Isolation and characterization of a novel glucosyltransferase involved in production of emodin-6-O-glucoside and rhaponticin in *Rheum palmatum*

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Abstract Anthraquinones are widely distributed in various organisms and known as bioactive ingredients. Some of the anthraquinones accumulate as glycosides in higher plants. Plant secondary product glycosyltransferases (PSPGs) are the well-characterized enzymes producing plant secondary metabolite glycosides. However, PSPGs involved in the formation of anthraquinone glycosides remains unclear. The rhizome of *Rheum palmatum* contains anthraquinones as laxative agents, some of which are accumulated as glucosides. We isolated a glucosyltransferase, *R. palmatum* UDP-glycosyltransferase (RpUGT) 1 from the rhizome of *R. palmatum*, and characterized functionally. RpUGT1 glucosylated emodin yielding emodin-6-O-glucoside, and it also glucosylated rhapontigenin, a compound belonging to stilbenes, yielding rhaponticin. The expression patterns of RpUGT1 and the accumulation of the metabolites revealed that RpUGT1 contributes to the production of these glucosides in *R. palmatum*. These results may provide important information for the substrate recognition of the PSPGs for anthraquinones and stilbenes.

Key words: anthraquinones, plant secondary product glycosyltransferase (PSPG), *Rheum palmatum*, stilbenes.

Introduction Anthraquinones are a large group of natural products with a quinone structure and are accumulated not only in higher plants but in bacteria and fungi (Caro et al. 2012). Anthraquinones have been reported to exhibit various biological activities including antimicrobial, antimalarial, and antioxidative functions (Cao et al. 2017; Duval et al. 2016; Fouillaud et al. 2016; Han et al. 2001). Anthraquinones are classified into emodin and alizarin types according to their biosynthetic pathways. Emodin-type anthraquinones are biosynthesized through the condensation of acetyl-CoA and malonyl-CoA, followed by modification reactions such as hydroxylation, methylation, oxidation, and glycosylation (Gessler et al. 2013) (Figure 1). Dianthrone glycosides such as sennoside A are derived from the polymerization of emodin-type anthraquinone monomers. Some of the emodin-type anthraquinones have been shown to accumulate as glycosides including glucoside, rhamnoside, and apioside in plants (Rosenthal et al. 2014). The content of emodin-8-O-glucoside was shown to be 8-fold higher than that of emodin in dried rhizomes of *Rheum palmatum* (Komatsu et al. 2006). However, the enzymes catalyzing the glycosylation step in the anthraquinone biosynthetic pathway have remained to be characterized. Plant secondary product glycosyltransferases (PSPGs) are the main enzymes for low-molecular glycosides biosynthesis in plants (Caputi et al. 2012; Gachon et al. 2005). To the best of our knowledge, no report characterizing PSPGs that glycosylate anthraquinone aglycones have been published.

Dried rhizomes of *R. palmatum* have been used as a laxative in traditional Asian and European medicine, and its active ingredients are anthraquinones such as emodin, rhein, aloe-emodin, chrysophanol, sennoside A/B, and so on (Cao et al. 2017; Zheng et al. 2013). In this study, we isolated three PSPG cDNAs from the rhizomes of *R. palmatum* using homology-based cloning and identified *R. palmatum* UDP-glycosyltransferase (RpUGT) 1 as an emodin glucosyltransferase. RpUGT1 showed glycosylation activity towards not only anthraquinones but also stilbenes, which are also contained in *R. palmatum*.

Abbreviations: PSPGs, plant secondary product glycosyltransferases; qRT-PCR, quantitative RT-PCR; UGT, UDP-glycosyltransferase.

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Figure 1. Biosynthetic pathway leading to anthraquinone glucosides. PSPG; plant secondary product glucosyltransferase.

