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Discovery of New Biocatalysts for the Glycosylation of Terpenoid Scaffolds

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Abstract: The synthesis of terpenoid glycosides typically uses a chemical strategy since few biocatalysts have been identified that recognise these scaffolds. In this study, a platform of 107 recombinant glycosyltransferases (GTs), comprising the multigene family of small molecule GTs of *Arabidopsis thaliana* have been screened against a range of model terpenoid acceptors to identify those enzymes with high activity. Twenty-seven GTs are shown to glycosylate a diversity of mono-, sesqui- and diterpenes, such as geraniol, perillyl alcohol, artemisinic acid and retinoic acid. Certain enzymes showing substantial sequence similarity recognise terpenoids containing a primary alcohol, irrespective of the linear or cyclical structure of the scaffold; other GTs glycosylate scaffolds containing secondary and tertiary alcohols; the carboxyl group of other terpenoids also represents a feature that is recognized by GTs previously known to form glucose esters with many different compounds. These data underpin the rapid prediction of potential biocatalysts from GT sequence information. To explore the potential of GTs as biocatalysts, their use for the production of terpenoid glycosides was investigated by using a microbial-based whole-cell biotransformation system capable of regenerating the cofactor, UDP-glucose. A high cell density fermentation system was shown to produce several hundred milligrams of a model terpenoid, geranyl-glucoside. The activities of the GTs are discussed in relation to their substrate recognition and their utility in biotransformations as a complement or alternative to chemical synthesis.

Keywords: biotransformations · enzymes · glycosides · glycosylation · terpenoids

Introduction

Terpenoids represent one of the major classes of natural products with more than 40,000 different chemical structures described in the literature.[1] The structures are oxygen-containing derivatives of terpenes, such as alcohols, aldehydes, ketones and carboxylic acids. In nature, synthesis occurs through either the mevalonic acid pathway or the 2-C-methyl-D-erythritol 4-phosphate/1-deoxy-D-xylulose 5-phosphate pathway (MEP/DOXP pathway).[2–4] In terms of utility, terpenoids are used in many different applications, from health care and pharmaceutical uses to colour, flavour and fragrance compounds in food and cosmetics. Terpenoids such as taxol already have established utility in cancer treatment[5] and the sesquiterpenoid artemisinin is the active pharmaceutical ingredient of choice for malaria treatment.[6]

Terpenoids extracted from plants typically occur as glycosides, with the sugars linked to the active groups OH and/or COOH.[7] Glycosylation influences the chemical properties, biological activity and utility of terpenoids. For example, increased hydrophilicity impacts on emulsification and detergent properties.[8,9] It is the glycosides rather than the aglycones that are extensively used as fragrance ingredients[10,11] and as food flavours.[12–14] The potential of using terpenoid glycoconjugates as slow-release aroma compounds in applications in which the glycosidic bond is cleaved either enzymatically or chemically has also been highlighted.[15]

Whilst terpenoid glycosides exist as natural products, their levels in plant extracts are often limited. This has led to an increasing interest in synthesis. Several terpenoid glycosides, such as menthyl-glucoside, linalyl-glucoside, borneoyl-glucoside and terpineoyl-glucoside have been synthesized chemically by using the Koenigs–Knorr reaction involving diverse catalysts under a range of conditions.[16–19] A limitation in the use of chemical synthesis for glycosides of terpenoids with secondary or tertiary alcohols has been observed in

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these works. This arose from steric hindrance of the glycosylation site with consequent low product yields.[16,19]

Typically, current approaches in chemical synthesis involve organic solvents and heavy-metal catalysts. Whilst the research on chemical glycosylation continues, nevertheless there is an increasing demand for natural products, directly extracted or manufactured by using biotechnologies that are environmentally benign. For example, recent legislation in the US and EU have classified "natural" flavours as only those that are extracted from natural sources or involve bioprocesses with precursors isolated from nature.[20] This classification has led to a preference for synthetic methods involving biotransformations rather than the use of chemistry.

