS mRNA in the petals is substantially lower than in the leaves of the same transformant (Fig. 2A). Extracts from the flower petals of these two as well as four other transformants were hydrolyzed to convert any genistein derivatives to the free form, and assayed by HPLC. These samples contained a peak not found in non-transformed flowers that has the retention time and UV spectrum of genistein (Fig. 3, A and B). Gas chromatography-mass spectrometry (GC-MS) analysis confirmed that the compound is genistein (Fig. 3, C and D). There was no detectable peak corresponding to naringenin in either non-transformed control or IFS transformant flower petal extracts. This result indicates that the naringenin intermediate is transient, and that IFS is capable of competing with endogenous enzymes for the limited amount of naringenin present in this tissue to produce genistein.

In progeny obtained from selfing six randomly selected positive transformants, the IFS transgene cosegregated with genistein production in the flowers (data not shown). The amount of genistein produced in the flower petals was approximately 2 ng mg^{-1} of fresh weight, as determined by comparison with the genistein standard, similar to the amount found in combined leaves and stems of transgenic Arabidopsis (Jung et al., 2000). To determine whether any genistein could be detected in tobacco leaves in a more sensitive assay, samples were analyzed by GC-MS. In this analysis it was possible to detect genistein in the transformed tobacco leaf samples (data not shown). To compare activities of the anthocyanin branch of the phenylpropanoid pathway in tobacco leaves and flowers, anthocyanin levels were assayed. The level in flowers was about 130-fold higher than in leaves (based on fresh weight normalized A_{530} minus A_{657} readings of 0.258 and 0.002, respectively). These results indicate that the production of genistein in transgenic tobacco is regulated with a positive correlation to activity of the anthocyanin branch of the phenylpropanoid pathway in specific tissues.

Production of Genistein in Arabidopsis Expressing IFS Can Be Enhanced by UV-B Stress-Induced Activation of the Phenylpropanoid Pathway

To further investigate the relationship of isoflavone production and phenylpropanoid pathway activity in non-legume plants expressing IFS, the effect of stress induction of the pathway on the level of genistein produced was investigated. As in other plant species, treatment of Arabidopsis plants with UV-B light has been shown to cause an increase in flavonoid accumulation in the leaves (Caldwell et al., 1983; Li et al., 1993). Thus the flavonoid branch of the phenylpropanoid pathway producing the naringenin intermediate is activated. Independent Arabidopsis transformants containing the 35S/P-IFS gene and producing genistein in the leaves and stems (Jung et al., 2000) were selfed, segregation ratios were determined based on kanamycin resistance, and then the process was repeated to identify lines homozygous for a single transgene locus. Seedlings of a homozygous line germinated on kanamycin containing medium were transferred after 2 weeks to soil, and after an additional 10 d of growth were placed directly under UV-B light for 36 h (258 nm, 46 mWatt cm^{-2}). Immediately following the UV treatment the aboveground tissues of each UV-treated plant, as well as of untreated control plants, were ground to a fine powder in liquid nitrogen, extracted with 80% (v/v) methanol, and analyzed for genistein content by HPLC. The same extracts were also assayed spectrophotometrically for the levels of both total UV-absorptive compounds (which includes flavonoids) and of anthocyanins. The untreated IFS transformants had slightly elevated levels of total UV-absorptive compounds over those in the non-transformed controls (Table I), indicating a change in accumulating compounds (including genistein and its derivatives) in the presence of IFS.

The anthocyanin and total UV-absorptive compound levels increased following UV-B treatment in the control plants (Table I), indicating that the UV treatment was effective in raising the activity of the phenylpropanoid pathway thereby producing these compounds. The levels of these compounds also increased in the IFS transformants and in addition, the level of genistein produced was more than doubled. This result indicates that in the presence of IFS activity some of the increased naringenin intermediate produced by UV-B treatment is redirected from flavonoids into isoflavone. Thus IFS can compete with endogenous enzymes for this substrate to produce more genistein when the pathway is activated.

Genistein Can Be Produced in Monocot BMS Cells by IFS when the Phenylpropanoid Pathway Is Activated by C1 and R Transcription Factors

We used the maize BMS cell system to determine whether isoflavones could be synthesized and accumulated in monocot cells. To enhance expression, the 35S/P-IFS gene construction prepared for monocot transformation additionally included intron 6 from the maize *Adh1*gene (Mascarenhas et al., 1990), and the entire chimeric gene was bounded by scaffold attachment regions (SARs) derived from the chicken lysozyme locus (Stief et al., 1989). Bombardment of BMS suspension culture cells with this construct, and an additional plasmid containing a 35S/P-bar gene, produced cell lines resistance to bialaphos. The presence of the IFS transgene was confirmed in 25 independent lines by PCR, and expression was confirmed in six randomly selected lines by reverse transcriptase-PCR (data not shown). All twenty-five independent lines were assayed by HPLC for production of genistein. In none of them could a peak with the properties of genistein be identified (Table II).

BMS culture cells are not pigmented, and it is known that genes encoding enzymes required for the synthesis of anthocyanin are not active in these cells due to a lack of expression of the regulating transcription factors (Dooner et al., 1991, and refs. therein; Grotewold, et al., 1998). A chimeric transcription factor containing maize C1 and R coding regions, called CRC, is able to activate expression of genes in BMS cells leading to the synthesis and accumulation of anthocyanins (Bruce et al., 2000), which can be visually detected by a reddish color. Because the naringenin intermediate for anthocyanin synthesis is the substrate for IFS, we tested the effect of CRC expression on making this substrate available for genistein synthesis.

Transformation of BMS cells with a *Nos*/P-CRC gene alone or in combination with the IFS gene described above produced stably transformed lines of both reddish and their natural "white" color, with

Table I. *Effects of UV-B treatment on phenylpropanoid pathway compounds*

Control and IFS-expressing Arabidopsis plants treated with UV-B light were assayed for levels of anthocyanin, total UV-absorptive compounds, and genistein. The average value and SD of data from four independent plants from each group are shown.