Materials and methods

**Plant materials and chemicals**

The *R. palmatum* plant used in this study was obtained from the Research Center for Medicinal Plant Resources, National Institutes of Biomedical Innovation, Health and Nutrition, Japan (Nayoro, Japan). The seeds were germinated under a 14/10-h light/dark photoperiod in a plant chamber at 20–25°C. Emodin was purchased from Sigma-Aldrich (St. Louis, MO, USA). UDP-glucose, resveratrol, and daidzein were from FUJIFILM Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Chrysophanol, piceatannol, rhapontigenin, genistein, and apigenin were from Tokyo Chemical Industry (Tokyo, Japan). Kaempferol was from Santa Cruz Biotechnology (Dallas, TX, USA). Quercetin dihydrate was from AdipoGen Life Sciences (San Diego, CA, USA). Aloe-emodin, emodin-1-O-glucoside, and emodin-8-O-glucoside were from Toronto Research Chemicals (North York, ON, Canada). All other chemicals were of commercial reagent-grade quality.

**cDNA cloning and heterologous expression**

Total RNA was prepared from rhizomes of *R. palmatum* using innuSPEED Plant RNA Kit (Analytik Jena AG, Jena, Germany). cDNA preparation and homology-based PCR cloning were performed as described in previous papers (Asada et al. 2013; Nagatoshi et al. 2011; Yamada et al. 2019). The expression vector pE-SUMO pro3 (LifeSensors, Malvern, PA, USA) was used for heterologous expression. The expression and purification of recombinant proteins were performed as previously described (Yamada et al. 2019). After purification, the recombinant protein was confirmed by SDS-PAGE and the concentration of the recombinant protein was determined by the Bradford method (Bradford 1976). Primers used in cDNA cloning and vector construction are indicated in Supplementary Tables S1 and S2.

**Gene expression analysis**

First-strand cDNAs for quantitative RT-PCR (qRT-PCR) were synthesized from 0.5 µg of total RNA using High-Capacity RNA-to-cDNA™ Kit (Thermo Fisher Scientific, Waltham, MA, USA). Real-time PCR was performed with the StepOne™ real-time system (Thermo Fisher Scientific) using SYBR Select Master Mix (Thermo Fisher Scientific) according to the manufacturer's instructions. Briefly, the reaction mixture consisted of cDNA template, 10 pmol primers, and 10 µl of

**Enzyme assay**

The glucosyltransferase activity was measured as described in the previous papers (Asada et al. 2013; Nagatoshi et al. 2011; Yamada et al. 2019). Briefly, the reaction mixture (total volume of 50 µl) contained 50 mM Tris–HCl (pH 7.5) or glycylglycine–NaOH (pH 8.4), 5 mM UDP-glucose, 1 mM acceptor substrates, and the enzyme preparation. The reaction products were analyzed by HPLC as described in Supplementary Methods. To determine kinetic parameters, enzyme assays were performed in triplicate at various substrate concentrations with 15 µg of the purified enzyme at 30°C for 2 or 4 min. The substrate concentrations used were 50 µM–1 mM emodin or rhapontigenin with UDP-glucose at 10 mM for acceptor kinetics, and 50 µM–1 mM UDP-glucose with emodin at 2.5 mM for donor kinetics. Initial velocity data were visualized by a Hanes–Woolf plot and kinetic parameters were calculated based on linear regression analysis.

**Purification and identification of reaction products**

The reaction products were obtained by a large-scale incubation and purified as described in Supplementary Methods. NMR spectra were recorded on an Agilent Varian VNS500 spectrometer (Santa Clara, CA, USA). Chemical shifts (ppm) were referenced to the residual solvent peaks (δH 3.31 and δC 49.0 for CD3OD). Positive-mode ESITOFMS was obtained on a JEOL JMS-T100LP AccuTOF LC-plus 4G spectrometer (Tokyo, Japan). Emodin-6-O-glucoside: 1H NMR (CD3OD, 500 MHz) δ 7.63 (1H, s, H-4), 7.46 (1H, d 2.5 Hz, H-5), 7.15 (1H, s, H-2), 6.95 (1H, d 2.5 Hz, H-7), 5.13 (1H, d 7.0 Hz, H-1′), 3.91 (1H, dd 12.0, 5.5 Hz, H-6b), 3.56 (1H, m, H-3′), 3.51 (1H, m, H-2′), 3.51 (1H, m, H-3′), 3.44 (1H, m, H-4′) and 13C NMR (CD3OD, 125 MHz) δ 192.4 (C-9), 182.9 (C-10), 166.1 (C-8), 165.7 (C-6), 163.8 (C-5), 150.2 (C-3), 136.7 (C-11), 134.7 (C-14), 125.4 (C-2), 122.0 (C-4), 114.9 (C-13), 112.4 (C-12), 110.5 (C-7), 110.3 (C-5), 101.5 (C-1′), 78.4 (C-5′), 77.8 (C-3′), 74.7 (C-2′), 71.1 (C-4′), 62.3 (C-6′), 22.1 (C-15); ESIMS m/z 455 [M+Na]+; HRESIMS m/z 455.0961 [M+Na]+ (calcd for C21H20O10Na, 455.0954).