There are a number of alternative glycosylation tools that can be applied in biotransformation strategies. These include the use of glycosidases,[21–24] glycosynthases[25] and glycosyltransferases (GTs)[26] and in each case their use can be combined with chemical synthesis to increase the flexibility of the approach.[27–29] In the context of terpenoid glycosylation, there have been no reports in the literature on the use of glycosynthases and only one report on the use of a glycosidase.[24] As yet, relatively few GTs have been identified that recognise terpenoid acceptors. In this context, by using a functional genomics strategy, we have identified the entire multigene family of GTs encoded in the genome of the model plant *Arabidopsis thaliana* that recognises small-molecule scaffolds. These enzymes belong to the Family 1 GTs in the CAZy classification.[39] GTs in this family catalyse inverting glycosylation reactions, and typically use UDP-α-glucose as a sugar donor to form β-glucoside products.[26] Other nucleotide sugars, such as UDP-α-galactose, UDP-α-glucuronic acid, UDP-α-xylose and UDP-β-rhamnose, are also known to be recognised by this family of GTs. We have used the plant GT sequences to establish a unique platform which can be screened for catalytic activities towards natural and non-natural scaffolds in vitro. The platform provides the basis for identifying and optimising novel biocatalysts, particularly for those reactions in which a chemical approach is neither possible nor appropriate.

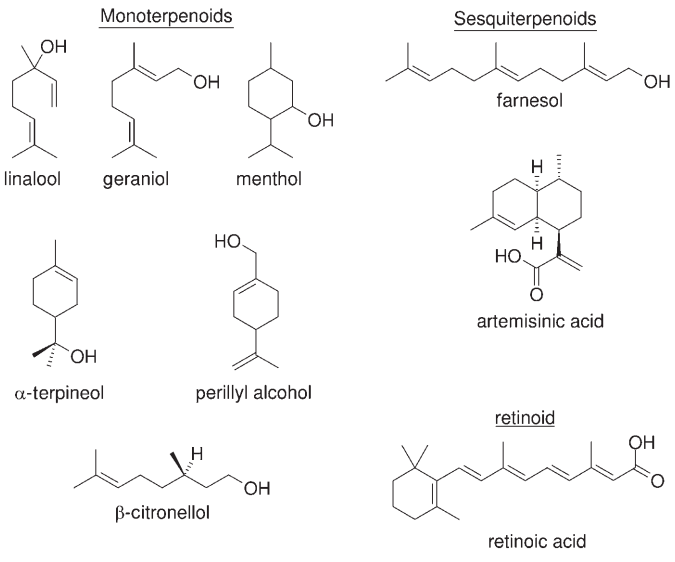
In this study, we have used our platform of 107 recombinant GTs to explore their activity towards a number of terpenoid scaffolds. The data provide insights into sequence-activity relationships and a means to identify relevant biocatalysts for natural and non-natural terpenoid scaffolds. We have also demonstrated that the biocatalysts can be used to prepare research-scale quantities of defined glycosides by means of a whole-cell biotransformation route.

Results and Discussion

Screen of *Arabidopsis* GT activities towards model terpenoids: To gain an insight into the activity of *Arabidopsis* GTs towards terpenoid scaffolds, members of the entire GT multigene family were first screened against model compounds in batches of six recombinant enzymes (Table 1).

Table 1. GT grouping in the initial screen.

Group	GTs
1	82A1, 92A1, 84A3, 84A4, 84A2, 84A1
2	84B2, 84B1, 75B2, 75B1, 75D1, 75C1
3	74E1, 74E2, 74C1, 74D1, 74F1, 74F2
4	74B1, 86A1, 86A2, 87A2, 87A1, 83A1
5	76B1, 76E5, 76E6, 76E3, 76E4, 76E1
6	76E2, 76E7, 76E11, 76E12, 76D1, 76C4
7	76C2, 76C3, 76C5, 76C1, 76F2, 76F1
8	85A3, 85A1, 85A2, 85A7, 85A5, 85A4
9	78D1, 78D3, 78D2, 71B8, 71B7, 71B6
10	71B5, 71B2, 71B1, 71D1, 71D2, 71C1
11	71C2, 88A1, 72E2, 72E3, 73E1, 72D1
12	72C1, 72B2, 72B3, 72B1, 71C5, 71C4
13	71C3, 73C3, 73C4, 73C2, 73C5, 73C6
14	73C1, 73B2, 73B3, 73B1, 73B4, 76E9
15	73B5, 73D1, 73C7, 90A1, 90A2, 90A4
16	89B1, 89C1, 89A2, 79B11, 79B10, 79B9
17	79B8, 79B7, 79B4, 79B5, 79B6, 79B2
18	79B3, 79B1, 91A1, 91C1, 91B1, GST