Sample	Anthocyanin		Total UV-Absorptive Compounds		Genistein (by HPLC)	
	Control	+UV	Control	+UV	Control	+UV
					ng mg^{-1}	
Control plants (no IFS)	0.468 ± 0.023	0.645 ± 0.018	1.808 ± 0.168	2.206 ± 0.086	0	0
A109-4 (35S/P-IFS)	0.432 ± 0.068	0.680 ± 0.040	2.587 ± 0.124	3.089 ± 0.099	3.63 ± 1.23	9.10 ± 1.74

Table II. *Summary of BMS line genotypes with corresponding phenotypes*

Each line is derived from an independent transformation event. The presence of genistein and naringenin was determined by HPLC analysis.

No. of Lines	Genotype	Tissue Phenotype	Genistein	Naringenin
5	Control	White	No	No
25	IFS	White	No	No
6	IFS + Nos/P-CRC	White	No	No
6	Nos/P-CRC	Red	No	Yes
7	IFS + Nos/P-CRC	Red	Yes	Yes
16	IFS + 35S/P-CRC	Red	Yes	Yes

about a 1:1 ratio, most likely due to variation among the lines in CRC gene expression level. Transformation with a 35S/P-CRC gene, which was introduced in combination with IFS, produced red lines at a rate of 95%. All lines selected for further analysis contained either the CRC gene alone, or the CRC and IFS genes that were introduced in combination, as determined by PCR analysis. Extracts were prepared from individual BMS cell lines and analyzed by HPLC to detect genistein.

Lines with CRC alone that were reddish in color indicating accumulation of anthocyanin did not produce genistein (Table II). Lines that received both IFS and CRC, but were white, also produced no genistein. In all 23 lines tested that received both IFS and CRC genes and showed anthocyanin accumulation, genistein was produced as shown by the characteristic peak present in the HPLC profile (Fig. 4B). The peak was confirmed as genistein by the 414 *m/z* and 399 *m/z* diagnostic ions of derivitized genistein in the GC-MS profile. Thus the synthesis of genistein in BMS cell lines is strictly correlated with the red color that is induced by CRC expression and the presence of the IFS gene. We attribute the production of genistein in every red CRC line that harbors the IFS gene to effects of the A element SAR on expression of the IFS gene in BMS cells. We previously found that bounding a gene with this SAR greatly enhanced both the number of expressing lines, and the level of expression in BMS cells (J.T. Odell and A.K. Luckring, unpublished data).

This experiment shows that the soybean IFS coding region is expressed and produces a functional enzyme that is active in monocot cells, producing genistein when its substrate is present. However, the introduced IFS is not able to utilize all of the naringenin produced as a result of CRC expression. Note that a large peak in the HPLC profile corresponding to naringenin is present in the red CRC lines, whether or not IFS is expressed (Fig. 4, A and B). Thus the naringenin is also not efficiently used in synthesizing anthocyanins. The naringenin peak is not present in non-hydrolyzed samples (data not shown) indicating that this naringenin is in a conjugated form.

Production of Daidzein in BMS Cells by Cotransformation of CRC, IFS, and CHR

A second substrate of IFS, liquiritigenin, is produced through the activity of CHR, thereby allowing the synthesis of the isoflavone daidzein (Fig. 1). CHR activity is present in legumes, but has not been reported in most non-legume plants including Arabidopsis, tobacco, and corn. We tested whether expression of CHR in cells engineered to produce genistein would allow the synthesis of daidzein in addition to genistein. A soybean CHR cDNA clone was identified from the DuPont EST sequencing program (DuPont, Wilmington, DE) by homology to known CHR sequences of soybean and alfalfa (Welle et al., 1991;

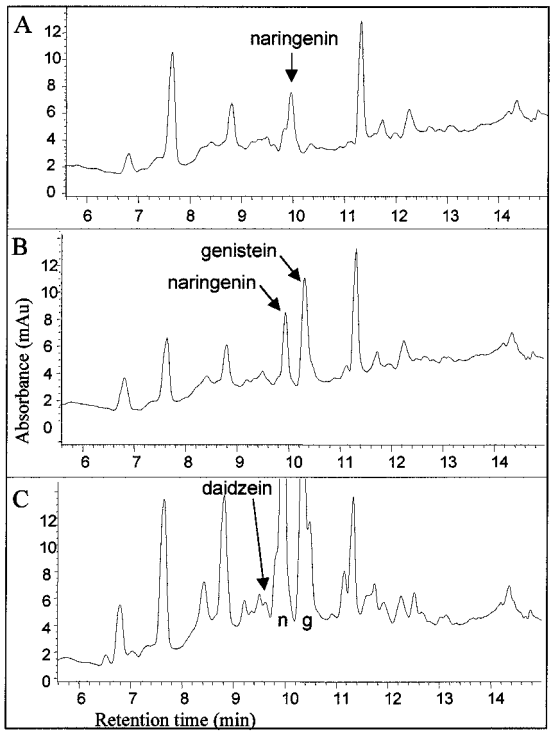

Figure 4. Production of genistein and daidzein in transformed BMS cell lines. HPLC chromatogram at 260 nm of extracts from BMS cell lines transformed with CRC (A), CRC and IFS (B), and CRC, IFS, and CHR (C). Peaks corresponding to retention times and spectra of naringenin, genistein, and daidzein are labeled. n and g designate naringenin and genistein peaks, respectively.

