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Accumulation of Isoflavone Genistin in Transgenic Tomato Plants Overexpressing a Soybean Isoflavone Synthase Gene

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Isoflavones are legume-specific flavonoids best known for their potential cancer preventive and phytoestrogenic properties. In this study, we attempted to engineer the isoflavone pathway in the popular fruit crop tomato (*Solanum lycopersicum* L). Tomato plants were transformed with a soybean (*Glycine max* L) isoflavone synthase (*GmIFS2*) cDNA under the control of the cauliflower mosaic virus 35S promoter. LC-MS/MS analysis demonstrated the presence of genistin (genistein 7-*O*-glucoside) as the major isoflavone metabolite in the transgenic plants. Substantial amounts of genistin (up to 90 nmol/g FW) were found in leaves, while the levels were marginally detectable (less than 0.5 nmol/g FW) in fruit peels. In either case, no drastic variations in endogenous phenolic contents were observed. Fruit peels were found to accumulate high levels of naringenin chalcone, implicating the limitation of naringenin substrates for isoflavone synthesis. Our results suggested that tomato plants could be engineered to produce isoflavones without comprising the levels of endogenous flavonols, which are also health-beneficial, but it may be necessary to enhance the expression levels of chalcone isomerase simultaneously to achieve significant yields in edible tissues such as fruit peels.

KEYWORDS: Genistin; isoflavone synthase; transgenic tomato; metabolic engineering

INTRODUCTION

Isoflavones (e.g., genistein and daidzein) are a class of flavonoids predominantly restricted to leguminous plants in which they play important roles in interactions with microorganisms. For example, they serve as phytoalexins, which are part of the complex defense mechanisms against fungal pathogens (*1–3*). In addition, they are signaling factors for establishing symbiotic relationships with *Rhizobium* spp. in roots through inducing the expression of nodulation genes in bacteria (*4*). In recent years, isoflavones have been demonstrated to show an impressive list of health benefits. The most notable benefit is their possible role as phytoestrogens. For example, studies have suggested that isoflavones could represent an alternative therapeutic to relieve vasomotor symptoms (*5, 6*) and increase bone mineral density in menopausal women (*7*). Their potential activities as chemopreventive and therapeutic agents for cancers, although controversial (*8–10*), have been supported by results from both in vitro and epidemiological investigations. For example, tests on different cancer cell lines (e.g., breast cancer, prostate cancer, hepatoma, leukemia, and lymphoma cells) demonstrated that isoflavones inhibited proliferation through cell cycle arrest and induction of apoptosis (*11–16*). In addition, dietary consumption of isoflavones has been positively correlated to reduced risks and incidences of lung, breast, prostate, and colorectal cancers, as well as cardiovascular diseases (*17–21*).

Soybeans and their food products represent the major dietary sources of isoflavones. However, soybean is not a regular western food item, and it is known to have more than 15 protein allergens (*22*). The perceived health benefits of isoflavones have prompted interests in engineering these compounds in nonleguminous plants (*23–26*). Using the flavanone substrate naringenin, isoflavone synthase (IFS), a cytochrome P450 oxygenase, catalyzes a reaction leading to the formation of genistein, which is often glycosylated to form genistin (genistein 7-*O*-glucoside) (**Figure 1**). Naringenin is ubiquitously present in plants since it is also a common precursor for different classes of flavonoids, including flavones, flavonols, and anthocyanins. Thus, the synthesis of isoflavones appears to be feasible with the introduction of IFS activities in transgenic plants. Following the identification of IFS-encoding genes in soybean (*27*), different attempts have been made to transfer the legume-specific pathway to several plant species, including *Arabidopsis*, maize cells, tobacco, petunia, and lettuce (*23, 26, 28*). Genetic engineering of secondary metabolism offers the opportunity to generate crop plants with improved agronomical performance and nutritional characteristics.

