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Ethylene Induced a High Accumulation of Dietary Isoflavones and Expression of Isoflavonoid Biosynthetic Genes in Soybean (*Glycine max*) Leaves

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Supporting Information

ABSTRACT: Dietary isoflavones, daidzein and genistein are of huge interest in the nutraceutical field due to their practical application to postmenopause complications. This study is the first report an efficient method to prepare isoflavone rich soybean leaves (soyleaves) which is an edible food stuff in Asian countries. The preharvest treatment of ethylene highly stimulated the level of isoflavone in soyleaves. Annotation and quantification of metabolites were determined by UPLC-Q-TOF-MS and HPLC. Phenolic metabolites of soyleaves are mostly kaempferol glycosides, but not dietary isoflavones. The accumulated isoflavones by ethylene treatment were determined to be daidzin **1**, genistin **2**, malonyldaidzin **3** and malonylgenistin **4**, which were easily hydrolyzed to daidzein and genistein by β-glucosidase. Total content of dietary isoflavones was increased up to 13854 μg/g. The most suitable condition was estimated to be 250 μg/g ethylene or 200 μg/g ethephon (ethylene donor) treatment at the R3 growth stage. The ratio of daidzein and genistein glycosides was approximately 5 to 3. The accumulated isoflavonoid biosynthesis pathway genes were identified within the transcriptome of soyleaves tissues at 1 day after treatment of ethephon. The quantitative RT-PCR analysis of these genes indicated significantly higher expression of *CHS, CHI, IFS, HID, IF7GT*, and *IF7MaT* compared to control leaves. These findings suggest that ethylene activates a set of structural genes involved in isoflavonoid biosynthesis, thereby leading to enhanced production of isoflavones in soybean plants.

KEYWORDS: *soybean leaves, dietary isoflavones, ethylene, genistein, daidzein, isoflavonoid biosynthetic genes*

INTRODUCTION

Plants constantly synthesize secondary metabolites to adapt to their environment and enable their survival and well-being. These metabolites can then confer health benefits to the plants' consumers. The hormone ethylene regulates multiple physiological and development processes in plants. This hormone leads to the activation of various transcription factors, some of which are involved in synthesis of metabolites.[1−3] For example, ethylene stimulates expression of the yellow or orange carotenoid pigments. The ripening process can be catalyzed by ethylene, and this observation is used in the agricultural industry to obtain desired flavors, quality, color, and other textural properties of produce.[4−6] There is also a report that ethephon, a source of ethylene, stimulates cannabinoid and plastidial terpenoid production in *Cannabis sativa* during flowering.[7]

Isoflavones are distinct secondary metabolites produced predominately in leguminous plants. The principal role of isoflavones is in establishing the symbiotic relationship between the plant and rhizobial bacteria.[8] Isoflavones are a subgroup of phytoestrogens, which are natural plant substances with structures similar to 17-β-estradiol. These compounds are able to bind to estrogen receptors (ERs). The ERs are found in two forms, ERα and ERβ. Interaction of isoflavones with ERs leads to the activation of so-called estrogen response elements located on the inner side of the nuclear membrane.[9,10] Unsurprisingly, isoflavones are also believed to confer benefits to human health when consumed as a dietary supplement. For instance, a large number of dietary intervention studies have demonstrated the positive effects of soy isoflavones on risk factors for osteoporosis, cardiovascular disease, and hormone dependent cancer, all diseases that are affected by estrogen levels.[11] When the representative isoflavone, genistein, was administered to postmenopausal women, a significant increase in bone density and reduction in the concentration of biochemical markers of bone resorption were observed.[12] Isoflavones were also found to inhibit growth and migration of vascular smooth muscle cells, which may protect against the development of atherosclerosis.[13]

Natural isoflavones occur in more than 300 kinds of plants, located mostly in the roots and seeds. Principal dietary isoflavones are daidzein, genistein, glycitein, and their glycosides. They are found in kudzu, red clover, alfalfa, linseed, and soybean. All these plants, apart from soybean, contain less than 1000 μg/g of dietary isoflavones. The richest source is soybean

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that shows total isoflavone content around 2300 μg/g.[14] Unsurprisingly, most of the classical efforts to provide functional foods with enhanced isoflavone content have focused on breeding studies aimed at increasing isoflavone levels in soybeans.[15] Another approach has been to manipulate isoflavone synthase (IFS) genes which are essential for the biosynthesis and accumulation of isoflavones in plants.[16] Elicitor was also used to enhance the productivity of isoflavones in plant cells or cultured tissue.[17] However, all approaches so far trialed have had a limited impact on isoflavone content. Furthermore, abiotic and biotic elicitors have been applied to soyplants to enhance isoflavone content.[18] Isoflavones are involved in plant−microbe interaction because pathogens might produce elicitor molecules that increase isoflavone content in plants as defense response.[18] Microbial pathogens are commonly examined to increase isoflavone. For example, *Pseudomanas syringae* pv *glycinea*, *Ps. pisi* (bacteria), and *Phytophthora megasperma* f. sp. *glycinea* (fungi) led to accumulation of isoflavones in soyplants: daidzein, genistein, formonetin, isoformonetin, glycitein, glyceollins, and their glycosides.[19−21] It is also reported that soybean cultivars affected accumulation of isoflavones after infection. On the other hand, a series of chemical compounds such as chitosan, salicylic acid, and jasmonic acid or their derivatives could affect metabolic processes including accumulation of isoflavone. Application of chitosan to soybean leaves increased total isoflavone content from 16 to 96%.[18] However, the enhancement of isoflavone contents in plant has a limit for practical use so far.

Successful attempts to increase isoflavone contents in soyleaves have not been previously reported. In this report, we describe how we found an effective method to produce soyleaves containing extremely high amount of dietary isoflavones. The key method to elicit changes in dietary isoflavones, flavonoid profile, and antioxidant activities of soyleaves was preharvest treatment of ethylene. The accumulated isoflavones in soyleaves were fully characterized and quantified by ultraperformance liquid chromatography coupled with photodiode array detection and quadrupole-time-of-flight mass spectrometry (UPLC-Q-TOF-MS). Furthermore, our real-time quantitative RT-PCR analysis shows that activation of a set of isoflavonoid biosynthetic genes by the ethylene donor, ethephon, induces an elevation in isoflavone biosynthesis genes in soybean plants.