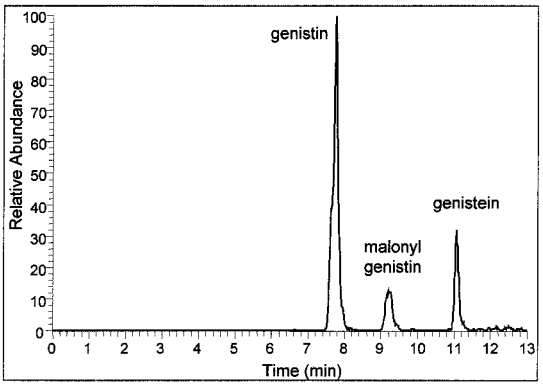

Figure 5. Genistein conjugates found in tobacco petals. LC-MS^2 analysis of extract from IFS transgenic tobacco petals showing detection of the singly protonated aglycone daughter ion (271.1; genistein) from genistin (432.9) and malonyl genistin (474.9) at the indicated elution times. The daughter ions of genistein (215 and 243) were also detected at the indicated elution time. NL, 1.44E^6.

Ballance and Dixon, 1995). The identified cDNA encodes a protein with 100% amino acid identity to the soybean p10 CHR (Swissprot accession no. P26690; Welle et al., 1991). The coding region was amplified using PCR and was used to construct a 35S/P-CHR-*Nos* 3′ chimeric gene. This CHR gene was cobombarded into BMS cells with the IFS, CRC, and 35S/P-bar genes and bialaphos-resistant lines selected. Thirty-two transformed lines were screened by PCR for the presence of all three trait genes. From the six lines shown to contain the CHR, IFS, and CRC genes and a control line carrying only the IFS and CRC genes, extracts were prepared, hydrolyzed, and analyzed by HPLC and GC-MS. In one line the HPLC profile showed a peak with the retention time of the daidzein standard (Fig. 4C) that was not present in the control. The GC-MS assay showed the diagnostic ions of derivatized daidzein: *m/z*: 398, 383, 218, and 97, confirming that daidzein was produced in this cell line.

Identification of Genistein Conjugates in Transgenic Tissues

The majority of the isoflavones found in soybean tissues are present as conjugated forms that are glucosides and malonyl-glucosides (Graham, 1991). In all of the analyses described above the samples were treated with hot HCl to hydrolyze any isoflavone conjugates. This treatment would convert possibly multiple derivatives into the single aglycone form for easier detection. Without the hydrolysis, free genistein was not detected by HPLC in any of the above experiments indicating that most of the genistein produced in tobacco, Arabidopsis, and BMS cells is in conjugated forms. A preliminary indication that Glc-conjugated derivatives were present came from the observation of shifting of peaks in the HPLC profiles of all three species following β-glucosidase treatment (data not shown). Analysis by liquid chromatography-mass spectrometry^2 (LC-MS^2) was used to determine whether the glucoside (genistin) and/or malonyl-glucoside (malonyl-genistin) forms of genistein are indeed present. The tobacco flower sample clearly contains both genistin and malonyl-genistin, with genistin being the major form detected (Fig. 5). The Arabidopsis and BMS samples both clearly contain genistin, but the presence of the malonyl form was unclear due to being near the limit of detection (data not shown). It is likely that other derivatives exist that we have not yet identified.

DISCUSSION

Isoflavone Synthesis Correlates with Phenylpropanoid Pathway Activity

In most plants other than legumes, isoflavones are not among the complex array of secondary metabolites synthesized by the phenylpropanoid pathway. This difference is due to the absence IFS, which would convert naringenin, a phenylpropanoid pathway intermediate that is common to most plants, into an isoflavone. The recent identification of the gene encoding IFS (Akashi et al., 1999; Steele et al., 1999; Jung et al., 2000) allows the introduction of isoflavone synthesizing capacity into a plant that does not naturally produce isoflavones. Our previous report on transformation of the soybean IFS gene regulated by a 35S promoter into Arabidopsis (Jung et al., 2000) showed that the isoflavone genistein could be synthesized in Arabidopsis seedlings grown under standard conditions of light and nutrient provision. Thus the naringenin substrate was present under these conditions, and it was available for IFS to convert to genistein.

Since genistein was detected in leaves of normally-grown Arabidopsis IFS transformants, it was surprising that we did not detect genistein by HPLC analysis in leaves of tobacco plants transformed with the same 35S/P-IFS gene. The more sensitive GC-MS assay was necessary to detect the small amount of genistein produced. This very low level of genistein could be due to low expression of the transgene, or to unavailability of the naringenin substrate due to inactivity of the flavonoid pathway. On the other hand, channeling of the substrate between enzymes, which has been proposed for several steps in the phenylpropanoid pathway (for review, see Winkel-Shirley, 1999) could be a factor. Tobacco plants have a natural tissue-specific activation of the phenylpropanoid pathway leading to production of anthocyanins in the flower petals. Though naringenin was not detected in the tobacco flowers, this tissue must produce naringenin as an intermediate for anthocyanin synthesis. We found that genistein was readily detected in the flowers of IFS tobacco transformants, demonstrating

the ability of the introduced IFS to compete for the pathway intermediate.

The low genistein production in the tobacco leaves was not caused by low transgene expression. The IFS mRNA was actually detected at a higher level in the leaves than in the flowers of tobacco transformants, and IFS protein and enzyme activity were also demonstrated in the leaves. Thus the low genistein synthesis most likely reflects the unavailability of the naringenin substrate. The ability of IFS to compete for naringenin in tobacco flowers, as well as in Arabidopsis leaves, makes unavailability of the substrate due to channeling unlikely. Because addition of cytochrome P450 reductase to the in vitro IFS assay increases genistein production, cytochrome P450 reductase limitation remains a formal possibility. However, the reductase would be expected to be present in leaves to interact with other P450s (Mizutani and Ohta, 1998). Thus inactivity of the flavonoid pathway in producing naringenin appears to be the limitation for genistein synthesis in tobacco leaves.