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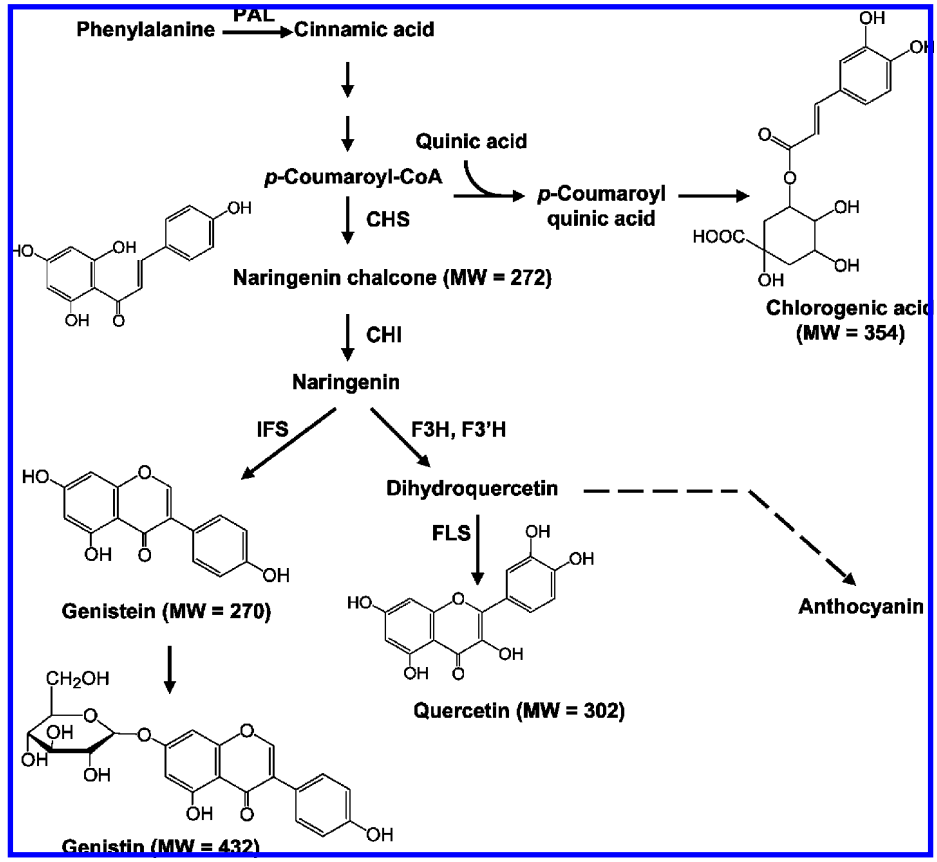

Figure 1. Biosynthesis pathway of phenylpropanoids and flavonoids described in this study. Phenylpropanoid pathway, initiated by the PAL reaction, provides the substrates for the synthesis of different phenolic compounds in plants. CHS is a key enzyme directing the flow of carbon to flavonoid biosynthesis. Naringenin is the common precursor for the synthesis of genistein and other flavonoids. Genistein is frequently glucosylated at C-7 to form genistin. Enzymes are abbreviated as follows: PAL, phenylalanine ammonia-lyase; CHS, chalcone synthase, F3H, flavanone 3-hydroxylase; F3′H, flavonoid 3′-hydroxylase; IFS, isoflavone synthase. The broken arrow represents multiple enzymatic steps.

Tomato (*Solanum lycopersicum*, formerly *Lycopersicon esculentum*) is a popular vegetable fruit crop around the world. Tomato fruits can be readily ingested without cooking, thus avoiding potential damage to their beneficial phytochemicals. They are known to contain carotenoids and some flavonoids, but the plants do not naturally produce isoflavones. Previously, successful modifications of secondary metabolism in tomato have been achieved through the introduction of structural genes in biosynthesis pathways for carotenoids, flavonoids, and stilbenes (*29, 30*). In this study, we expressed a soybean IFS gene under the control of the cauliflower mosaic virus (CaMV) 35S promoter in tomato, which was used for the first time as a host for isoflavone engineering. We demonstrated the accumulation of genistin in leaves and fruit peels of different transgenic tomato lines. Expression of endogenous genes encoding enzymes in phenolic biosynthesis pathways was examined to understand the variations in genistin contents among different tissues. To our knowledge, this is the first report of isoflavone accumulation in transgenic tomato plants.

