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Journal

Journal of chemical ecology, 42(12)

ISSN

0098-0331

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Publication Date

2016-12-01

DOI

10.1007/s10886-016-0786-8

Peer reviewed

Inducible *De Novo* Biosynthesis of Isoflavonoids in Soybean Leaves by *Spodoptera litura* Derived Elicitors: Tracer Techniques Aided by High Resolution LCMS

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Received: 24 February 2016 / Revised: 18 September 2016 / Accepted: 30 September 2016 / Published online: 8 November 2016
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Abstract Isoflavonoids are a characteristic family of natural products in legumes known to mediate a range of plant-biotic interactions. For example, in soybean (*Glycine max*: Fabaceae) multiple isoflavones are induced and accumulate in leaves following attack by *Spodoptera litura* (Lepidoptera: Noctuidae) larvae. To quantitatively examine patterns of activated *de novo* biosynthesis, soybean (Var. Enrei) leaves were treated with a combination of plant defense elicitors present in *S. litura* gut content extracts and L- α -¹³C₉, ¹⁵N]phenylalanine as a traceable isoflavonoid precursor. Combined treatments promoted significant increases in ¹³C-labeled isoflavone aglycones (daidzein, formononetin, and genistein), ¹³C-labeled isoflavone 7-*O*-glucosides (daidzin, ononin, and genistin), and ¹³C-labeled isoflavone 7-*O*-(6''-*O*-malonyl- β -glucosides) (malonyldaidzin, malonylononin, and malonylgenistin). In contrast levels of ¹³C-labeled flavones and flavonol (4',7-dihydroxyflavone, kaempferol, and apigenin) were not significantly altered. Curiously, application of fatty acid-amino acid conjugate (FAC) elicitors present in *S. litura* gut contents, namely *N*-linolenoyl-L-glutamine and *N*-linoleoyl-L-glutamine, both promoted the induced accumulation of isoflavone 7-*O*-glucosides and isoflavone 7-*O*-(6''-*O*-malonyl- β -glucosides), but not isoflavone aglycones in the leaves. These results demonstrate that at least two separate

reactions are involved in elicitor-induced soybean leaf responses to the *S. litura* gut contents: one is the *de novo* biosynthesis of isoflavone conjugates induced by FACs, and the other is the hydrolysis of the isoflavone conjugates to yield isoflavone aglycones. Gut content extracts alone displayed no hydrolytic activity. The quantitative analysis of isoflavone *de novo* biosynthesis, with respect to both aglycones and conjugates, affords a useful bioassay system for the discovery of additional plant defense elicitor(s) in *S. litura* gut contents that specifically promote hydrolysis of isoflavone conjugates.

Keywords Plant-insect interaction · Plant induced resistance · Insect-produced elicitors · Fatty acid-amino acid conjugates (FACs) · Isoflavones · Insect herbivory · Secondary metabolites · Lepidoptera · Noctuidae

Introduction

Flavonoids are predominant bioactive natural products in plants, with over 7000 related compounds identified to date (Anderson and Markham 2006). Flavonoids are divided into many subclasses that include but are not limited to flavones, isoflavones, and flavonols. Diverse bioactivities of flavonoids have been reported and span a range of complex roles in plant physiology including floral pigments, modulators of auxin transport, and defense (Harborne et al. 1975; Kumar and Pandey 2013). The structural and biological complexity is due in part to the fact that plants commonly store flavonoids as highly soluble glycoside conjugates (Barz and Welle 1992; Edwards et al. 1997; Jones and Vogt 2001; Lin et al. 2000).

Isoflavonoids are confined largely to the Fabaceae subfamily Faboideae. These compounds have diverse roles in environmental adaptation and are useful in understanding legume specific physiology. We previously demonstrated that

Electronic supplementary material The online version of this article (doi:10.1007/s10886-016-0786-8) contains supplementary material, which is available to authorized users.

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daidzein, formononetin, and their conjugates (glucosides and malonylglucosides) are induced and accumulate in soybean leaves [*Glycine max* (L.) Merr. (Fabaceae)] following attack by common cutworm larvae [*Spodoptera litura* (Lepidoptera: Noctuidae)] (Murakami et al. 2014). Given that daidzein inhibits the growth of *S. litura* larvae, it has been suggested that induction of soybean isoflavones is a defensive reaction against lepidopteran herbivores (Zhou et al. 2011). This dynamic soybean response is triggered not only by herbivory but also by application of complex blend of oral secretion elicitors present in *S. litura* gut content extracts (Murakami et al. 2014).