Synthesis of flavonoids has been shown to be activated by UV-B light in many plant species as a means of selectively filtering damaging radiation (Hahlbrock, 1981; Caldwell et al., 1983). In Arabidopsis, the flavonoid pathway is activated by UV-B irradiation (Li et al., 1993) and other environmental stress conditions such as phosphorus starvation (Trull et al., 1997). UV-B treatment of Arabidopsis can result in up to 2-fold increases in levels of UV-absorptive compounds, which include flavonoids, and mutants that are unable to synthesize flavonoids are hypersensitive to UV irradiation (Li et al., 1993). It was therefore possible that stress conditions activating this pathway branch would provide increased levels of the naringenin substrate to be converted to genistein by introduced IFS. Treatment of Arabidopsis transformants expressing the soybean IFS gene with UV-B irradiation did lead to a 2.5-fold higher level of genistein accumulation. The elevated phenylpropanoid pathway activity in treated plants was detected by assaying levels of total UV-absorptive compounds and anthocyanins, which increased in control and IFS transformed plants. Naringenin, which was not detected in untreated control or IFS plants, was also not detected in the treated plants. Thus naringenin is a transient intermediate and IFS is able to compete with the endogenous pathway enzymes for this substrate. However, IFS does not capture all of the increased amount of naringenin produced because the anthocyanin level increases in addition to the genistein level in the UV-B treated IFS transformants.

A positive correlation between the activity of the anthocyanin branch of the phenylpropanoid pathway and the ability to synthesize genistein was more directly demonstrated in maize BMS cells. These cell cultures are white, but the synthesis and accumulation of anthocyanins, which leads to a reddish color in the tissue, can be activated by the maize transcription factors C1 and R (Grotewold et al., 1998). Expression of soybean IFS in the white BMS cells where no naringenin is detected did not lead to genistein synthesis. However, IFS expression in conjunction with expression of the chimeric CRC gene creating red tissue did lead to synthesis of genistein. So in maize cells, as in Arabidopsis and tobacco, the naringenin intermediate in anthocyanin synthesis could be captured by IFS. However, a relatively large amount of naringenin accumulates in the CRC transformants, even when IFS is expressed. This accumulating naringenin is actually present in a conjugated form. Conjugation of naringenin and transport for storage in the vacuole, as is the case for anthocyanins in maize and isoflavones in soybean, would reduce substrate availability in BMS cells. This competing storage pathway would explain the reduced availability of naringenin for both isoflavone and anthocyanin synthesis.

Synthesis of Daidzein in BMS Cells

The isoflavone daidzein is synthesized in legumes from liquiritigenin, a phenylpropanoid pathway intermediate that is not found in most non-legumes. CHR is the key enzyme that creates the pathway branch containing liquiritigenin and daidzein. Expression of a soybean CHR cDNA in BMS cells in conjunction with CRC and IFS led to the synthesis of daidzein in addition to genistein. However, daidzein was present at about a 10-fold lower level than genistein. Our previous characterization of the IFS enzyme showed that it converts liquiritigenin to daidzein more efficiently than converting naringenin to genistein in vitro (Jung et al., 2000), suggesting that substrate specificity is not the reason for the low production of daidzein. Therefore synthesis of the liquiritigenin substrate may be limiting. A transient complex between CHR and chalcone synthase is required for the synthesis of trihydroxychalcone (Welle and Grisebach, 1988), which is then converted to liquiritigenin. If the soybean CHR were unable to interact efficiently with the endogenous maize chalcone synthase, liquiritigenin synthesis would be limited.

Other possible explanations for the low daidzein accumulation include low CHR transgene expression and further metabolism of daidzein. It is interesting to note that expression of CHR in BMS cells leads to changes in the HPLC profile in addition to the new daidzein peak. The novel CHR activity appears to have additional effects on the levels of as yet unidentified compounds of the phenylpropanoid pathway.

Genistein Conjugates in Transgenic Non-Legume Tissues

The genistein that we detected in the transgenic IFS plant tissues was seen in hydrolyzed samples where

the aglycone was liberated from any conjugates. In soybean the isoflavones are present predominantly as glucosyl and malonyl-glucosyl conjugates (Graham, 1991). The conjugates are generally less active for plant defense and nodulation, and conversion to the more active aglycones occurs readily by bacterial and fungal glycosidases in the plant's environment (Rivera-Vargas et al., 1993; Van Rhijn and Vanderleyden, 1995). Following human consumption of soy products containing isoflavone conjugates, the active aglycones are released by acid hydrolysis in the stomach or by intestinal bacteria (Davis et al., 1999). Conjugation of isoflavones in the plant cell is probably related to storage of these compounds in the vacuole, and thus is important for their accumulation.

In the non-legume tissues studied here, genistein was also found to be conjugated. In BMS cells and Arabidopsis tissue, genistein was conjugated to Glc forming the compound genistin. In tobacco flowers, malonyl-genistin was detected, as well as genistin. Other conjugate forms of genistein may also be present in these three species, judging from the disappearance of additional peaks in the HPLC profiles following β-glucosidase treatment. However, the identities of these other conjugates were not determined. Since in Arabidopsis, rhamnose or rhamnose and Glc are the predominant conjugates of flavonols (Graham, 1998), genistein may also be conjugated with these sugars.

The presence of genistein conjugates in these plant tissues that do not naturally synthesize isoflavones indicates that endogenous conjugation enzymes are able to use this novel compound as a substrate. Plants have a broad capability for glucosylation of both xenobiotic and endogenous chemicals. Secondary metabolites produced by pathogens, as well as foreign compounds such as herbicides, can be glucosylated as part of a detoxification mechanism (Sandermann, 1994). Malonyl conjugates of anthocyanins are commonly found in many non-legume dicots and malonyltransferase activities have been studied in flowers of several plants (Yamaguchi et al., 1999). These types of activities may be responsible for producing the conjugates in tissues that do not naturally encounter genistein.

