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Exploiting the aglycon promiscuity of glycosyltransferase Bs-YjiC from *Bacillus subtilis* and its application in synthesis of glycosides

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ABSTRACT

Glycosylation is a prominent biological mechanism for structural and functional diversity of natural products. Uridine diphosphate-dependent glycosyltransferases with aglycon promiscuity are generally recognised as effective biocatalysts for glycodiversification of natural products for practical applications. In this study, the aglycon promiscuity of glycosyltransferase Bs-YjiC from *Bacillus subtilis* 168 was explored. Bs-YjiC, with uridine diphosphate glucose (UDPG) as sugar donor, exhibited robust capabilities to glycosylate 19 structurally diverse types of drug-like scaffolds with regio- and stereospecificities and form *O*-, *N*- and *S*-linkage glycosides. Twenty-four glycosides of 17 aglycons were purified from scale-up reactions using Bs-YjiC as a biocatalyst, and their structures were confirmed by nuclear magnetic resonance spectra. Furthermore, a one-pot reaction by coupling Bs-YjiC to sucrose synthase from *Arabidopsis thaliana* was applied to glycosylate pterostilbene. Without adding the costly UDPG as sugar donor, 9 mM (3.8 g/L) pterostilbene 4′-*O*-*β*-glucoside was obtained by periodic feeding of pterostilbene. These results suggest the aglycon promiscuity of Bs-YjiC and demonstrate its significant application prospect in biosynthesis of valuable natural products.

1. Introduction

Glycosylation is one of the most common and important modifications in nature because it plays a central role in cellular communication, molecular recognition, chemical defence and orchestration of life in general (Bruyn et al., 2015; Thibodeaux et al., 2009). Sugar moieties, as part of many valuable natural products, exert considerable influence on their solubility, stability, bioactivity, taste perception, target recognition and pharmacological properties (Dai et al., 2015; Liang et al., 2016; Xie et al., 2014). Furthermore, variation in glycosylation patterns, such as the number, and position and type of sugar moieties attached to natural products, can remarkably extend their structural diversification, which can therefore facilitate the development of a range of new and/or incrementally bioactive products and drug leads (Kim et al., 2015; Simkhada et al., 2010).

Attachment of sugar moieties to natural products can be engineered by chemical or biological approaches. However, the chemical preparation of glycosides is remarkably complicated by disadvantages, such as poor stereo- and regiospecificities and a series of complicated protecting and deprotecting steps (Chen et al., 2015). In addition, scale-up production is hampered by the low atom efficiency, low yield, environmental waste generation, and use of highly expensive catalysts (Bruyn et al., 2015; Dewitte et al., 2016). In this respect, enzymatic glycosylation mediated by uridine diphosphate-dependent glycosyltransferases (UGTs), which drive the formation of glycosidic bonds from nucleotide-activated donor molecules and specific aglycon acceptors (Lairson et al., 2008), can alleviate these disadvantages and have gained considerable interest. In the past decades, a considerable number of UGTs were isolated from plants or microbes and applied to decorate numerous natural or unnatural products for drug development (Thibodeaux

Abbreviations: *A.thaliana*, *Arabidopsis thaliana*; *B.subtilis*, *Bacillus subtilis*; CAZy, carbohydrate-active enzyme database; MGT, macrolide glycosyltransferase; *E. coli*, *Escherichia coli*; HPLC-ESI–MS, high–performance liquid chromatography–electrospray ionisation–mass spectrometry; IPTG, isopropyl-β-D-thiogalactopyranoside; NMR, nuclear magnetic resonance spectra; SDS-PAGE, sodium dodecyl sulphate polyacrylamide gel electrophoresis; SuSy, sucrose synthase; UDPG, UDP-glucose; UGT, uridine diphosphate-dependent glycosyltransferase.

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et al., 2009; Tiwari et al., 2016). Nevertheless, the glycosylation process remains severely restricted by enzyme specificity, thereby highlighting the need for discovery of UGTs with novel specificity for the targets of interest.

UGTs with aglycon promiscuity are generally recognised as effective tools for glycodiversification of natural products through both *in vitro* and *in vivo* strategies (Dewitte et al., 2016). Compared with plant UGTs, UGTs originated from microorganisms are thought to possess more open and large acceptor binding pocket and thus endow themself with broader acceptor tolerance (Lu et al., 2013; Yang et al., 2005). To date, more than 6000 microbial UGT genes have been deposited to the carbohydrate-active enzyme database (CAZy; www.cazy.org/GlycosylTransferases.html), but no more than 1% of these UGTs have been functionally characterised yet. Notably, several microbial macrolide glycosyltransferases (MGTs) isolated from *Streptomyces* or *Bacillus* strains, such as OleD and OleI from *Streptomyces antibioticus* (Gantt et al., 2008; Yang et al., 2005), Sl-MGT from *Streptomyces lividans* (Yang et al., 2005), BcGT1 from *Bacillus cereus* (Chiu et al., 2016) and Bl-YjiC from *Bacillus licheniformis* (Pandey et al., 2014, 2013b; Wu et al., 2012), show remarkable acceptor plasticity. These MGTs have been used to glycodiversify a variety of important microbial antibiotics or botanical natural products, which motivated us to seek substrate-flexible UGTs from *Streptomyces* or *Bacillus* strains.

