Diurnal metabolic regulation of isoflavones and soyasaponins in soybean roots

AUTHOR(S):

Matsuda, Hinako; Nakayasu, Masaru; Aoki, Yuichi; Yamazaki, Shinichi; Nagano, Atsushi J.; Yazaki, Kazufumi; Sugiyama, Akifumi

CITATION:

Matsuda, Hinako ...[et al]. Diurnal metabolic regulation of isoflavones and soyasaponins in soybean roots. Plant direct 2020, 4(11): e00286.

ISSUE DATE:

2020-11

URL:

http://hdl.handle.net/2433/259809

Received: 23 April 2020 | Revised: 23 September 2020 | Accepted: 14 October 2020

DOI: 10.1002/pld3.286

ORIGINAL RESEARCH

Diurnal metabolic regulation of isoflavones and soyasaponins in soybean roots

Hinako Matsuda[1] | Masaru Nakayasu[1] | Yuichi Aoki[2] | Shinichi Yamazaki[2] | Atsushi J. Nagano[3] | Kazufumi Yazaki[1] | Akifumi Sugiyama[1]

[1]Research Institute for Sustainable Humanosphere, Kyoto University, Gokasho, Uji, Japan

[2]Tohoku Medical Megabank Organization, Tohoku University, Sendai, Japan

[3]Faculty of Agriculture, Ryukoku University, Otsu, Japan

Correspondence
Akifumi Sugiyama, Laboratory of Plant Gene Expression, Research Institute for Sustainable Humanosphere, Kyoto University, Uji 611-0011, Japan.
Email: akifumi_sugiyama@rish.kyoto-u.ac.jp

Funding information
JST-CREST, Grant/Award Number: JPMJCR17O2 and JPMJCR15O2; JSPS KAKENHI, Grant/Award Number: 18H02313

Abstract

Isoflavones and soyasaponins are major specialized metabolites accumulated in soybean roots and secreted into the rhizosphere. Unlike the biosynthetic pathway, the transporters involved in metabolite secretion remain unknown. The developmental regulation of isoflavone and soyasaponin secretions has been recently reported, but the diurnal regulation of their biosynthesis and secretion still needs to be further studied. To address these challenges, we conducted transcriptome and metabolite analysis using hydroponically grown soybean plants at 6-hr intervals for 48 hr in a 12-hr-light/12-hr-dark condition. Isoflavone and soyasaponin biosynthetic genes showed opposite patterns in the root tissues; that is, the former genes are highly expressed in the daytime, while the latter ones are strongly induced at nighttime. *GmMYB176* encoding a transcription factor of isoflavone biosynthesis was upregulated from ZT0 (6:00 a.m.) to ZT6 (12:00 a.m.), followed by the induction of isoflavone biosynthetic genes at ZT6. The isoflavone aglycone content in the roots accordingly increased from ZT6 to ZT18 (0:00 a.m.). The isoflavone aglycone content in root exudates was kept consistent throughout the day, whereas that of glucosides increased at ZT6, which reflected the decreased expression of the gene encoding beta-glucosidase involved in the hydrolysis of apoplast-localized isoflavone conjugates. Co-expression analysis revealed that those isoflavone and soyasaponin biosynthetic genes formed separate clusters, which exhibited a correlation to ABC and MATE transporter genes. In summary, the results in this study indicated the diurnal regulation of isoflavone biosynthesis in soybean roots and the putative transporter genes responsible for isoflavone and soyasaponin transport.

KEYWORDS

diurnal variability, isoflavones, soyasaponins, soybean roots, transcriptome analysis, transporters

1 | INTRODUCTION

Plants change their metabolisms and physiological functions during the day to adapt to external biotic and abiotic stresses (Gil & Park, 2019; Grundy et al., 2015; Lu et al., 2017; Seo & Mas, 2015). Specialized metabolites play important roles in adapting to diurnally changing external environments. The glucosinolate content in the leaves of Arabidopsis (*Arabidopsis thaliana*) and cabbage (*Brassica oleracea*) peaks in the morning and decreases in the evening to midnight to protect their leaves against pests, such as the cabbage looper (*Trichoplusia ni*), which is active during the daytime (Goodspeed et al., 2013). In an iron-deficient condition, barley (*Hordeum vulgare*) roots secrete mugineic acids, which are iron-chelating phytosiderophores, 2–3 hr after dawn to presumably avoid the degradation by microbes (Nagasaka et al., 2009; Römheld & Marschner, 1990; Takagi et al., 1984). The flowers of the rose plant (*Rosa hybrida L.*) secrete geranyl acetate and germacrene D before and at dawn to attract pollinators that are active at dawn (Hendel-Rahmanim et al., 2007).

Transcription factors, such as circadian clock associated 1 (CCA1), late elongated hypocotyl (LHY), timing of CAB expression 1/ pseudo-response regulator 1 (TOC1/PRR1), pseudo-response regulator 3/5/7/9 (PRR3/5/7/9), REVEILLE 4/6/8 (RVE4/6/8), and night-light-inducible and clock-regulated 1/2 (LNK1/2), coordinately regulate the plant circadian clock (Srivastava et al., 2019). These transcription factors also regulate specialized metabolism (Nguyen & Lee, 2016). For example, RVE8 positively regulates anthocyanin biosynthesis (Pérez-García et al., 2015), and PRR5/7/9 negatively regulates carotenoid and abscisic acid biosynthesis in Arabidopsis (Fukushima et al., 2009). In soybeans, CCA1-like MYB transcription factor GmMYB133 (*Glyma.07G066100*) stimulates isoflavone biosynthesis by inducing *chalcone synthase 8* (*CHS8*) and *isoflavone synthase 2* (*IFS2*) (Bian et al., 2018), suggesting the diurnal regulation of isoflavone biosynthesis.

Isoflavones are major specialized metabolites in soybeans and function as phytoalexins in defense against pathogens (Subramanian et al., 2005). Isoflavones work not only inside a plant but also in the rhizosphere, a soil region close to the roots. Daidzein, the major isoflavone secreted from soybean roots, acts as a signal for the nodulation (Kosslak et al., 1987) and also modulates the rhizosphere microbiota (Okutani et al., 2020). In addition to isoflavones, soybean roots secrete an equivalent amount of soyasaponins, which was first demonstrated by Tsuno et al. (2018). Recently, soyasaponin Bb was shown to enrich *Novosphingobium*, commonly found in the soybean rhizosphere (Fujimatsu et al., 2020). The secretion of these specialized metabolites changes dramatically in quality and quantity depending on the developmental stage of the soybean plant (Sugiyama et al., 2016; Tsuno et al., 2018); however, the diurnal regulation of these soybean metabolites remains to be described.

Isoflavone and soyasaponin biosynthesis occur in the cytosol (Figure 1) (Augustin et al., 2011; Nakayama et al., 2019). Conceivably, both isoflavones and soyasaponins accumulate in the vacuoles as glycosides and are secreted *via* transporters (Figure 1) (de Brito Francisco & Martinoia, 2018; Mylona et al., 2008; Sawai & Saito, 2011; Sugiyama, 2019; Yazaki et al., 2008; Yoo et al., 2013). ATP-binding cassette (ABC) and multidrug and toxic compound extrusion (MATE) transporters are involved in the intracellular transport of isoflavones. For example, in *Medicago truncatula*, MtMATE1 and MtMATE2 are responsible for the transport of isoflavone glycosides, such as daidzin and genistin, into the vacuole (Zhao & Dixon, 2009; Zhao et al., 2011). Biochemical analysis has suggested the involvement of ABC-type transporters and apoplast-localized isoflavone conjugate-hydrolyzing beta-glucosidase (ICHG) in isoflavone aglycone secretion to the rhizosphere (Sugiyama et al., 2007; Suzuki et al., 2006), although these processes have not been genetically characterized (Figure 1a). In contrast to isoflavones, the secretory mechanism involving the transporters that are responsible for the vacuolar accumulation and secretion to the rhizosphere are still unknown for soyasaponins, whereas the soyasaponin biosynthetic pathway in soybeans has been intensively studied and characterized (Krishnamurthy et al., 2019; Sundaramoorthy et al., 2019) (Figure 1b).

In this study, we performed transcriptomic and metabolic analyses using hydroponically grown soybean plants to characterize the diurnal regulation of biosynthesis and secretion of two major classes of specialized metabolites in soybeans. We also narrowed down candidate transporter genes that are responsible for the accumulation and secretion of isoflavone and soyasaponin through co-expression network analysis, which highlighted the members of ABC and MATE transporters that reveal a tight correlation with the biosynthetic gene expression pattern of isoflavone and soyasaponin.

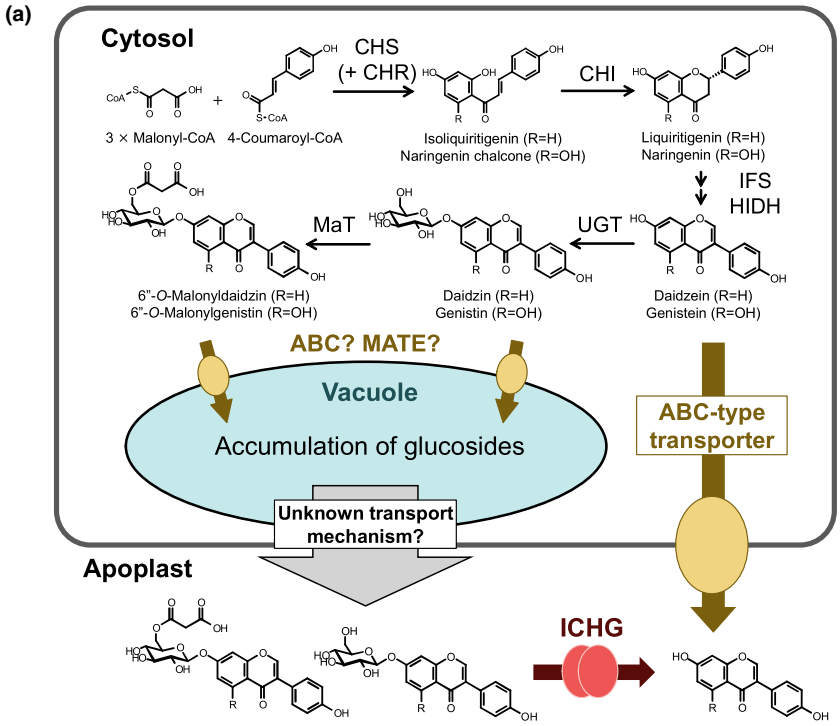
2 | MATERIALS AND METHODS

2.1 | Chemicals

Malonyldaidzin and malonylgenistin were purchased from Nagara Science. Soyasaponin Ab, soyasapogenol A, and soyasapogenol B were purchased from Funakoshi. Soyasaponin Bb was purchased from ChromaDex. The other chemicals were purchased from Wako Pure Chemical Industries Ltd. or Nacalai Tesque Inc., unless otherwise stated.