Prospects for Isoflavone Metabolic Engineering

We have shown that through introducing IFS enzyme activity, monocot and dicot plant tissues that do not naturally produce isoflavones can acquire the potential to synthesize this compound. However, since the availability of the substrate for IFS relies on the activity of the flavonoid branch of the phenylpropanoid pathway (Fig. 1), isoflavones are produced only in specific tissues or under certain environmental conditions where this branch pathway is active. UV stress-induced expression of the relevant pathway and using transcription factors that activate a set of pathway genes were shown to be viable strategies for providing the required substrate for isoflavone synthesis. Further engineering to more specifically increase the level of IFS substrate, reduce activities of endogenous competing pathways, and enhance the effectiveness of the introduced IFS enzyme activity may allow higher levels of isoflavone accumulation in novel target tissues.

MATERIALS AND METHODS

Tobacco and Arabidopsis IFS Transformants

The construction of binary vector pOY204, which contains a chimeric gene consisting of the cauliflower mosaic virus 35S promoter, the soybean IFS1 coding region, and the nopaline synthase terminator (*Nos* 3′), together with an *nptII* gene cassette for kanamycin selection in plants, was described previously (Jung et al., 2000). The transformation of Arabidopsis ecotype WS was also reported. For tobacco (*Nicotiana tabacum*) transformation, the pOY204 vector was transformed into the *Agrobacterium tumefaciens* strain LBA4404, and subsequently introduced into tobacco SR1 by leaf disc cocultivation following standard procedures (DeBlock et al., 1984). Primers used to detect the IFS transgene by PCR with genomic DNA templates from putative transformants and their progeny were: 5′-GACGCCTCACTTACGACAACTCTGTG-3′ and 5′-CCTCTCGGGACGGAATTCTGATGGT-3′. These primers produce an 840-bp fragment of the IFS coding region.

Constructions for Maize BMS Cell Transformation

A chimeric gene containing the 35S promoter and chlorophyll *a/b*-binding protein gene 22L leader (CabL; Harpster et al., 1988), a 490-bp fragment containing the sixth intron from the maize (*Zea mays*) *Adh1* gene (Mascarenhas et al., 1990), the soybean IFS1 coding region (Jung et al., 2000), and the *Nos* 3′ was constructed. This gene cassette was cloned between duplicate 3-kb A element SAR fragments from the chicken lysozyme locus (Stief et al., 1989) in pGEM9Zf to produce pOY206.

The plasmid pDP7951, obtained from W. Bruce (Pioneer Hi-Bred International, Johnston, IA), contains a chimeric gene consisting of the *Nos* promoter, the omega leader of tobacco mosaic virus, the first intron of the maize *Adh1* gene, the CRC coding region, and polyadenylation signal sequence from the potato *PinII* gene (An et al., 1989). CRC is a fusion of the maize C1 and R coding regions, with R placed between the DNA-binding domain and the activation domain of C1, as described in Bruce et al. (2000). An additional CRC gene construction, pOY163, was made that contains the CaMV 35S promoter, *Adh1* intron 6, CRC coding region, and the *Nos* 3′.

Soybean ESTs generated in the DuPont Genomics Program (S.V. Tingey, G.H. Miao, and M. Dolan, personal communication) were screened by BLAST (Altschul et al., 1990) searching with the sequences of known CHR genes of

soybean and alfalfa (Welle et al., 1991; Ballance and Dixon, 1995). A cDNA encoding CHR was identified in a library made from mRNA of 8-d-old soybean roots, 4 d following inoculation with cyst nematode. The full-length coding region from the identified clone was amplified by PCR using the primers: 5′-GTTACCATGGCTGCTGCTATTG-3′ and 5′-TTAAACGTAAAATGAAACAAGAGG-3′. The 1.3-kb PCR product was subcloned into the pCR2.1 vector (Invitrogen, Carlsbad, CA). The CHR coding region fragment was cloned as an *Nco*I/*Kpn*I fragment between the 35S/CabL promoter and *Nos* 3′ to produce the plasmid pCHR40.

The plasmid pDETRIC, obtained from J. Botterman (Aventis, Gent, Belgium) contains a chimeric selection marker gene for bialaphos resistance (Thompson et al., 1987) consisting of the CaMV 35S promoter, *bar* gene coding region, and *Ocs* 3′.

Transformation of BMS Cells

Cell cultures originally derived from the maize inbred BMS were maintained in MS2D medium (Murashige and Skoog salts with vitamins [Life Technologies, Gaithersburg, MD], 20 g L^{-1} Suc, and 2 mg L^{-1} 2,4-dichlorophenoxyacetic acid, pH 5.8) as a suspension culture incubated with shaking (125 rpm) at 26°C in the dark, and subcultured with fresh medium every 5 d. Transformations were performed by microprojectile bombardment (Klein et al., 1987) using a Biolistic PDS 1000/He system (DuPont). Gold particles (0.6 microns) were coated with mixtures of DNAs containing 3 μg of the pDETRIC selection construct and 6 μg of each other plasmid in each experimental combination. Transformed BMS cell lines were obtained following the procedure of Klein et al. (1989). Transformed lines identified by their resistance to 1.5 mg L^{-1} bialaphos (Shinyou Sangyou Kabushiki-Kais, Tokyo) selection were tested by PCR analysis of genomic DNA. Primers used to detect the IFS gene were the same as above. The primers for the CRC gene were in the R region and the 3′-untranslated region of *PinII*: 5′-GCGGTGCACGGGCGGACTCTTCTTC-3′, and 5′-CGCCCAATACGCAAACCGCCTCTCC-3′. The primers for the CHR gene were 5′-GACACTTCGACACTGCTGCTGCTTAT-3′ and 5′-TCTCAAACTCACCTGGGCTATGGAT-3′.