The screen used UDP-[^{14}C]-glucose as the sugar donor, which was incubated with each batch of enzymes and terpenoid acceptor molecule, followed by analysis of the reaction mixture by using a TLC system known to separate the aglycones from glycosides. The presence of radioactively labelled compounds was detected by using phosphor-imaging screens and provided an indication of putative product formation. The data for two model terpenoids, geraniol and farnesol, are illustrated in Figure 1A and B, respectively. For example, in the geraniol screen, incorporation of [^{14}C]-glucose into putative products could be detected in reaction mixtures from batch groups 4, 6, 8, 11, 13 and 14. In the farnesol screen, only four groups displayed potential activity (groups 8, 11, 13 and 14). The comprehensive screening results of GT batches against other terpenoid scaffolds are provided in Figure S1 in the Supporting Information.

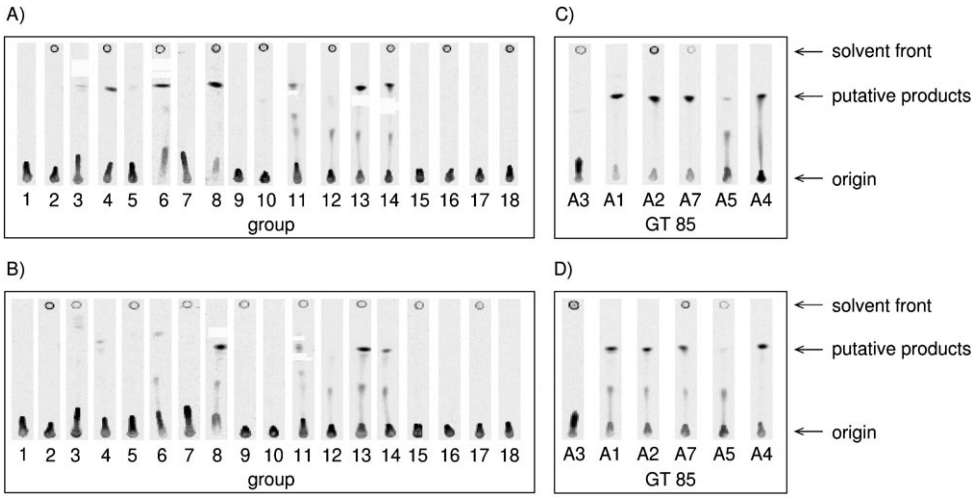


Figure 1. TLC analysis of the reaction mixtures from the initial activity screen. Two examples of the activity screen are shown, A) geraniol screen and B) farnesol screen. The groups that displayed putative activity were further analysed with the individual enzymes assayed towards the substrates. C) and D) show the detailed analysis of group 8 towards geraniol and farnesol, respectively.

When putative products were detected in the batch screen, individual GTs from those batches were assayed separately against the acceptors and the reaction mixtures analysed as before. In Figure 1C, the activities of the six GTs comprising group 8 are illustrated; this shows that only four of the six enzymes were potentially active towards geraniol. Similarly, in Figure 1D, when batch group 8, displaying activity towards farnesol was further analysed, incorporation of [^{14}C]-glucose into putative products was found in only four reaction mixtures.

In total, this screening strategy revealed that twenty-seven *Arabidopsis* GTs, from a total number of 107 enzymes, displayed potential activity in vitro towards the nine model terpenoids. The activities were restricted to groups D, E, G, H and L of the phylogenetic tree of the GT1 multigene family of *Arabidopsis*.[30] These data are summarized in Table 2, which provides the GT, the phylogenetic group of the tree within which it is located and its activity towards the acceptor. The activities from this screen are defined as the percentage incorporation of [^{14}C] glucose into putative product and are colour-coded as low (1–10%), medium (10–40%) or

Table 2. *Arabidopsis* GT activities towards model terpenoids.[a]