MATERIALS AND METHODS

Construction of Transformation Vector. A full-length cDNA clone of the soybean IFS gene *GmIFS2* (GenBank accession AF195799) was ordered from the Soybean Genomic Research Institute (Iowa State University, Ames, IA). The *GmIFS2* coding region was placed under the control of the CaMV 35S promoter and the nopaline synthase 3-terminator. The cassette was then cloned into the binary vector EM104A (E Lam, Rutgers University, USA), which harbors the neomycin phosphotransferase II gene for kanamycin selection and the

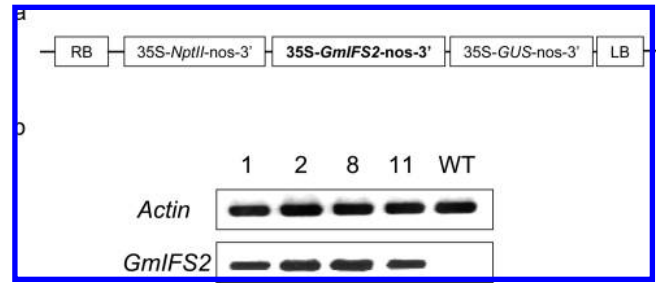

Figure 2. Transformation of tomato plants. (**a**) Binary vector construction for tomato transformation. The CaMV 35S-*GmIFS2* overexpression cassette was subcloned into a binary vector that carried the neomycin phosphotransferase (*npt*) II selection marker and the *gus* reporter gene; nos-3′, nopaline synthase terminator. (**b**) Genomic PCR analysis of four independent transgenic tomato lines (1, 2, 8, and 11) by *GmIFS2*-specific primers. An endogenous gene (Actin) was amplified in all samples including the wild type (WT).

β-glucuronidase (GUS) reporter gene (**Figure 2a**). Subsequently, the *GmIFS2* transformation vector was transferred into *Agrobacterium tumefaciens* strain GV3101 by freeze−thaw treatment.

Tomato Transformation and Growth Conditions. Seeds of an "ornamental small tomato" (*S. lycopersicum*) cultivar (Brighten seeds A02411) were obtained locally (Brighten Floriculture, Hong Kong). Cotyledons from 7-day-old seedlings were first cultured on modified Murashige and Skoog (MS) agar plates (MS salt, 200 mg/L KH_2PO_4, 0.2 mg/L 2−4-D, 3% sucrose, pH 5.5) for one day. Afterward, they were submerged for 5 min in suspensions of the transformed *Agrobacterium* strain described above, followed by dark incubation on the original agar plates for 2 days. The cotyledons were then transferred

Table 1. Primers Used in RT-PCR Experiments

Gene	accession no.	forward primer	reverse primer	amplicon length (bp)
Actin	BT012695	5′-gaatggaagctgcaggaatc	5′-ttcctttcaccaacattgtca	359
CyP	M55019	5′-gatcccagggttcatgtgtc	5′-tgaccgcagtcagcaataac	321
PAL	M900692	5′-acccttgatgcagaagctg	5′-gtccattgcaaaatgctgtg	327
CHS	X55194	5′-ccaaactcttgtccccgata	5′-ggcctttctcatttcatcca	328
CHI	BI928340	5′-gatgcaggccattgagaagt	5′-ggacatggtctatttctcttcgat	335
F3H	BT014352	5′-cagaggcaatgggcttagag	5′-ggtgctggattctggaatgt	361
FLS	BM413323	5′-cctgatttggctcttggagt	5′-cttgaattttggtgggttgg	322
GmIFS2	AF195799	5′-ggttccaaacctcagccata	5′-cgttctttctcctcctcacg	471

to S1 agar plates (MS salt, 3% sucrose, 2 mg/L zeatin, and 100 mg/L kanamycin, pH 6.0) for selection and regeneration for 3 weeks. S1 agar plates were subsequently replaced by S2 agar plates (identical to S1 plates except for 1 mg/L of zeatin). Calli with shoots of about 2 cm long were transferred to rooting medium (MS salt, 3% sucrose, 0.5 mg/L naphthalene acetic acid, and 100 mg/L kanamycin, pH 6.0). A total of 14 resistant plantlets were obtained and later transplanted to soil and grown to maturity in a greenhouse. All chemicals used in tissue culture and transformation were purchased from Sigma (St Louis, MO, USA).