Northern Analysis of IFS mRNA in Transgenic Tobacco

Total RNA was extracted from leaves and flowers of two randomly selected tobacco transformants using the RNeasy Plant Mini Kit (Qiagen, Valencia, CA). Northern-blot analysis was performed following standard procedures (Lessard et al., 1997). The radioactive probe was made from a purified PCR product of the soybean IFS1 coding region (with primers: 5′-TTGCTGGAACTTGCACTTGGT-3′ and 5′-GTATATGATGGATCCCTTAATTAAGAAAGGAG-3′) using the RadPrime DNA labeling system (Life Technologies).

Western Analysis of IFS Transgenic Tobacco

An IFS antibody was prepared using a peptide sequence derived from the IFS coding region. The peptide NH2-DPKYWDRPSEFRPER conjugated with KLH was produced by Quantum Biotechnologies (Montreal) and injected into a rabbit by Covance Research Products (Denver, PA) using their suggested immunization schedule. Serum from a bleed 60 d after initial immunization was used to carry out western-blot analysis. In brief, microsomes were made from two independent transformants and one wild-type tobacco plant as described below and resuspended with the addition of 0.1% (v/v) Triton X-100. The samples were run on a Novex NuPage 4% to 12% (w/v) bis-tris gel and transferred to 0.2-micron nitrocellulose (Novex, Carlsbad, CA) using a transfer apparatus (Bio-Rad, Hercules, CA) according to the manufacturer's instructions. The resulting membrane was blocked overnight in Tris [tris(hydroxymethyl)aminomethane]-buffered saline-0.5% (v/v) Tween 20 containing 5% (w/v) non-fat dry milk and then probed for 1 h with antibody diluted 1:5,000 in Tris-buffered saline-0.5% (v/v) Tween 20 containing 1% (w/v) non-fat dry milk. The membrane was then probed with antirabbit IgG conjugated to horseradish peroxidase (Promega, Madison, WI) at a dilution of 1:100,000 for 1 h. The membrane was washed four times for 5 min. The blot was developed using SuperSignal West Femto Maximum Sensitivity Substrate (Pierce, Rockford, IL) and exposed to x-ray film for 25 s before processing.

In Vitro IFS Enzyme Activity Assay

Tobacco leaf microsomes were prepared according to Kochs and Grisebach (1986). The protein content of each microsome preparation was assayed using the Bradford protein microassay (Bio-Rad). Approximately 100 μg of microsomal proteins were incubated at room temperature in 80 mM K_2HPO_4, 0.5 mM glutathione, and 20% (w/v) Suc, pH 8.0, with 100 μM naringenin substrate and 0.4 mM NADPH. Also included in the incubation was 20 μg of microsomes prepared from the yeast strain WHT1 expressing a plant cytochrome P450 reductase (Pompon et al., 1996; Jung et al., 2000). Following a 16-h incubation, reactions were extracted with ethyl acetate and analyzed by HPLC as described below.

Identification of Free Isoflavones in Transgenic Plant Tissues

Plant tissues were ground in liquid nitrogen, extracted with 80% (v/v) methanol at 100 mg mL^{-1}, and filtered through Acrodisc CR-PTFE syringe filters (Gelman Sciences, Ann Arbor, MI). To hydrolyze any possible isoflavone conjugates, 3 mL of 1 N HCl were added to 1 mL of the extract and the sample was incubated at 95°C for 2 h, followed by extraction using 1 mL ethyl acetate. The HPLC protocol was similar to that previously reported by Jung et al. (2000). In brief, samples were assayed on an HPLC system (model 1100, Hewlett-Packard, Palo Alto, CA) on a Phenomenex Luna C18 (2) column (3 μ, 150 × 4.6 mm), separated by using a 10-min linear gradient from 20% methanol/80% 100 mM ammonium acetate (pH 5.9) to 100% methanol at a flow rate of 1 mL min^{-1}. Genistein and daidzein were monitored by A_{260} and naringenin and

liquiritigenin were monitored by A_{290}. For calibration, authentic naringenin, genistein, and genistin (Sigma, St. Louis), liquiritigenin (Indofine Chemical Company, Somerville, NJ), and daidzein (Research Biochemicals International, Natick, MA) were dissolved in ethanol and used as standards. Peak areas were converted to nanograms.

For GC-MS analysis, extracts were prepared as described above, except that after hydrolysis, the dried ethyl acetate extracts were derivatized with 100 μL of *N*,*O*-bis(trimethylsilyl)-trifluoroacetamide (Supelco, Bellefonte, PA) at 37°C for 30 min. The samples were dried and then dissolved in chloroform immediately before GC analysis, using a GC system (6890, Hewlett-Packard) with a mass-selective detector (5973, Hewlett-Packard). Samples were resolved in a 15-m × 0.25-mm i.d. DB-1ht column (J&W, Jones Chromatography, Mid Glamorgan, UK) through an injector port operated in the split mode (5:1). The column was set at a linear temperature gradient from 200°C to 300°C over 20 min with a helium gas flow rate of 1.5 mL min^{-1}. The mass spectrum was monitored at an ionization potential of 70 eV. Isoflavone-trimethylsilyl derivatives in plant extracts were identified by comparison of retention times and mass spectra with those of genistein or daidzein standards derivatized with *N*,*O*-bis(trimethylsilyl)-trifluoroacetamide.

Analysis of Anthocyanin and Total UV-Absorptive Compounds

Anthocyanin analysis was performed as described in Bariola et al. (1999), as follows. Plant tissues were ground in liquid nitrogen and extracted with 80% (v/v) methanol as described above. The methanol extracts were mixed with an equal volume of 0.5% (v/v) HCl, then extracted with 2 mL of chloroform. The aqueous/methanol phase was assayed by spectrophotometer at A_{530}, with A_{657} subtracted, and the resulting absorbance value was normalized to the fresh weight for each sample.