GT	Group	Terpineol	Linalool	Citronellol	Menthol	Geraniol	Perillyl alcohol	Farnesol	Artemisinic acid	Retinoic acid
76E2	H									
76E11	H									
76E12	H									
76D1	H									
84A3	L									
84A4	L									
84B2	L									
84B1	L									
75B2	L									
75B1	L									
75D1	L									
74E2	L									
74F1	L									
74F2	L									
74D1	L									
74B1	L									
85A1	G									
85A5	G									
85A4	G									
85A2	G									
85A7	G									
73C3	D									
73C5	D									
73C6	D									
73C1	D									
71C2	E									
88A1	E									

[a] : 1–10%; : 10–40%; : >40%.

high (>40%). The products from these reactions were confirmed by HPLC-MS, as described in the next section.

Characterization of terpenoid glycosides: To investigate the nature of the putative products in the reaction mixtures from the initial screen of *Arabidopsis* GTs, assays were repeated with unlabelled UDP-glucose and products were analysed by using HPLC-MS. Two representative examples are illustrated in Figure 2 in which the reaction mixtures from an incubation of GT 73C5 with geraniol and GT 85A1 with farnesol were analysed. The products were confirmed to be geranyl-glucoside (Figure 2A and B) and farnesyl-glucoside (Figure 2C and D). The positive ion-mode MS spectrum of geranyl-glucoside gave two ions at *m*/*z*: 334.23 and 339.19, which corresponded to the NH_4^+ and the Na^+ adducts, respectively (Figure 2A). Further in-source fragmentation resulted in the loss of the glucose moiety and gave rise to the product ion at *m*/*z*: 137.15 (Figure 2B), which indicated that a fragmentation had occurred at the glucosidic linkage. The positive ion-mode MS spectrum of the farnesyl-glucoside was very similar to that of geranyl-glucoside, showing the NH_4^+ adduct (*m*/*z*: 402.16) and Na^+ adduct (*m*/*z*: 407.11) (Figure 2C) and the in-source fragmentation leading to the breakage of the glucosidic bond and formation of the aglycone product ion at *m*/*z*: 205.22 (Figure 2D). Examples of HPLC-MS confirmation of other terpenoid products produced by GTs from the in vitro reactions are shown in Figure S2 in the Supporting Information.

In summary, the presence of a radioactively labelled product in the reaction mixtures from the initial TLC screen could be confirmed to represent the corresponding glycoside of each terpenoid acceptor. The fragmentation data indicated that *O*-glucosides were formed.

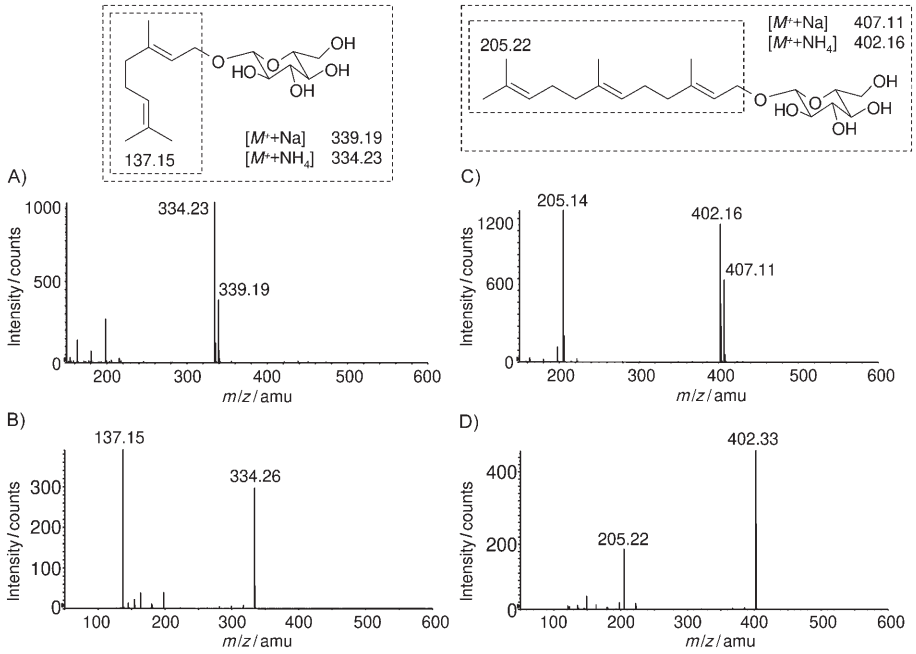


Figure 2. HPLC-MS analysis. A) and B) geranyl-glucoside. C) and D) farnesyl-glucoside.