MATERIALS AND METHODS

Plant Material and Experimental Design. Soybeans, *Glycine max* (L.) Merrill, were obtained from the National Institute of Crop Science (NICS), Miryang, Republic of Korea. They were cultivated in pots in a greenhouse over a period of about 60 days until the plant reached a maximum growth stage R3 for R1, R2, R3, or R4, respectively. This was carried out from April to June with daily temperature ranging between 15 and 35 °C. The experiment consisted of totally 5 treatments with 5 replications. Two different chemicals (ethylene and ethephon) were tested in the current study. In the case of ethylene, the treatments included five different concentrations of ethylene in μg/mL as follows: T1 = 100, T2 = 150, T3 = 200, T4 = 250, and T5 = 300. Ethylene was applied twice to the chamber (80 × 60 × 80 cm). Every 24 h and afterward plants were maintained in normal conditions. The chamber was sealed airtight and opened about every 24 h to supply carbon dioxide. At every 24 h, viz., 24, 48, 72, 96, and 120 h post treatment of ethylene, plant leaves from each pot were harvested randomly, cut into small pieces using a laboratory blade cutter, dried at 35 °C, and used for further quantitative analysis. In the case of ethephon, the treatments consisted of five ethephon concentrations (μg/mL): T1 = 50, T2 = 100, T3 = 150, T4 = 200, and T5 = 250. Ethephon was sprayed to the plants until the solution started dripping. Controls were maintained each time. Control pots were sprayed with water lacking the respective test chemical. Plant leaves from the ethephon treated pots were sampled at every 24 h and used for further assays as described for ethylene treatment. Some of the harvested leaves were immediately frozen at −78 °C for biosynthetic gene quantification. All sample masses were based on dry weight basis. Value of each treatment is a mean of five replicate samples.

Preparation of Crude Sample and Standard. Phenolic metabolite extraction was performed in five replicates from soybean leaves with different treatments. Sample extraction for UPLC-Q-TOF-MS and HPLC analysis was carried out using the following method. The dried and chopped sample (1.0 g) was extracted with 50 mL of 70% methanol for 24 h at room temperature. For hydrolysis of isoflavone glucosides, 1 mL of extract solution was treated with 4 mL of 50 mM phosphate buffer (pH 6.8) with 0.1 unit of β-glucosidase. The reaction mixture was incubated at 37 °C for 2 h to hydrolyze the glucose moiety completely. The fluid was filtered through a syringe filter (0.2 μm) and injected directly into the UPLC-Q-TOF-MS or HPLC system. Standards for the 6 isoflavones (daidzin, genistin, malonyldaidzin, malonylgenistin, daidzein, genistein) were purchased from LC Laboratories (Woburn, MA, USA). All solvents used were of HPLC grade. The isoflavone standards were dissolved in methanol at several concentrations (5, 10, 20, 50, and 100 μg/mL), and high linearity (r^2 > 0.999) was obtained for each compound. The 6 isoflavones were identified by their retention time and mass, and their concentrations were calculated by comparing the peak areas of the samples with those of the standards.

Identification of Phenolic Metabolites by UPLC-Q-TOF-MS. The Q-TOF mass spectrometer was operated in the ESI-positive mode. The ionization source conditions were as follows: capillary voltage of 2.5 kV, source temperature of 100 °C, and desolvation temperature of 300 °C. The sampling cone voltage was set at 40 V, extraction cone 0.8 V, trap collision energy 6.0, transfer collision energy 4.0; the trap gas flow was 1.50 mL/min, ion energy 1.0 V, collision energy 4.0 V. Mass spectrometric data were collected in the range of 50−1200 m/z with a scan time of 0.2 s and interscan delay time of 0.02 s. The MS/MS experiments were performed at variable collision energies (15−40 eV), which were optimized for each individual compound. The accurate mass and composition of the precursor and fragment ions were calculated and sequenced using the MassLynx software (Waters Corp.), which was incorporated into the instrument.

To analyze changes in the content of methanol-soluble isoflavones during different stages of ethephon treatment, a UPLC system (Waters Corp., Milford, MA, USA) equipped with a binary solvent delivery system, autosampler, and photodiode array (PDA) detector was used. An ACQUITY UPLC BEH C18 column (2.1 × 100 mm, 1.7 μm; Waters Corp.) was used for quantitative analysis, and the analysis was performed at a UV wavelength of 254 nm. The mobile phase was composed of A (0.5% aqueous formic acid, v/v) and B (acetonitrile) with a linear gradient elution: 0−1 min, B, 3%; 1−5 min, B, 3%−15%; 5−10 min, B, 15%−25%; 10−11 min, B, 25%−30%; 11−12 min, B, 30%−45%; 12−15 min, B, 45%−57%; 15−18 min, B, 57%−100%; 18−20 min, back to 3% B. The flow rate of the mobile phase was 0.4 mL/min, and the column temperature was maintained at 30 °C.

HPLC-DAD Analysis of Phenolic Metabolites. HPLC of extracts was performed using an Agilent 1200 series HPLC system consisting of Agilent G1311 A quaternary pump (Agilent Technologies, Santa Clara, CA), using a Zolbax Bonus-RP (150 × 4.6 mm, 5 μm) column (Agilent Technologies, Santa Clara, CA) connected to Agilent G1315B diode array detector. Data were collected and analyzed using Chem Station software (Agilent Technologies). Gradient elution was carried out with water/0.1% acetic acid (solvent A) and acetonitrile (solvent B). The linear gradient elution program was as follow: 5 min, 15% B; 20 min, 20% B; 50 min, 50% B; 65 min, 60% B; 75 min, 100% B. The flow rate was 1.0 mL/min, and injection volume was 10 μL. Absorbance was detected at 254 nm using the