2.2 | Plant materials and growth conditions

The soybean seeds (cv. Enrei) used in the study were purchased from Tsurushin Shubyo (Matsumoto, Japan). The growth condition of the soybeans in hydroponic cultures was set up according to the description of Sugiyama et al. (2016). After 7 days of the growth in autoclaved vermiculite containing water at 25°C with 16/8-hr photoperiods, the seedlings were rinsed and transferred to a hydroponic culture system where the soybeans were grown in 450-mL plastic containers filled with a mineral nutrient medium consisting of 3.0 mM $MgSO_4$, 6.3 mM KNO_3, 0.87 mM KCl, 1.4 mM KH_2PO_4, 2.5 mM $Ca(NO_3)_2$, 21 μM Fe–EDTA, 4.5 μM KI, 28 μM $MnCl_2$, 19 μM



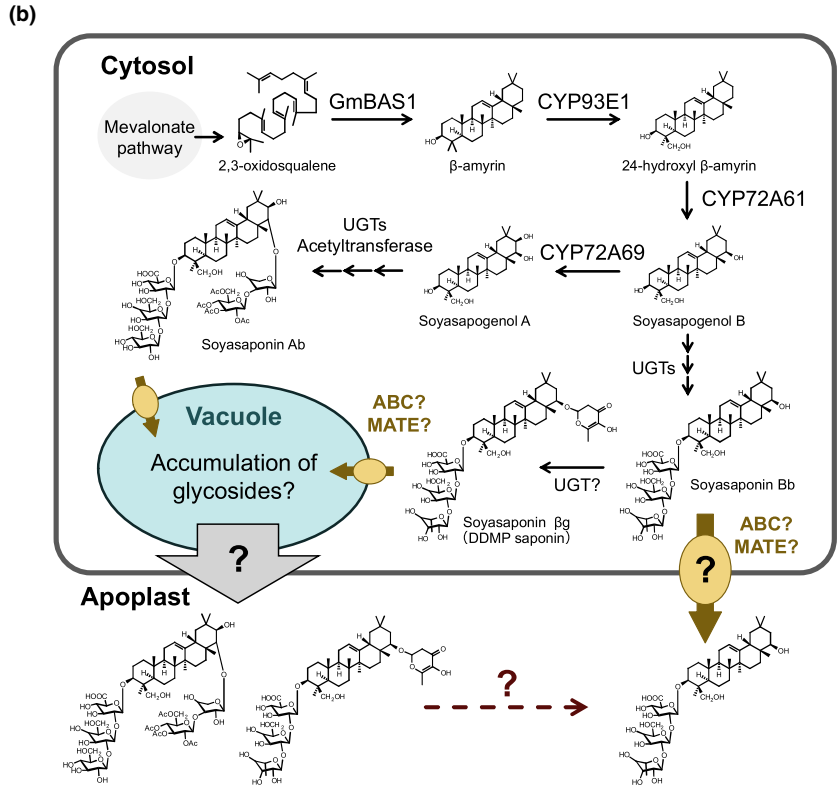


FIGURE 1 Isoflavone and soyasaponin biosynthesis, and their proposed secretion pathways in soybean root. (a) Isoflavones and (b) soyasaponins. ABC, ATP-binding cassette transporter; BAS1, beta-amyrin synthase 1; CHI, chalcone isomerase; CHR, chalcone reductase; CHS, chalcone synthase; CYP, cytochrome P450 (CYP); HIDH, 2-hydroxyisoflavanone dehydratase; ICHG, isoflavone conjugate-hydrolyzing beta-glucosidase; IFS, isoflavone synthase; MaT, malonyl transferase; MATE, multidrug and toxic compound extrusion transporter; UGT, UDP-glucuronosyltransferase

H_3BO_3, 2.3 μM $ZnSO_4$, 0.5 μM $CuSO_4$, and 0.003 μM Na_2MoO_4, with pH 6.0. The soybeans were kept in a cultivation room set at 25°C with a 12-hr light/dark cycle. After 2 weeks, the plants were transferred to a new medium 6 hr before sampling for analysis. The leaf and root tissues and root exudates were sampled at ZT0 (6:00 a.m.), ZT6 (0:00 p.m.), ZT12 (6:00 p.m.), and ZT18 (0:00 a.m.) for 48 hr with five biological replicates.

2.3 | RNA extraction and transcriptome sequencing

The total RNA was derived from soybean leaves and roots using RNeasy Plant Mini Kits (Qiagen, CA) according to the manufacturer's instruction. The DNA in each total RNA sample was digested using DNase I (RNase-free DNase sets, Qiagen). The RNA-seq library was prepared through the Lasy-Seq v1.1 protocol (Kamitani et al., 2019;

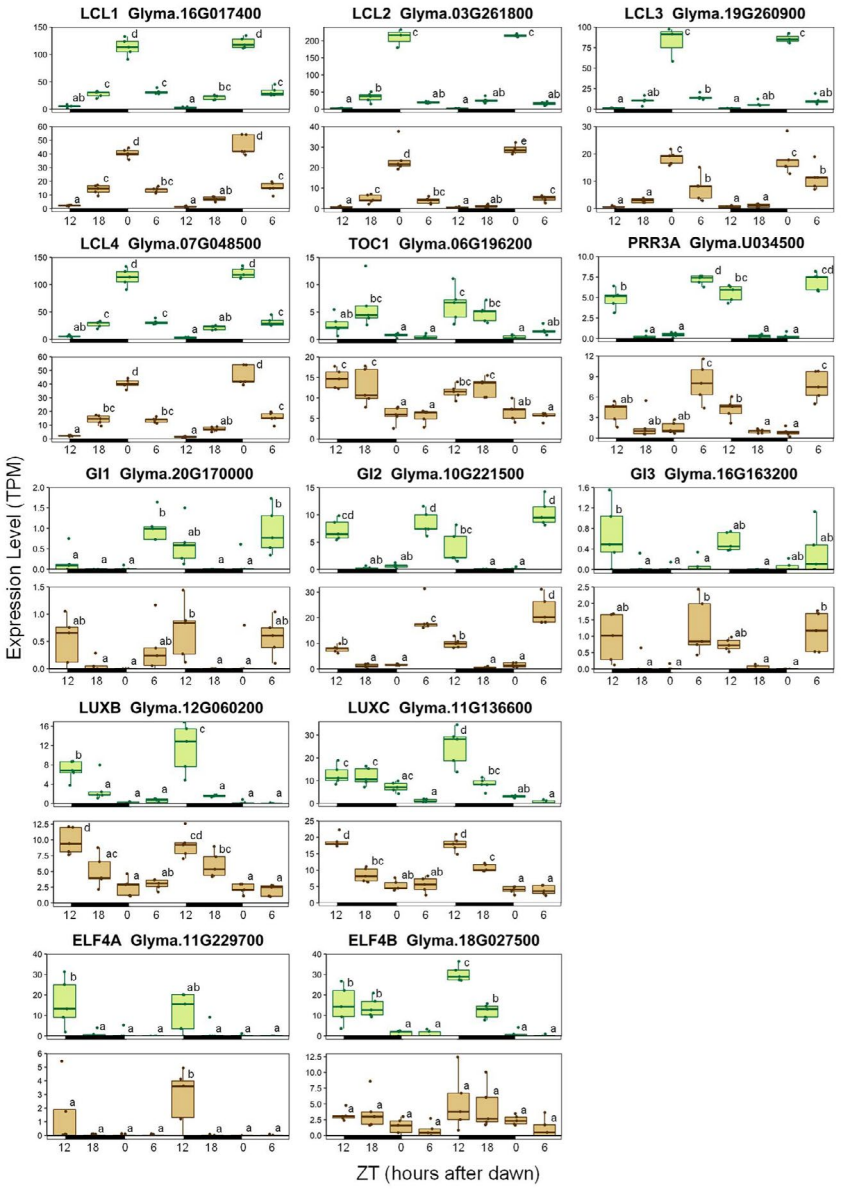


FIGURE 2 Diurnal variations of circadian rhythm-related genes. Each boxplot was constructed by five replicates. The individual dots indicate raw data. The outliers were identified using the 1.5*IQR (interquartile range) rule. The green and brown boxplots exhibit gene expression levels in the leaves and roots. Tukey's HSD test was used for statistical analysis ($p < 0.05$). ELF, Early flowering; GI, GIGANTEA; LCL, Late elongated hypocotyl/circadian clock associated; LUX, LUX ARRHYTHMO; PRR, Pseudo-response regulator; TOC, Timing of CAB expression; TPM, Transcripts per million. Correspondence between gene IDs and their names was referenced from NCBI (https://www.ncbi.nlm.nih.gov/)

https://sites.google.com/view/lasy-seq/) using 500 ng total RNA. The library was sequenced by paired-end 150 bp + 150 bp mode of HiSeqX platform (Illumina).

2.4 | Transcriptome data analysis

The raw-reads data were quality controlled by removing low-quality bases using Trimmomatic (Bolger et al., 2014) with default parameters. The trimmed reads were aligned to the soybean genome (Glycine_max_v2.1 assembly) (Schmutz et al., 2010) using STAR v2.7.0f (Dobin et al., 2013) based on Ensembl Plants release 43 (Monaco et al., 2014) gene annotations. Gene expression levels were estimated as transcripts per million (TPM) (Wagner et al., 2012) using RSEM v1.3.1 (Li & Dewey, 2011) with default parameters. The transcriptome data set supporting the results of this study is publicly available at the DNA Data Bank of Japan (https://www.ddbj.nig.ac.jp) (DRA010744). Principal component analysis (PCA) was performed using the whole transcriptome data set (30,362 genes; TPM > 1) after removing low-expression genes. Diurnally rhythmic genes were detected using the JTK_CYCLE algorithm (Hughes et al., 2010) with a MetaCycle package (Wu et al., 2016) in R environment, with 24-hr periodicity and false discovery rate (FDR) < 0.01. Rhythmic genes were classified into eight patterns based on the phase obtained by JTK_CYCLE. Gene Ontology (GO) enrichment analysis of gene set was performed using the SoyBase GO Term Enrichment Tool (http://www.soybase.org) in accordance with a diurnal pattern of interests. Gene IDs for isoflavone and soyasaponin biosynthesis, ABC and MATE transporters, and ICHG were collected based on the procedures described in previous literature (Ahmad et al., 2017; Krishnamurthy et al., 2019; Liu et al., 2016; Mishra et al., 2019; Sundaramoorthy et al., 2019; Yoo et al., 2013). The networks were

constructed using the network visualization software Cytoscape (v. 3.7.2; Shannon et al., 2003).