Based on evidence from multiple plant-insect systems, it is established that during attack by Lepidopteran larvae, trace amounts of specific elicitors present in foregut-derived oral secretions (or gut contents) can contact the wounded leaf surface and activate plant defense responses (reviewed in Schmelz 2015). Moreover, it has been demonstrated that specific Lepidopteran oral secretion elicitors can be used to predict elicitation of legume defense responses following natural bouts of herbivory (Schmelz et al. 2006, 2012). First identified from beet armyworm *S. exigua* larvae, fatty acid-amino acid conjugates (FACs) remain the most extensively examined insect-produced elicitors (Alborn et al. 1997). The FACs and especially volicitin [*N*-(17-hydroxylinolenoyl)-L-glutamine] elicit maize volatile emissions that serve as host location cues for predators and parasitoids (Huffaker et al. 2013; Turling et al. 2000). A further example of FAC-mediated plant defense is the induced accumulation of tobacco (*Nicotiana attenuata*) trypsin inhibitors in both locally treated leaves and untreated systemic leaves (Roda et al. 2004). With respect to direct roles in Lepidoptera physiology, FACs play important roles in nitrogen assimilation by *S. litura* larvae (Yoshinaga et al. 2008). The growth benefits to herbivores of enhanced nitrogen assimilation, mediated by FAC cycling, are likely to outweigh the costs associated with plant defense activation mediated by FAC.

In the current study, we investigated *de novo* biosynthesis of inducible soybean isoflavonoids by using exogenous L- α -[$^{13}\text{C}_9$, ^{15}N]phenylalanine in combination with *S. litura* gut content elicitors or specific FACs. Although it is known that most soybean isoflavonoids accumulate as glucosides and malonylglucosides, the relative contribution of conversion from the pre-existing pools compared to production via *de novo* biosynthesis has not been established. In a preliminary experiment, we detected the qualitative incorporation of L- α -[$^{13}\text{C}_9$, ^{15}N]phenylalanine into [$^{13}\text{C}_9$]flavonoid skeletons following soybean leaf treatments with a combination of *S. litura* gut content extracts and labeled phenylalanine (Murakami et al. 2014). The present study sought to gain a more systematic and quantitative assessment of isotopic incorporation as a marker for *de novo* biosynthesis. Using a combination of stable isotope flavonoid precursors and gut content

elicitors, we traced the induction of several soybean isoflavones and demonstrated a significant role for *de novo* biosynthesis. Given the complexity of flavonoid conjugate profiles, our analytical scheme used acid hydrolysis of the glycoside conjugates and focused on the analyses on the flavonoid aglycones. Using synthetic FACs as elicitors, we revealed activation of a distinct subset of the gut-content elicited responses, and we provide indirect evidence for additional, as yet unidentified, elicitors in *S. litura* gut contents.

Methods and Materials

Plants and Insects Soybean (Var. Enrei) and *S. litura* larvae were reared as previously described (Murakami et al. 2014).

Chemicals L- α -[$^{13}\text{C}_9$, ^{15}N]Phenylalanine was purchased from Cambridge Isotope Laboratories, Inc. (Tewksbury, MA, USA). Daidzein and formononetin were purchased from LKT laboratories, Inc. (St. Paul, MN, USA). Genistein and apigenin were purchased from Tokyo Chemical Industry Co, Ltd. (Tokyo, Japan). Kaempferol and 4',7-dihydroxyflavone were purchased from Extrasynthese (Genay, France). 7-Hydroxyflavone, daidzin, linolenic acid, linoleic acid, L-glutamine, trimethylamine, and ethyl chloroformate were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Tetrahydrofuran was purchased from Kanto Chemical Co., Inc. (Tokyo, Japan). Gut extracts and elicitor solutions were prepared in phosphate buffer (50 mM Na_2HPO_4 - NaH_2PO_4 , pH 8).

Gut Content Treatment To standardize the quality, quantity, and the contents of the elicitor preparation, *S. litura* larvae ($N=10$) were frozen at -20°C , gut tissues were quickly removed with razor on ice, and further separated to collect only gut contents (foregut and midgut). The goal of this preparation was to maximize the complexity of oral secretion elicitors present. Gut contents were homogenized in 2 ml of phosphate buffer and then heated at 80°C for 15 min. After centrifugation at 5,000 g for 5 min, the supernatant was stored at -80°C for future use. For select experiments, L- α -[$^{13}\text{C}_9$, ^{15}N]phenylalanine was dissolved in gut content extracts at a concentration of 5 $\mu\text{g}/\mu\text{l}$ for the elicitation treatments. Using V3 stage soybean plants, approximately 50 pin holes were created in area of the center of the central trifoliolate of the first true leaves, and then 10 μl of gut content extracts (0.05 larval equivalents) were applied over the wounded area (16 plants). After 48 hr, all treated leaves were harvested and frozen at -80°C . Control leaves were wounded similarly, treated with 10 μl of phosphate buffer containing L- α -[$^{13}\text{C}_9$, ^{15}N]phenylalanine, and harvested as described above.

Leaf Extraction Soybean (about 100–200 mg) leaves were extracted with 80 % MeOH solution (MeOH/H₂O, v/v) (1 mg tissue in 5 µl MeOH solution; 0.2 mg fresh weight/µl) containing 7-hydroxyflavone (10 ng/µl) as an internal standard as previously described (Murakami et al. 2014). To reduce observed variation, each chemical replicate ($N=4$) consisted of individual pools of 4 separate identically treated leaves. Thus, 16 leaves were required for each set of 4 chemical replicates analyzed by a low resolution LC-MS (LR-LC-MS; LCMS-2020, Shimadzu, Kyoto, Japan).