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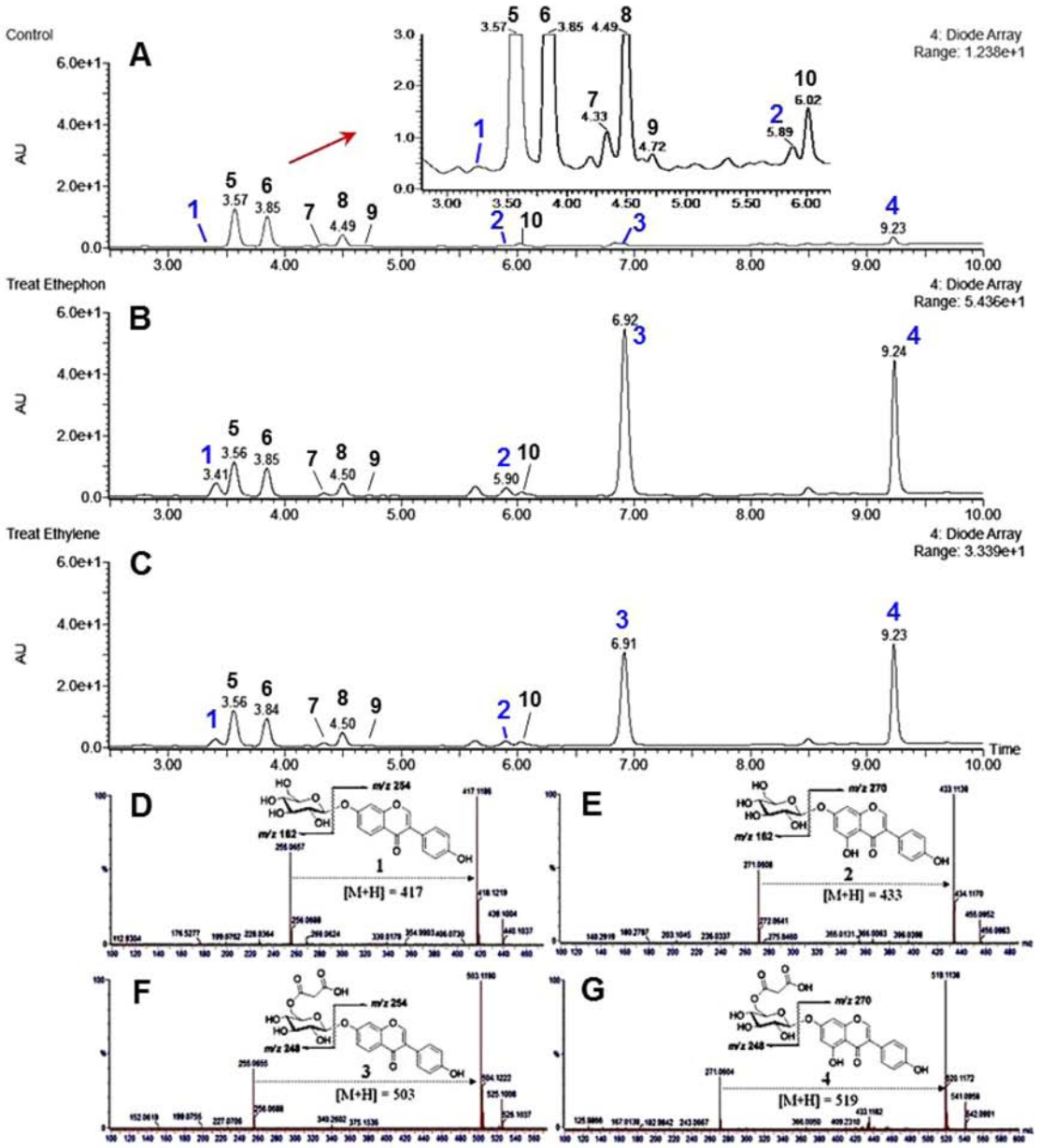

Figure 1. Effect of ethylene on isoflavone accumulation in soyleaves. Samples were extracted by 70% methanol for 6 h. UPLC-PDA chromatograms are shown at 254 nm: (A) control leaves; (B) leaves treated with ethephon; (C) leaves treated with ethylene. Positive ion mass spectrum acquired by UPLC-ESI/MS analysis of isoflavone glycosides (D−G) from soyleaves treated with ethylene.

Agilent diode array detector. Phenolic compounds in the extracts were identified by comparison of their retention times (t_R) values with the spectra of known standards and quantified by using the calibration curve for each phenolic compound obtained by peak areas from the chromatogram.

Determination of Total Phenolic and Total Flavonoid Content. Total phenolic content (TPC) of the extracts was determined according to the Folin−Ciocalteu (FC) procedure with slight modifications.[22] Briefly, 20 μL of diluted crude extracts was mixed with 160 μL of sodium carbonate solution (7.5%, w/v). After 3 min, 20 μL of FC reagent was added and vortexed. The absorbance at 765 nm against blank was determined using a SpectraMax M3 multimode microplate reader (Molecular Devices, CA, USA) after 1 h incubation in the dark at room temperature. Measurements were calibrated to a standard curve of prepared gallic acid solution (5−100 μg/mL) with $y = 0.0396x$ ($r^2 = 0.9985$), and TPC was then expressed as milligrams of gallic acid equivalents (GAE) per 100 g of dry weight (DW). Estimation of the total flavonoid content (TFC) in crude extracts was performed according to the procedures described by Chun et al.[23] with slight modifications. The reaction mixture contained 10 μL of crude extract, 120 μL of deionized water, and 10 μL of 5% sodium nitrite. After 6 min, 10 μL of 10% aluminum chloride 6-hydrate was added. In the next 5 min, 50 μL of 1 M sodium hydroxide solution was added and mixed. Immediately, the reaction mixture absorbance was measured at 415 nm against a blank and used to calculate TFC using quercetin (5−100 μg/mL) as standard ($y = 0.0033x$; $r^2 = 0.9986$). The TFC was then expressed as quercetin equivalents (QE), in milligrams of QE per 100 g DW.

RNA Isolation, cDNA Synthesis and Real-Time Quantitative RT-PCR (qRT-PCR). Total RNA was extracted from soybean leaves (0.1 g of fresh weight) using FavorPrepTri-RNA reagent (Favorgen), and further purified using an RNeasy Plant Mini Kit (Qiagen) according to the manufacturer's instructions. First-strand cDNAs were synthesized from 1 μg of total RNA using a RevertAid first-strand cDNA synthesis kit (Thermoscientific). For qRT-PCR, soybean gene specific primers were designed using the Beacon designer software

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Table 1. Quantitative Analysis of Dietary Isoflavones in Soyleaves

		isoflavone content (μg/g)[b]					
treatment	isoflavone	0 h[a]	24 h	48 h	72 h	96 h	120 h
ethylene[c]	daidzin (**1**)	nd[e]	1649 ± 119	1784 ± 285	1996 ± 266	2331 ± 259	2395 ± 183
	genistin (**2**)	nd	955 ± 102	1280 ± 204	1402 ± 216	1452 ± 172	1465 ± 229
	malonyldaidzin (**3**)	<100	2706 ± 153	5971 ± 274	6158 ± 484	6232 ± 502	6461 ± 419
	malonylgenistin (**4**)	<100	1969 ± 178	3734 ± 382	3279 ± 384	3301 ± 359	3533 ± 346
	total	<200	7279 ± 552	12769 ± 1145	12835 ± 1350	13316 ± 1292	13854 ± 1177
ethephon[d]	daidzin (**1**)	nd	1311 ± 87	1342 ± 163	2585 ± 104	2784 ± 189	2863 ± 196
	genistin (**2**)	nd	868 ± 64	1035 ± 178	1708 ± 171	1797 ± 116	1663 ± 165
	malonyldaidzin (**3**)	<100	3133 ± 103	5563 ± 201	5756 ± 318	6456 ± 391	6430 ± 389
	malonylgenistin (**4**)	<100	1980 ± 121	3323 ± 262	4056 ± 375	4186 ± 323	4295 ± 324
	total	<200	7292 ± 375	11265 ± 804	14105 ± 968	15223 ± 1019	15251 ± 1074

[a]Untreated. [b]All values are mean ± SD of in five independent experiments. [c]Ethylene treatments at 250 μg/mL. [d]Ethephon treatments at 200 μg/mL. [e]Not detected.

(Premier Biosoft) and presented in Table S1. All reactions were performed in 10 μL volumes containing 2 μL of 10-fold diluted cDNA (total 10 ng), 1 μL of primer mix (1 μM of each forward and reverse primer), and 5 μL of iQ SYBR Green Supermix reagent (Bio-Rad) with the CFX96 real-time system (Bio-Rad). The reactions were subjected to an initial denaturation of 95 °C/3 min, followed by 40 cycles of 95 °C/10 s, primer specific annealing, and extension 55 °C/30 s. A melting curve analysis was performed at the end of PCR with temperature range of 65−95 °C in 0.5 °C increment/5 s. Data were analyzed using the CFX manager software 3.1 (Bio-Rad). Transcript level was calculated using the normalized against soybean beta-tubulin (*β-TUB*) gene used as a reference control. Relative expression of the different genes was analyzed at three time points after ethephon treatment.

