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Corresponding Author: M. Y. Hirai; RIKEN Center for Sustainable Resource Science, Yokohama, 230-0045, Japan, Telephone, +81-45-503-7040: Fax, +81-45-503-9489; E-mail, masami.hirai@riken.jp.

Subject Areas: Proteins, enzymes, and metabolism

Number of black and white figures: 1

Colour figures: 6

Supplementary Data: 2 figures, 9 tables, and supplementary methods

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Identification of a Unique Type of Isoflavone O-methyltransferase, GmIOMT1, based on Multi-omics Analysis of Soybean under Biotic Stress

**Running Title:** Isoflavone methyltransferase in fermented soybean

**Authors:** Kai Uchida\(^1\), Yuji Sawada\(^1\), Koji Ochiai\(^2\), Muneo Sato\(^1\), Jun Inaba\(^1\) and Masami Yokota Hirai\(^1\)*

\(^1\) RIKEN Center for Sustainable Resource Science, Yokohama, 230-0045, Japan

\(^2\) DAIZ Inc., Kumamoto, 860-0812, Japan

**Corresponding Author:** M. Y. Hirai: E-mail, masami.hirai@riken.jp; Fax, +81-45-503-9489.

**Footnotes:** RNA-sequencing reads are available in the DDBJ Sequence Read Archive database with accession numbers DRA010476.
Abstract

Isoflavonoids are commonly found in leguminous plants. Glycitein is one of the isoflavones produced by soybean. The genes encoding the enzymes in isoflavone biosynthetic pathway have mostly been identified and characterized. However, the gene(s) for isoflavone O-methyltransferase (IOMT), which catalyses the last step of glycinein biosynthesis, has not yet been identified. In this study, we conducted multi-omics analyses of fungal-inoculated soybean and indicated that glycinein biosynthesis was induced in response to biotic stress. Moreover, we identified a unique type of IOMT which participates in glycinein biosynthesis.

Soybean seedlings were inoculated with *Aspergillus oryzae* or *Rhizopus oligosporus* and sampled daily for 8 days. Multi-omics analyses were conducted using liquid chromatography-tandem mass spectrometry and RNA sequencing. Metabolome analysis revealed that glycinein derivatives increased following fungal inoculation. Transcriptome co-expression analysis identified two candidate IOMTs that were co-expressed with the gene encoding flavonoid 6-hydroxylase (F6H), the key enzyme in glycinein biosynthesis. The enzymatic assay of the two IOMTs using respective recombinant proteins showed that one IOMT, named as GmIOMT1, produced glycinein. Unlike other IOMTs, GmIOMT1 belongs to the cation-dependent OMT family and exhibited the highest activity with Zn$^{2+}$ among cations tested. Moreover, we demonstrated that GmIOMT1 overexpression increased the levels of glycinein derivatives in soybean hairy roots when F6H was co-expressed. These results strongly suggest that GmIOMT1 participates in inducing glycinein biosynthesis in response to biotic stress.
Keywords: Isoflavone O-methyltransferase, Omics analysis, Soybean
Introduction

Flavonoids are an important class of plant specialised metabolites derived from phenylalanine. They have various biological functions, including antioxidant action and interaction with other organisms. Isoflavonoids are a group of flavonoids that are mostly produced by leguminous plants. In soybean, three types of isoflavone aglycones: genistein (5,7,4′-trihydroxyisoflavone), daidzein (7,4′-dihydroxyisoflavone), and glycitein (7,4′-dihydroxy-6-methoxyisoflavone) are produced via two different pathways (Fig. 1). Genistein is synthesised via the pathway shared with other flavonoids, in which chalcone synthase (CHS) and type I and type II chalcone isomerase (CHI) are involved. Daidzein and glycitein are produced via the specific pathway involving polyketide reductase, also called chalcone reductase, and CHS and type II CHI (Akashi et al. 1997; Mameda et al. 2018; Welle et al. 1991). Soybean accumulates genistein, daidzein, glycitein, and their glucosides and malonylglucosides (Fig. 1). Glycitein and its derivatives are accumulated exclusively in the hypocotyl, and the others are found in the whole body but most accumulated in the seed (Kudou et al. 1991). Till date, many functions of daidzein and genistein have been clarified for soybean. For example, daidzein and genistein are required for symbiosis with rhizobia (Subramanian et al. 2006), and daidzein is a precursor of glyceollin, a major phytoalexin in soybean (Fig. 1).
However, the physiological function of glycitein is almost unknown. The amount of glycitein and its derivatives is very low, and varies among cultivars when compared to other isoflavones (Artigot et al. 2013). Glycitein derivatives are accumulated only in the hypocotyl under normal condition (Kudou et al. 1991), and their accumulations are notably induced by biotic and abiotic elicitors (Al-Tawaha et al. 2006; Landini et al. 2003). These observations suggest that glycitein plays a role in defence reaction against pathogen infection.