Complete and Partial Hydrolysis of Flavonoid Glycosides

The samples (50 µl each) were evaporated with a centrifugal evaporator (EYELA CVE-2000; Tokyo Rikakikai Co., Ltd., Tokyo, Japan), re-suspended in 200 µl of 2 N HCl, and heated at 90 °C for 1 hr. After cooling the samples to room temperature, 400 µl of 1 M NaHCO₃ were added for neutralization. The sample solutions were applied to MonoSpin C18 extraction columns (GL sciences, Inc., Tokyo, Japan). After adsorption of the samples, the columns were rinsed with H₂O (3,000 g, 3 min, 25 °C). The hydrophobic compounds, retained on the columns, were eluted with acetonitrile (5,000 g, 2 min, 25 °C) and evaporated with the centrifugal evaporator. The residues were dissolved in 50 µl of 80 % MeOH and analyzed by LR-LC-MS and high resolution LC-MS (HR-LC-MS; LCMS-IT-TOF, Shimadzu, Kyoto, Japan).

Partial hydrolysis was used to assess malonylglucosides, which are specifically converted into the corresponding glucosides upon heating. For the hydrolysis of malonated conjugates to yield glucosides, filtered extracts (200 µl) were heated at 80 °C for 16 hr and left at room temperature for 1 hr (Farag et al. 2007; Lin et al. 2000). After centrifuging at 10,000 g for 10 min, the supernatants were analyzed by both LR- and HR-LC-MS.

FAC Synthesis *N*-Linolenoyl-L-glutamine and *N*-linoleoyl-L-glutamine were synthesized as described (Koch et al. 1999). Wakogel C-200 (Wako Pure Chemical Industries, Ltd., Osaka, Japan) was used for silica gel column chromatography. ¹H-NMR (400 MHz) spectra were recorded with TMS as internal standard in methanol-*d*₄ by using a Bruker AV-III NMR spectrometer. Chemical shifts are summarized in Supplemental Information 1.

FAC Treatments The target concentrations of synthetic FACs used to treat leaves were 55.7 and 47.3 ng/µl for *N*-linolenoyl-L-glutamine and *N*-linoleoyl-L-glutamine, respectively (Supplemental Information 2).

Each synthetic purified FAC solution (55 ng/µl) in phosphate buffer containing L-α-[¹³C₉, ¹⁵N]phenylalanine (5 µg/µl) was separately prepared and used for tests. Soybean leaves were treated with 10 µl of the mixture, and after 48 hr the

leaves were extracted and analyzed by LR-LC-MS as described above ($N=4$, 4 pooled leaves per chemical replicate).

β-Glucosidase Activity of Gut Content Extracts

Spodoptera litura gut content extracts were prepared as described above. The substrate daidzin (daidzein 7-*O*-β-D-glucoside) was dissolved in a mixture of 490 µl of phosphate buffer and 10 µl of the gut contents extract (0.05 larval equivalents) to give a final daidzin concentration of 2 µM (Day et al. 1998). After incubation for at 37 °C for up to 12 hr, the reaction was stopped by heating at 80 °C for 10 min and cooling at room temperature for 1 hr. The reaction mixture was purified by Sep-pak plus C18 (1 g) (Waters, Milford, MA, USA) that was eluted with MeOH. After evaporating under N₂, 500 µl of 80 % MeOH containing 7-hydroxyflavone (10 ng/µl) as an internal standard were added to the residue before filtering through DISMIC-13HP (0.45 µm) (Tokyo Roshi Kaisha, Ltd., Tokyo, Japan) and analysis by LR-LC-MS.

Chemical Analyses LR-LC-MS was conducted with a Prominence HPLC system coupled with the LCMS-2020 (Shimadzu, Kyoto, Japan). For analysis of leaf extracts and β-glucosidase reaction solutions, separations were performed with an ODS column (Mightysil RP-18GP 250 × 2.0 mm I.D.; Kanto Chemical Co., Inc., Tokyo, Japan) at 40 °C with 0.200 ml/min flow rate. The solvent program was 25 % (0–10 min), 25–60 % (10–55 min), 60–99 % (55–65 min) MeOH containing 0.08 % acetic acid in H₂O containing 0.05 % acetic acid. MS operating parameters are described in detail (Supplemental Information 3A).

For the analysis of gut content extracts, chromatography was conducted under the same conditions, but the solvent program was 30–99 % (0–7 min), 99 % (7–15 min) acetonitrile containing 0.08 % acetic acid in H₂O containing 0.05 % acetic acid. The MS was operated as described (Supplemental Information 3B).