Statistical Analysis. In this study, statistical analysis was undertaken using the general linear model procedure (GLM) from SAS statistical software Institute (Version 9.1, 2002, SAS Cary, NC, USA). All extraction runs and analyses were carried out at least in duplicate and in a randomized order with the mean values being reported. Differences between the means of sample were analyzed by Tukey's test at a probability level of 0.05.

RESULTS AND DISCUSSION

Dietary Isoflavones Accumulation on Soyleaves. Consistent with the drive to create new functional foods, a significant effort has been targeted to improving the content of bioactive components in edible plants. In particular, soy isoflavones have received much attention as a nutraceutical supplement because there is significant evidence for their biological benefits to humans.[11] We found that treatment of soybean plants with ethylene or ethephon (an ethylene donor) can hugely stimulate levels of dietary isoflavones in soyleaves that are not typically considered a source of isoflavones (Figure 1 and Table 1). These stimulated soyleaves are one of the most abundant sources of isoflavones: they are 5-fold richer than soy beans, and although leguminous plants contain dietary isoflavones, their concentrations are relatively low.

Indeed, many efforts to boost production of isoflavones in both nonlegume and legume plants have been attempted by applying metabolic engineering or cell culture technology. Cell cultures of lupine, *Glycyrrhiza echinata*, *Cicer arietinum*, and *P. lobata* have been evaluated for elicitor-induced manipulation of isoflavonoid production. For instance, ethylene promoted production of dietary isoflavones up to 12 mg/L in suspension cultures of *Pueraria tuberose*. However, these low concentrations effectively limit its commercialization so far.[24]

The isoflavones that accumulated upon ethylene treatment were investigated by UPLC-Q-TOF-MS, which serves as a powerful tool for simultaneous identification of complicated secondary metabolites owing to its HRESI-MS function, which can give information regarding the molecular formula of the molecular ion as well as its fragmentation pattern. As presented in Figure 1, complete chromatographic separation of the phenolic compounds within soyleaves was achieved within 10 min monitoring elution by UV detection at a wavelength of 254 nm. A comparison of a UPLC-PDA chromatogram of control and ethylene treated soyleaves in Figure 1 (A and C) showed that four increased peaks (**1**−**4**) appeared upon ethylene treatment. The first peak **1** (t_R 3.41, λ_{max} 259.7) was detected by HRESI-MS as its protonated ion $[M + H]^+$ (m/z 417.1186) with a molecular formula $C_{21}H_{21}O_9$ that coincides with daidzin. Additionally, the fragment ion (m/z 255.0657 ($M + H^+$, −162 amu)) having molecular formula $C_{15}H_{11}O_4$ showed the loss of a glucose moiety (Figure 1D) to give daidzein. Full HRESI-MS analysis of peak **3** (t_R 6.92, λ_{max} 257.4) showed a $[M + H]^+$ ion at m/z 503.1190 having molecular formula $C_{24}H_{23}O_{12}$, which coincides with malonyldaidzin. The major fragment ion (m/z 255.0655 ($M + H^+$, −248 amu)) results from the loss of a malonyl-glucose moiety. On the basis these observation, this peak was identified as malonyldaidzin (**3**) and this assignment was also consistent with previously reported data.[25]

Peak **2** (t_R 5.89, λ_{max} 259.8) showed a protonated molecular ion $[M + H]^+$ at m/z 433.1139 having the molecular formula $C_{21}H_{21}O_{10}$. The major fragment ion had a m/z 271.0608 (the mass of genistein aglycon) of $C_{15}H_{11}O_5$ as shown Figure 1E, which was formed by the loss of the glucose moiety (−162 amu). Based on this evidence as well as previous data, peak **2** was assigned as genistin (**2**). The molecular ion $[M + H]^+$ of peak **4** was detected at m/z 519.1138 having molecular formula $C_{24}H_{23}O_{13}$, which coincides with malonylgenistin. The major fragmentation at 271.0604 ($[M + H]^+$, −248 amu) showed the loss of a malonyl-glucose moiety and had the characteristic of genistein, like peak **3**. Thus, peak **4** was confirmed to be malonylgenistin (**4**), and this assignment was consistent with previous data.[26]

Peaks **5**−**10** were kaempferol glycosides because fragment ions with the molecular weight/formula of kaempferol were found in all peaks (**5**−**10**) ($C_{15}H_{11}O_6$, calculated m/z 287.0556) by HRESI-Q-TOF-MS (Supporting Information). Each peak was confirmed by mass fragmentation pattern and comparison to previous retention times.[27] To illustrate the method used, the structures of peaks **5** and **6** were confirmed as follows. The mass profile of peaks **5** (t_R = 3.57 min) and **6** (t_R = 3.85 min) showed a maximal absorption at 264.7 nm, and their