Although genistein and daidzein are biosynthesised from naringenin and liquiritigenin, respectively, the reactions are both catalysed by 2-hydroxyisoflavanone synthase (IFS) and 2-hydroxyisoflavanone dehydratase (HID) (Fig. 1). IFS belong to the CYP93C subfamily of the cytochrome P450 family and is essential in isoflavonoid biosynthesis. Two IFS cDNAs: CYP93C1 (IFS1) and CYP93C5 (IFS2), have been identified in soybean (Jung et al. 2000; Steele et al. 1999). A HID cDNA has also been identified in soybean (Akashi et al. 2005). Isoflavone biosynthesis from liquiritigenin diverges to enter the glycitein biosynthetic pathway in a reaction catalysed by flavonoid 6-hydroxylase (F6H; CYP71D9). A F6H cDNA was first isolated as the elicitor-inducible gene (Schopfer and Ebel 1988). Later, an in vitro assay using a heterologously expressed protein in yeast revealed that F6H recognises liquiritigenin as
a substrate, but not daidzein (Latunde-Dada et al. 2001). Following the production of 6-hydroxyliquiritigenin by the F6H, IFS and HID convert it to 6-hydroxydaidzein. Glycitein has been hypothesised to be produced by isoflavone O-methyltransferase (IOMT) but has not been experimentally confirmed so far.

The plant S-adenosyl-L-methionine (SAM)-dependent OMTs are grouped into two major types: cation-independent type (type 1) and cation-dependent type (type 2) (Noel et al. 2003). Type 1 OMTs form a large family that comprised the OMTs involved in specialised (secondary) metabolism such as phenylpropanoid and flavonoid biosynthesis. This family contains all the OMTs identified in the isoflavonoid biosynthesis, namely: (+)-6a-hydroxymaackiain 3-OMT (Wu et al. 1997), daidzein 7-OMT (He et al. 1998), 2-hydroxyisoflavanone 4′-OMT (Akashi et al. 2003), and isoflavone 3′-OMT (Li et al. 2016). Most of the type 2 OMTs identified in plants so far are the caffeoyl-CoA OMTs (CCoAOMT), which are the key enzymes of lignin biosynthesis. Among the OMTs involved in flavonoid biosynthesis, only two kinds of OMTs belong to the type 2 family; they are the phenylpropanoid and flavonoid OMTs (PFOMT) from ice plant (Mesembryanthemum crystallinum) (Ibdah et al. 2003), rice (Oryza sativa) (Lee et al. 2008), sweet basil (Ocimum basilicum) and anthocyanin OMTs (AOMTs) from grape (Vitis vinifera) (Hugueney et al. 2009), soybean (Kovinich (2001)).
et al. 2011), tomato (Gomez Roldan et al. 2014), *Paeonia* (Du et al. 2015), and *Nemophila menziesii* (Okitsu et al. 2018).

In this study, we aimed to understand isoflavonoid biosynthesis under biotic stress and identify undiscovered enzymes/genes in the pathway. We analysed transcriptomic and metabolomic changes of soybean seedlings inoculated with *Aspergillus oryzae* or *Rhizopus oligosporus* based on the finding that isoflavonoids were increased by the inoculation of fungi in soybean (Aisyah et al. 2013; Simons et al. 2011a; Simons et al. 2011b). We clarified that the biosynthesis of glycitein and its derivatives was induced by inoculation of these fungi. In addition, we identified an IOMT catalysing the last step of glycitein biosynthesis, which has a unique characteristic in terms of cation dependency. The findings in this study will contribute to understanding of physiological importance of glycitein.

**Results**

*Changes in isoflavone contents and gene expression in fungal inoculated soybean seedlings*

We analysed time-dependent changes in isoflavonoid contents in soybean seedling,
inoculated with *A. oryzae* or *R. oligosporus*, for 8 days. Targeted metabolome analysis using liquid chromatography-tandem mass spectrometry (LC-QqQ-MS) was used to analyse genistein, daidzein, glycitein, and their derivatives. Under normal conditions without inoculation, developmental increase in daidzein was observed (Fig. 2A). When the seedlings were inoculated with fungi, particularly *R. oligosporus*, metabolic flow was redirected to the biosynthesis of glyceollin I, glycitein, and glycitin derivatives (Fig. 2A). Of these compounds, glyceollin I was detected only in fungal-inoculated soybean, and its amount increased in a time-dependent manner (Fig. 2B). In addition, glycitein accumulation was induced by fungal inoculation. Although two glycitin derivative levels increased following inoculation, glycitin level did not. No obvious trend was observed in other metabolic pathways (Supplementary Tables S1, S2).

Transcriptome analysis was also performed via RNA sequencing (Supplementary Table S3). We focused on the expression patterns of the genes annotated to be involved in flavonoid biosynthesis (Fig. 3 and Supplementary Table S3). We found that several genes were induced by fungal inoculation. Among the phenylpropanoid and flavonoid biosynthetic genes, *phenylalanine ammonia-lyase* 2.3, *CHS7*, and *CHS8*, which are involved in isoflavone production (Dhaubhadel et al. 2007), were induced. *IFS1* and *IFS2*, which are essential for isoflavone biosynthesis, were also induced. Their
expression peaked at 3 days after soaking (DAS) in A. oryzae-inoculated seedlings (Fig 3). F6H was also induced by fungal inoculation, but its expression peaked at 8 DAS in R. oligosporus-inoculated seedlings (Fig 3). The expression of F6H was suppressed to a certain low level under the non-inoculated condition (Supplementary Fig. S1). As F6H is the key enzyme at the branching point of the glycitein biosynthetic pathway (Fig 1), the induction of F6H might be responsible for redirecting isoflavonoid biosynthesis in response to fungal inoculation.