Whole-cell biotransformations of terpenoids into glycosides: GTs are known to carry out whole-cell biotransformations in microbial cells to form glycosides of the substrates added to the culture medium.[26] The chemicals applied to this approach in earlier studies were all phenolic compounds.[26] This study is the first to investigate the utility of GTs as biocatalysts to synthesize research-scale quantities of terpenoid glycosides. Each of the model terpenoid scaffolds was provided as a substrate in a whole-cell biocatalysis system, which consisted of *E. coli* expressing a recombinant GT with known activity towards the specific scaffold. Control batches, in which *E. coli* expressed only the empty vector, were used to confirm that the glycoside products were formed by the recombinant GTs. After 18 h of incubation, the glycosides produced through these processes were purified from the culture medium by using preparative HPLC, and were analyzed by HPLC-MS for purity assessment and NMR spectroscopy for identity confirmation. The analyses showed that the purity of the products was >95%. These purified glycosides were used to generate the standard curves for glycoside quantification. An example of these analyses is provided in Figure S3 and Table S1 in the Supporting Information.

Samples from the whole-cell biotransformations were reanalyzed and quantified by using the standard curves, and the process yields and productivity are summarized in Table 3. Thus, after 18 h of incubation, the molar yields of substrates converted into products ranged from 5% for farnesyl-glucoside to 52.8% for menthyl-glucoside. Essentially,

Table 3. Small-scale (50 mL) whole-cell biotransformations after 18 h incubation.

Product	GT	Total yield			Productivity
		[μmol]	[mg]	[%]	[μg mL^{-1} h^{-1}]
geranyl-glucoside	73C5	10.7	3.4	21.4	3.8
citronellyl-glucoside	73C5	3.6	1.1	7.2	1.3
farnesyl-glucoside	73C5	2.3	0.9	4.6	1.0
terpineoyl-glucoside	73C5	15.8	5.0	31.6	5.6
perillyl-glucoside	73C5	10.3	3.2	20.7	3.6
linalyl-glucoside	73C5	10.7	3.4	21.4	3.8
menthyl-glucoside	73C5	26.4	8.4	52.8	9.7
artemisinic acid glucose ester	75D1	9.3	3.7	18.6	4.1

whole-cell biocatalysis using *Arabidopsis* GTs was successful in producing milligram quantities of glycosides of each of the monoterpenoid scaffolds and two model sesquiterpenoids. This whole-cell system was not able to produce the glucose ester of retinoic acid; this may due to the instability of the compound in the culture medium.[31]

To test the possibility of a larger-scale production of terpenoid glycosides in a more controlled process, glycosylation of geraniol by *E. coli* expressing GT 73C5 was studied in a 3L bioreactor. During the transformation process 247 mg of geranyl-glucoside were formed from 764 mg of geraniol added to the bioreactor. The highest conversion was 41% (0.62 mmol, 196 mg of product), obtained at 6 h of the production phase (Figure 3).

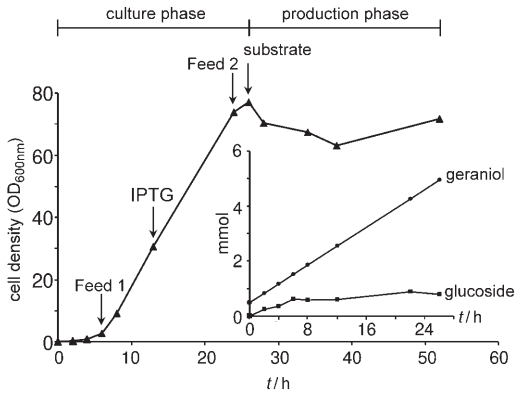


Figure 3. Production of geranyl-glucoside through fed-batch fermentation. The fermentation process was divided into a growth phase and a production phase. Bacterial cells were grown to a high density in the culture phase, and were fed with geraniol substrate during the production phase. The quantities of geranyl-glucoside produced in the fermenter and the amount of geraniol added during the production phase are plotted in the insert figure.