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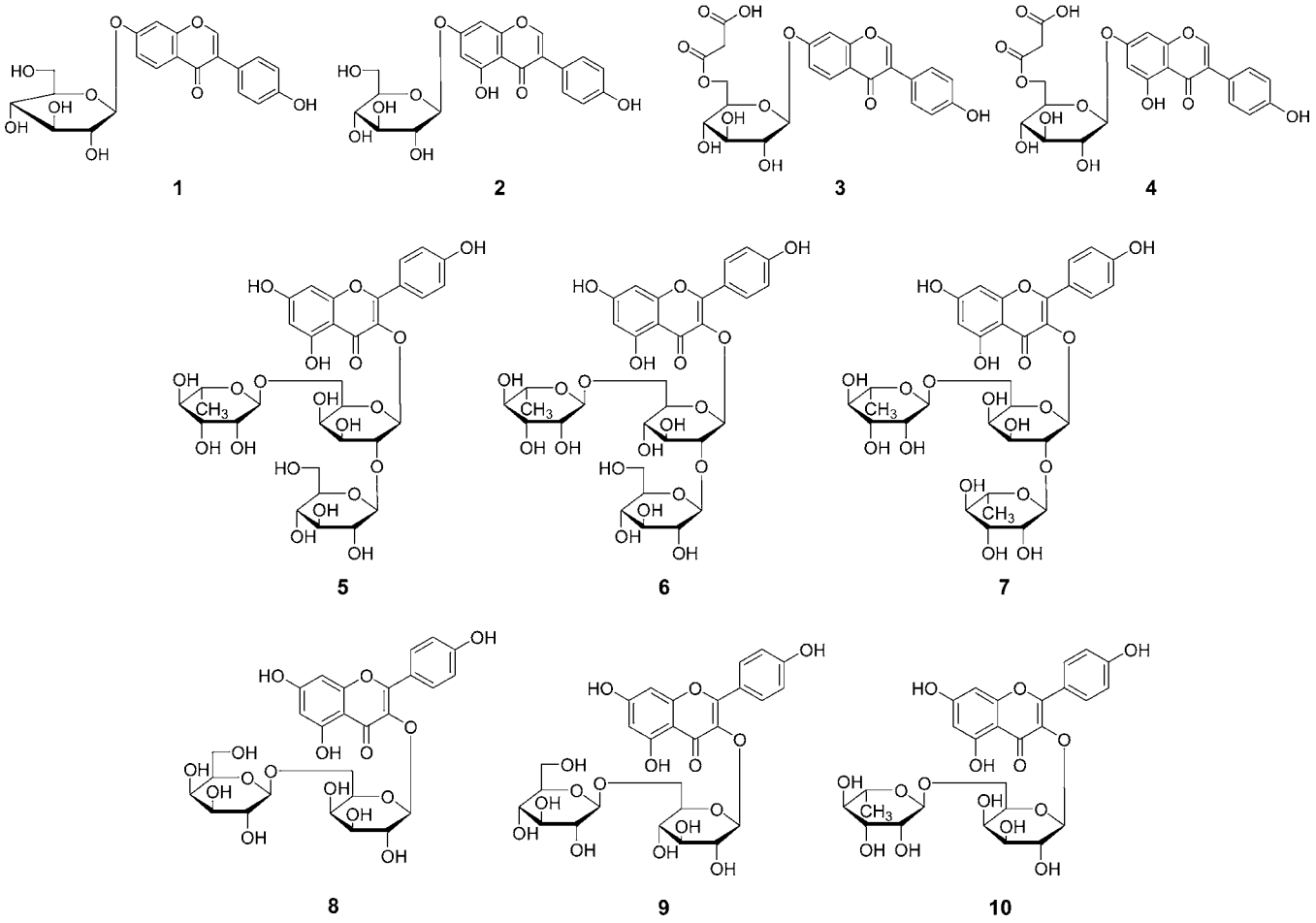

Figure 2. Structure of annotated secondary metabolites in soybean leaves.

molecular ion $[M + H]^+$ had a mass of 757.2181 ($C_{33}H_{41}O_{20}$, calculated m/z 757.2191), respectively. The major fragment of both peaks had a m/z 287.0559 $[M - (glc + gal + rham)]^+$, which corresponds to kaempferol. By comparison with previously published data, peaks **5** and **6** were identified to be kaempferol-3-*O*-*β*-D-glucopyranosyl (1→2)-*α*-L-rhamnopyranosyl (1→6)-*β*-D-galactopyranoside and kaempferol-3-*O*-*β*-D-galactopyranosyl (1→2)-*α*-L-rhamnopyranosyl (1→6)-*β*-D-glucopyranoside, respectively. It is generally accepted that galactopyranoside had a shorter retention time than glucopyranoside.[28] Figure 2 represents the identified polyphenol in soyleaves. Table 2 shows UV−vis absorption maxima, retention times, and mass spectral data, including molecular and fragment ions of the peaks (**5**−**10**). A PDA chromatogram of extract in Figure 1 showed that most dietary isoflavones were accumulated as malonyl glycosides (**3** and **4**). Usually malonyl glycosides are unstable and can be easily converted to their corresponding glycosides by hydrolysis under different conditions. The abundance of malonyl glycosides (**3** and **4**) varied according to extraction conditions.

We started our experiments with a soy plant (*Glycine max*) in the R3 growth stage. Thus, we set out initially to identify the secondary metabolites present in the control at this stage. The principal isoflavones in soybean are well documented to be genistein, daidzein, and their glycosides. It has been reported that kaempferol glycosides are the most abundant polyphenols in soyleaves.[29] In addition, it is known that the soyleaves contain only small amount of isoflavones and accumulate pterocarpan derivatives as they progress to later growth stages.[30]

Quantitation of Dietary Isoflavones and Accumulation Pattern. To get maximum extraction of isoflavones from soyleaves 70% methanol was found to be the appropriate solvent. As we mentioned above, the proportion of malonyl isoflavone glycoside and isoflavone glycoside in the sample depended on extraction conditions, because malonyl isoflavone glucosides (**3** and **4**) were likely converted into the corresponding isoflavone glucosides (**1** and **2**) due to the effect of water portion and extraction temperature (Figure 3A,B). The extraction for quantification was carried out with 70% methanol at room temperature for 24 h, which gave the most stable ratio. The contents of dietary isoflavones (**1**−**4**) in the soyleaves were analyzed with HPLC at 254 nm.

In our study we found that ethylene and ethephon (ethylene donor) significantly affected dietary isoflavone (**1**−**4**) levels in soyleaves. The major influencing factors were doses of ethylene or ethephon, growth stages, and post-treatment harvest time. Soybean reproductive growth and development can be divided into 8 stages according to morphological features: R1 and R2 describing flowering; R3 and R4 describing pod development.[31] After the treatment of different concentrations of ethylene (100−300 μg/g) and ethephon (50−200 μg/g) at multiple growth stages (R1−R4) for several hours (24 to 120 h), we found the most reliable and best conditions for maximum outcome to be 300 μg/g of ethylene or 250 μg/g of ethephon concentrations, R3 growth stage, and 120 h as harvest time of

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Table 2. Characterization of Phenolic Compounds in Soyleaves Identified by UPLC-Q-TOF/MS