Identification of candidate OMTs responsible for glycitein biosynthesis by transcriptome co-expression analysis

Among the enzymes responsible for soybean isoflavonoid biosynthesis, only IOMT, which is the last step of glycitein biosynthesis, was unidentified. We planned to isolate the candidates of IOMT genes based on ‘guilt-by-association’ principle (Saito et al. 2008; Gillis and Pavlidis 2012) using transcriptome data. This principle is based on the assumption that the genes involved in the same metabolic pathway are co-ordinately expressed when the product metabolite(s) is accumulated. To this end, we carried out Weighted Gene Co-expression Network Analysis (WGCNA) (Langfelder and Horvath 2008). After removing the transcripts with sum of TPM less than 1 in all samples, the
remaining 59,343 transcripts were subjected to WGCNA for clustering based on expression pattern. The transcripts were classified into 382 classes (Supplementary Table S4). The $F6H$ gene belonged to the class No. 229. This class contained three transcripts annotated as methyltransferase (Glyma.05G147000.1, Glyma.05G147000.2, and Glyma.07G048900.1), and their coding sequences (CDSs) were predicted to encode 236, 185, and 372 amino acids, respectively. Since Glyma.05G147000.2 encoded the shorter amino acid sequence, lacking two exons found in Glyma.05G147000.1, we selected Glyma.05G147000.1 and Glyma.07G048900.1 as the $IOMT$ candidates and tentatively named them $OMT1$ and $OMT2$, respectively. $OMT1$ and $OMT2$ showed very similar expression pattern to that of $F6H$ (Supplementary Fig. S1). We conducted phylogenetic analysis with the known OMTs, which were involved in the flavonoid and isoflavonoid biosynthesis. The known OMTs were classified into type 1 (cation-independent) OMT clade and type 2 (cation-dependent) OMT clade. The previously-identified isoflavonoid OMTs formed a subclade within the type 1 OMT clade (Fig. 4A). Unexpectedly, $OMT1$ belonged to the type 2 OMT clade, while $OMT2$ was a member of the type 1 OMT clade but did not belong to the isoflavonoid OMT subclade. In addition, $OMT1$ had a catalytic triad (– Lys-Asn-Asp –), which is essential for the catalysis of the methyl transfer in cation-dependent OMT (Fig 4B) (Brandt et al.)
Enzyme assay of candidate OMTs

The CDSs of OMT1 and OMT2 were cloned and expressed with *Escherichia coli* expression system. Recombinant proteins were extracted as the cell-free extract, and the enzyme assay was carried out using 6-hydroxydaidzein and SAM as substrates. Reaction products were analysed using high-performance liquid chromatography-ultraviolet detection (HPLC-UV). Interestingly, the peak coinciding with glycitein was detected only when using OMT1 in the reaction (Fig. 5). The reaction product was chromatographically purified and confirmed to be glycitein by NMR analysis (Supplementary methods). This result indicated that OMT1 has the activity of converting 6-hydroxydaidzein to glycitein. Thus, we renamed OMT1 as GmIOMT1 and used it for further analyses.

Biochemical properties of GmIOMT1

The pH dependence of GmIOMT1 was examined in the reaction mixture containing 20 mM MgCl₂. The optimum pH range was neutral (pH 7.0-7.6) (Fig. 6A) as it was with the other OMTs reported previously. Cation dependency was analysed according to a
previous study (Hugueney et al. 2009). Generally, cation-dependent OMTs contain cation inside, and their enzymatic activities are decreased in the presence of EDTA by chelating effect. GmIOMT1 activity was decreased in the presence of EDTA (Fig. 6B). GmIOMT1 activity was enhanced by all the divalent cations tested at 5 mM: Zn$^{2+}$, Mg$^{2+}$, Ca$^{2+}$, and Mn$^{2+}$ by 3-, 2-, 2-, and 1.5-fold, respectively; then, we checked Zn$^{2+}$-concentration dependency. The GmIOMT1 activity was almost the same at the range of 2.5–10 mM Zn$^{2+}$ (Fig. 6C). Finally, the kinetic parameters against 6-hydroxydaidzein was determined at 10 mM Zn$^{2+}$ as follows: $K_m = 2.1 \pm 0.5 \mu$M, $V_{max} = 411 \pm 20$ pkat mg$^{-1}$, $k_{cat} = 1.1 \times 10^{-2}$ s$^{-1}$, and $k_{cat} / K_m = 5.2$ s$^{-1}$ mM$^{-1}$ (Fig. 6D).

Since the known cation-dependent OMTs showed broad substrate specificity against the catechol structure-containing compounds, we analysed the specific activity of GmIOMT1 against catechol structure-containing isoflavones: 6-hydroxydaidzein, 8-hydroxydaidzein, and 3′-hydroxydaidzein. The result showed that GmIOMT1 displayed almost the same activity with 6-hydroxydaidzein and 8-hydroxydaidzein; however, the activity with 3′-hydroxydaidzein was two-fold weaker (Fig. 6E).