DNA and RNA Analysis. For PCR confirmation of transformants, genomic DNA was extracted from leaf tissues using 2 M cetyltrimethyl ammonium bromide. For gene expression analysis, total RNA was extracted from fully expanded leaves as well as peels and flesh of red fruits by the Trizol method (Invitrogen). Peels were separated as 2 mm-thick outer layers from fruits. RNA samples were DNase-I treated (Invitrogen) and reverse-transcripted by M-MLV reverse transcriptase (Promega). Primers used for PCR amplification of *GmIFS2* and tomato genes encoding Actin, phenylalanine ammonia lyase (*PAL*), chalcone synthase (*CHS*), chalcone isomerase (*CHI*), flavanone 3-hydroxylase (*F3H*), flavonol synthase (*FLS*), and cyclophilin (*CyP*) are listed in **Table 1**. PCR amplifications were programmed as follows: preincubation (95 °C for 10 min), followed by 30 cycles of denaturation (95 °C for 30 s), annealing (55 °C for 30 s), and extension (72 °C for 1 min), followed by a final extension step at 72 °C for 7 min. Identities of the DNA fragments amplified by PCR were confirmed by sequencing.

Metabolite Analysis of Tomato Extracts. Approximately 1 g of tissues was ground in liquid nitrogen and lyophilized. Freeze-dried samples were then extracted three times with 10 mL of 100% methanol. The extracts were dried using a vacuum concentrator (Concentrator 5301, Eppendorf) and resuspended in 2 mL of 50% methanol (v/v, MeOH/H_2O) for metabolite analysis. Samples (20 μL) were injected onto an HP 1100 HPLC system (Agilent Technologies, Palo Alto, CA) connected with a Zorbax Eclipse XDB-C18 column (5 μm, 150 × 2.1 mm; Agilent Technologies) using 0.5% formic acid (v/v) in water as solvent A and 0.5% formic acid (v/v) in acetonitrile as solvent B. A linear gradient with a flow rate of 0.2 mL/min was established by increasing solvent B from 5% to 50% over 50 min. The elution was monitored by a diode-array detector (200−600 nm), followed by an online LCQ ion trap mass spectrometer (Thermo Finnigan, CA, USA) equipped with an electrospray ionization (ESI) source operating in positive mode. The mass spectrometry parameters were optimized and programmed as follows: a capillary temperature of 350 °C, a sheath gas flow rate of 70 arbitrary units, an auxiliary gas flow rate of 40 arbitrary units, an ESI spray voltage of 4.8 kV, a capillary voltage of 18 V, a tube lens offset voltage of 30 V, an octapole 1 offset voltage of −8 V, an octapole 2 offset voltage of −8.5 V, and an interoctapole lens voltage of −10 V. Helium was used as damping and collision gas at a pressure of 0.1 Pa. The Xcalibur 1.4 software (Thermo Finnigan) was used for instrument control, data acquisition, and processing. Authentic standards of genistin (Alexis Biochemicals, CA), naringenin chalcone (Chromadex, CA), quercetin, and chlorogenic acid (Sigma, MO) were used for compound identification and quantification. Triplicate samples for each transgenic line were used for LC-MS/MS analysis.

RESULTS AND DISCUSSION

Generation of Transgenic Tomatoes. Following *Agrobacterium*-mediated transformation, plantlets regenerated from kanamycin resistant calli were first screened by GUS staining using small leaf discs, and GUS-positive plantlets were transplanted to soil. **Figure 2b** shows the genomic PCR data for four independent transgenic lines, each showing the expected signal for the *GmIFS2* transgene, which was not amplified in the wild-type sample. T_1 seeds collected from primary transformants were germinated under kanamycin selection. Three T_1 plants for each independent transformant line were selected and grown to maturity for metabolite and gene expression analyses. All of the transgenic tomato plants did not differ significantly from the wild type in their germination and growth rates, flowering time, or fruit size and yield.

Detection of Genistin in Transgenic Tomato Plants. Accumulation of *GmIFS2*-derived metabolites in the transgenic lines harboring the *GmIFS2* gene was first examined in methanol extracts prepared from leaf tissues by LC-UV-MS/MS analysis. Results revealed the presence of a distinct peak (32 min) in the transgenic plant samples when compared to the wild-type samples (**Figure 3**). This peak coeluted with a genistin (genistein 7-*O*-glucoside) standard, and they have identical UV spectra (data not shown). Under positive ESI mode, the compound eluted at 32 min generated an $[M + H]^+$ ion at *m/z* 433, which is consistent with the molecular weight of a $[\text{genistin} + H]^+$ ion (**Figure 3**). The MS/MS spectrum of the *m/z* 433 ion showed the diagnostic $[\text{genistein} + H]^+$ ion at *m/z* 271, indicating a neutral loss of a hexosyl unit (162 Da) from the parent ion. The MS^3 experiment for the *m/z* 271 ion produced a fragmentation pattern identical to that generated for the authentic standard (**Figure 3**), thus allowing unambiguous confirmation of isoflavone accumulation in our transgenic tomato plants. Similarly, LC-MS/MS analysis detected the presence of genistin in fruit peel extracts prepared from all of the different transgenic lines (data not shown), while none was detected in the fruit flesh (data not shown). Accumulation of genistin indicated that genistein can be recognized by an endogenous glucosyl-transferase enzyme in tomato. Conjugation of sugars commonly occurs to constitutively accumulated phenolic compounds and may serve to protect the plant cells from their potential toxic effects (*31*).