2.5 | Preparation of root extracts and exudates

The preparation of root extracts and exudates was performed according to the procedure previously described by Sugiyama et al. (2016). The medium containing root exudates was filtered through Omnipore membrane filters (Millipore). The medium was passed through a Sep-Pak C18 Plus short cartridge (Waters), which was eluted with 2 ml of MeOH. The eluant was dried under nitrogen and reconstituted in 50 μl of MeOH for LC-MS/MS analysis.

2.6 | LC-MS/MS analysis

The samples were separated using an ACQUITY UPLC BEH C18 Column (2.1 × 50 mm, 1.7 μm, Waters) on an LC system (ACQUITY H-Class System, Wnters). The LC mobile phase consisted of (C) water containing 0.1% (v/v) formic acid and (D) acetonitrile. The gradient program was isocratic at 10% D, Initial; linear 1at 0%–85% D, 0–15 min; isocratic at 100% D, 15–16 min; and isocratic at 100% D, 16–20.5 min. The injection volume of each sample was 5 μl, and the flow rate was 0.2 ml min. The isolated samples were detected using a tandem quadrupole MS (Xevo TQ-S, Waters) in the Multiple Reaction Monitoring (MRM) mode. The MRM conditions for the respective compounds are listed in Table S1.

2.7 | Statistical analysis

Statistical differences were calculated using the Tukey's HSD test at $p < 0.05$ implemented in R (v. 3.6.1; R Core Team, 2019). The outliers (defined as >1.5*IQR) in the boxplots were excluded from the calculated data.

3 | RESULTS AND DISCUSSION

3.1 | Time-dependent transcriptome analysis

The RNA-seq analysis yielded over 1.5 billion reads from 80 samples, that is, eight time points, two tissues, and five replicates. We conducted PCA and found that the leaf and root transcriptomes were clearly separated (Figure S1). The circadian clock-related transcription factors in soybeans include Late elongated hypocotyl/circadian clock associated (LCL), TOC1, PRR, GIGANTEA (GI), LUX ARRHYTHMO (LUX), and Early flowering 4 (ELF4) (Cheng et al., 2019; Li et al., 2013; Liew et al., 2017; Wang et al., 2020). The expression of these key circadian clock-related genes showed diurnal changes both in the leaves and roots and displayed a similar expression tendency as observed in previous studies (Cheng et al., 2019; Li

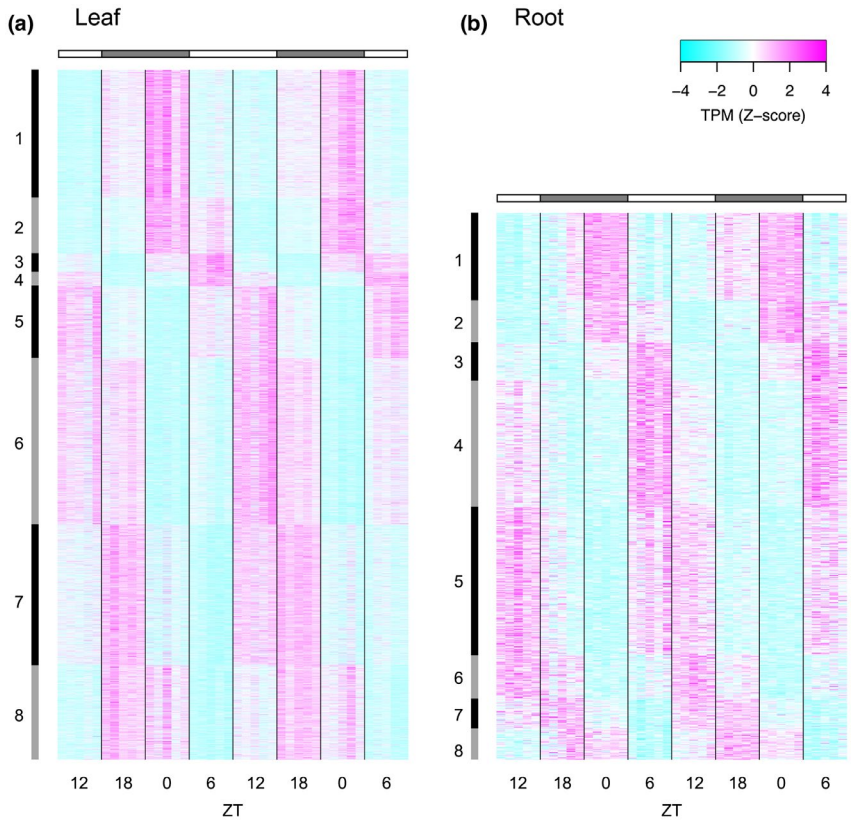


FIGURE 3 Phase sorted heatmap showing diurnally oscillating gene expressions in leaves (a) and roots (b). Each row represents a rhythmically expressed gene with 24-hr periodicity (FDR < 0.01). Data from five replicates are grouped in adjacent columns. Eight phases are sorted by the peak time of each gene expression in a day. For each sample, color corresponds to the z-score given by the sample's TPM. ZT, hours after dawn. TPM, transcripts per million

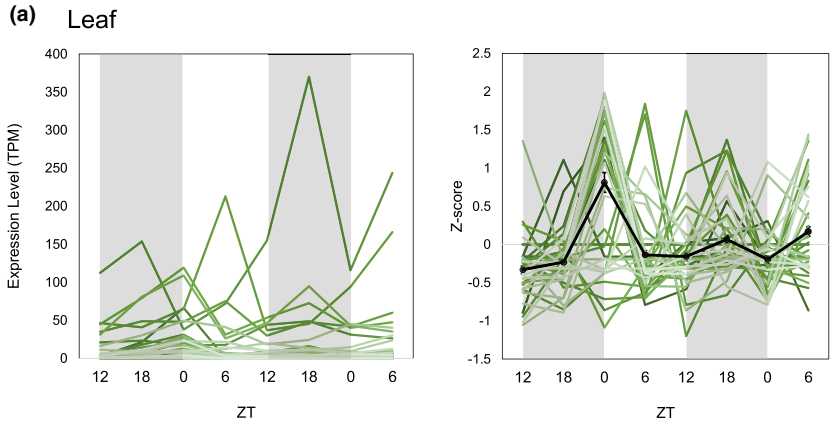
FIGURE 4 Enriched GO terms for genes with diurnally rhythmic expression patterns in soybean leaves (a) and roots (b) ($p < 0.01$). Cells with red gradient color represent the time that each GO term is enriched and a darker red corresponds to a greater fold enrichment. ZT, hours after dawn

et al., 2013; Liew et al., 2017; Locke et al., 2018; Marcolino-Gomes et al., 2014; Wang et al., 2020) (Figure 2).

To provide an overview of the diurnal changes of gene expressions in soybean, rhythmic genes with 24-hr periodicity were examined by the JTK_CYCLE algorithm (FDR < 0.01) (Hughes et al., 2010). Rhythmic genes were sorted into eight phases according to the peak time of the gene expression. A total of 6,777 and 1,240 genes were detected as diurnally oscillating genes among soybean genome (55,897 genes) in leaves and roots, respectively (Figure 3, Figure S2, Table S2 and Table S3). To understand the characteristic biological processes according to time of day, we carried out GO enrichment analysis for the diurnally rhythmic genes (Table S2 and Table S3). In the leaves, the GO terms related to light response were represented at ZT0 and the GO terms associated with photosynthesis were frequently represented from ZT3 to ZT9, which consistent with the GO enrichment analysis on diurnal transcripts in rice leaves (Xu et al., 2011) (Figure 4a, Table S4). During nighttime, the GO terms involved in protein synthesis, respiration, and transport were enriched in chronological order (Figure 4a, Table S4). In roots, GO terms related to transport were highlighted at ZT0 and the GO terms associated with circadian rhythm were enriched at ZT9 (Figure 4b, Table S5). The GO terms linked to secondary metabolism were not enriched at any time of day, although Xu et al. (2011) represented that secondary metabolic process was induced from ZT4 (10:00 a.m.) to ZT6.

3.2 | Diurnal variation of biosynthetic gene expression for isoflavone and soyasaponin

To analyze the diurnal metabolic changes of isoflavones and soyasaponins in soybean, we investigated the fluctuations of gene expression for all the reported genes involved in isoflavone and soyasaponin biosynthesis. No diurnal variation pattern common to the isoflavone or soyasaponin biosynthetic genes was observed in the leaves (Figures 5a and 6a). In contrast, the expression profiles in the roots exhibited clear diurnal variation patterns for the genes involved in isoflavone and soyasaponin biosynthesis (Figures 5b and 6b, Table S6 and Table S7). Isoflavone biosynthetic genes,