The relation of substrate recognition and protein primary structures: In this study, we explored the terpenoid features recognized by the *Arabidopsis* GTs by using scaffolds with different structures (linear and cyclic), sizes (C_{10}-C_{20}) and functional groups (primary, secondary and tertiary alcohol, and carboxyl group). Through the evaluation of their in vitro activity, twenty-seven enzymes from five different phylogenetic groups were identified as potential terpenoid biocatalysts. The activity screen highlighted several distinct features. First, and most significantly, the entire group G recognized terpenoids with primary alcohols as substrates irrespective of linear or cyclic structure. This activity can be found in other groups but is restricted to only a few members, for example 4 out of 13 GTs in group D and 4 out of 19 GTs in group H. The preference of group G for primary alcohols is also reflected in our earlier study reporting the glycosylation of the primary alcohol on the N^6 side chain of *trans*-zeatin and dihydrozeatin (adenine scaffolds) by GT 85A1.[32] Thus, the group G enzymes can be prioritized candidates for future activity screening towards compounds carrying primary alcohol groups. Second, for the glycosylation of terpenoid scaffolds containing secondary and tertiary alcohols, the major activities were detected in groups D and H. GTs from these groups are also known to glycosylate secondary alcohols present in a diverse range of compounds, such as benzoates,[33] phenylpropanoids[34] and flavonoids.[35] The broad substrate range of these GTs enhances their potential utility in biocatalysis involving natural products or non-natural scaffolds carrying secondary or tertiary alcohols. Third, enzymes in one of the phylogenetic groups, group L, all shared the ability to form ester linkages. This ability of group L enzymes to recognize carboxyl groups has been previously reported for other scaffolds, such as benzoates and phenylpropanoids,[34] and has again been demonstrated in this study towards terpenoids (i.e. artemisinic acid and retinoic acid).

The aim of this study has been to establish a foundation for the rapid prediction and selection of biocatalyst candidates towards this class of substrates. This advanced knowledge will aid future research in the identification of GT biocatalysts for terpenoid glycoside production.

Conclusion

This study has demonstrated that plant GTs can be identified that recognize terpenoid scaffolds and can be used to produce a diverse range of natural product glycosides. The biotransformation route of the synthesis is simple, involves a single-step and is a useful complement or alternative to the chemical route of production.

In comparison to the chemical route, enzymatic synthesis does not require organic solvents and heavy-metal catalysts. Biotransformation thereby allows terpenoid glycosides to be made by using a "green" process that can meet regulations for products to be classified as "natural".[20] Also, it is significant that GTs have been identified in this study that glycosylate terpenoids with secondary and tertiary alcohols. Use of these biocatalysts overcomes the recognized difficulties in chemical glycosylation of terpenoids, such as menthol and linalool.[16,18,19]

The bioprocess can be readily scaled-up by using microbial whole-cell biocatalysis systems that regenerate sugar donors and enable good yields of glycosides without the requirement for cofactor addition.[35–37] Our data underpin the future design of novel routes of terpenoid glycoside synthesis for those applications that increasingly require green chemistry, such as those in the fine chemical, flavour, fragrance and food additive sectors.

Experimental Section

Model substrates: (±)-Linalool, menthol, farnesol (mixed isomers), (±)-β-citronellol, (S)-(−)-perillyl alcohol, terpineol (mixed isomers), geraniol and *all-trans*-retinoic acid were purchased from Sigma-Aldrich. Artemisinic acid (arteannuic acid) was purchased from Apin Chemicals.

Preparation of recombinant GTs: Recombinant GTs were expressed as fusion proteins with glutathione-*S*-transferase (GST) attached to the N-terminus of the GTs, by using the GST gene fusion vector pGEX-2T (Amersham Biotech). The recombinant proteins were prepared as previously described[30] and quantified by using the Bradford method with bovine serum albumin as the reference.