*Functional analysis of GmIOMT1 in hairy root*

We further analysed the *in vivo* function of GmIOMT1 using soybean hairy root.
Since $F6H$ is not expressed in hairy root and thus in the substrate of GmIOMT1, 6-hydroxydaidzein was expected to be absent; we expressed not only GmIOMT1 but also F6H. $F6H$-GmIOMT1- or GFP-overexpressed hairy roots were analysed by real-time PCR and LC-QqQ-MS. Real-time PCR revealed that the expression level of $F6H$ and GmIOMT1 in $F6H$-GmIOMT1-overexpressed lines were approximately 15,000-35,000 and 1,000-3,500 folds higher than that in GFP-overexpressed lines, respectively (Fig. 7A). Glycitein derivatives in hairy root were detected by LC-QqQ-MS, and $6''$-O-malonylglycitin and glycitin were significantly increased in the $F6H$-GmIOMT1 lines except for $F6H$-GmIOMT1-2 (Fig. 7B). In contrast, $6''$-O-acetylglycitin and glycine showed no significant difference. Despite the significant increase in the expression level, the increase in glycine-related metabolites was only about twice that of the control.

**Discussion**

*Usefulness of integrated omics analysis for identification of novel genes in soybean*

Although glycine was first isolated from soybean in 1973 (Naim et al. 1973), it was not until 2001 that the involvement of F6H in its biosynthesis was identified (Latunde-Dada et al. 2001). IOMT catalysing the last step in glycine biosynthesis had
not been revealed for more than 40 years. The characteristics of soybean genome make it difficult to analyse genes: the genome size is relatively large (approximately 1.1 Gbp); a half of the genome has highly repetitive sequences; and more than 90% of the genes, which do not have repetitive sequences have over 2 copies (Schmutz et al. 2010). Identification of IOMT gene by biochemical assay screening or functional prediction from genome sequences was particularly difficult because soybean has more than 500 genes annotated as methyltransferase in Phytozome (https://phytozome.jgi.doe.gov/pz/portal.html). In this study, we could efficiently narrow down the candidate genes to two from more than 500 putative methyltransferase genes based on the co-occurrence analysis of metabolome and transcriptome using F6H, a key gene in glycitein biosynthesis, as the query. The omics data obtained in this study can be further used for isolation of other unknown genes such as the transcription factor involved in glycitein and glyceollin biosynthesis as well as the elucidation of the mechanism of biotic stress response in soybean.

Characteristics of GmIOMT1

Although soybean isoflavonoid OMT is expected to be a member of leguminous plant-specific IOMTs, GmIOMT1 belongs to the cation-dependent OMT (type 2 OMT)
clade (Fig. 5). The internal cation of cation-dependent OMTs is considered necessary for binding with the hydroxyl group of the substrate (Hugueney et al. 2009; Ibdah et al. 2003). Cation type affects substrate specificity and kinetic parameters (Lukacin et al. 2004). The assay using the purified GmIOMT1 indicated that the optimum pH was similar to that for other cation-dependent OMTs, and the activity decreased in the absence of cations and presence of EDTA (Fig. 6A, B). Although the activity of previously reported cation-dependent OMTs, such as AOMT and PFOMT, was inhibited by Zn$^{2+}$ (Hugueney et al. 2009; Ibdah et al. 2003), GmIOMT1 activity was rather increased by Zn$^{2+}$ to a greater extent than Mg$^{2+}$ (Fig. 6B, C). The mechanism, by which the enzyme activity changes, depending on the type of cation, is still unknown. Crystal structure analysis of GmIOMT1, which has different cation requirements, would help elucidate the underlying mechanism in future studies.

GmIOMT1 showed the activity not only towards 6-hydroxydaidzein, which is the direct precursor of glycine, but also to other isoflavones possessing catechol structure (Fig. 6E). Although 3’-hydroxydaidzein and 8-hydroxydaidzein were isolated from fungi-fermented soybean, they have not been detected in non-fermented soybean (Chang 2014). Since fungi have cytochrome P450, which catalyses the hydroxylation of isoflavone, 3’-hydroxydaidzein and 8-hydroxydaidzein cannot be produced by soybean
but by fungi; thus, the reaction toward these isoflavones is not expected to occur in soybean. Such a wide range of substrate specificities would be useful in metabolic engineering for material production.

The expression levels of F6H and GmIOMT1 in their overexpressing hairy root lines were much higher than that in GFP control (Fig. 7A), and 6"'-O-malonylglycitin and glycitin were increased in lines nos. 1 and 3 (Fig. 7B). However, despite the significant increase in the expression level, the increase in glycine-related metabolites was only about twice that of control. This may be because the translational efficiency of those transcripts was very low and/or that there was an unknown mechanism for adjusting the amount of glycine, such as feedback regulation.