peak no.	t_R (min)	UV	$[M + H]^+$ (MF, ppm)	other fragmental ion $[M + H]^+$ (MF, ppm)	identification
1	3.41	259.7	417.1186 (calcd 417.1186) ($C_{21}H_{21}O_9$, 0.0)	269.0624 ($C_{12}H_{13}O_7$, −13.7), 256.0688 ($C_{15}H_{12}O_4$, −18.7)	daidzin
				255.0657 ($C_{15}H_{11}O_4$, 0.0), 228.0364 ($C_{13}H_8O_4$, −25.8)	
				199.0762 ($C_{13}H_{11}O_2$, 1.5)	
2	5.89	259.8	433.1139 (calcd 433.1135) ($C_{21}H_{21}O_{10}$, 0.9)	271.0608 ($C_{15}H_{11}O_5$, 0.7), 236.0337 ($C_{11}H_8O_6$, 6.8)	genistin
3	6.92	257.4	503.1190 (calcd 503.1190) ($C_{24}H_{23}O_{12}$, 0.0)	255.0655 ($C_{15}H_{11}O_4$, −0.8), 227.0706 ($C_{14}H_{11}O_3$, −0.9)	malonyldaidzin
				199.0755 ($C_{13}H_{11}O_2$, −2.0), 152.0619 ($C_5H_{12}O_5$, −43.4)	
4	9.23	259.8	519.1138 (calcd 519.1139) ($C_{24}H_{23}O_{13}$, −0.2)	433.1182 ($C_{21}H_{21}O_{10}$, −10.8), 271.0604 ($C_{15}H_{11}O_5$, −0.7)	malonylgenistin
				243.0667 ($C_{14}H_{11}O_4$, 4.1)	
5	3.57	264.7	757.2181 (calcd 757.2191) ($C_{33}H_{41}O_{20}$, −1.3)	595.1655 ($C_{27}H_{31}O_{15}$, −0.8), 511.1464 ($C_{23}H_{27}O_{13}$, 2.3)	kaempferol-3-*O*-*β*-D-glucopyranosyl (1→2)-*α*-L-rhamnopyranosyl (1→6)-*β*-D-galactopyranoside
		344.8		487.1217 ($C_{24}H_{23}O_{11}$, −4.7), 398.0864 ($C_{17}H_{18}O_{11}$, 3.8)	
				287.0559 ($C_{15}H_{11}O_6$, 1.0)	
6	3.85	264.5	757.2177 (calcd 757.2191) ($C_{33}H_{41}O_{20}$, −1.8)	611.1599 ($C_{27}H_{31}O_{16}$, −2.1), 595.1654 ($C_{27}H_{31}O_{15}$, −1.5)	kaempferol-3-*O*-*β*-D-galactopyranosyl (1→2)-*α*-L-rhamnopyranosyl (1→6)-*β*-D-glucopyranoside
		342.7		471.1714 ($C_{18}H_{31}O_{14}$, 0.0), 449.1088 ($C_{21}H_{21}O_{11}$, 0.9)	
				398.0867 ($C_{17}H_{18}O_{11}$, 4.5), 287.0557 ($C_{15}H_{11}O_6$, 0.3)	
7	4.49	264.7	611.1605 (calcd 611.1612) ($C_{27}H_{31}O_{16}$, −1.1)	449.1078 ($C_{21}H_{21}O_{11}$, −1.3), 447.1291 ($C_{22}H_{23}O_{10}$, 0.0)	kaempferol-3-*O*-*β*-digalactopyranoside
		346.8		325.0578 ($C_{14}H_{13}O_9$, 5.5), 287.0558 ($C_{15}H_{11}O_6$, 0.7)	
8	4.33	264.8	741.2236 (calcd 741.2242) ($C_{33}H_{41}O_{19}$, −0.8)	595.1659 ($C_{27}H_{31}O_{15}$, −0.7), 449.1086 ($C_{21}H_{21}O_{11}$, 0.4)	kaempferol-3-*O*-(2,6-di-*O*-*α*-L-rhamnopyranosyl)-*β*-D-galactopyranoside
		346.7		380.0896 ($C_{19}H_{18}O_9$, −13.8), 287.0561 ($C_{15}H_{11}O_6$, 1.7)	
9	4.72	264.8	611.1612 (calcd 611.1612) ($C_{27}H_{31}O_{16}$, 0.0)	449.1088 ($C_{21}H_{21}O_{11}$, 0.9), 447.1291 ($C_{22}H_{23}O_{10}$, 0.0)	kaempferol-3-*O*-*β*-diglucopyranoside
		346.8		325.0580 ($C_{14}H_{13}O_9$, 6.1), 287.0561 ($C_{15}H_{11}O_6$, 1.7)	
10	6.02	264.7	595.1664 (calcd 595.1663) ($C_{27}H_{31}O_{15}$, 0.2)	450.1122 ($C_{21}H_{22}O_{11}$, −8.9), 449.1088 ($C_{21}H_{21}O_{11}$, 0.9)	kaempferol-3-*O*-*α*-L-rhamnopyranosyl (1→6)-*β*-D-galactopyranoside
		346.8		412.0352 ($C_{20}H_{12}O_{10}$, −18.9), 317.0607 ($C_{16}H_{13}O_7$, −17)	
				287.0561 ($C_{15}H_{11}O_6$, 1.7), 177.0558 ($C_{10}H_9O_3$, 3.4)	

post-treatment (Figure 4 and Table 1). Using these optimal conditions, we observed a drastic increase in the total isoflavone contents starting from 140 μg/g (control leaves) reaching to 13854 μg/g. Furthermore, the contents of individual isoflavones like daidzin (**1**), genistin (**2**), malonyl daidzin (**3**), and malonyl genistin (**4**) were quantified to be 2395, 1465, 6461, and 3533 μg/g, respectively. From these results it appears that all the individual isoflavones (**1**−**4**) were significantly affected by ethylene treatment. Ethephon treatment also elicited a high accumulation of dietary isoflavones (vide infra). We proceeded to measure the isoflavone content dependence on ethylene dose and harvest time postinitiation of treatment. Different concentrations of ethylene (100−300 μg/g) were tested. 100 μg/g ethylene treatment led to 3545 μg/g of isoflavones; whereas 250 μg/g ethylene gave 13854 μg/g of isoflavones as shown Figure 4. However, there was no significant difference in total isoflavone content in soyleaves treated with more than 250 μg/g ethylene. Ethylene concentrations higher than 300 μg/g were detrimental, leading to rapid leaf senescence and yellow coloration. We continuously monitored this increase of malonyldaidzin at different time intervals starting from 24 h until 120 h, and quantified the concentrations of malonyldaidzin to be 5971 μg/g at 48 h, 6158 μg/g at 72 h, 6232 μg/g at 96 h, and 6461 μg/g at 120 h. This result indicated that the best harvest time was 120 h of post-treatment, but no significant difference was observed between 72 h (6158 μg/g) and 120 h (6461 μg/g). The contents of the other three isoflavones (**1**, **2**, and **4**) showed very similar increasing pattern like malonyldaidzin by harvest times. In order to establish optimal conditions for isoflavone accumulation the next step was to investigate the level of isoflavones within soyleaves as a function of growth stage. The stages of R1, R2, and R3 produced a high content of isoflavones, 12307, 13324, and 13508 μg/g, respectively. Notably, ethylene treatment was a less effective way to increase isoflavone content in the R4 stage because isoflavone levels diminished to 6869 μg/g as shown in Figure 5.

Ethephon is a representative ethylene donor that produces ethylene in aqueous solution above pH 4. Soyleaves treated with ethephon also showed a dose dependent increase in isoflavone content, similar to the increase observed upon ethylene treatment. A 200 μg/g dose was the most effective at

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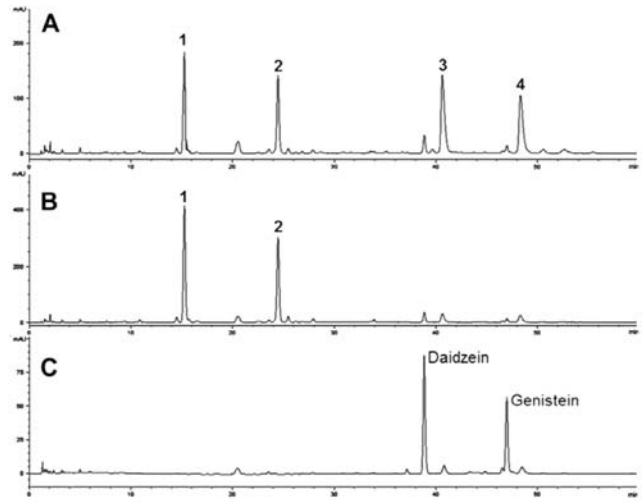

Figure 3. Relative abundance of dietary isoflavones according to extraction conditions or enzymatic hydrolysis assessed using HPLC chromatogram (254 nm): (A) 70% methanol for 24 h at room temperature; (B) 70% methanol for 10 h at reflux; (C) hydrolysis of extract with 0.1 unit of β-glucosidase.