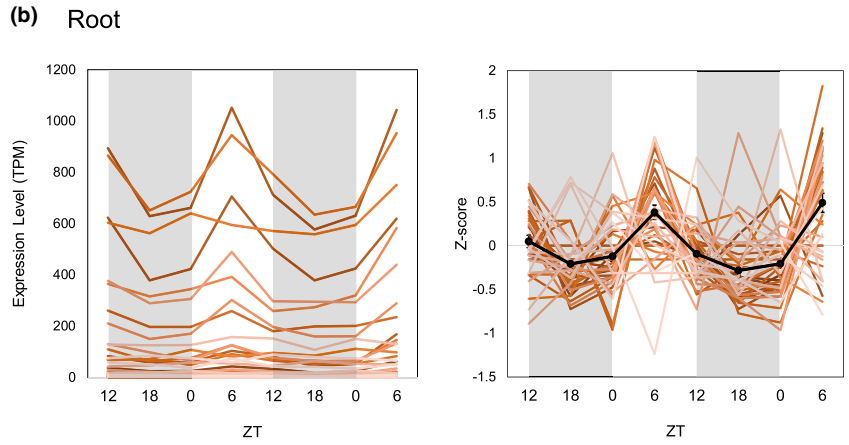
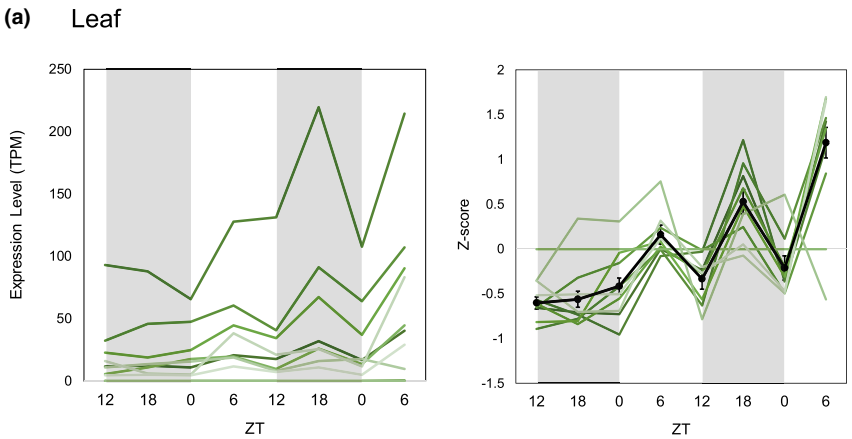
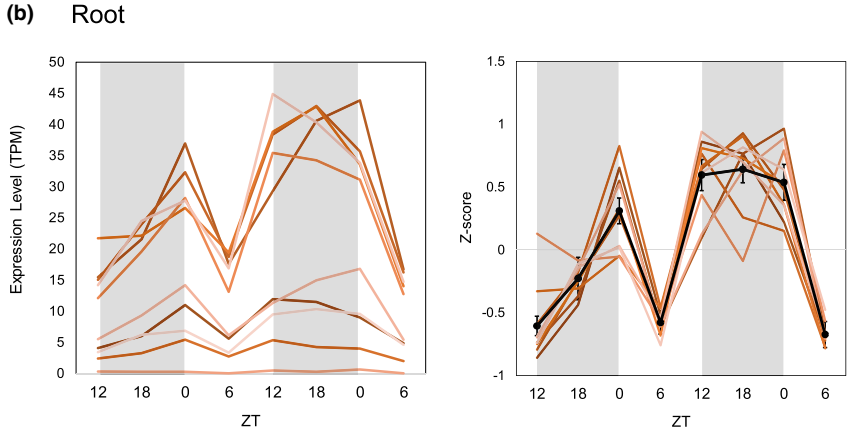


FIGURE 5 Diurnally differentially expressed genes related to isoflavone biosynthesis in soybean leaves (a, green lines) and roots (b, brown lines). The genes displayed are listed in Table S6. The shaded areas show nighttime. The data points indicate the average of expression levels and z-scores of five replicates. The black lines and error bars in the z-score graphs depict the average and SEM of z-scores of all displayed genes. ZT, hours after dawn. TPM, transcripts per million

FIGURE 6 Diurnally differentially expressed genes related to soyasaponin biosynthesis in soybean leaves (a, green lines) and roots (b, brown lines). The genes displayed are listed in Table S7. The shaded areas show nighttime. The data points indicate the average of expression levels and z-scores of five replicates. The black lines and error bars in the z-score graphs depict the average and SEM of z-scores of all displayed genes. ZT, hours after dawn. TPM, transcripts per million





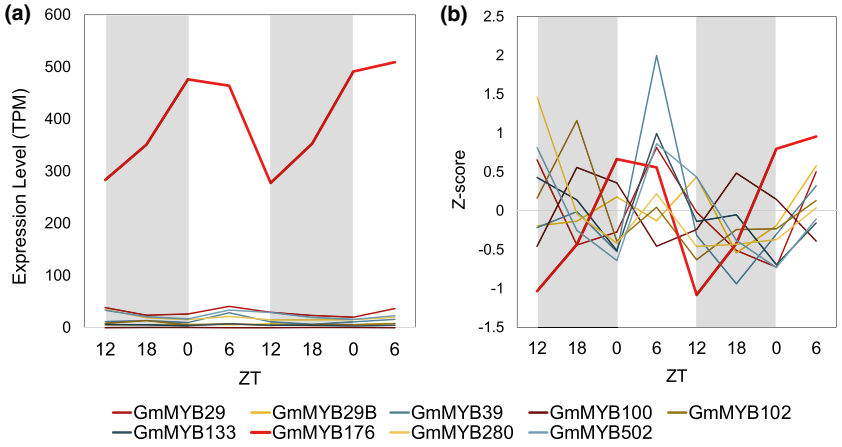


FIGURE 7 Diurnally differentially expressed genes of MYB transcription factors of isoflavone biosynthesis in soybean roots. The genes displayed are listed in Table S8. The shaded areas show nighttime. The data points indicate the average of expression levels (a) and z-scores (b) of five replicates. ZT, hours after dawn. TPM, transcripts per million

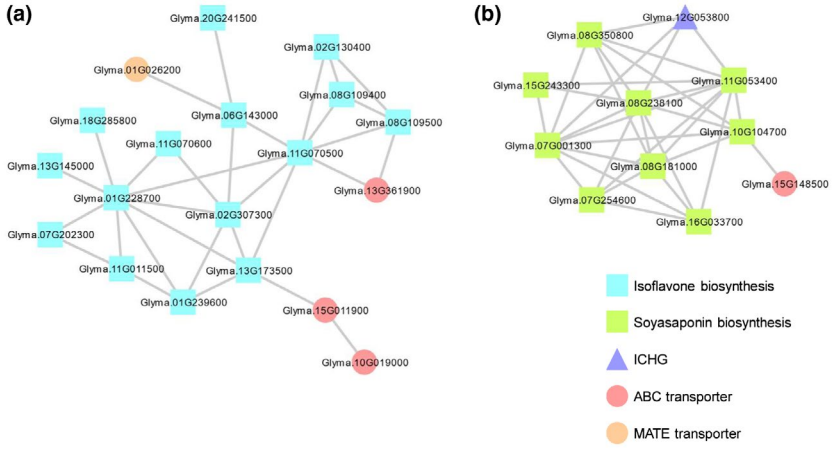


FIGURE 8 Co-expression network analysis in soybean roots. Isoflavone and soyasaponin biosynthetic genes, ICHG, ABC, and MATE transporters were analyzed. (a) Isoflavone and (b) soyasaponin biosynthetic gene clusters. The exhibited relationships have the Spearman's rank correlation coefficient >0.8. Light blue square, isoflavone biosynthetic gene; green square, soyasaponin biosynthetic gene; blue triangle, ICHG gene; red circle, ABC transporter gene; orange circle, MATE transporter gene. ABC, ATP-binding cassette transporter; ICHG, isoflavone conjugate-hydrolyzing beta-glucosidase; MATE, multidrug and toxic compound extrusion transporter

such as chalcone reductase (*CHR*), chalcone synthase (*CHS*), isoflavone synthase (*IFS*), and 2-hydroxyisoflavanone dehydratase (*HIDH*) showed high expression at ZT6 and low expression at night (Figure 5b, Table S6). This variation pattern is inconsistent with that of the flavonoid biosynthetic genes in Arabidopsis (Harmer et al., 2000), which were highly expressed at night under constant light conditions after 7 days of culture in a 12-hr light/dark condition. The expression levels of genes encoding β-Amyrin synthase (*BAS*), cytochrome P450 (*CYP*) for triterpenes, and UDP-glucuronosyltransferase (*UGT*) involved in soyasaponin biosynthesis (Krishnamurthy et al., 2019; Sundaramoorthy et al., 2019) increased from ZT18 (0:00 a.m.) to ZT0 (6:00 a.m.) and decreased at ZT6, which was an inverse pattern to that of isoflavone biosynthesis (Figure 6b, Table S7).

The transcription factors of the MYB family play crucial roles in the regulation of isoflavone biosynthesis. GmMYB29, GmMYB102, GmMYB133, GmMYB176, GmMYB280, and GmMYB502 are positive regulators of isoflavone biosynthetic genes (Anguraj Vadivel et al., 2019; Bian et al., 2018; Chu et al., 2017; Sarkar et al., 2019; Yi et al., 2010), while GmMYB29B, GmMYB39, and GmMYB100 negatively regulate those genes (Jahan et al., 2020; Liu et al., 2013; Yan et al., 2015). Among these MYBs, *GmMYB176* showed the highest expression level in the roots (Figure 7, Table S8). *GmMYB176* exhibited a remarkable diurnal variation, with a higher expression level at ZT0 and ZT6 than at ZT12 (Figure 7, Table S8), indicating the roles of this MYB member in the induction of isoflavone biosynthetic genes during the daytime.

3.3 | Co-expression network of isoflavone and soyasaponin metabolism-related genes

The transporters engaged in the accumulation or secretion of both isoflavones and soyasaponins in soybeans have not been

TABLE 1 Co-expressed biosynthetic and transporter genes in soybean roots (Spearman correlation coefficient >0.8). The genes are exhibited in Figure 8.

Cluster	GeneID	Category	Symbol	Description
Isoflavone	Glyma.10G019000	ABC Transporter	ABCC	ABC transporter C family member 4-like
	Glyma.13G361900	ABC Transporter	ABCG	Pleiotropic drug resistance 1
	Glyma.15G011900	ABC Transporter	ABCG	Pleiotropic drug resistance 1
	Glyma.01G026200	MATE Transporter	GmMATE3	Protein DETOXIFICATION
	Glyma.01G228700	Isoflavone Biosynthesis	GmCHS7	Chalcone synthase 7
	Glyma.01G239600	Isoflavone Biosynthesis	HIDH	2-Hydroxyisoflavanone dehydratase
	Glyma.02G130400	Isoflavone Biosynthesis	GmCHS10	Chalcone synthase 10
	Glyma.06G143000	Isoflavone Biosynthesis	GmCHI4A, CHI4A	Chalcone isomerase 4A
	Glyma.07G202300	Isoflavone Biosynthesis	IFS1	Isoflavone synthase 1
	Glyma.08G109400	Isoflavone Biosynthesis	GmCHS1	Chalcone synthase 1
	Glyma.08G109500	Isoflavone Biosynthesis	CHS9	Chalcone synthase 9
	Glyma.11G011500	Isoflavone Biosynthesis	CHS8	Chalcone synthase 8
	Glyma.11G070500	Isoflavone Biosynthesis	IFR3	Isoflavone reductase
	Glyma.11G070600	Isoflavone Biosynthesis	IFR4	NmrA-like family domain-containing protein
	Glyma.13G173500	Isoflavone Biosynthesis	IFS2	2-Hydroxyisoflavanone synthase
	Glyma.18G285800	Isoflavone Biosynthesis	GmCHR5	Chalcone reductase CHR5
	Glyma.20G241500	Isoflavone Biosynthesis	CHI1A, CHI1	Chalcone--flavonone isomerase 1A
soyasaponin	Glyma.15G148500	ABC Transporter	ABCC	ABC transporter C family member 14-like
	Glyma.12G053800	Isoflavone Secretion	GmICHG	Isoflavone conjugate-specific beta-glucosidase
	Glyma.07G001300	Soyasaponin Biosynthesis	GmBAS1	Beta-amyrin synthase
	Glyma.07G254600	Soyasaponin Biosynthesis	UGT73F2, UGT73F4	Glucosyltransferase
	Glyma.08G181000	Soyasaponin Biosynthesis	UGT91H4	Soyasaponin III rhamnosyltransferase
	Glyma.08G238100	Soyasaponin Biosynthesis	CYP72A61	Hydroxylates the C-22 of β-amyrin or other intermediates
	Glyma.08G350800	Soyasaponin Biosynthesis	CYP93E1	Hydroxylates the C-24 of β-amyrin or other intermediates
	Glyma.10G104700	Soyasaponin Biosynthesis	UGT91H9	Putative glycosyltransferase UGT91H9
	Glyma.11G053400	Soyasaponin Biosynthesis	UGT73P2	Soyasapogenol B glucuronide galactosyltransferase
	Glyma.15G243300	Soyasaponin Biosynthesis	CYP72A69	Hydroxylates the C-21 of β-amyrin or other intermediates
	Glyma.16G033700	Soyasaponin Biosynthesis	UGT73K	UDP-glycosyltransferase UGT73K