For accurate mass measurements, HR-LC-MS was carried out using the LCMS-IT-TOF (Shimadzu, Kyoto, Japan) equipped with the Prominence HPLC system (Shimadzu, Kyoto, Japan). HPLC conditions were the same as above. The MS was operated as described in Supplemental Information 3C. The multiple-stage mass spectrometry was operated as described in Supplemental Information 3D. Accurate mass measurements enabled the calculation of elemental compositions using the Formula Predictor (Shimadzu, Kyoto, Japan) with following condition assumptions: elements, C_{0–50}H_{0–100}O_{0–50}; polarity, positive; error margin, 10 ppm; DBE range, 9–20; electron ions, odd configuration only; HC ratio, 0.0–3.0; using nitrogen rule; using fragment information; charge 1. The combination of the Accurate mass calculator (Shimadzu, Kyoto, Japan) and the Formula

Predictor software enabled objective identification of analytes based on accurate mass, isotope pattern and MS/MS spectra.

Quantification of [$^{13}\text{C}_9$]Flavonoids in Soybean Leaves The amount of each [$^{13}\text{C}_9$]aglycone in the completely hydrolyzed extracts ($A_{\text{complete-hyd}}$) (pmol/mg leaf FW) was estimated with calibration curves (Supplemental Information 4), which were made by using non-labeled aglycones for the standard. The peak areas of selected ion chromatograms of each analyte were used for the quantitation.

Total aglycones in completely hydrolyzed extracts are the sum of original aglycones, glucosides, and malonylglucosides. The amounts of [$^{13}\text{C}_9$]glycosides in soybean leaves were estimated as follows:

Amount of [$^{13}\text{C}_9$]glycoside (pmol/mg leaf FW)

$$= A_{\text{complete-hyd}} - A_{\text{intact}}$$

The amount of each [$^{13}\text{C}_9$]aglycone in intact extracts (A_{intact}) (pmol/mg leaf FW) was estimated with calibration curves as described above.

The amount of glucoside in heated extracts is the sum of original glucosides and malonylglucosides. The amount of [$^{13}\text{C}_9$]glucoside and malonylglucoside in soybean leaves was estimated from the equation:

Amount of [$^{13}\text{C}_9$]glucoside (pmol/mg leaf FW)

$$= (A_{\text{complete-hyd}} - A_{\text{intact}}) \times \frac{G_{\text{intact}}}{G_{\text{heat}}}$$

Amount of [$^{13}\text{C}_9$]malonylglucoside (pmol/mg leaf FW)

$$= (A_{\text{complete-hyd}} - A_{\text{intact}}) \times \frac{G_{\text{heat}} - G_{\text{intact}}}{G_{\text{heat}}}$$

where G_{heat} is the amount of [$^{13}\text{C}_9$]glucoside in heated extracts (LCMS peak area in a selected ion chromatogram); G_{intact} is the amount of [$^{13}\text{C}_9$]glucoside in intact extracts (LCMS peak area in a selected ion chromatogram).

Fig. 1 Qualitative analysis of flavonoids by LCMS in negative ion mode. LCMS selected ion chromatogram profiles of flavonoid aglycones (**a**), authentic samples (**b**), and [$^{13}\text{C}_9$]flavonoid aglycones (**c**) in completely hydrolyzed extracts of soybean leaves treated with gut content extracts. Reference numbers (1–6, 1'–6') of compounds identified in the current study are as follows: (1) daidzein, (2) 4',7-dihydroxyflavone, (3)

genistein, (4) kaempferol, (5) apigenin, (6) formononetin, (1') [$^{13}\text{C}_9$]daidzein, (2') [$^{13}\text{C}_9$]4',7-dihydroxyflavone, (3') [$^{13}\text{C}_9$]genistein, (4') [$^{13}\text{C}_9$]kaempferol, (5') [$^{13}\text{C}_9$]apigenin, and (6') [$^{13}\text{C}_9$]formononetin. The structures are summarized in (**d**) and $^{13}\text{C}_9$ labeled positions are shown with bold lines

Table 1 Non-labeled flavonoids identified by HR-LC-MS in complete-hydrolyzed extracts of soybean leaves

Peak No.	Identification	t_R /min	Measured m/z [M+H] ⁺	Calculated m/z [M+H] ⁺	Elemental composition	Observed product ions m/z (%) base peak ^a
1	Daidzein	39.2	255.065	255.065	C ₁₅ H ₁₀ O ₄	227.068 (14); 199.075 [M+H-C ₂ O ₂] ⁺ (55); 137.023 [RDA] (100)
2	4',7-Dihydroxyflavone	41.8	255.066	255.065	C ₁₅ H ₁₀ O ₄	N.D. ^b
3	Genistein	45.7	271.060	271.060	C ₁₅ H ₁₀ O ₅	243.065 (15); 215.070 [M+H-C ₂ O ₂] ⁺ (31); 153.018 [RDA] (100)
4	Kaempferol	49.4	287.055	287.055	C ₁₅ H ₁₀ O ₆	241.048 [M+H-CH ₂ O ₂] ⁺ (35); 213.054 [M+H-C ₂ H ₂ O ₃] ⁺ (45); 165.018 (100); 153.018 [RDA] (59)
5	Apigenin	50.5	271.060	271.060	C ₁₅ H ₁₀ O ₅	153.020 [RDA] (100)
6	Formononetin	55.2	269.079	269.081	C ₁₆ H ₁₂ O ₄	254.056 (100); 237.047 [M+H-CH ₄ O] ⁺ (67); 213.086 [M+H-C ₂ O ₂] ⁺ (54); 137.021 [RDA] (8);

^a NLS formulae calculated by the Formula Predictor were also shown^b Not detected

Statistical Analyses Data were analyzed by the Tukey-Kramer's H.S.D. test, using JMP 3.2.6. (SAS Institute, Cary, NC, USA).