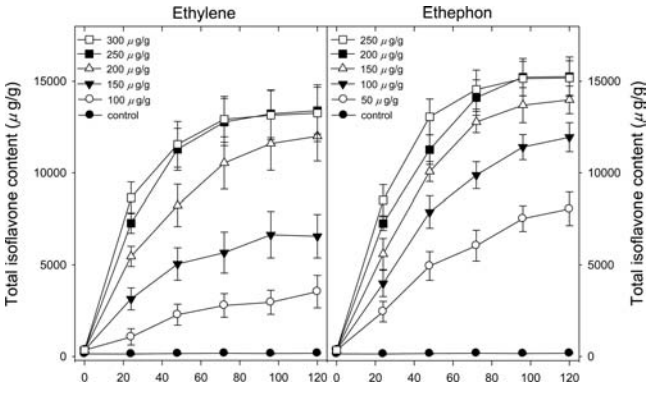

Figure 4. Evolution of total isoflavone content of soyleaves as affected by ethylene and ethephon treatments at five concentrations applied at five different harvest times (24, 48, 72, 96, and 120 h).

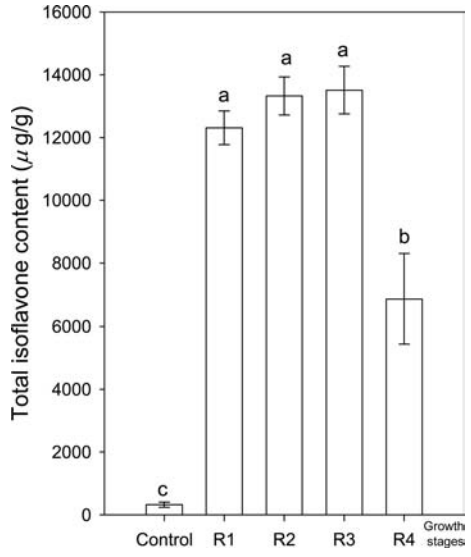

Figure 5. Effects of ethylene treatment on total isoflavone content across growth stages. Different letters indicate significant differences, as determined by Tukey's (LSD) test with $p < 0.05$.

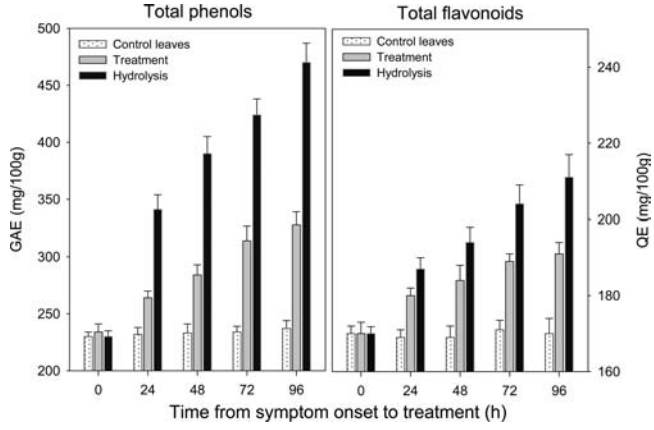

Figure 6. Evolution of total phenol and total flavonoid contents of soyleaves as affected by ethylene at 250 μg/mL treatment. Data are the mean ± SD of determinations performed in five replicates in 5 samples.

increasing isoflavones (Figure 4). However, as we observed with ethylene, larger doses of ethephon (>250 μg/mL) rapidly led to leaf senescence. Under optimal ethephon treatment conditions, levels of total isoflavones in soyleaves increased up to 15251 μg/g. Individual isoflavones were quantified such as **1** (daidzin, 2863 μg), **2** (genistin, 1663 μg), **3** (malonyldaidzin, 6430 μg), and **4** (malonylgenistin, 4295 μg) as shown in Table 1. The increased efficacy of ethephon over ethylene can be ascribed to ethephon being in solution and hence the liberated ethylene has more chance to be absorbed than in the use of free ethylene.

The concentrations of total phenolics and total flavonoids also increased over the 96 h period post ethylene treatment, reaching 328 mg GAE/100 g and 191 mg QE/100 g of concentrations in soyleaves, respectively as shown Figure 6. This phenomenon is in agreement with the increment of isoflavone concentrations due to ethylene treatment. On the other hand, isoflavone glycosides (**1**−**4**) in soyleaf extract could be hydrolyzed to their derivative isoflavones. These products have more phenolic hydroxyl groups, which may endow them with more potent antioxidant potential. Thus, to hydrolyze malonyl isoflavone glycosides to their corresponding derivatives like genistein and daidzein (Figure 3C), the soyleaf extract was treated with β-glucosidase, 2 h. Pleasingly, the hydrolyzed soyleaves showed higher total phenolic and flavonoid content as 470 mg GAE/100 g and 211 mg QE/100 g, respectively (Figure 6), compared to control leaves. These higher concentrations of antioxidants presumably are due to hydrolysis of accumulated isoflavones.

It is well-known that ethylene plays a crucial role in the activation of plant defense responses against various biotic stresses through cross-talk between complex signaling networks, such as jasmonic acid (JA), salicylic acid (SA), and abscisic acid (ABA).[32] Although ethylene is not a common signal for induction of plant secondary metabolism, some instances have been reported where accumulation of plant secondary metabolites is affected by ethylene. For example, ethylene treatment increased flavonoid, anthocyanin, and stilbenoid production via upregulation of their respective biosynthetic genes in grape.[33,34] These studies together with our results suggest that ethylene (or ethylene produced from elicitor) can act as an integral signal that is sufficient to

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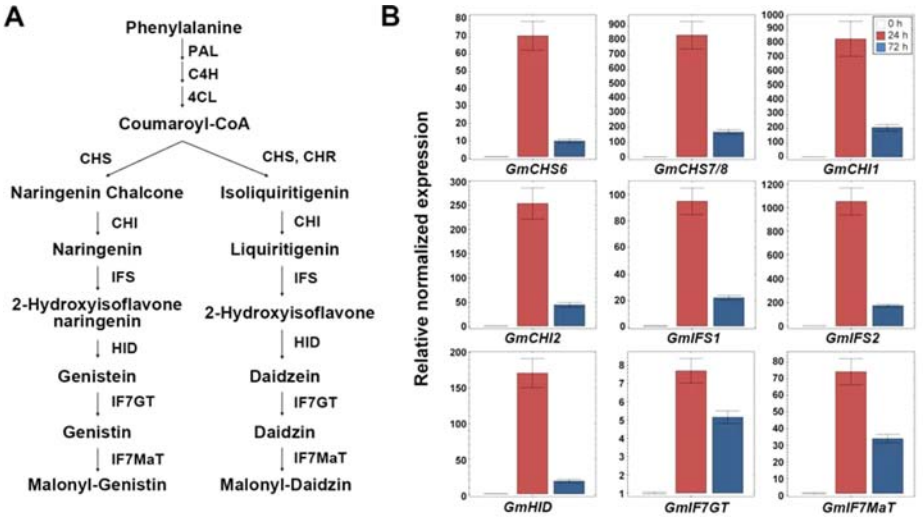