GT activity assay: In the initial screen, each reaction mixture (20 μL) contained Tris-HCl (100 mM, pH 7.0), UDP-[^{14}C] glucose (3.7 μM, 11.6 GBq/mmol, Amersham), substrate (1 mM) and a pool of six different recombinant GTs (300 ng each). The reaction mixture was incubated at 30 °C for 2 h and stored at −20 °C before TLC analysis. The reaction mixtures that showed positive signals in the TLC analysis were further analyzed by using the same conditions but were incubated with one enzyme (300 ng) in each assay to identify the GTs active in the initial screen. The active enzymes were also assessed by HPLC methods. The reaction mixture (200 μL) for HPLC analysis contained Tris-HCl (100 mM, pH 7.0), UDP-glucose (2.5 mM), substrate (1 mM) and enzyme (1 μg). The reaction was incubated at 30 °C for 2 h and stored at −20 °C prior to HPLC analysis.

TLC analysis of the GT reaction mixture: TLC analysis was performed on Silica gel 60 TLC plates in a solvent system consisting of ethylacetate/acetone/dichloromethane/methanol/water (20:15:6:5:4, v/v/v/v/v). TLC plates were dried and exposed to phosphor-imaging screens (Molecular Dynamics) for 24 h. The screens were read by using a Molecular Imager FX scanner (BioRad) supplied with Quantity One software (BioRad). The amount of UDP-[^{14}C]-glucose transferred by the enzymes to the substrates was calculated by using a regression equation obtained by analysing UDP-[^{14}C]-glucose standards (0.008–0.555 kBq) with the TLC method described above.

HPLC analysis of the GT reaction mixture: Reverse-phase HPLC (SpectraSYSTEM HPLC system and UV6000LP photodiode array detector, ThermoQuest) was carried out by using a Columbus 5μ C_{18} column (250×4.6 mm, Phenomenex) at a flow rate of 1 mL min^{-1} with a linear gradient of solvent A (methanol, 10–50 %) against solvent B (10 mM ammonium acetate) over 10 min, followed by a linear gradient A (50–100 %) over 20 min against B. The column was then washed with A (100 %) for 5 min. Chromatography was monitored at 210 nm.

HPLC-MS analysis of glycosides: Glycosides formed in the enzymatic reactions were confirmed by using an Agilent 1100 Series HPLC system (Agilent Technologies) coupled with a QSTAR hybrid quadrupole-TOF mass spectrometer (Applied Biosystems). HPLC was performed with a Columbus 5 μ C_{18} column (150×3.2 mm, Phenomenex) at a flow rate of 0.5 mL min^{-1} by following the gradient described in the previous section. MS analysis was carried out in a positive-ion mode. The mass spectrometer was operated with a capillary voltage of 4.5 kV, by using nitrogen as the drying gas at 200 °C. Ion-source fragmentation was achieved by using declustering potentials of 10, 20 and 30 V. Full-scan spectra were recorded by scanning from *m*/*z*: 150 to 600 for MS analysis and from *m*/*z*: 50–600 for MS-MS studies. Total ion current and ion traces for specific [M^++H], [M^++NH_4] and [M^++Na] adduct ions were used to detect the compounds. MS and MS-MS analysis were performed simultaneously by using the Information Dependent Acquisition (IDA) software tool. Data were collected and processed by using ANALYST QS (Applied Biosystems) software.

Production of terpenoid glycosides by whole-cell biotransformations: *E. coli* BL21 cultures for whole-cell biotransformations were grown overnight at 37 °C in 2×YT medium (50 mL) containing ampicillin (50 μg mL^{-1}). Cells were harvested by centrifugation (5,000×g, 5 min), and suspended in M9 minimal medium (50 mL, pH 7.0) containing glucose (1 %) to an $OD_{600\,nm}$ reading of 1.0. IPTG (1 mM) was added to the bacterial cultures and terpenoid substrate (50 μmol) was added 6 h later. The biotransformation processes were carried out for 3 days at 25 °C in a shaker set at 150 rpm. Samples were harvested at intervals and analyzed by HPLC-MS for the presence of glycosides in the medium.