**Glycitein biosynthesis probably involves different enzymes depending on the situation**

In this study, we identified GmIOMT1 as the gene induced by fungal inoculation and increased glycine biosynthesis using metabolome and co-expression analysis. Glycitein is induced by conditions such as fungal infection and localises specifically in the hypocotyl of soybean (Al-Tawaha et al. 2006; Kudou et al. 1991). In our study, glycine was detected via highly sensitive LC-QqQ-MS (Fig. 2B), although GmIOMT1 and F6H were hardly expressed in non-fermented soybean (Supplementary Fig. S1).
This result suggests that glycitein biosynthesis occurs via two routes, inducible and constitutive routes, which consist of different sets of enzymes with the same activities. In fact, previous research suggests a possibility that F6H paralogues, CYP71D101 and CYP71D102, participate in constitutive glycitein biosynthesis (Artigot et al. 2013). In addition, CYP71D101 and CYP71D102 were not or were hardly expressed in all samples of soybean seedlings regardless of fungal inoculation (Supplementary Fig. 1). A recent study reported a quantitative trait locus for 6′′-O-malonylglycitin accumulation (Watanabe et al. 2019) but did not determine its match with the positions of GmIOMT1, F6H, and their paralogues. Thus, the genes participating in glycitein biosynthesis might be adequately used depending on organs, growth stage, stress response, and so on. Identification and characterisation of other enzymes and transcription factors involved in glycitein biosynthesis will help understand the biological functions of glycitein and its derivatives, which should be different from those of other isoflavonoids such as genistein, daidzein, and their derivatives.

**Materials and methods**

*Chemicals*
6-Hydroxydaidzein and 3’-hydroxydaidzein were purchased from INDOFINE Chemical Company, Inc (Hillsborough, NJ, USA). 8-Hydroxydaidzein and glycitein were purchased from Nagara Science (Gifu, Japan) and Sigma-Aldrich (St Louis, MO, USA), respectively. Glyceollin I was purchased from FUJIFILM Wako Pure Chemical Corporation (Osaka, Japan). S-adenosyl-L-methionine solution (32 mM) was purchased from New England Biolabs (Ipswich, MA, USA).

Plant materials

A soybean cultivar Otofuke-Osodefuri, which is known for its high isoflavonoid accumulation (Tanifuji et al. 2009), was used. A. oryzae strain AOK139 and R. oligosporus were purchased from AKITA KONNO CO., LTD (Akita, Japan). Soybean seeds were germinated in a chamber at 26°C, and sprinkled with water (26°C) for 12 min every 4 h. After 48 h when germination started, sprinkling was stopped; germinated soybeans were inoculated with 1% (w/w) of R. oligosporus or A. oryzae spore and then incubated under a humidity of 100%. Soybeans seedlings were sampled every 24 h for 8 day from the start of germination, and then frozen at −80°C.

Sample preparation for widely targeted metabolomics

Frozen samples were powdered using dry ice powder via a mill cutter (Tube Mill
control and MT 40, IKA, Staufen, Germany) and were then lyophilised by a freeze-dryer (dry chamber, DRC-1000; freeze-drying instrument, FDU-2100; EYELA, Tokyo, Japan). Four milligrams of powdered samples were weighed (AP324W, Shimadzu Corporation, Kyoto, Japan) and placed into a 2-mL tube with 5 mm zirconia beads. One millilitre of extraction solvent with 0.1% (v/v) formic acid in 80% (v/v) methanol and internal standards (8.4 nM of lidocaine and 210 nM of 10-camphorsulfonic acid) was added into the tube, and the metabolites were extracted using a bead-shocker (Shake Master NEO, Biomedical Science, Tokyo, Japan) for 2 minutes at 1000 rpm, followed by centrifugation at 9,100 × g for 1 min. The extracted solutions were evaporated using a liquid handling system (MicrolabSTARplus, Hamilton Company, Reno, NV, USA), and the residual extract was re-dissolved with LC-MS grade water (FUJIFILM Wako Pure Chemical Corporation) so that the final diluted solution would be 40-fold. The re-dissolved solution was filtered (MZHVN0W50, Merck Millipore, Darmstadt, Germany). One μL of the solution including 100 ng of the sample was subjected to widely targeted metabolomics.

*Widely targeted metabolomics*

We modified the method of widely targeted metabolomics (Sawada et al., 2009) as
follows. The selective reaction monitoring (SRM) conditions of 490 standard metabolites were optimised by flow injection analysis, and the retention time (RT) was determined using LC-QqQ-MS (Nexera MP/LCMS-8050, Shimadzu Corporation) (Supplementary Table S5, SRM and RT; Supplementary Table S6, LC conditions; Supplementary Table S7, QqQ-MS conditions). The raw data of peak area values of 13 target isoflavonoids and 477 metabolites were collected using the LabSolution software (Shimadzu Corporation). The raw data (lcd file) were converted into Abf file using Reifys Abf (Analysis base file) Converter (https://www.reifys.com/AbfConverter/). The peak area values of LC-QqQ-MS data were calculated using MRMPROBS (Tsugawa et al. 2013, http://prime.psc.riken.jp/compms/mrmprobs/main.html). The data matrix (72 samples × 490 metabolites) is provided in Supplementary Table S1, S2, and S8. The data matrix of metabolome data was analysed using R script with pheatmap package. Z-score was calculated using the average peak area of biological replicates.