Contents of Genistin and Endogenous Phenolic Metabolites in Transgenic Tomato Plants. Quantification analysis showed that the four transgenic tomato lines accumulated comparable amounts of genistin (**Table 2**). However, the levels of accumulation were appreciably higher (up to 270 fold) in leaves compared to those in fruit peels in all transgenic lines tested. Line 1 showed the highest yield of genistin, with 90.56 nmol/g fresh weight (FW) and 0.45 nmol/g FW detected in leaves and fruit peels, respectively. Previously, overexpressing an IFS gene alone generally resulted in relatively low isoflavone yields in transgenic plants. For example, transgenic *Arabidopsis* accumulated only up to 5.4 nmol/g FW of genistein in leaves (*28*), whereas transgenic petunia accumulated similar amounts of genistein in leaves (3.4 nmol/g FW) and petals (7.4 nmol/g FW) (*23*). Manipulations of endogenous flavonoid pathways have

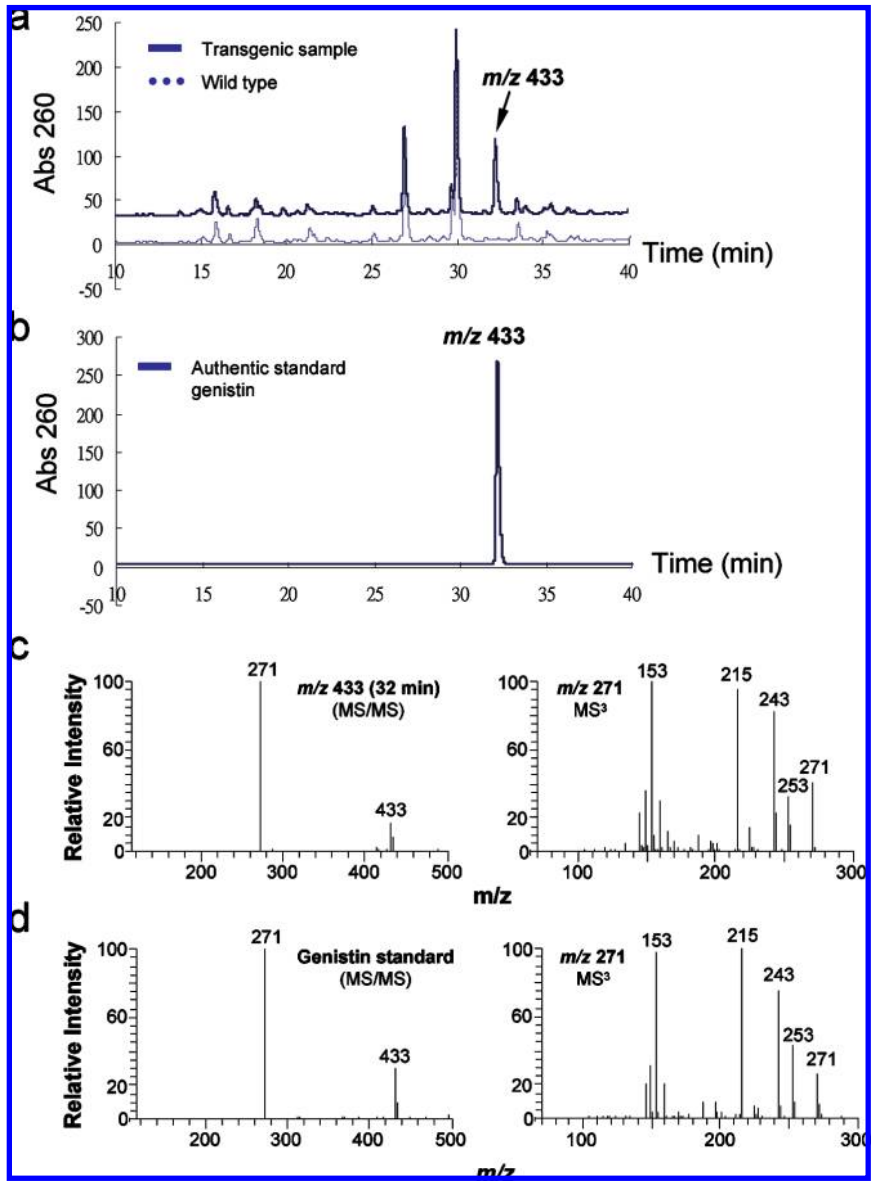

Figure 3. LC-UV-MS/MS characterization of genistin accumulation in transgenic tomato plants. (**a**) Representative LC-UV profiles of methanol leaf extracts prepared from wild-type and transgenic tomato plants. A distinct peak was identified at 32 min, and it gave an *m*/*z* 433 ion under positive ESI mode. (**b**) An LC-UV profile for an authentic genistin standard, which generated an *m*/*z* 433 ion under positive ESI mode. (**c**) MS/MS spectrum for the *m*/*z* 433 ion eluted at 32 min and MS^3 spectrum for the *m*/*z* 271 product ion. (**d**) MS/MS and MS^3 spectra for the genistin standard and the *m*/*z* 271 product ion, respectively.

been used as a strategy to elevate isoflavone production in transgenic plants. Naringenin is a common precursor for different classes of flavonoids, including isoflavones, flavones, flavonols, and anthocyanins. Transformation of IFS gene into *Arabidopsis tt3*/*tt6* double mutants had resulted in larger amounts of genistein (31−169 nmol/g) in leaves (*28*). The double mutants were defective in