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SYBR Select Master Mix in a total volume of 20 µl. The standard PCR condition was as follows: 95°C for 10 min, 40 cycles of 95°C for 15 s, and 60°C for 1 min. Gene-specific primers are listed in Supplementary Table S3.

**Metabolite analysis**

The metabolites of *R. palmatum* plants were measured as described in the previous papers (Yamada et al. 2019). Briefly, fresh plant tissues of *R. palmatum* were ground in liquid nitrogen to a fine powder using a mortar and pestle, and the powdered tissues (0.1 g) were extracted with 1 ml of 70% (v/v) methanol by sonication. The methanol extracts were analyzed by HPLC. The gradient elution program was described in Supplementary Methods. The amounts of metabolite compounds were calculated based on a calibration curve prepared using standards.

**Results**

**Molecular cloning of glucosyltransferase genes from Rheum palmatum**

Unlike other model plants, sequence databases are not available in *R. palmatum*. To explore an anthraquinone glucosyltransferase, we tried comprehensive cloning using the homology-based PCR (Asada et al. 2013; Nagatoshi et al. 2011). PSPGs have highly conserved 44 amino acid sequences defined as PSPG-box in C-terminal region (Caputi et al. 2012). PCR cloning of PSPGs expressed in *R. palmatum* rhizomes was performed using degenerate primers designed from the amino acid sequences within PSPG-box (Nagatoshi et al. 2011). Three full-length cDNAs were cloned as *RpUGT1*–3 (Supplementary Figure S1). These clones were subsequently assigned to UGT73BE14 (*RpUGT1*), UGT72B49 (*RpUGT2*), and UGT71AQ1 (*RpUGT3*) by the UGT Nomenclature Committee. Molecular phylogenetic analysis showed that *RpUGT1* belonged to Group D and *RpUGT2* and 3 belonged to Group E (Figure 2).

**Characterization of recombinant RpUGT1**

The ORFs of the three cDNAs were expressed in *E. coli* (Yamada et al. 2019). Glucosylation activities towards five anthraquinone substrates (aloe-emodin, chrysophanol, emodin, rheochrysidin, and rhein) were screened using the crude enzyme preparations in the presence of UDP-glucose. The crude recombinant *RpUGT1* converted emodin to its glucoside (Figure 3). This product was purified by preparative HPLC and identified as emodin-6-O-glucoside based on 1H-, 13C-, and 2D NMR, and ESITOFMS analyses. No other products were detected upon prolonged incubation. *RpUGT1* didn't show glucosylation activities towards aloe-emodin, chrysophanol, rheochrysidin, or rhein. *RpUGT2* and 3 had no glucosylation activity towards any of these anthraquinones. The glucosyl acceptor specificities of *RpUGT1* were then examined using stilbenes contained in *R. palmatum*, and flavonoids known as substrate of PSPGs belonging to the UGT73 subfamily (Figures 2, 4). *RpUGT1* converted rhapontigenin, daidzein, genistein, apigenin, kaempferol, and quercetin to these glucosides. *RpUGT1* glucosylated a hydroxy group at the 3-position of rhapontigenin to generate rhaponticin. It also glucosylated flavonol compounds including kaempferol and quercetin to multiple products of which only kaempferol-3-O-glucoside and quercetin-3-O-glucoside were tentatively identified by comparison with standard compounds. (Figure 5). *RpUGT1* had no activity towards other stilbenes, resveratrol and piceatannol, showing selective substrate preference of *RpUGT1* for rhapontigenin. Comparison of *RpUGT1* glucosylation activity exhibited approximately 40% relative activity for rhapontigenin compared to emodin (Table 1). *RpUGT1* also showed 80% relative activity for quercetin, but less than 60% for other substrates. The kinetic parameters of *RpUGT1* for emodin and rhapontigenin, using UDP-glucose as a sugar donor substrate, were determined using the affinity-purified protein. The $K_m$ value for emodin was about 0.22 mM, similar to that of rhapontigenin (Table 2).