Total RNA isolation and cDNA synthesis

Total RNAs were isolated from the powdered frozen samples using RNeasy Plant Mini Kit (Qiagen, Hilden, Germany). cDNAs for cloning and real-time PCR were synthesised using the SuperScript IV FirstStrand Synthesis System (Thermo Fisher
Transcriptomics

RNA samples for RNA sequencing were prepared by mixing the RNAs of three biological replicates. The process from library preparation to RNA sequencing was performed by TAKARA BIO INC. (Shiga, Japan). RNA sequencing was carried out using HiSeq 2500 (Illumina) under the conditions of read length (100 bp) and paired-end. The obtained reads were cleaned by FaQCs (Lo and Chain 2014). Quantification of transcript (transcripts per million [TPM]) was carried out by Kallisto (Bray et al. 2016) using soybean transcript sequence (Williams 82 Assembly 2 Annotation 1). Data of 88,647 transcripts were obtained. For each transcript, the sum of TPM values in all 24 samples (3 treatments x 8 time points) was calculated. After removing the transcripts with the sum of less than 1, the remaining 59,343 transcripts were subjected to WGCNA using R package (Langfelder and Horvath 2008).

Construction of expression vector for plant

The construction methods and simplified schematic of plant expression vectors are described in the Supplementary methods and Supplementary Figure S2.
**Cloning**

All CDSs were amplified by PCR using PrimeSTAR MAX DNA Polymerase (TAKARA) with primer sets and cDNA (Aspergillus-fermented soybean at DAS 5) as the template. Primer sequences were as follows: *GmiOMT1* (Glyma.05G147000.1); 5′-CACCATGTCGGGTGATTTAGCATACAAG-3′ and 5′-TCACAGACGTCTACACAGGG-3′, *OMT2* (Glyma.07G048900.1); 5′-ATGGCTCCATCATTGGAAACC-3′ and 5′-TTACTTATAAAATTCATAACCCAG-3′, *F6H* (Glyma.18G080400.1); 5′-ATGGATCTTCAACTTCTCTACTTC-3′ and 5′-CTAATTATGAACAGTTTTGGGAATG-3′. Amplified fragments were introduced into pENTR-D-TOPO vector (Thermo Fisher Scientific) for GmIOMT1 or pCR8/GW/TOPO vector (Thermo Fisher Scientific) for OMT2 and F6H. CDSs of OMT were introduced into the pET-53-DEST vector (Merck Millipore) using a Gateway LR Clonase II Enzyme Mix (Thermo Fisher Scientific). For *GmiOMT1* and *F6H* expression in hairy root, the genes were introduced into pASG-GW and pAKG-GW vectors (supplementary method) using a Gateway LR Clonase II Enzyme Mix.

**Crude enzyme assay of OMT candidates**

For the assay using crude protein extract, *E. coli* strain C41 (DE3) (Lucigen, Middleton,
WI, USA) was transformed with pET-53-DEST vector (Merck Millipore), and protein induction was conducted at an OD$_{600}$ adjusted to approximately 0.4. The culture was supplemented with 0.5 mM IPTG and cultured for 3 h at 37°C. *E. coli* cells were collected by centrifugation, re-suspended in 100 mM sodium phosphate buffer (pH 7.6), disrupted by vortex with glass beads for 5 min, and centrifuged at 13,000 × g at 4°C for 5 min. An aliquot of 200 μL of supernatant was incubated with 10 μg 6-hydroxydaidzein, dissolved in 4 μL of 2-methoxyethanol and 0.32 mM SAM at 30°C for 1 h. Reaction products were extracted using ethyl acetate and analysed using LC-UV (Nexera X2, Shimadzu). LC-UV conditions were as follows: Column, Kinetex 2.6 μm XB-C18 50×2.1 mm (Shimadzu); detector, UV/VIS detector (SPD-20A) at 260 nm; solvent, A: water (0.1% formic acid), B: acetonitrile (0.1% formic acid); liner gradient, B: 10% to 70% (5 min); flow rate, 0.36 mL/min; and column temperature, 40°C.

*Enzyme purification*

*E. coli* strain KRX (Promega, Madison, WI, USA) was transformed with *GmiOMT1* (pET-53-DEST), and precultured in 20 mL liquid LB medium (50 mg L$^{-1}$ carbenicillin) at 37°C overnight. Two millilitres of pre-culture and final concentration of 0.15% glucose, 0.1% rhamnose, and 0.5 mM IPTG were added into a 500 mL Erlenmeyer flask...
containing 200 mL of same medium and cultured at 16°C overnight. Cell pellet was collected by centrifuge, re-suspended in lysis buffer (300 mM KCl, 50 mM KH₂PO₄, 5 mM imidazole, pH 8.0), and then disrupted by sonication. The supernatant collected by centrifuge and His-tagged protein was purified using Profinia protein purification system (Bio-Rad, München, Germany) with Profinia IMAC Purification Kit (Bio-Rad) and cOmplete His-Tag Purification Column (Roche Diagnostics, Rotkreuz, Switzerland).