Results

Identification of Non-Labeled Flavonoids in Soybean Leaves

Induced leaf extracts were completely hydrolyzed with acid and analyzed by LR-LC-MS (Fig. 1a). Peak numbers 1–6 were detected as proton desorbed ions {[M-H]⁻ (t_R min): 1, m/z 253 (39.2 min); 2, m/z 253 (41.8 min); 3, m/z 269 (45.7 min); 4, m/z 285 (49.4 min); 5, m/z 269 (50.5 min); 6, m/z 267 (55.2 min)}. The respective proton adduct ions [M+H]⁺ also were detected in positive ion mode, but the negative ion mode sensitivity was higher for LR-LC-MS.

Peak 1 was confirmed as daidzein (Fig. 1a, b). The MS/MS spectrum was obtained by multiple-stage mass spectrometry using HR-LC-MS, and revealed prominent proton adduct [M+H]⁺ ions with greater sensitivity (with HR-LC-MS) than proton desorbed [M-H]⁻ ions. Thus, we conducted MS/MS analyses in the positive ion mode. Daidzein yielded a m/z 255.065 [M+H]⁺ parent ion and characteristic m/z 137.023 product ion (Supplemental Fig. 1A), consistent with a retro-

Diels-Alder reaction (RDA) commonly detected following collision induced dissociation (CID) of flavonoids (Farag et al. 2007; Wu et al. 2003). Additional predominant product ions likewise corresponded to the MS/MS spectrum of authentic daidzein (Supplemental Fig. 1B). The elemental composition was calculated as C₁₅H₁₀O₄ by the Formula Predictor further supporting the identification of daidzein. Similarly, peaks 2–6 were identified as 4',7-dihydroxyflavone, genistein, kaempferol, apigenin, and formononetin, respectively (Fig. 1a, b and d). These compounds were analyzed in positive ion mode using HR-LC-MS/MS. LC retention times, MS spectrum data and calculated results are summarized in Table 1.

Each of the initially identified soybean flavonoid aglycones was expected to exist as several glycoside conjugates differentiated by glycosylation pattern. These “satellites sets” (de Rijke et al. 2004) can be recognized by their identical aglycone masses (in ESI-MS positive ion mode) and identical fragment ions associated with the corresponding aglycone mass. For example, daidzein-based satellite sets exhibit fragment ions at m/z 255, which are detected at both 17.0 and 34.3 min in the selected ion chromatograms of intact extracts (Supplemental Fig. 2). These ions originate from the fragmentation of [M+H]⁺ parent ions at m/z 417 (peak 7; t_R , 17 min)

Fig. 2 Qualitative analysis of flavonoid glycosides by LCMS in positive ion mode showing the conversion of malonylglucoside into corresponding glucoside by heat treatment. LCMS $[M+H]^+$ selected ion chromatogram profiles of glucosides and malonylglucosides in intact extracts of soybean leaves (**a**) and heated extracts (**b**). The chromatograms of m/z 239 was reduced to 0.5 times. Reference

numbers (7–14) of compounds identified in the current study are as follows: (7) $[^{13}C_9]$ daidzin, (8) $[^{13}C_9]$ genistin, (9) $[^{13}C_9]$ apigenitrin, (10) $[^{13}C_9]$ malonyldaidzin, (11) $[^{13}C_9]$ ononin, (12) $[^{13}C_9]$ malonylgenistin, (13) $[^{13}C_9]$ malonylapigenitrin, and (14) $[^{13}C_9]$ malonylononin. Their structures are summarized in (**c**)

and 503 (peak 10; t_R , 34.3 min), respectively. We predicted that peak 7 was daidzein glucoside (daidzin) and peak 10 was daidzein malonylglucoside (malonyldaidzin). Malonylglucosides are thermally unstable and readily converted to their related glucosides by heating and cleavage of the malonyl ester bonds (Farag et al. 2007; Lin et al. 2000). To confirm these predicted metabolite relationships, heat treatment promoted a comparative loss in peak 10 and commensurate increase in peak 7 supporting the identification of peak 10 as malonyldaidzin.