**Relationship between RpUGT1 expression and concentration of metabolite in Rheum palmatum**

The transcript levels of *RpUGT1* were compared among root, rhizome, petiole, leaf, and seedling tissues by qRT-PCR. As shown in Figure 6A, the *RpUGT1* expression was observed in all the organs. The expression
levels in the above-ground parts were higher than those in the underground parts. The accumulation of emodin, emodin glucosides, rhapontigenin, and rhaponticin in each organ was quantified by HPLC analysis. As shown in Figure 6B, emodin and emodin-8-O-glucoside were found in all the organs, and emodin-1-O-glucoside and emodin-6-O-glucoside were accumulated mostly in the underground parts of *R. palmatum*. Rhapontigenin and rhaponticin were highly accumulated in the roots and rhizomes (Figure 6C). No spatial correlation was found between the expression of *RpUGT1* and the accumulation of emodin-6-O-glucoside.

**Discussion**

In this study, we isolated three cDNA clones *RpUGT1*–3
encoding glucosyltransferases by homology-based cloning from *R. palmatum* and screened the glucosyltransferase activity of the recombinant proteins towards emodin. RpUGT1 (UGT73BE14) catalyzed glucosylation of emodin to emodin-6-O-glucoside, and of rhapontigenin to rhaponticin, respectively. Neither RpUGT2 (UGT72B49) nor RpUGT3 (UGT71AQ1) glucosylated these substrates. RpUGT1 may be involved in the production of these anthraquinone and stilbene glucosides in *R. palmatum*.

Molecular phylogenetic analysis revealed that RpUGT1 belonged to UGT73 subfamily of Group D PSPGs. Various PSPGs belonging to the UGT73 subfamily are known as flavonoid glucosyltransferases (Yonekura-Sakakibara and Hanada 2011). For example, UGT73C6 of *Arabidopsis thaliana* was shown to glucosylate the 7 and 3-positions of flavonol, flavone, and flavonol glycoside in vitro (Jones et al. 2003). UGT73B1 from *A. thaliana* was also shown to glucosylate the 7 and 3-positions of quercetin (Lim et al. 2004). UGT73B23 from strawberry was shown to catalyze glucosylation of a diverse group of phenolic compounds including flavonols, flavanones, naphthols, pyrogallol, epicatechin and catechin (Song et al. 2016). These are consistent with the finding that RpUGT1 (UGT73BE14) showed glucosylation activity towards various flavonoids in vitro. RpUGT1 glucosylated regio-selectively at hydroxyl group of the 6-position of emodin. Because of partial structural similarity between emodin, flavones, and isoflavones, this regio-selectivity of RpUGT1 also seems to show glucosylation activity towards a hydroxyl group at the 7-position of flavonoids (Hirotani et al. 2000). Among the stilbene compounds examined, RpUGT1 glucosylated rhapontigenin but not resveratrol or piceatannol. Glucosylation of resveratrol has been investigated by plant tissue and cell cultures since the nutraceutical function of resveratrol has attracted attention (Härtl et al. 2017; Hüskens et al. 2005; Iwakiri et al. 2013; Ozaki et al. 2012; Weis et al. 2016).