Total UV-absorptive compounds were assayed according to Li et al. (1993), as follows. Plant tissues were ground in liquid nitrogen and extracted with 80% (v/v) methanol/1% (v/v) HCl. The extracts were cleared by centrifugation at 14,000*g* for 10 min, followed by filtration through nylon centrifuge tube filters (Corning Inc., Corning, NY). The flavonoid compounds were assayed by spectrophotometer at A_{330} and absorbance values normalized to the fresh weight of each sample.

Analysis of Genistein Conjugates by LC-MS²

Extracts prepared as described above were dried and resuspended in one-fourth of the original volume in 80% (v/v) methanol. Samples were analyzed on an HPLC (model 1100, Hewlett-Packard) using the same column as above, with an ion-trap mass spectrometer (LCQ, Finnigan, San Jose, CA). The column was run with a 0.5-min linear gradient from 95% of 0.1% (v/v) formic acid/5% methanol to 70% of 0.1% (v/v) formic acid/30% methanol; then a 14.5-min linear gradient to 100% methanol at a flow rate of 1 mL min^{-1}. A post-column split delivered approximately 700 μL to a UV detector monitoring 260 nm and the remaining approximately 300 μL was delivered to the electrospray mass spectrometer ion source. The mass spectrometer was operating in positive ion selected reaction monitoring mode. The characteristic daughter ion product of 271.1 *m/z* for genistein was monitored for the 432.9 *m/z* genistin in the 0- to 8.3-min time segment, and for the 474.9 *m/z* acetyl genistin and the 518.9 *m/z* malonyl genistin in the 8.3- to 10.9-min time segment. The characteristic 215 and 243 daughter ions of genistein were monitored in the 10.9- to 13-min time segment. Optimization of the ion optics was done by infusing the malonyl genistin standard (Nacalai Tesque, Kyoto) with a capillary temperature of 210°C, sheath gas flow of 60, auxiliary gas flow of 7 arbitrary units, and a spray voltage of 5 kV.

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LITERATURE CITED

Akashi T, Aoki T, Ayabe S (1999) Cloning and functional expression of a cytochrome P450 cDNA encoding 2-hydroxyisoflavanone synthase involved in biosynthesis of the isoflavonoid skeleton in licorice. Plant Physiol **121:** 821–828

Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ (1990) Basic local alignment search tool. J Mol Biol **215:** 403–410

An G, Mitra A, Choi HK, Costa MA, An K, Thornburg RW, Ryan CA (1989) Functional analysis of the 3′-control region of the potato wound-inducible proteinase inhibitor II gene. Plant Cell **1:** 115–122

Ballance GM, Dixon RA (1995) *Medicago sativa* cDNAs encoding chalcone reductase. Plant Physiol **107:** 1027–1028

Bariola PA, MacIntosh GC, Green PJ (1999) Regulation of S-like ribonuclease levels in Arabidopsis: antisense inhibition of *RNS1* or *RNS2* elevates anthocyanin accumulation. Plant Physiol **119:** 331–342

Blount JW, Dixon RA, Paiva NL (1992) Stress response in alfalfa (*Medicago sativa* L.): XVI. Antifungal activity of medicarpin and its biosynthetic precursors: implications for the genetic manipulation of stress metabolites. Physiol Mol Plant Pathol **41:** 333–349

Bolwell GP, Bozac K, Zimmerlin A (1994) Plant cytochrome P450. Phytochemistry **37:** 1491–1506

Bruce W, Folkerts O, Garnaat C, Crasta O, Roth B, Bowen B (2000) Expression profiling of the maize flavonoid pathway genes controlled by estradiol-inducible transcription factors CRC and P. Plant Cell **12:** 65–79

Caldwell MM, Robberecht R, Flint S (1983) Internal filters: prospects for UV-acclimation in higher plants. Physiol Plant **58:** 445–450

Davis SR, Dalais FS, Simpson ER, Murkies AL (1999) Phytoestrogens in health and disease. Recent Prog Horm Res **54:** 185–210

DeBlock M, Herrera-Estrella L, Van Montague M, Schell J, Zambryski P (1984) Expression of foreign genes in regenerated plants and their progeny. EMBO J **3:** 1681–1686

Dixon RA, Harrison MJ, Paiva NL (1995) The isoflavonoid phytoalexin pathway: from enzymes to genes to transcription factors. Physiol Plant **93:** 385–392

Dixon RA, Paiva NL (1995) Stress-induced phenylpropanoid metabolism. Plant Cell **7:** 1085–1097

Dooner HK, Robbins TP, Jorgensen RA (1991) Genetic and developmental control of anthocyanin biosynthesis. Annu Rev Genet **25:** 173–199

Eldridge AC, Kwolek WF (1983) Soybean isoflavones: effect of environment and variety on composition. J Agric Food Chem **31:** 394–396

Garcia-Arenal F, Fraile A, Sagasta EM (1978) The multiple phytoalexin response of bean (*Phaseolus vulgaris*) to infection by *Botrytis cinerea*. Physiol Plant Path **13:** 151–156

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

Graham TL (1995) Cellular biochemistry of phenylpropanoid responses of soybean to infection by *Phytophthora sojae. In* M Daniel, RP Purkayastha, eds, Handbook of Phytoalexin Metabolism and Action. Marcel Dekker, New York, pp 85–116

Graham TL (1998) Flavonoid and flavonol glycoside metabolism in Arabidopsis. Plant Physiol Biochem **36:** 135–144

Graham TL, Graham MY (2000) Defense potentiation and competancy: redox conditioning effects of salicylic acid and genistein. *In* N Keen, G Stacey, eds, Plant-Microbe Interactions, Vol. 5. APS Press, St. Paul

Grotewold E, Chamberlin M, Snook M, Siame B, Butler L, Swenson J, Maddock S, Clair GS, Bowen B (1998) Engineering secondary metabolism in maize cells by ectopic expression of transcription factors. Plant Cell **10:** 721–740