flavonol and anthocyanin biosynthesis, thus allowing more naringenin substrates for the introduced IFS enzyme. A similar approach was employed in tobacco in which the F3H gene was antisense-silenced, leading to accumulation of up to 70 nmol/g FW of genistein (*28*). Interestingly, without genetic manipulation of endogenous metabolic pathways, our transgenic tomato harboring the *GmIFS2* gene alone accumulated comparable levels of genistin in leaves (up to 90 nmol/g FW). However, the levels of genistin accumulation in fruit peels were far from satisfactory (<0.5 nmol/g FW). The amounts of different endogenous phenolic compounds in both wild-type and transgenic lines were also determined (**Table 2**). Tomato plants accumulated two glycosides of quercetin (a flavonol) in both leaves and fruit peels, but no detectable amounts were found in fruit flesh (data not shown). Quercetin is a health-beneficial flavonoid with strong antioxidant activities and cardiovascular protection properties (*32*). Considerable amounts of quercetin glycosides (>200 nmol/g FW, expressed as quercetin equivalents) were detected in transgenic leaf and fruit peel samples (**Table 2**). In addition, chlorogenic acid (a quinic acid ester of phenylpropanoid) and naringenin chalcone were also found in all fruit peel samples (**Table 2**), as reported previously (*33, 34*). However, naringenin accumulation was not detectable in leaves and fruit peels, suggesting that most of them had been converted to downstream flavonoid metabolites.

Gene Expression Analysis. To understand the differences in genistin accumulation in leaves and fruit peels, we investigated the expression of *GmIFS2* as well as endogenous genes encoding different enzymes along the phenolic biosynthesis pathways. RNA samples were prepared from different tissues for RT-PCR experiments using different sets of gene-specific primers. Expression of the *Cyp* gene was constant in all tomato tissues (*35*) and was used as a positive control in our study.