Additional structural information was obtained by multiple-stage mass spectrometry using HR-LC-MS. In positive ion mode, the $[M+H]^+$ ion at m/z 417.118 (peak 7) showed a product ion at m/z 255.064 in the 1st generation product ion spectrum. A neutral loss (NLS) of 162 Da, representing glucose, is indicative of glucoside conjugates (Farag et al. 2007; Lin et al. 2000; Wu et al. 2003). The CID

of $[M+H-Glc]^+$ ion at m/z 255.064 resulted in RDA to yield the product ion m/z 137.023 in the 2nd generation product ion spectrum (Supplemental Fig. 3A). Predominant product ions in the 2nd generation product ion spectrum corresponded to those in MS/MS spectrum of daidzein. Consistent with daidzin, the elemental composition was calculated as $C_{21}H_{20}O_9$ by Formula Predictor. Similarly, the peak 10 was identified as malonyldaidzin with the $[M+H-248]^+$ ion at m/z 255.064 observed in the 1st generation product ion spectrum (Supplemental Fig. 3B). NLS of 248 Da is characteristic of a loss of malonylglucose (162 + 86) (Farag et al. 2007; Klejduš et al. 2001; Lin et al. 2000; Wu et al. 2003).

Similarly, genistin (8), malonylgenistin (12), apigenitrin (9), malonylapigenitrin (13), ononin (11), and malonylononin (14) were identified (Fig. 2a, b and c). LC retention times, MS spectra and calculated results are summarized in Table 2.

Table 2 Non-labeled flavonoids glycoisdes identified by HR-LC-MS in intact and heated extracts of soybean leaves

Peak No.	Identification	t_R / min	Measured m/z	Calculated m/z	Elemental composition	Observed product ions m/z (%) base peak ^a	
			[M+H] ⁺	[M+H] ⁺		in 1st generation product ion spectrum	in 2nd generation product ion spectrum
7	Daidzin	17.0	417.118	417.118	C ₂₁ H ₂₀ O ₉	255.064 [M+H-Glc] ⁺ (100)	227.067 (25); 199.075 [M+H-C ₂ O ₂] ⁺ (100); 181.062 (58); 137.023 [RDA] (90)
8	Genistin	24.7	433.113	433.113	C ₂₁ H ₂₀ O ₁₀	271.059 [M+H-Glc] ⁺ (100)	243.062 (26); 215.069 [M+H-C ₂ O ₂] ⁺ (53); 153.018 [RDA] (100); 149.023 (26)
9	Apigetrin	34.0	433.113	433.113	C ₂₁ H ₂₀ O ₁₀	271.058 [M+H-Glc] ⁺ (100)	153.019 [RDA] (100)
10	Malonyldaidzin	34.3	503.118	503.118	C ₂₄ H ₂₂ O ₁₂	255.064 [M+H-Malonylgc] ⁺ (100)	227.068 (29); 199.076 [M+H-C ₂ O ₂] ⁺ (97); 181.065 (29); 137.024 [RDA] (100)
11	Ononin	37.0	431.134	431.134	C ₂₂ H ₂₂ O ₉	269.080 [M+H-Glc] ⁺ (100)	254.056 (36); 253.050 (65); 237.055 [M+H-CH ₄ O] ⁺ (100); 213.092 [M+H-C ₂ O ₂] ⁺ (45); 137.024 [RDA] (5);
12	Malonylgenistin	40.4	519.114	519.113	C ₂₄ H ₂₂ O ₁₃	433.109 [M+H-Malonyl] ⁺ (6); 271.059 [M+H-Malonylgc] ⁺ (100)	243.070 (23); 215.069 [M+H-C ₂ O ₂] ⁺ (100); 153.019 [RDA] (94); 149.023 (94)
13	Malonylapigetrin	46.7	519.114	519.113	C ₂₄ H ₂₂ O ₁₃	433.115 [M+H-Malonyl] ⁺ (23); 271.059 [M+H-Malonylgc] ⁺ (100)	153.017 [RDA] (100)
14	Malonylononin	50.5	517.135	517.134	C ₂₅ H ₂₄ O ₁₂	269.080 [M+H-Malonylgc] ⁺ (100)	254.058 (68); 253.049 (61); 237.052 [M+H-CH ₄ O] ⁺ (100); 213.090 [M+H-C ₂ O ₂] ⁺ (75); 137.013 [RDA] (4);

^a NLS formulae calculated by Formula Predictor were also shown

Investigation of [¹³C₉]Flavonoids in Induced Soybean Leaves [¹³C₉]Daidzein (1'), [¹³C₉]genistein (3'), and [¹³C₉]formononetin (6') were clearly observed in the completely hydrolyzed extracts (Fig. 1c). [¹³C₉]4',7-Dihydroxyflavone (2'), [¹³C₉]kaempferol (4'), and [¹³C₉]apigenin (5') were comparatively minor components compared to the [¹³C₉]isoflavones detected. Confirmation of isotopic flavonoid identities was obtained by HR-LC-MS, LC retention times, and MS spectra as summarized in Supplemental Table 1. [¹³C₉]Satellite sets also were observed in the same samples by comparing intact extracts and heated extracts (Supplemental Fig. 4). LC retention times, MS spectral data, and calculated results for [¹³C₉]flavonoid glycosides are summarized in Supplemental Table 2.

Quantification of the Amounts of [¹³C₉]Flavonoids in Soybean Leaves Following the treatment of soybean leaves with gut content extracts mixed with L-α-[¹³C₉, ¹⁵N]phenylalanine, the amounts of [¹³C₉]isoflavones [daidzein (1'), genistein (3'), and formononetin (6')] were significantly increased compared with control leaves. In contrast, levels of [¹³C₉]flavones and flavonol [4',7-dihydroxyflavone (2'), kaempferol (4'), and apigenin (5')] were not significantly increased (Fig. 3).