Hahlbrock K (1981) Flavonoids. *In* PK Stumpf, EE Conn, eds, Biochemistry of Plants, Vol 7. Academic Press, New York, pp 425–456

Harpster MH, Townsend JA, Jones JDG, Bedbrook J, Dunsmuir P (1988) Relative strengths of the 35S cauliflower mosaic virus, 1′, 2′, and nopaline synthase promoters in transformed tobacco, sugarbeet, and oilseed rape callus tissue. Mol Gen Genet **212:** 182–190

Jin H, Martin C (1999) Multifunctionality and diversity within the plant MYB-gene family. Plant Mol Biol **41:** 577–585

Jung W, Yu O, Lau SC, O'Keefe DP, Odell J, Fader G, McGonigle B (2000) Identification and expression of isoflavone synthase, the key enzyme for biosynthesis of isoflavones in legumes. Nat Biotechnol **18:** 208–212; erratum Jung W, Yu O, Lau SC, O'Keefe DP, Odell J, Fader G, McGonigle B (2000) Nat Biotechnol **18**: 559

Klein TM, Kornstein L, Sanford JC, Fromm ME (1989) Genetic transformation of maize cells by particle bombardment. Plant Physiol **91:** 440–444

Klein TM, Wolf ED, Wu T, Sanford JC (1987) High-velocity microprojectiles for delivery of nucleic acids to living cells. Nature **85:** 70–73

Kochs G, Grisebach H (1986) Enzymic synthesis of isoflavones. Eur J Biochem **155:** 311–318

Lessard P, Decroocq V, Thomas M (1997) Extraction of RNA, cloning and subtractive hybridization. *In* MS Clark, ed, Plant Molecular Biology: A Laboratory Manual. Springer-Verlag, Heidelberg, pp 154–220

Li J, Ou-Lee TM, Raba R, Amundson RG, Last RL (1993) Arabidopsis flavonoid mutants are hypersensitive to UV-B irradiation. Plant Cell **5:** 171–179

Mascarenhas D, Mettler IJ, Pierce DA, Lowe HW (1990) Intron-mediated enhancement of heterologous gene expression in maize. Plant Mol Biol **15:** 913–920

Messina MJ (1999) Legumes and soybeans: overview of their nutritional profiles and health effects. Am J Clin Nutr **70:** 439S–450S

Mizutani M, Ohta D (1998) Two isoforms of NADPH: cytochrome P450 reductase in *Arabidopsis thaliana*: gene structure, heterologous expression in insect cells, and differential regulation. Plant Physiol **116:** 357–367

Pompon D, Louerat B, Bronne A, Urban P (1996) Yeast expression of animal and plant P450s in optimized redox environments. Methods Enzymol **272:** 51–64

Pueppke JL (1996) The genetics and biochemical basis for nodulation of legumes by rhizobia. Crit Rev Biotechnol **16:** 1–51

Rivera-Vargas LI, Cshmitthenner AF, Graham TL (1993) Soybean flavonoid effects on and metabolism by *Phytophthora sojae*. Phytochemistry **32:** 851–857

Sandermann H Jr (1994) Higher plant metabolism of xenobiotics: the "green liver" concept. Pharmacogenetics **4:** 225–241

Steele CL, Gijzen M, Qutob D, Dixon RA (1999) Molecular characterization of the enzyme catalyzing the aryl migration reaction of isoflavonoid biosynthesis in soybean. Arch Biochem Biophys **367:** 146–150

Stief A, Winter DM, Stratling WH, Sippel AE (1989) A nuclear DNA attachment element mediates elevated and position-independent gene activity. Nature **341:** 343–345

Tamagnone L, Merida A, Parr A, Mackay S, Culianez-Macia FA, Roberts K, Martin C (1998) The AmMYB308 and AmMYB330 transcription factors from *Antirrhinum* regulate phenylpropanoid and lignin biosynthesis in transgenic tobacco. Plant Cell **10:** 135–154

Thompson CJ, Movva NR, Tizard R, Crameri R, Davies JE, Lauwereys M, Botterman J (1987) Characterization of the herbicide-resistance gene *bar* from *Streptomyces hygroscopicus*. EMBO J **6:** 2519–2523

Trull MC, Guiltinan MJ, Lynch JP, Deikman J (1997) The responses of wild-type and ABA mutant *Arabidopsis thaliana* plants to phosphorus starvation. Plant Cell Environ **20:** 85–92

Tsukamoto C, Shimada S, Igita K, Kudou S, Kokubun M, Okubo K, Kitamura K (1995) Factors affecting isoflavone content in soybean seeds: changes in isoflavones, saponins and composition of fatty acids at different temperatures during seed development. J Agric Food Chem **43:** 1184–1192

Van Rhijn R, Vanderleyden J (1995) The *Rhizobium*-plant symbiosis. Microbiol Rev **59:** 124–142

Welle R, Grisebach H (1988) Isolation of a novel NADPH-dependent reductase which coacts with chalcone synthase in the biosynthesis of 6′-deoxychalcone. FEBS Lett **236:** 221–225

Welle R, Schroder G, Schiltz E, Grisbach H, Schroder J (1991) Induced plant responses to pathogen attack: analysis and heterologous expression of the key enzyme in the biosynthesis of phytoalexins in soybean (*Glycine max* L. Merr. cv. Harosoy 63). Eur J Biochem **196:** 423–430

Winkel-Shirley B (1999) Evidence for enzyme complexes in the phenylpropanoid and flavonoid pathways. Physiol Plant **107:** 142–149

Yamaguchi M-A, Oshida N, Nakayama M, Koshioka M, Yamaguchi Y, Ino I (1999) Anthocyanidin 3-glucoside malonyltransferase from *Dahlia variabilis*. Phytochemistry **52:** 15–18