Characterisation of enzymatic properties

Enzymatic assay was carried out using 900 ng purified His-tagged GmIOMT1 protein, 320 μM SAM, and 100 mM sodium phosphate buffer of pH 7.6 with divalent cations or EDTA in total of 200 µL for 30 min at 30°C. The optimum pH of GmIOMT1 was determined with 100 μM 6-hydroxydaidzein and 20 mM MgCl₂ in potassium phosphate buffers (FUJIFILM Wako Pure Chemical Corporation) at pH 6.0, 6.4, 7.0, 7.2, 7.6, and 8.0. To determine the cation requirement of GmIOMT1, enzyme assay was performed in the presence of 5 mM MgCl₂, CaCl₂, MnCl₂, or ZnCl₂ or 50 mM EDTA. According to a previous study (Hugueny et al. 2009), 5 mM MgCl₂ was also added to test the effect of Zn²⁺, Ca²⁺, and Mn²⁺. To determine the relative activity of GmIOMT1, 100 µM
6-hydroxydaidzein, 8-hydroxydaidzein, or 3′-hydroxydaidzein was incubated in the presence of 10 mM ZnCl₂ with recombinant enzyme for 10 min. To determine the kinetic parameters of GmIOMT1, 0.5, 5, 10, 25, and 50 μM 6-hydroxydaidzein were used. \( K_m \), \( V_{max} \), and \( k_{cat} \) values were calculated using non-linear regression in SigmaPlot14 (Systat Software, Inc, San Jose, CA, USA).

**Phylogenetic tree analysis**

Amino acid sequence of OMTs was aligned using ClustalW v2.1 in DDBJ (https://clustalw.ddbj.nig.ac.jp/) with default settings and phylogenetic tree was drawn using MEGA7 (Kumar et al. 2016) using default setting of neighbour-joining method with amino acid sequence and 1,000 bootstrap replicates. Accession numbers were as follows: HI4′OMT (G. echinata), BAC58011.1; HMM (Pisum sativum), AAC49856.1; D7OMT (G. echinata), BAC58012.1; F3′5′OMT (Chrysosplenium americanum), AAA80579.1; F3′OMT (Arabidopsis thaliana), AAB96879.1; CCoAOMT (M. sativa), AAC28973.1; HI4′OMT (G. max), XP_003542715.1; NOMT (Oryza sativa), XM_015763455.1; IOMT6 (M. truncatula), XP_013455257.1; IOMT2 (M. truncatula), ABD83942.1; COMT (M. truncatula), XP_003626614.1; GmIOMT1 (G. max), NP_001237478.1 (Glyma.05G147000); PFOMT (M. crystallinum), AAN61072.1;
IF3′OMT (Pueraria lobata), KP057887.1; OMT2 (G. max), XM_003528712.3 (Glyma.07G048900); AOMT (G. max), NP_001242455.1; and Catechol OMT (Homo sapiens), XP_011528188.1.

Production of transformed hairy root

Soybean (‘enrei’) seeds were sterilised using the chlorine gas method (Paz et al. 2005). Sterilised seeds were putted on a Gamborg B5 medium (B5 vitamin, 20 g L⁻¹ sucrose, and 7 g L⁻¹ agar) and then cultured at 26°C under 16-h light and 8-h dark condition for 1 week. Agrobacterium rhizogenes strain K599 was transformed by overexpressing GFP or F6H-GmIOMT1 on LB medium (15 g L⁻¹ agar and 100 mg L⁻¹ spectinomycin); the strain was then cultured in liquid medium at 28°C overnight. The cells were collected by centrifuge and re-suspended in the infection buffer (10 mM MES, 10 mM MgCl₂, 250 mg L⁻¹ acetylsyringone, and 0.02% (v/v) silwet l-77, pH 5.7) at OD₆₀₀ of 0.1-0.2. The cotyledons of soybean seedlings were divided, leaving 5 mm hypocotyl, and putted on MS medium (MS vitamin, 30 g L⁻¹ sucrose, and 9 g L⁻¹ agar). Two microlitres of Agrobacterium-suspension buffer was placed at the cut ends of the cotyledon, followed by culturing under condition similar to those for seed germination. Three weeks after infection, the hairy root from which GFP fluorescence was confirmed was transferred to
Gamborg B5 medium (B5 vitamin, 30 g L\(^{-1}\) sucrose, 2 g L\(^{-1}\) gellite, and 250 mg L\(^{-1}\) carbenicillin) and cultured at 25°C under dark condition. After 4 weeks, two or three hairy root tips (approximately 1 cm) were transferred to a 125 mL polycarbonate Erlenmeyer flask with vent cap (Corning) containing 50 mL Gamborg B5 liquid medium and then cultured under gyratory conditions (120 rpm) at 25°C for 2 weeks. Three root tips were transferred into three flasks containing same medium and gyratory cultured under same conditions.