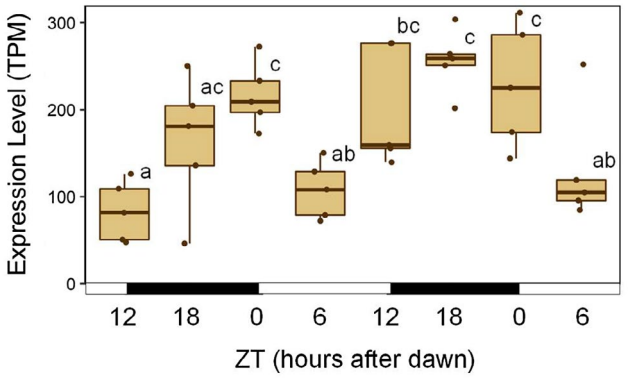


FIGURE 9 Diurnal variation of the ICHG gene in soybean roots. Each boxplot was constructed by five replicates. The individual dots indicate raw data. The outliers were identified using the 1.5*IQR (interquartile range) rule. Tukey's HSD test was used for statistical analysis ($p < 0.05$). ICHG, isoflavone conjugate-hydrolyzing beta-glucosidase; TPM, transcripts per million

reported to date. In this study, co-expression network analysis was performed using Spearman's correlation coefficient threshold value of 0.8 to explore the candidate genes responsible for isoflavone and soyasaponin transport. Isoflavone biosynthetic genes displayed a separated co-expression network from those of soyasaponin biosynthesis (Figure 8). The isoflavone biosynthetic gene cluster included three genes coding for ABC transporters (*Glyma.10G019000*, *Glyma.13G361900*, and *Glyma.15G011900*), and a MATE-type transporter gene *Glyma.01G026200* (Figure 8a, Table 1). Moreover, the cluster of soyasaponin biosynthetic genes only contained an ABC transporter gene, *Glyma.15G148500* (Figure 8b, Table 1). When co-expression network analysis was performed using a Spearman's correlation coefficient threshold value of 0.7, the isoflavone biosynthetic gene cluster contained five additional ABC transporter genes, namely *Glyma.01G008200*, *Glyma.03G101000*, *Glyma.07G233900*, *Glyma.13G043800*, and *Glyma.20G242000*, whereas the soyasaponin biosynthetic gene cluster included four more ABC transporter genes, namely *Glyma.04G069800*, *Glyma.08G101500*, *Glyma.19G021500*, and *Glyma.19G184300* (Figure S2, Table S9). The correlation analysis using publicly open transcriptome data has recently become available in SoyCSN for soybean (Wang et al., 2019). The co-expression of the ABC transporter genes (*Glyma.10G019000*, *Glyma.13G043800*, *Glyma.13G361900*, and *Glyma.15G011900*) with CHS7 (*Glyma.01G228700*) and IFS1 (*Glyma.07G202300*), and the co-expression of two other ABC transporter genes (*Glyma.15G148500* and *Glyma.19G021500*) with BAS1 (*Glyma.07G001300*) and CYP93E1 (*Glyma.08G350800*) were also obtained from SoyCSN (Wang et al., 2019). These findings suggest that the ABC transporter genes are co-expressed with the biosynthetic genes of isoflavone or soyasaponin not only in diurnal variations but also in the symbiosis or other tissues. It is thus expected that those transporters mediate in the vacuolar accumulation and secretion into the rhizosphere of isoflavones or soyasaponins.

The coordinated gene expression for biosynthesis and transport has been reported in several studies. For instance, MtMATE1 of *Medicago truncatula* was reported as an epicatechin 3'-*O*-glucoside transporter identified from the co-expression with its biosynthetic genes (Zhao & Dixon, 2009). A glucosinolate transporter 1 (GTR1) in Arabidopsis was identified as jasmonoyl-isoleucine and gibberellin transporter based on its co-expression with jasmonate biosynthetic genes (Saito et al., 2015). Furthermore, *Catharanthus roseus* CrNPF2.9 was identified by co-expression analysis of the biosynthetic genes of monoterpene indole alkaloids (Payne et al., 2017), and saffron (*Crocus sativus*) CsABCC4a was revealed as a transporter for crocins by narrowing down the candidate transporter genes that were highly expressed in pistils (Demurtas et al., 2019). These reports prompted us to genetically and biochemically investigate candidate transporter genes for isoflavone and soyasaponin transport further.

ICHG is grouped within the soyasaponin biosynthetic gene cluster, although it hydrolyzes isoflavone glucosides and not soyasaponins, presumably (Suzuki et al., 2006). Nevertheless, the expression level of *ICHG* in the roots from ZT18 to ZT0 was high, whereas it was low at ZT6 (Figure 9), which was consistent with the pattern of the soyasaponin biosynthetic genes (Table 1, Figures 6 and 8). This could be a coincidence because *ICHG* is the most downstream gene in the secretion of isoflavones and could be expressed later than those for isoflavone biosynthesis, while there remains a possibility that ICHG hydrolyzes the glucoside linkage of soyasaponins in the apoplast.

3.4 | Diurnal variation of isoflavones and soyasaponins in roots and root exudates

The gene expression in isoflavone and soyasaponin biosynthesis displayed diurnal variations in the roots. We analyzed the contents of these specialized metabolites in both the roots and root exudates using LC-MS/MS. The daidzein content was lowest at ZT0 and increased from ZT6 to ZT18 (Figure 10a), followed by the highest expression of isoflavone biosynthetic genes at ZT6 (Figure 5b). This temporal difference between gene expression and metabolite accumulation was consistent with the flavonoid biosynthesis in Arabidopsis (Nakabayashi et al., 2017). The contents of its glycosides, daidzin, and malonyldaidzin, differed among time points, but did not have a variation pattern. The contents of genistein and its glucosides were about 5- to 10-fold lower than those of daidzein and its glucosides (Figure 10a). Genistein and genistin contents exhibited similar trends with that of daidzein, while malonylgenistin did not show apparent diurnal variations (Figure 10a). In contrast to the accumulation of isoflavone glucosides in the roots, the aglycone daidzein was the predominant isoflavone form found in root exudates as observed previously (Sugiyama et al., 2016). The amount of daidzein and genistein in root exudates did not show

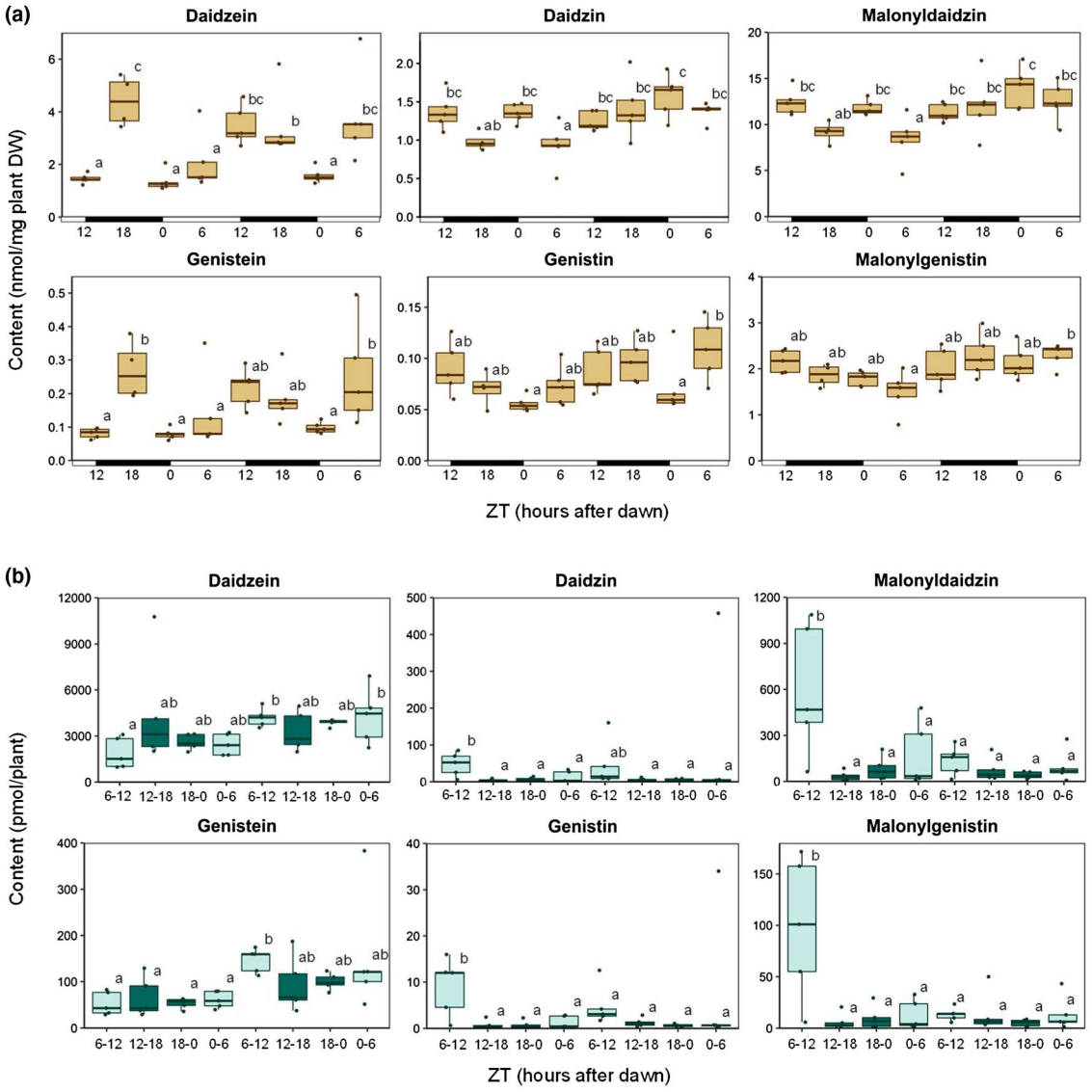


FIGURE 10 Isoflavone content in soybean roots and root exudates. Isoflavone content in (a) soybean roots and (b) root exudates. The brown boxplots exhibit root content. Pale blue and dark green boxplots show root exudate content in the light and dark time. Each boxplot was constructed by five replicates except for the first ZT18 of the root content, which lacked one replicate due to a technical error. The individual dots indicate raw data. The outliers were identified using the 1.5*IQR (interquartile range) rule. Tukey's HSD test was used for statistical analysis ($p < 0.05$)

clear diurnal variations (Figure 10b). In contrast, the contents of glucosides, such as daidzin, malonyldaidzin, genistin, and malonylgenistin, showed a tendency to increase during ZT6 to ZT12 (Figure 10b), which is in concordance with the decreased *ICHG* expression at ZT6 (Figure 9). These findings implicate the possible involvement of two modes in the secretion of isoflavones to the rhizosphere, that is, one for the secretion of aglycones and another for the secretion of glucosides, because daidzein content constantly remained at its highest among isoflavones even at ZT6 when *ICHG* expression was suppressed.