Purification of glycosides from the culture medium: The culture broths containing the glucoside products were harvested by centrifugation (5000×g, 5 min). The supernatants were applied to an Amberlite XAD-2 column (100×15 mm). The column was then washed with 3 column volumes of water, followed by 3 column volumes of methanol (25 %). To elute the glycosides, the column was titrated with methanol (70 %). The eluents were evaporated to dryness, suspended in methanol (50 %, 2 mL) and subjected to preparative HPLC (Äkta purifier 10, Pharmacia) equipped with a fraction collector. Preparative HPLC was performed by using a Luna 5 μ C_{18} column (250×10 mm, Phenomenex) with a linear gradient of solvent A (methanol, 10–100 %) against solvent B (10 mM ammonium acetate) over 25 min at a flow rate of 3 mL min^{-1}. The column was then washed with A (100 %) for 5 min. Chromatography was monitored at 210 nm. Fractions containing the glycosides were pooled, evaporated to dryness and stored at 4 °C.

Quantification of terpenoid glycosides by HPLC-MS: The quantification of glycosides was carried out by interpolation of the peak areas obtained by HPLC-MS with the standard curves. The standard curves were prepared by HPLC-MS analysis of glycosides (0.1–1000 ng/50 μL) produced by whole-cell transformations and purified by preparative HPLC (>95 % purity by HPLC). The HPLC-MS analysis for glucoside quantification was performed with a SpectraSYSTEM HPLC system (ThermoQuest) coupled with a LCQ ion trap mass spectrometer equipped with a APCI source (Finnigan MAT). The chromatographic separation was performed by using a Columbus 5 μ C_{18} (150×4.6 mm, Phenomenex) column, at a flow rate of 0.5 mL min^{-1} with a linear gradient of solvent A (methanol, 10–50 %) against solvent B (10 mM ammonium acetate) over 10 min, followed by a linear gradient A (50–100 %) against B over 20 min. The column was then washed with A (100 %) for 5 min and re-equilibrated for 5 min. The mass spectrometry analysis was performed in positive mode (source voltage, 5.09 kV; source temperature, 450 °C; nebulizing sheath gas flow rate 63.48; auxiliary gas flow rate 29.27; capillary voltage 10.24 V; capillary temperature 514.6 °C). The instrument was operated at unit resolution in full-scan MS-MS mode, scanning the product ion spectrum from *m*/*z*: 50–800. The LCQ was interfaced to a computer workstation running Xcalibur 2.0 software.

Fermentation: Fed-batch fermentation was performed in a stirrer tank bioreactor (3 L, Applikon). The fermentation broth (1 L) contains KH_2PO_4 (2 g), K_2HPO_4 (5.79 g), $(NH_4)_2SO_4$ (0.5 g), glucose (5 g), $MgSO_4{\cdot}7H_2O$ (0.31 g), trace element solution (1.25 mL), vitamin solution (1.25 mL) ampicillin (50 μg mL^{-1}).[38] Prior to inoculation, the bioreactor was conditioned (37 °C, 150 rpm, 50 % dO_2 relative to air, pH 7.4) and the pH was maintained by using NH_4OH (20 %) and H_2SO_4 (2 N). An overnight culture was prepared by inoculating 2×YT (30 mL) containing ampicillin (50 μg mL^{-1}) with a freshly streaked colony and incubating at 37 °C with agitation (180 rpm). The overnight culture (10 mL) was added to the conditioned fermenter at the beginning of the fermentation process. After approximately 8 h, when the glucose in the bioreactor was completely consumed, the culture was fed started Feed 1 (60 % glucose, 1.2 % $MgSO_4$, 0.6 % $(NH_4)_2SO_4$, 15 mL L^{-1} trace element solution and 15 mL L^{-1} vitamin solution) at a rate of 200 mL day^{-1}. After 12 h of culturing time, the dO_2 was set to 25 % and the temperature at 25 °C. IPTG (1 mM) was then added to the culture. After 25 h post-inoculation, the feed was changed to Feed 2 (4 % glycerol, 1.2 % $MgSO_4$, 15 mL L^{-1} trace element solution and 15 mL L^{-1} vitamin solution) at a rate of 200 mL day^{-1} and 1 h later geraniol was added into the bioreactor at a rate of 26.3 mg h^{-1}. Samples were harvested at intervals for HPLC analysis.

Acknowledgements

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