**Table 1.** Relative activity of recombinant RpUGT1.

| Substrate     | Product                     | Relative activity (%) |
|---------------|-----------------------------|-----------------------|
| Emodin        | emodin-6-O-glucoside        | 100 ± 3               |
| Rhapontigenin | rhapontigenin-3-O-glucoside | 37.0 ± 1.2            |
| Daidzein      | daidzein-7-O-glucoside      | 14.0 ± 1.0            |
| Genistein     | genistein-7-O-glucoside     | 55.6 ± 5.3            |
| Apigenin      | apigenin-7-O-glucoside      | 2.70 ± 0.25           |
| Kaempferol    | kaempferol-3-O-glucoside    | 4.97 ± 2.38           |
| Quercetin     | quercetin-3-O-glucoside     | 79.4 ± 29.6           |

RpUGT1 activity was measured as described in Materials and Methods. Specific activity of RpUGT1 for emodin was 62.0 ± 1.6 pkat/mg protein. Data are expressed as mean ± standard deviation in triplicate measurements.

**Table 2.** Kinetic parameters of recombinant RpUGT1 for emodin, rhapontigenin, and UDP-glucose.

| Substrate  | $K_m$ (mM) | $k_{cat}$ ($\times 10^{-3}$ s$^{-1}$) | $k_{cat}/K_m$ ($\times 10^4$ M$^{-1}$ s$^{-1}$) |
|------------|------------|--------------------------------------|-----------------------------------------------|
| Emodin     | 0.22 ± 0.01| 1.9 ± 0.1                            | 8.5 ± 0.7                                     |
| Rhapontigen| 0.32 ± 0.01| 0.6 ± 0.04                           | 1.9 ± 0.04                                    |
| UDP-glucose| 0.63 ± 0.17| 3.1 ± 0.3                            | 4.9 ± 0.6                                     |

RpUGT1 activity was measured as described in Materials and Methods. The alternative substrate concentrations used for UDP-glucose or emodin were 10 and 2.5 mM, respectively, for saturation curves. Data are expressed as mean ± standard deviation in triplicate measurements.

Figure 5. Glucosylation activity of RpUGT1. HPLC analysis of recombinant RpUGT1 incubated with rhapontigenin (A), daizein (B), genistein (C), apigenin (D), kaempferol (E), and quercetin (F). RpUGT1 activity was measured as described in Materials and Methods.
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2006). However, cDNA cloning of glycosyltransferase for resveratrol from resveratrol-producing plants has so far been unsuccessful. The molecular insight of substrate recognition and specificity of RpUGT1 towards rhapontigenin might be useful for the isolation of resveratrol glucosyltransferases.

To our knowledge, this is the first report on the presence of emodin-6-O-glucoside in *R. palmatum*. However, the distribution of emodin-6-O-glucoside among plant organs was not consistent with the *RpUGT1* expression (Figure 6A). Especially, emodin-8-O-glucoside was accumulated more abundantly than other emodin glucosides in the petiole and leaf (Figure 6B). These results suggest that another PSPG with higher affinity for emodin than RpUGT1 might be involved in the production of emodin-8-O-glucoside in the petiole and leaf of *R. palmatum* (Figure 6B). Moreover, unidentified transporters might be involved in the translocation of emodin glucosides from the petiole and leaf to the root and rhizome. Furthermore, RpUGT1 and emodin might be localized in different cellular compartment in the petiole and leaf of *R. palmatum*. In any cases, isolation, characterization, and expression analysis of the PSPG(s) specific to emodin-8-O-glucosylation await further investigation. It may be effective to perform RNA-seq comparative analysis using the organs with differential accumulation of emodin glucoside as petioles in *R. palmatum*.

In summary, we isolated a glucosyltransferase, RpUGT1, catalyzing glucosylation of emodin, rhapontigenin, and flavonoids from *R. palmatum* although accumulation of flavonoid glucosides in rhizomes of *R. palmatum* has not been reported. RpUGT1 might contribute to the production of emodin-6-O-glucoside and rhaponticin in *R. palmatum*. The present results may provide the useful information for isolation of the PSPGs specific to anthraquinones or stilbenes.

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