We also determined the contents of soyasaponins using LC-MS/MS. We focused on two major soyasaponins in root exudates of soybean, soyasaponin Ab and soyasaponin Bb, and their aglycones, soyasapogenol A and soyasapogenol B (Tsuno et al., 2018), due to the limited availability of authentic samples for soyasaponins. The root content of soyasaponin Bb was low at ZT0 but was significantly increased at ZT6 and maintained until ZT18 (Figure 11a). The root content of soyasaponin Ab did not exhibit a clear diurnal variation pattern. In root exudates, contents of soyasaponin Ab and Bb slightly varied but showed no apparent diurnal pattern (Figure 11b). The amount of soyasaponin aglycones in the roots was much lower than that of their glucosides. Soyasapogenol A was not detected. Soyasapogenol B was present in small quantities, and it was high at ZT6 and low at night (Figure 11a). The content of soyasapogenol B in the root exudates was relatively high from ZT0 to ZT6, which would reflect the induction of soyasaponin biosynthetic genes during

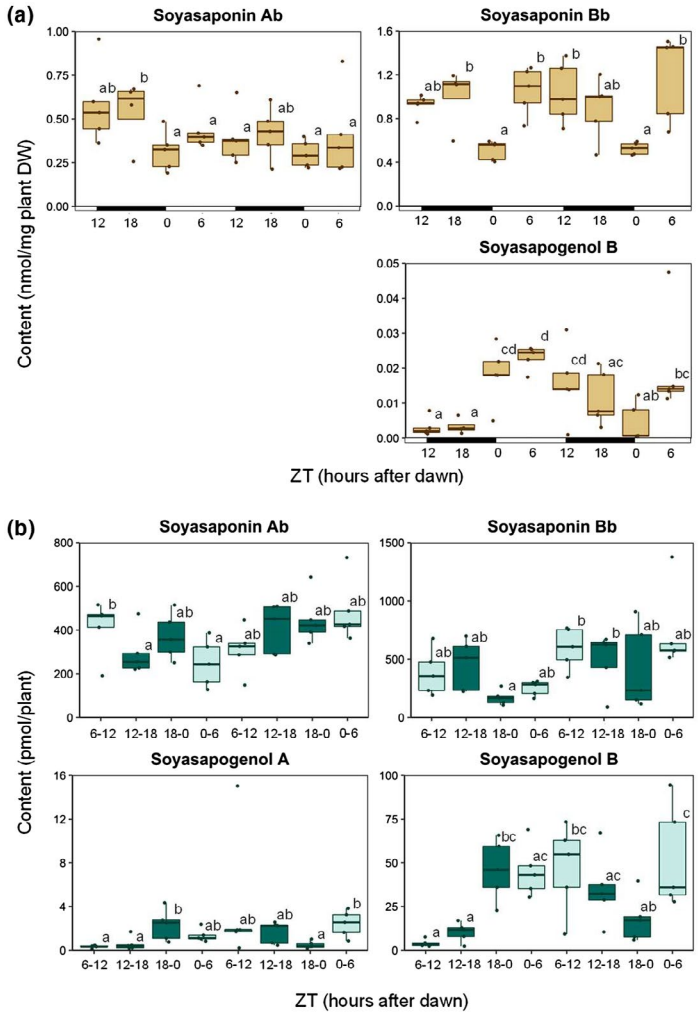


FIGURE 11 Soyasaponin content in soybean roots and root exudates. The brown boxplots exhibit root content. Pale blue and dark green boxplots show root exudate content in the light and dark time. Soyasaponin content in (A) soybean roots and (B) root exudates. Each boxplot was constructed by five replicates except for the first ZT18 of the root content, which lacked one replicate due to a technical error. The individual dots indicate raw data. The outliers were identified using the 1.5*IQR (interquartile range) rule. Soyasapogenol A was not detected in soybean root. Tukey's HSD test was used for statistical analysis ($p < 0.05$)

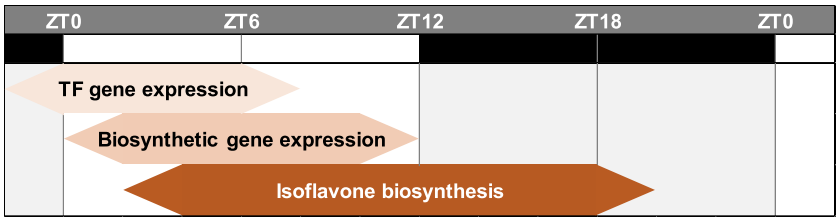


FIGURE 12 Diurnal cycle of isoflavone biosynthesis in soybean roots. ZT, hours after dawn. TF, transcription factor

the night and the slight increase of soyasapogenol B in roots at ZT6 (Figure 11b).

4 | CONCLUSIONS

In this study, we elucidated the diurnal variability of isoflavone biosynthesis in soybean roots. GmMYB176, a major transcription factor of isoflavone biosynthesis, stimulates the isoflavone biosynthetic genes from ZT0 to ZT6, followed by the induction of isoflavone biosynthetic genes at ZT6, the increment of daidzein content from ZT6 to ZT18 (Figure 12). In contrast, soyasaponin biosynthetic genes were highly expressed from ZT18 to ZT0. Co-expression network analysis revealed that the clusters for isoflavone and soyasaponin biosynthesis were separated; that is, the former was induced during daytime, and the latter was activated at nighttime. The network analyses highlighted several genes encoding ABC and MATE transporters, which showed closely correlated expression patterns with isoflavone and soyasaponin biosynthetic genes. These genes are promising candidates for further characterization in future studies.

ACKNOWLEDGMENTS

This study was supported in part by grants from JST-CREST (JPMJCR17O2 to A.S. and Y.A., JPMJCR15O2 to A.J.N.), and JSPS KAKENHI (18H02313 to A.S.); from the Research Institute for

Sustainable Humanosphere (Mission 1). We thank Ms. Keiko Kanai and Ms. Kyoko Y-Mogami for technical assistance. We also thank DASH/FBAS, the Research Institute for Sustainable Humanosphere, Kyoto University, for supporting the institutional setting.

CONFLICT OF INTEREST

The authors have no conflicts of interest directly relevant to the content of this article.

AUTHOR CONTRIBUTIONS

H.M., M.N., and A.S. conceived and designed the research; K.Y. and A.S. supervised the experiments; H.M., M.N., and A.S. conducted plant sampling and extraction; A.J.N. conducted RNA-seq experiments; Y.A. and S.Y. performed RNA-seq data analysis; H.M. and M.N. conducted LC-MS/MS analysis; H.M. and Y.A. constructed the correlation network and performed data analysis; H.M., Y.A., A.J.N. and A.S. wrote the article with contributions of all the authors; A.S. agrees to serve as the author responsible for contact and ensures communication.

RESPONSIBILITIES OF THE AUTHOR FOR CONTACT

The author responsible for the distribution of materials integral to the findings presented in this article in accordance with the policy described in the Instructions for Authors (www.plantdierct.org) is Akifumi Sugiyama (akifumi_sugiyama@rish.kyoto-u.ac.jp).

ORCID

Hinako Matsuda https://orcid.org/0000-0002-5271-6583
Shinichi Yamazaki https://orcid.org/0000-0003-0824-5873
Akifumi Sugiyama https://orcid.org/0000-0002-9643-6639

REFERENCES

Ahmad, M. Z., Li, P., Wang, J., Rehman, N. U., & Zhao, J. (2017). Isoflavone malonyltransferases GmiMaT1 and GmiMaT3 differently modify isoflavone glucosides in soybean (Glycine max) under various stresses. *Frontiers in Plant Science*, *8*, 1–17. https://doi.org/10.3389/fpls.2017.00735

Anguraj Vadivel, A. K., Renaud, J., Kagale, S., & Dhaubhadel, S. (2019). GmMYB176 regulates multiple steps in isoflavonoid biosynthesis in soybean. *Frontiers in Plant Science*, *10*, 562. https://doi.org/10.3389/fpls.2019.00562

Augustin, J. M., Kuzina, V., Andersen, S. B., & Bak, S. (2011). Molecular activities, biosynthesis and evolution of triterpenoid saponins. *Phytochemistry*, *72*, 435–457. https://doi.org/10.1016/j.phytochem.2011.01.015

Bian, S., Li, R., Xia, S., Liu, Y., Jin, D., Xie, X., Dhaubhadel, S., Zhai, L., Wang, J., & Li, X. (2018). Soybean CCA1-like MYB transcription factor GmMYB133 modulates isoflavonoid biosynthesis. *Biochemical and Biophysical Research Communications*, *507*, 324–329. https://doi.org/10.1016/j.bbrc.2018.11.033

Bolger A. M., Lohse M., & Usadel B. (2014). Trimmomatic: a flexible trimmer for Illumina sequence data. *Bioinformatics*, *30*, (15), 2114–2120. https://doi.org/10.1093/bioinformatics/btu170

Cheng, Q., Dong, L., Su, T., Li, T., Gan, Z., Nan, H., Lu, S., Fang, C., Kong, L., Li, H., Hou, Z., Kou, K., Tang, Y., Lin, X., Zhao, X., Chen, L., Liu, B., & Kong, F. (2019). CRISPR/Cas9-mediated targeted mutagenesis of GmLHY genes alters plant height and internode length in soybean. *BMC Plant Biology*, *19*, 562. https://doi.org/10.1186/s12870-019-2145-8