Although UGTs with exceptional regio- and stereoselectivities are generally recognised as highly valuable glycosylation catalysts, synthetic applications of UGTs are frequently restricted due to the high cost and poor availability of uridine diphosphate glucose (UDPG) (Schmölzer et al., 2015). Sucrose synthase (SuSy), which can catalyse the conversion of cheap sucrose and uridine diphosphate (UDP) into UDPG and fructose, is an attractive biocatalyst for economic production of costly UDPG (Gutmann and Nidetzky 2016; Schmölzer et al., 2016). Furthermore, when SuSy is coupled with UGT in a one-pot reaction, the UDP produced by UGT can be instantly regenerated to UDPG by SuSy; consequently, the SuSy-UGT cascade reaction is allowed to synthesise glycosylated natural products as fine chemicals by only using cheap sucrose and catalytic amount of UDP (Bungaruang et al., 2013; Dewitte et al., 2016).

In the present study, a MGT (Bs-YjiC) from *Bacillus subtilis* 168 was selected as a candidate, and its aglycon promiscuity was explored using a series of structurally diverse types of natural or unnatural products containing hydroxyl, amino, or sulfhydryl group. The regio- and stereospecificities of Bs-YjiC were confirmed by analysing the glycosylated products using high–performance liquid chromatography–electrospray ionisation–mass spectrometry (HPLC–ESI–MS) and nuclear magnetic resonance (NMR) spectra. In addition, a one-pot reaction by coupling Bs-YjiC to AtSuSy from *Arabidopsis thaliana* was also probed using pterostilbene as substrate.

2. Materials and methods

2.1. Chemicals and reagents

Mogrol, mogroside IA and mogroside IE were prepared according to our previous study (Dai et al., 2015). All other chemicals and reagents were purchased from Sigma–Aldrich (St. Louis, MO, USA), Chengdu Biopurify Phytochemicals Ltd. (Chengdu, China) and J

2.2. Analytical procedures

Agilent 1260 HPLC system coupled to Bruker-micrOTOF mass spectrometer equipped with an electrospray ionisation (ESI) probe was used for HPLC-ESI–MS analysis. Enzymatic products (20 μL) were subjected to a reverse-phase Ultimate C18 column (4.6 mm × 250 mm, 5 μm particle, Welch, Shanghai, China) at a flow rate of 1 mL/min at 30 °C. ESI parameters were established as described previously (Dai et al., 2015). The UV wavelengths and gradient programs of the mobile phases used for analysis of diverse types of acceptors and their glycosylated products are listed in Table S1. Purified products dissolved in methanol-d_4 or dimethyl sulfoxide-d_6 were characterised by ^{1}H NMR and ^{13}C NMR using Bruker AVANCE III 400 MHz spectrometer.

2.3. Heterologous expression of Bs-YjiC and AtSuSy

The coding regions of *Bs-YjiC* (**NP_389104**) and *AtSuSy* (**NM_001036838**) were PCR amplified from the genomic DNA of *B. subtilis* 168 and cDNA of *A. thaliana* using gene-specific primer pairs (Bs-YjiC-F: 5′-CGCGGATCCATGAAAAAGTACCATATTTCGAT-3′ and Bs-YjiC-R: 5′-CGCGTCGACTTACTGCGGGACAGCGGATTTT-3′; AtSuSy-F: 5′-GCGTCGACAAATGGCAA

ACGCTGAACGTATGATAA-3′ and AtSuSy-R: 5′-TTGCGGCCGCTTATCATACGTTCAGCGTTTG CCAT-3′), respectively. Subsequently, Bs-YjiC and AtSuSy were inserted into the pET32a expression vector (Novagen, Darmstadt, Germany) to construct recombinant vectors pET32a-Bs-YjiC and pET32a-AtSuSy, respectively. After verified by sequencing, the recombinant plasmid and empty plasmid pET-32a were transformed into *Escherichia coli* BL21 (DE3) (Cwbiotech, Beijing, China) for heterologous expression.

Transformants containing pET32a-Bs-YjiC or pET32a-AtSuSy were incubated with Luria–Bertani medium containing ampicillin (100 μg/mL). The cultures were incubated at 37 °C and 200 rpm until the final optical density at 600 nm (OD_{600}) reached 0.6–0.8, and the expression of Bs-YjiC and AtSuSy was induced with 0.2 mM of isopropyl-β-D-thiogalactopyranoside. Afterwards, the cultures were further incubated at 16 °C and 200 rpm for 16–20 h. The cell pellets containing the recombinant protein were harvested by centrifugation at 5000 g for 15 min at 4 °C, resuspended in lysis buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl and 25 mM imidazole) and disrupted with a French Press. The homogenate was centrifuged at 17000 g for 60 min at 4 °C to remove cell debris. The soluble fraction was subjected to a Ni-NTA agarose affinity column using an AKTA Purifier system (GE Healthcare, Piscataway, NJ, USA) and eluted using a 25–250 mM imidazole gradient. Finally, the recombinant proteins were dialysed thrice against 50 mM Tris-HCl (pH 8.0) and concentrated using Amicon Ultra–10 K centrifugal filters (Millipore, Billerica, MA, USA). The purity and molecular mass of recombinant proteins were confirmed by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). Protein concentration was measured by the Bradford method with BSA as standard. The final proteins were stored at −80 °C for use and they were stable for more than six months at −80 °C.

2.4. In vitro glycosyltransferase activity assays

UGT activity assays (300 μL) were conducted with 0.2 mM aglycon, 1 mM UDPG, 50 mM Tris-HCl (pH 8.0), 10 mM $MnCl_2$ and 10 μg of purified protein. The reaction mixtures were incubated at 35 °C for 6 h and terminated by adding equal volume of methanol. Subsequently, the reactants were centrifuged at 10000 g for 15 min and filtered by a 0.22 μm filter prior to analysis by HPLC or HPLC-ESI–MS. The relative amount of individual products of the total glycosylated products was analysed based on percentage peak area relative to total peak area.