In non-hydrolyzed methanolic leaf extracts, the amounts of [¹³C₉]daidzein (1'), genistein (3'), and formononetin (5') aglycones were low (Fig. 4A) compared to corresponding levels in completely hydrolyzed extracts (Fig. 3). Further, the amounts of glycosides were much greater than those of aglycones

Fig. 3 The total amount of soybean leaf [$^{13}\text{C}_9$]flavonoid aglycone following complete hydrolysis. The data show the average ($N=4$; $\pm$ S.E.M.) levels of [$^{13}\text{C}_9$]flavonoid aglycones in soybean leaves challenged with *Spodoptera litura* gut content extracts. The data were analyzed using Welch's t -test and asterisks denote significant differences (*, $P<0.005$; **, $P<0.001$) relative to controls. The numbers represent (1') [$^{13}\text{C}_9$]daidzein, (2') [$^{13}\text{C}_9$]daidzein-7-O-glucoside, (3') [$^{13}\text{C}_9$]genistein, (4') [$^{13}\text{C}_9$]genistein-7-O-glucoside, (5') [$^{13}\text{C}_9$]apigenin, and (6') [$^{13}\text{C}_9$]formononetin

(Fig. 4A, B). This indicated that isoflavones are predominantly stored as either glucosides and/or malonylglucosides. In addition, the amounts of [$^{13}\text{C}_9$]malonylglucosides (10', 12',

Fig. 4 Quantitative estimates of [$^{13}\text{C}_9$]isoflavone aglycones (A) and glycosides (B) in soybean leaves treated with gut content extracts of *Spodoptera litura*. In B, the upper segment of the bar represents glucosides and the lower portion of the bar malonylglucosides. The numbers beside compound names correspond with the numbers in Tables 1 and 2. Each data value is shown as the mean $\pm$ S.E.M. of 4 replicates. The data were analyzed using Welch's t -test and asterisks denote significant differences (*, $P<0.05$; **, $P<0.005$; ***, $P<0.001$) relative to controls

Fig. 5 The amount of [$^{13}\text{C}_9$]isoflavone aglycones (A) and glycosides (B) in soybean leaves treated with *N*-linolenoyl-L-glutamine and *N*-linoleoyl-L-glutamine. In B, the upper segment of the bar represents glucosides and the lower portion of the bar malonylglucosides. The numbers beside compound names correspond with the numbers in Tables 1 and 2. Each data value is shown as the mean $\pm$ S.E.M. of 4 replicates. Different letters represent significant differences ($P<0.05$, Tukey-Kramer's H.S.D.)

and 14') were greater than those of [$^{13}\text{C}_9$]glucosides (7', 8', and 11') (see stacks of Fig. 4B).

Investigation of the Eliciting Activity of FACs LC-MS analyses of FACs in gut content extracts of *S. litura* revealed peaks 15–17 detected as proton desorbed ions $\{[M-H]^{-}\}$ (t_R min): 15, m/z 421 (5.0 min); 16, m/z 405 (7.3 min); 17, m/z 407 (8.0 min), (Supplemental Fig. 5A)}. Based on previous work, these analytes correspond to volicitin [*N*-(17*S*-hydroxylinolenoyl)-L-glutamine], *N*-linolenoyl-L-glutamine, and *N*-linoleoyl-L-glutamine, respectively (Supplemental Fig. 5B) (Mori et al. 2003; Yoshinaga et al. 2010). Based on the composition of FACs in gut contents, synthetic purified *N*-linolenoyl-L-glutamine and *N*-linoleoyl-L-glutamine were used to evaluate eliciting activity in soybeans. Leaves were treated with FACs separately, extracted, and analyzed by LR-LC-MS. In the intact extracts, the accumulation of [$^{13}\text{C}_9$]isoflavone aglycones following FACs treatment was not significantly different from controls (Fig. 5A). In contrast, analysis of completely and partially hydrolyzed extracts of aglycones representing the combination of glucosides and malonylglucosides demonstrated that each FAC selectively induced the biosynthesis of [$^{13}\text{C}_9$]isoflavone glycosides (Fig. 5B).