Chu, S., Wang, J., Zhu, Y., Liu, S., Zhou, X., Zhang, H., Wang, C.-E., Yang, W., Tian, Z., Cheng, H., & Yu, D. (2017). An R2R3-type MYB transcription factor, GmMYB29, regulates isoflavone biosynthesis in soybean. *PLoS Genetics*, *13*, e1006770. https://doi.org/10.1371/journal.pgen.1006770

de Brito, F. R., & Martinoia, E. (2018). The vacuolar transportome of plant specialized metabolites. *Plant and Cell Physiology*, *59*, 1326–1336. https://doi.org/10.1093/pcp/pcy039

Demurtas, O. C., Francisco, R., Diretto, G., Ferrante, P., Frusciante, S., Pietrella, M., Aprea, G., Borghi, L., Feeney, M., Frigerio, L., Coricello, A., Costa, G., Alcaro, S., Martinoia, E., & Giuliano, G. (2019). ABCC transporters mediate the vacuolar accumulation of crocins in saffron stigmas. *The Plant Cell*, *31*, 2789–2804. https://doi.org/10.1105/tpc.19.00193

Dobin A., Davis C. A., Schlesinger F., Drenkow J., Zaleski C., Jha S., Batut P., Chaisson M., & Gingeras T. R. (2013). STAR: ultrafast universal RNA-seq aligner. *Bioinformatics*, *29*, (1), 15–21. https://doi.org/10.1093/bioinformatics/bts635.

Fujimatsu, T., Endo, K., Yazaki, K., & Sugiyama, A. (2020). Secretion dynamics of soyasaponins in soybean roots and effects to modify the bacterial composition. *Plant Direct.*, *4*, e00259. https://doi.org/10.1002/pld3.259.

Fukushima, A., Kusano, M., Nakamichi, N., Kobayashi, M., Hayashi, N., Sakakibara, H., Mizuno, T., & Saito, K. (2009). Impact of clock-associated Arabidopsis pseudo-response regulators in metabolic coordination. *Proceedings of the National Academy of Sciences of the United States of America*, *106*, 7251–7256. https://doi.org/10.1073/pnas.0900952106.

Gil, K.-E., & Park, C.-M. (2019). Thermal adaptation and plasticity of the plant circadian clock. *New Phytologist*, *221*, 1215–1229. https://doi.org/10.1111/nph.15518

Goodspeed, D., Liu, J. D., Chehab, E. W., Sheng, Z., Francisco, M., Kliebenstein, D. J., & Braam, J. (2013). Postharvest circadian entrainment enhances crop pest resistance and phytochemical cycling. *Current Biology*, *23*, 1235–1241. https://doi.org/10.1016/j.cub.2013.05.034

Grundy, J., Stoker, C., & Carré, I. A. (2015). Circadian regulation of abiotic stress tolerance in plants. *Frontiers in Plant Science*, *6*, 648. https://doi.org/10.3389/fpls.2015.00648

Harmer, S. L., Hogenesch, J. B., Straume, M., Chang, H. S., Han, B., Zhu, T., Wang, X., Kreps, J. A., & Kay, S. A. (2000). Orchestrated transcription of key pathways in Arabidopsis by the circadian clock. *Science (80-)*, *290*, 2110–2113. https://doi.org/10.1126/science.290.5499.2110

Hendel-Rahmanim, K., Masci, T., Vainstein, A., & Weiss, D. (2007). Diurnal regulation of scent emission in rose flowers. *Planta*, *226*, 1491–1499. https://doi.org/10.1007/s00425-007-0582-3

Hughes, M. E., Hogenesch, J. B., & Kornacker, K. (2010). JTK-CYCLE: An efficient nonparametric algorithm for detecting rhythmic components in genome-scale data sets. *Journal of Biological Rhythms*, *25*, 372–380. https://doi.org/10.1177/0748730410379711.

Jahan, M. A., Harris, B., Lowery, M., Infante, A. M., Percifield, R. J., & Kovinich, N. (2020). Glyceollin transcription factor GmMYB29A2 regulates soybean resistance to Phytophthora sojae. *Plant Physiology*, *183*, 530–546. https://doi.org/10.1104/pp.19.01293.

Kamitani, M., Kashima, M., Tezuka, A., & Nagano, A. J. (2019). Lasy-Seq: A high-throughput library preparation method for RNA-Seq and its application in the analysis of plant responses to fluctuating temperatures. *Scientific Reports*, *9*, 1–14. https://doi.org/10.1038/s41598-019-43600-0

Kosslak, R. M., Bookland, R., Barkei, J., Paaren, H. E., & Appelbaum, E. R. (1987). Induction of Bradyrhizobium japonicum common nod genes by isoflavones isolated from Glycine max. *Proceedings of the National Academy of Sciences*, *84*, 7428–7432. https://doi.org/10.1073/pnas.84.21.7428

Krishnamurthy, P., Fujisawa, Y., Takahashi, Y., Abe, H., Yamane, K., Mukaiyama, K., Son, H.-R., Hiraga, S., Kaga, A., Anai, T., Tsukamoto, C., & Ishimoto, M. (2019). High-throughput screening and characterization of a high-density soybean mutant library elucidate the biosynthesis pathway of triterpenoid saponins. *Plant and Cell Physiology*, *60*, 1082–1097. https://doi.org/10.1093/pcp/pcz025

Li, B., & Dewey, C. N. (2011). RSEM: Accurate transcript quantification from RNA-Seq data with or without a reference genome. *BMC Bioinformatics*, *12*, 1–16. https://doi.org/10.1186/1471-2105-12-323

Li, F., Zhang, X., Hu, R., Wu, F., Ma, J., Meng, Y., & Fu, Y. (2013). Identification and Molecular Characterization of FKF1 and GI Homologous Genes in Soybean. *PLoS One*, *8*, e79036. https://doi.org/10.1371/journal.pone.0079036

Liew, L. C., Singh, M. B., & Bhalla, P. L. (2017). A novel role of the soybean clock gene LUX ARRHYTHMO in male reproductive development. *Scientific Reports*, *7*, 1–16. https://doi.org/10.1038/s41598-017-10823-y

Liu, J., Li, Y., Wang, W., Gai, J., & Li, Y. (2016). Genome-wide analysis of MATE transporters and expression patterns of a subgroup of MATE genes in response to aluminum toxicity in soybean. *BMC Genomics*, *17*, 1–15. https://doi.org/10.1186/s12864-016-2559-8

Liu, X., Yuan, L., Xu, L., Xu, Z., Huang, Y., He, X., Ma, H., Yi, J., & Zhang, D. (2013). Over-expression of GmMYB39 leads to an inhibition of the isoflavonoid biosynthesis in soybean (Glycine max. L). *Plant Biotechnol Rep*, *7*, 445–455. https://doi.org/10.1007/s11816-013-0283-2

Locke, A. M., Slattery, R. A., & Ort, D. R. (2018). Field-grown soybean transcriptome shows diurnal patterns in photosynthesis-related processes. *Plant Direct*, *2*, e00099. https://doi.org/10.1002/pld3.99

Lu, H., McClung, C. R., & Zhang, C. (2017). Tick Tock: circadian regulation of plant innate immunity. *Annual Review of Phytopathology*, *55*, 287–311. https://doi.org/10.1146/annurev-phyto-080516-035451

Marcolino-Gomes, J., Rodrigues, F. A., Fuganti-Pagliarini, R., Bendix, C., Nakayama, T. J., Celaya, B., Molinari, H. B. C., de Oliveira, M. C. N., Harmon, F. G., & Nepomuceno, A. (2014). Diurnal oscillations of soybean circadian clock and drought responsive genes. *PLoS One*, *9*, e86402. https://doi.org/10.1371/journal.pone.0086402

Mishra A. K., Choi J., Rabbee M. F., & Baek K.-H. (2019). In Silico Genome-Wide Analysis of the ATP-Binding Cassette Transporter Gene Family in Soybean (Glycine max L.) and Their Expression Profiling. *BioMed Research International*, *2019*, 1–14. https://doi.org/10.1155/2019/8150523.

Monaco, M. K., Stein, J., Naithani, S., Wei, S., Dharmawardhana, P., Kumari, S., Amarasinghe, V., Youens-Clark, K., Thomason, J., Preece, J., Pasternak, S., Olson, A., Jiao, Y., Lu, Z., Bolser, D., Kerhornou, A., Staines, D., Walts, B., Wu, G., … Ware, D. (2014). Gramene 2013: comparative plant genomics resources. *Nucleic Acids Research*, *42*(D1), D1193–D1199. https://doi.org/10.1093/nar/gkt1110

Mylona, P., Owatworakit, A., Papadopoulou, K., Jenner, H., Qin, B., Findlay, K., Hill, L., Qi, X., Bakht, S., Melton, R., & Osbourn, A. (2008). Sad3 and Sad4 are required for saponin biosynthesis and root development in oat. *The Plant Cell*, *20*, 201–212. https://doi.org/10.1105/tpc.107.056531.

Nagasaka, S., Takahashi, M., Nakanishi-Itai, R., Bashir, K., Nakanishi, H., Mori, S., & Nishizawa, N. K. (2009). Time course analysis of gene expression over 24 hours in Fe-deficient barley roots. *Plant Molecular Biology*, *69*, 621–631. https://doi.org/10.1007/s11103-008-9443-0.

Nakabayashi, R., Mori, T., Nishizawa, T., & Saito, K. (2017). Temporal lag between gene expression and metabolite accumulation in flavonol biosynthesis of Arabidopsis roots. *Phytochemistry Letters*, *22*, 44–48. https://doi.org/10.1016/j.phytol.2017.09.001

Nakayama, T., Takahashi, S., & Waki, T. (2019). Formation of flavonoid metabolons: functional significance of protein-protein interactions and impact on flavonoid chemodiversity. *Frontiers in Plant Science*, *10*, 821. https://doi.org/10.3389/fpls.2019.00821

Nguyen, N. H., & Lee, H. (2016). MYB-related transcription factors function as regulators of the circadian clock and anthocyanin biosynthesis in *Arabidopsis*. *Plant Signal Behav*, *11*, e1139278. https://doi.org/10.1080/15592324.2016.1139278.