Figure 7. Relative expression of isoflavonoid biosynthetic genes in soyleaves treated with ethephon. (A) The sequential actions of CHS, CHI, IFS, HID, IF7GT, and IF7MaT result in the conversion of phenylalanine to isoflavones: *CHS*, chalcone synthase; *CHI*, chalcone isomerase; *IFS*, isoflavone synthase; *HID*, 2-hydroxyisoflavanone dehydratase; *IF7GT*, UDP-glucose: isoflavone 7-*O*-glucosyltransferase; *IF7MaT*, malonyl-CoA: isoflavone 7-*O*-glucoside-6-*O*-malonyltransferase. (B) Relative expression of *CHS*, *CHI*, *IFS*, *HID*, *IF7GT*, and *IF7MaT* mRNAs were analyzed by real-time quantitative RT-PCR. Data were normalized against expression of the housekeeping gene beta-tubulin (*β-TUB*). All the values shown are mean ± SE.

upregulate production of some secondary metabolites including isoflavones. Thus, our results obtained with ethylene application would provide a useful strategy for increasing the isoflavone content in soyleaves.

Expression Analysis of Isoflavonoid Biosynthetic Genes by qRT-PCR. To gain more knowledge of what occurs at the molecular level during ethephon/ethylene application, quantitative real-time PCR was performed using RNA from soyleaves. Soybean plants at R3 growth stage were sprayed with ethephon, and soyleaves were harvested at 1 and 3 days postspraying. Isoflavonoids are synthesized by a legume specific branch of the general phenylpropanoid pathway (Figure 7A). Various structural genes within phenylpropanoid and isoflavonoid pathways such as chalcone synthase (*CHS*), chalcone isomerase (*CHI*), isoflavone synthase (*IFS*), 2-hydroxyisoflavone dehydratase (*HID*), isoflavone 7-*O*-uridine diphosphate glycosyltransferase (*IF7GT*), and isoflavone 7-*O*-glucoside-6-*O*-malonyltransferase (*IF7MaT*) are known to be involved in isoflavonoid biosynthetic pathways.[16,35−37] We thus tested whether these genes were upregulated upon ethylene treatment. Pleasingly, all genes were upregulated by severalfold (*GmIF7GT*) to 1000-fold (*GmIFS2*) when soyleaves were treated with ethephon relative to untreated soyleaves. The transcript levels decreased at 3 days after treatment of ethephon. These results suggest that ethephon/ethylene induces the transcriptional expression of structural genes involved in isoflavonoid biosynthesis, thereby leading to the enhancement of isoflavone accumulation in soyleaves. We realized that expression levels of the structural genes decreased upon prolonged treatment with ethylene (72 h) (Figure 7B). However, the isoflavone contents steadily increased up to 72 h as shown in Figure 4. Such a discrepancy is not unexpected if, for instance, expressed protein has a long half-life such that protein levels would be maintained at steady levels with their enzyme stability and activity over 72 h. The enzyme CHS catalyzes the first committed step in the flavonoid and isoflavonoid biosynthetic pathways.[38] Among nine *CHS* (*CHS1* to *CHS9*) genes present in the soybean genome, *CHS7* and *CHS8*, two proteins that share a high degree of sequence identity, were shown to be important for isoflavonoid biosynthesis.[39] Both naringenin and isoliquiritigenin chalcones are then converted to their corresponding flavanones by CHI. In our qRT-PCR analysis, *CHS7/8* and *CHI1* genes were more responsive to ethephon treatment than *CHS6* and *CHI2*, respectively. The enzyme IFS, cytochrome P450 monooxygenase enzyme, is the branch point enzyme that introduces isoflavonoid specific branch in the phenylpropanod pathway. IFS catalyzes the conversion of the naringenin and liquiritigenin flavanones to their corresponding 2-hydroxyisoflavones.[16,35] Isoflavones are synthesized by HID through the elimination of water from 2-hydroxyisoflavanones.[37] Here, the *IFS2* gene was more highly induced by ethephon than *IFS1*, indicating that IFS2 enzyme likely plays a more active role in isoflavonoid biosynthesis in response to ethylene signals. In addition, IF7GT and IF7MaT catalyze the sequential glycosylation and malonylation of isoflavone aglycons (genistein, daidzein), thereby leading to the production of glycosides (genistin, daidzin) and malonyl forms (malonylgenistin, malonyldaidzin), respectively.[35,36] This decoration of the isoflavone core provides the molecules with enhanced water solubility and reduced chemical reactivity.

MYB family transcriptional factors (TFs) are known to be involved in transcriptional regulation of the expression of flavonoid biosynthetic genes.[40,41] For example, AtMYB12 was reported to affect the flavonol production by regulating the transcriptional expression of *CHS*, *CHI*, *F3H*, and *FLS1* genes.[41,42] In addition, GmMYB176 was shown to regulate the expression of *GmCHS8* gene and affect the isoflavonoid biosynthesis in soybean.[43] In the context of isoflavonoid biosynthesis, we speculate that certain soybean MYB TFs play important roles in regulating the isoflavonoid biosynthesis by upregulation of the expression of *GmCHS*, *GmCHI*, *GmIFS*, *GmHID*, *GmIF7GT*, and *GmIF7MaT* genes. Thus, although beyond the scope of the present study, it will be very interesting to find out the key MYB TFs involved in regulating the isoflavonoid biosynthesis in soybean.

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In conclusion, this study focused on elevating dietary isoflavones in soyleaves. We found that treatment of soybean plants with ethylene or ethephon stimulates soyleaves to produce unprecedented levels of isoflavones. The principal isoflavones were confirmed to be dietary isoflavones such as daidzin (**1**), genistin (**2**), malonyldaidzin (**3**), and malonylgenistin (**4**). Under our best conditions, the amount of total isoflavones was up to 13854 μg/g. This method will help to improve dietary consumption of beneficial phytochemicals that confer known health benefits to their consumers. Furthermore, our qRT-PCR analysis suggests that ethephon application stimulates the transcription of a set of structural genes involved in isoflavonoid biosynthesis and thus leads to high-level accumulation of isoflavones in soybean plants.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jafc.6b02543.

Characterization data, materials, and methods (PDF)

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Author Contributions

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Notes

The authors declare no competing financial interest.

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