Discussion

In the current study, we demonstrate that elicitors present in *S. litura* gut extracts increase *de novo* biosynthesis of soybean isoflavones (daidzein, genistein, formononetin, and their

Fig. 6 The biosynthetic pathway of flavonoids. The biosynthesis of isoflavones is activated by gut content extracts of *Spodoptera litura* and FACS, and hydrolysis of isoflavone glycosides is activated by unknown elicitors. PAL, phenylalanine ammonia lyase; C4H, cinnamate 4-hydroxylase; 4CL, 4-coumarate:CoA ligase; CHS, chalcone synthase;

IFS, 2-hydroxyisoflavanone synthase; HID, 2-hydroxyisoflavanone dehydrogenase; GT, glucosyltransferase; malonyltransferase; HI4'OMT, 2-hydroxyisoflavanone 4'-O-methyltransferase; FNSII, flavone synthase II; F3H, flavone 3-hydroxylase

conjugates). Selective pathway activation was demonstrated by a comparative lack of [$^{13}\text{C}_9$]isotope incorporation into flavones and flavonol. This metabolic regulation is likely controlled by the activation of key enzymes in isoflavone biosynthesis, namely 2-hydroxyisoflavanone synthase (IFS) (Hashim et al. 1990; Kochs and Grisebach 1986) and 2-hydroxyisoflavanone dehydrogenase (HID) (Hakamatsuka et al. 1998) (Fig. 6). IFS (CYP93C) (Jung et al. 2000; Steele et al. 1999) catalyzes the migration of the flavonoid C ring from C2 to C3 and the hydroxylation at C2 to give 2-hydroxyisoflavanone, which is converted to the isoflavone by dehydration by HID. Both enzymes are found only in legumes (Akashi et al. 2005; Sawada et al. 2002; Sawada and Ayabe 2005). Therefore, the characteristic activation of isoflavone biosynthesis induced by *S. litura* elicitors is an example of legume-specific physiology.

In the present study, elicitation with *S. litura* gut content extracts increased the total amount of [$^{13}\text{C}_9$]5-deoxyisoflavones {[$^{13}\text{C}_9$]daidzein (1') and [$^{13}\text{C}_9$]formononetin (6')} more than the amount of [$^{13}\text{C}_9$]5-hydroxyisoflavone {[$^{13}\text{C}_9$]genistein (3')} (see Fig. 3); 5-deoxyisoflavones were more induced by *S. litura* derived elicitors. Many 5-deoxyisoflavones and their derivatives have phytoalexin activity (Tahara and Ibrahim 1995). Examples of soybean 5-deoxyisoflavones and their derivatives related to defenses include coumestrol (5-deoxyisoflavone derivative), phaseol (5-deoxyisoflavone derivative), and afrormosin (5-deoxyisoflavone) that may promote resistance against soybean looper *Chrysodeixis includens* (Lepidoptera: Noctuidae)

herbivory (Caballero et al. 1986). As a well-established antibiotic, glyceollin (5-deoxyisoflavone derivative) can significantly reduce feeding by southern corn rootworm *Diabrotica undecimpunctata howardi* (Coleoptera: Chrysomelidae) and Mexican bean beetle *Epilachna varivestis* (Coleoptera: Coccinellidae) larvae (Fischer et al. 1990). More recently, daidzein has been demonstrated to inhibit the growth of *S. litura* (Zhou et al. 2011). The bioactivity of formononetin against insects remains unexplored, but this compound may display a negative impact similar to other 5-deoxyisoflavones.

Among the elicitors produced by insects that are known to activate plant defense production, FACS are active in the largest number of plants and are the most studied class (Alborn et al. 1997). In the specific context of soybean, FACS elicit the production of defense related phytohormones such as ethylene and jasmonic acid which subsequently promote induced volatile emission (VOCs) (Schmelz et al. 2009). In the present study, we demonstrate a new activity of FACS, namely the induction of isoflavone glucosides and malonylglucosides in soybean leaves. Interestingly, while the elicitation of induced volatile emission in maize requires the linolenoyl moiety (18:3) (Turling et al. 2000), our current work demonstrates that isoflavone glycoside accumulation in soybean is promoted by either *N*-linolenoyl-L-glutamine (18:3) or *N*-linoleoyl-L-glutamine (18:2). However, FACS did not directly promote the accumulation of any flavonoid aglycones, although *S. litura* gut content extracts did stimulate production of flavonoid aglycones. This result suggests that additional elicitors exist in *S. litura* gut contents. Examples of insect elicitor

classes identified in other systems include inceptin-related peptide fragments from fall armyworm *Spodoptera frugiperda* (Lepidoptera: Noctuidae) (Schmelz et al. 2006), disulfoxy fatty acid (caeliferins) from American bird grasshopper *Schistocerca americana* (Orthoptera: Acrididae) (Alborn et al. 2007), β -glucosidase from *Pieris brassicae* (Lepidoptera: Pieridae) (Mattiacci et al. 1995), and β -galactofuranose polysaccharides from African cotton leafworm *Spodoptera littoralis* (Lepidoptera: Noctuidae) (Bricchi et al. 2013). Glucosides and malonylglucosides are considered to be latent forms of isoflavone aglycones that are hydrolyzed on demand (Suzuki et al. 2006). Given these dynamics, we anticipate that gut contents contain unknown elicitors that activate a β -glucosidase. Gut content extracts had no direct β -glucosidase activity (Supplemental Fig. 6), thus supporting the importance of *S. litura*-associated elicitors that activate soybean β -glucosidase(s) to cleave isoflavones glycosides to aglycones.

Acknowledgments This research was funded by Grants-in-Aids for Scientific Research (No. 24120006) from the Ministry of Education, Culture, Sports, Science, and Technology of Japan.

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