Okutani, F., Hamamoto, S., Aoki, Y., Nakayasu, M., Nihei, N., Nishimura, T., Yazaki, K., & Sugiyama, A. (2020). Rhizosphere modelling reveals spatiotemporal distribution of daidzein shaping soybean rhizosphere bacterial community. *Plant, Cell & Environment*, *43*(4), 1036–1046. https://doi.org/10.1111/pce.13708

Payne, R. M. E., Xu, D., Foureau, E., Teto Carqueijeiro, M. I. S., Oudin, A., Bernonville, T. D. D., Novak, V., Burow, M., Olsen, C.-E., Jones, D. M., Tatsis, E. C., Pendle, A., Ann Halkier, B., Geu-Flores, F., Courdavault, V., Nour-Eldin, H. H., & O'Connor, S. E. (2017). An NPF transporter exports a central monoterpene indole alkaloid intermediate from the vacuole. *Nature Plants*, *3*, 1–9. https://doi.org/10.1038/nplants.2016.208

Pérez-García, P., Ma, Y., Yanovsky, M. J., & Mas, P. (2015). Time-dependent sequestration of RVE8 by LNK proteins shapes the diurnal oscillation of anthocyanin biosynthesis. *Proceedings of the National Academy of Sciences of the United States of America*, *112*, 5249–5253. https://doi.org/10.1073/pnas.1420792112

R Core Team (2019). R: A language and environment for statistical computing. Vienna, Austria: R Foundation for Statistical Computing. https://www.R-project.org/

Römheld, V., & Marschner, H. (1990). Genotypical differences among graminaceous species in release of phytosiderophores and uptake of iron phytosiderophores. In *Plant Soil*, *123*, 147–153). https://doi.org/10.1007/BF00011260

Saito, H., Oikawa, T., Hamamoto, S., Ishimaru, Y., Kanamori-Sato, M., Sasaki-Sekimoto, Y., Utsumi, T., Chen, J., Kanno, Y., Masuda, S., Kamiya, Y., Seo, M., Uozumi, N., Ueda, M., & Ohta, H. (2015). The jasmonate-responsive GTR1 transporter is required for gibberellin-mediated stamen development in Arabidopsis. *Nature Communications*, *6*, 1–11. https://doi.org/10.1038/ncomms7095

Sarkar, M. A. R., Watanabe, S., Suzuki, A., Hashimoto, F., & Anai, T. (2019). Identification of novel MYB transcription factors involved in the isoflavone biosynthetic pathway by using the combination screening system with agroinfiltration and hairy root transformation. *Plant Biotechnology*, *36*, 241–251. https://doi.org/10.5511/plantbiotechnology.19.1025a

Sawai, S., & Saito, K. (2011). Triterpenoid biosynthesis and engineering in plants. *Frontiers in Plant Science*, *2*, 25. https://doi.org/10.3389/fpls.2011.00025

Schmutz, J., Cannon, S. B., Schlueter, J., Ma, J., Mitros, T., Nelson, W., Hyten, D. L., Song, Q., Thelen, J. J., Cheng, J., Xu, D., Hellsten, U., May, G. D., Yu, Y., Sakurai, T., Umezawa, T., Bhattacharyya, M. K., Sandhu, D., Valliyodan, B., … Jackson, S. A. (2010). Genome sequence of the palaeopolyploid soybean. *Nature*, *463*, 178–183. https://doi.org/10.1038/nature08670

Seo, P. J., & Mas, P. (2015). STRESSing the role of the plant circadian clock. *Trends in Plant Science*, *20*, 230–237. https://doi.org/10.1016/j.tplants.2015.01.001

Shannon, P, Markiel, A, Ozier, O, Baliga, NS, Wang, JT, Ramage, D, Amin, N, Schwikowski, B, & Ideker, T (2003). Cytoscape: A software Environment for integrated models of biomolecular interaction networks. *Genome Res*, *13*, 2498–2504.

Srivastava, D., Shamim, M., Kumar, M., Mishra, A., Maurya, R., Sharma, D., Pandey, P., & Singh, K. N. (2019). Role of circadian rhythm in plant system: An update from development to stress response. *Environmental and Experimental Botany*, *162*, 256–271. https://doi.org/10.1016/j.envexpbot.2019.02.025

Subramanian, S., Graham, M. Y., Yu, O., & Graham, T. L. (2005). RNA interference of soybean isoflavone synthase genes leads to silencing in tissues distal to the transformation site and to enhanced

susceptibility to Phytophthora sojae. *Plant Physiology*. *137*, 1345–1353. https://doi.org/10.1104/pp.104.057257.

Sugiyama, A. (2019). The soybean rhizosphere: Metabolites, microbes, and beyond—A review. *Journal of Advanced Research*, *19*, 67–73. https://doi.org/10.1016/j.jare.2019.03.005

Sugiyama, A., Shitan, N., & Yazaki, K. (2007). Involvement of a soybean ATP-binding cassette-type transporter in the secretion of genistein, a signal flavonoid in legume-Rhizobium symbiosis. *Plant Physiology*, *144*, 2000–2008.

Sugiyama, A., Yamazaki, Y., Yamashita, K., Takahashi, S., Nakayama, T., & Yazaki, K. (2016). Developmental and nutritional regulation of isoflavone secretion from soybean roots. *Bioscience, Biotechnology, and Biochemistry*, *80*, 89–94. https://doi.org/10.1080/09168451.2015.1062714

Sundaramoorthy, J., Park, G. T., Komagamine, K., Tsukamoto, C., Chang, J. H., Lee, J., Kim, J. H., Seo, H. S., & Song, J. T. (2019). Biosynthesis of DDMP saponins in soybean is regulated by a distinct UDP-glycosyltransferase. *New Phytologist*, *222*, 261–274.

Suzuki, H., Takahashi, S., Watanabe, R., Fukushima, Y., Fujita, N., Noguchi, A., Yokoyama, R., Nishitani, K., Nishino, T., & Nakayama, T. (2006). An isoflavone conjugate-hydrolyzing β-glucosidase from the roots of soybean (Glycine max) seedlings: Purification, gene cloning, phylogenetics, and cellular localization. *Journal of Biological Chemistry*, *281*, 30251–30259.

Takagi, S. I., Nomoto, K., & Takemoto, T. (1984). Physiological aspect of mugineic acid, a possible phytosiderophore of graminaceous plants. *Journal of Plant Nutrition*, *7*, 469–477. https://doi.org/10.1080/01904168409363213

Tsuno, Y., Fujimatsu, T., Endo, K., Sugiyama, A., & Yazaki, K. (2018). Soyasaponins: A new class of root exudates in soybean (Glycine max). *Plant and Cell Physiology*, *59*, 366–375. https://doi.org/10.1093/pcp/pcx192

Wagner, G. P., Kin, K., & Lynch, V. J. (2012). Measurement of mRNA abundance using RNA-seq data: RPKM measure is inconsistent among samples. *Theory in Biosciences*, *131*, 281–285. https://doi.org/10.1007/s12064-012-0162-3

Wang, J., Hossain, M. S., Lyu, Z., Schmutz, J., Stacey, G., Xu, D., & Joshi, T. (2019). SoyCSN: Soybean context-specific network analysis and prediction based on tissue-specific transcriptome data. *Plant Direct*, *3*(9), e00167. https://doi.org/10.1002/pld3.167

Wang, Y. U., Yuan, L. I., Su, T., Wang, Q., Gao, Y. A., Zhang, S., Jia, Q., Yu, G., Fu, Y., Cheng, Q., Liu, B., Kong, F., Zhang, X., Song, C.-P., Xu, X., & Xie, Q. (2020). Light- and temperature-entrainable circadian clock in soybean development. *Plant, Cell and Environment*, *43*, 637–648. https://doi.org/10.1111/pce.13678

Wu, G., Anafi, R. C., Hughes, M. E., Kornacker, K., & Hogenesch, J. B. (2016). MetaCycle: An integrated R package to evaluate periodicity in large scale data. *Bioinformatics*, *32*, 3351–3353. https://doi.org/10.1093/bioinformatics/btw405

Xu, W., Yang, R., Li, M., Xing, Z., Yang, W., Chen, G., Guo, H., Gong, X., Du, Z., Zhang, Z., Hu, X., Wang, D., Qian, Q., Wang, T., Su, Z., & Xue, Y. (2011). Transcriptome phase distribution analysis reveals diurnal regulated biological processes and key pathways in rice flag leaves and seedling leaves. *PLoS One*, *6*(3), e17613. https://doi.org/10.1371/journal.pone.0017613

Yan, J., Wang, B., Zhong, Y., Yao, L., Cheng, L., & Wu, T. (2015). The soybean R2R3 MYB transcription factor GmMYB100 negatively regulates plant flavonoid biosynthesis. *Plant Molecular Biology*, *89*, 35–48. https://doi.org/10.1007/s11103-015-0349-3

Yazaki, K., Sugiyama, A., Morita, M., & Shitan, N. (2008). Secondary transport as an efficient membrane transport mechanism for plant secondary metabolites. *Phytochemistry Reviews*. Springer, 513–524. https://doi.org/10.1007/s11101-007-9079-8

Yi, J., Derynck, M. R., Li, X., Telmer, P., Marsolais, F., & Dhaubhadel, S. (2010). A single-repeat MYB transcription factor, GmMYB176, regulates CHS8 gene expression and affects isoflavonoid biosynthesis in soybean. *The Plant Journal*, *62*(6), 1019–1034. https://doi.org/10.1111/j.1365-313X.2010.04214.x

Yoo, D. C., Hara, T., Fujita, N., Waki, T., Noguchi, A., Takahashi, S., & Nakayama, T. (2013). Transcription analyses of GmICHG, a gene coding for a β-glucosidase that catalyzes the specific hydrolysis of isoflavone conjugates in Glycine max (L.) Merr. *Plant Science*, *208*, 10–19. https://doi.org/10.1016/j.plantsci.2013.03.006

Zhao, J., & Dixon, R. A. (2009). MATE transporters facilitate vacuolar uptake of epicatechin 3′-O-glucoside for proanthocyanidin biosynthesis in medicago truncatula and arabidopsis. *The Plant Cell*, *21*, 2323–2340. https://doi.org/10.1105/tpc.109.067819.

Zhao, J., Huhman, D., Shadle, G., He, X. Z., Sumner, L. W., Tang, Y., & Dixon, R. A. (2011). MATE2 mediates vacuolar sequestration of flavonoid glycosides and glycoside malonates in Medicago truncatula. *The Plant Cell*, *23*, 1536–1555. https://doi.org/10.1105/tpc.110.080804.

SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

How to cite this article: Matsuda H, Nakayasu M, Aoki Y, et al. Diurnal metabolic regulation of isoflavones and soyasaponins in soybean roots. *Plant Direct*. 2020;00:1–15. https://doi.org/10.1002/pld3.286