**Analysis of F6H and GmIOMT1 expression by real-time PCR**

Primers for real-time PCR were designed using the Primer 3 programme. Real-time PCR was conducted using the StepOnePlus system (Thermo Fisher Scientific) with PowerUp SYBR Master Mix (Thermo Fisher Scientific), cDNAs, and 300 nM primers. Relative expression was analysed using the ΔΔCt method and the housekeeping gene *SKIP16* (GenBank accession No. NM_001255441) as internal standard. Primer sequences were as follows: *GmIOMT1*, 5′- AAAAGGCAGGAATGGAGCAC -3′ and 5′- TCTGCTTTATCCGATCCAC -3′; *F6H*, 5′-ACCGAAGCCGCAGGATTTCC-3′ and 5′-TTCAAGCCAGGATGCGTCG-3′; and *Skip16*, 5′-AGATAGGGAAATGGTGAGGT-3′ and 5′-CTATGGCAATTGCAGCTCCTG-3′.
Supplementary data

Supplementary data are available at PCP online.

Funding

This work was supported by Japan Science and Technology Agency-Core Research for Evolitional Science and Technology (JST-CREST) (https://www.jst.go.jp/kisoken/crest/en/index.html) [grant Number JPMJCR16O2] and Japan Society for the Promotion of Science KAKENHI Grant-in-Aid for Early-Career Scientists [grant Number JP19K15821] to K.U.

Disclosures

Conflicts of interest: No conflicts of interest declared.

Acknowledgements

We thank Dr. Akashi Tomoyoshi (Nihon University) for NMR spectroscopic measurements; Dr. Mami Okamoto for optimisation of LC-QqQ-MS MRM; Ms. Akane Sakata, Ms. Junko Takanobu, and Ms. Tomomi Sawada for technical support; Ms. Anna
Kuwahara and Ms. Ryoko Araki for sample preparation; and Dr. Keiichi Mochida, Dr. Hiroshi Tsugawa, and Mr. Yutaka Yamada (RIKEN CSRS) for information technology support. *Agrobacterium rhizogenes* strain K599 was provided as strain JCM 20922 by RIKEN BRC through the National Bio-Resource Project of the Ministry of Education, Culture, Sports, Science, and Technology, Japan.

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Legends to Figures

**Figure 1: Isoflavone biosynthesis pathway in soybean**

Acetylated isoflavone glucosides are considered artifacts produced from malonylated isoflavone glucosides (Horowitz and Asen 1989).

**Abbreviations:** PAL, phenylalanine ammonia lyase; C4H, cinnamate 4-hydroxylase; 4CL, 4-coumarate-CoA ligase; PKR or CHR, polyketide reductase or chalcone reductase, respectively; CHS, chalcone synthase; CHI, chalcone isomerase; F6H, flavonoid 6-hydroxylase; IFS, 2-hydroxyisoflavanone synthase; HID, 2-hydroxyisoflavanone dehydratase; IF7GT, isoflavone 7-O-glucosyltransferase; IF7MaT, isoflavone 7-O-glucoside 6'-O-malonyltransferase; ICHG, isoflavone conjugates hydrolysing beta-glucosidase; IOMT, isoflavone O-methyltransferase.

**Figure 2: Time-dependent changes in isoflavonoid contents in fungal inoculated soybean seedlings.**

A: Heatmap representation, B: Peak area value of glycinein derivatives and glyceollin analysed by LC-QqQ-MS and the mean and standard deviation (SD) of three bulked
(approximately 5 individuals) replicates are shown. Inoculation was conducted after 2
days of soaking (DAS2). n.d., not detected.

**Figure 3: Time-dependent changes in expression of putative isoflavonoid biosynthetic
genes.**

References of gene names are described in Supplementary Table S9.

**Figure 4: Comparison of GmIOMT1 with other OMTs.**

A: Phylogenetic tree of representative type 1 (cation-independent) and type 2
(cation-dependent) OMTs. The tree was created using the Neighbour-Joining method
with amino acid sequence and 1,000 bootstrap replicates. Percentages next to enzyme
names indicate the amino acid identity with GmIOMT1.

B: Multiple alignments of cation-dependent OMTs.

* indicates the residues of catalytic triad

**Abbreviation:** AOMT, anthocyanin O-methyltransferase; CCoAOMT, caffeoyl-CoA
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**Figure 5: LC-UV chromatogram of the reaction mixture of OMT1 assay.**

The assay was conducted using the cell-free extract from OMT1-expressing *E. coli* with 6-hydroxydaidzein as a substrate. The cell-free extract from β-glucuronidase (uidA)-expressing *E. coli* was used as negative control.

**Figure 6: Characteristics of GmIOMT1.**

A: pH dependence, B: cation dependence, C: Zn\(^{2+}\)-concentration, D: Michaelis-Menten kinetics, and E: substrate specificity of GmIOMT1 and substrate structure.
The mean ± standard error \((n = 3\) technical replicates\) are shown.

**Figure 7: Analysis of GmIOMT1 function in cultured hairy roots.**

A: transcript levels of \(F6H\) and \(GmIOMT1\) in hairy roots.

The means ± standard errors \((n = 3\), biological replicates\) are shown. Transcript levels were analysed using the \(\Delta\Delta C_t\) method, and \(skip16\) was used as the internal standard. Transcript levels were normalised against the GFP-1 value, which was set at 1.

B: Results of LC-QqQ-MS analysis of glycine derivatives in hairy roots.

The means ± SD \((n = 3\), biological replicates\) are shown. Different letters indicate significant differences according to Tukey–Kramer test \((p < 0.05)\).
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