Table 2. Contents of Genistin and Endogenous Phenolic Compounds in Selected Transgenic Tomato Lines[a]

	leaf samples					peel samples				
transgenic lines	WT	1	2	8	11	WT	1	2	8	11
genistin		90.56 ± 15.76	77.90 ± 24.34	64.92 ± 9.97	69.93 ± 15.32		0.451 ± 0.052	0.436 ± 0.107	0.234 ± 0.042	0.334 ± 0.013
quercetin[b]	426.84 ± 61.48	377.91 ± 52.66	352.60 ± 48.82	273.59 ± 28.16	303.43 ± 65.01	300.57 ± 41.34	246.48 ± 33.30	224.87 ± 11.45	227.73 ± 16.32	217.71 ± 42.40
naringenin chalcone						3616.72 ± 1047.8	2424.53 ± 623.5	3009.94 ± 814.2	2403.13 ± 815.6	2056.4 ± 406.5
chlorogenic acid						129.56 ± 6.45	104.51 ± 12.29	108.72 ± 9.68	127.38 ± 14.25	101.86 ± 3.94

[a] Triplicate samples were analyzed for each line. Data are presented as mean ± SD (nmol/g FW). [b] Levels of quercetin glycoside accumulation were quantified as quercetin equivalents.

Figure 4. RT-PCR analysis of the expression of *GmIFS2* and endogenous genes encoding different flavonoid enzymes in leaves and fruit peels of wild-type (WT) and transgenic plants (lines 1, 2, 8, and 11). The expression of the tomato *CyP* gene was used as internal control.

Expression of *GmIFS2*, driven by the CaMV 35S promoter, was detected at significant levels in both leaves and fruit peels of all transgenic lines (**Figure 4**). On the other hand, strong transcript accumulation was demonstrated for the endogenous genes *PAL*, *CHS*, *F3H*, and *FLS* in all of the wild-type and transgenic plant samples. In contrast, *CHI* transcript accumulation was detected in leaves but not in fruit peels, which accumulated considerably high amounts of naringenin chalcone (**Table 2**). CHI catalyzes the isomerization of naringenin chalcone to naringenin. Apparently, the weak expression of *CHI* gene in fruit peels (**Figure 4**) was responsible for the high-level accumulation of naringenin chalcone (>2 μmol/g FW). Consequently, the amounts of naringenin, which is isomerized from naringenin chalcone by CHI, would be limiting for the synthesis of both isoflavone and flavonol. It should be noted that significant amounts of quercetin still accumulated in transgenic fruit peels (>200 nmol/g FW), while genistin was barely detectable. For quercetin formation, naringenin is first hydroxylated at the C-3 position by F3H, followed by the enzymatic activities of FLS (**Figure 1**). Naringenin is known to be converted from naringenin chalcone by spontaneous isomerization, but it occurs at a lower rate (*36*). F3H was likely to have stronger affinity than IFS for the limiting naringenin substrates under such conditions, allowing substantial accumulation of quercetin but poor yield of genistin in fruit peels as demonstrated by our metabolite data (**Table 2**). However, the differences in genistin levels in leaves and peels may be attributed to variations in IFS enzyme activities in these tissues.

In summary, our work represents the first attempt to engineer isoflavones in tomato, which is one of the most important cash crops worldwide. We successfully transformed tomato with a soybean IFS cDNA under the control of the CaMV 35S promoter and conclusively identified genistin as the major form of isoflavone metabolite in the transgenic plants. Our results from transgenic leaf analysis suggested that tomatoes have the potential to accumulate significant levels of genistin (up to 90 nmol/g FW) without the need to manipulate endogenous metabolic pathways. This is particularly attractive since flavonols like quercetin are also health-beneficial because of their highly potent antioxidant activities (*37*). However, for the development of functional food products, it would be essential to have significant yields in tomato fruits, at least in the fruit peels. The

lack of expression of endogenous flavonoid genes in fruit flesh (data not shown) makes it more challenging to engineer a novel flavonoid metabolite in this tissue. Transgenic expression of maize regulatory genes *LC* and *C1* was recently demonstrated to be necessary and sufficient to induce the flavonoid biosynthesis pathway in tomato fruit flesh (*33*). However, the consumption of the whole fruit is increasingly perceived as health-beneficial, and it would be highly desirable to enrich the phytochemical constituents of fruit skins. Recently, processing of tomato fruits with enhanced quercetin in fruit peels showed that 65% of flavonols were retained in the concentrated paste (*34*). However, it may be necessary to elevate the expression of the *CHI* gene in fruit peels to levels comparable to those in leaf tissues (**Figure 4**) in which substantial amounts of genistin accumulated as a result of *GmIFS2* overexpression (**Table 2**).

NOTE ADDED AFTER ASAP PUBLICATION

The original posting of June 10, 2008, contained an incorrect version of Figure 3, which has been corrected with the posting of June 20, 2008.

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