Identification of sucrose synthase from Micractinium conductrix to favor biocatalytic glycosylation

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Abstract

Sucrose synthase (SuSy, EC 2.4.1.13) is a unique glycosyltransferase (GT) for developing cost-effective glycosylation processes. Up to now, some SuSys derived from plants and bacteria have been used to recycle uridine 5'-diphosphate glucose in the reactions catalyzed by Leloir GTs. In this study, after sequence mining and experimental verification, a SuSy from Micractinium conductrix (McSuSy), a single-cell green alga, was identified. In the direction of sucrose cleavage, the optimum temperature and pH of the recombinant McSuSy were 60 °C and pH 7.0. The mutations of the predicted N-terminal phosphorylation site (S31D) and the QN motif (K684T and N685D) significantly stimulated the activity of McSuSy. When the mutant S31D/684T/685D of McSuSy, with the highest activity, was applied by coupling the engineered yeast glycosyltransferase UGT51 in a one-pot two-enzyme reaction, 8 mM protopanaxadiol was transformed into 6.02 mM (3.75 g/L) ginsenoside Rh2 within 3 h at 37 °C. The yield was comparable to the control reaction of AtSuSy1 from Arabidopsis thaliana. This work reveals the lower eukaryotes as a promising resource for SuSys of industrial interest.

Identification of sucrose synthase from *Micractinium conductrix* to favor biocatalytic glycosylation

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Abbreviations: **ADP** , adenosine 5'-diphosphate; **HEPES** , N-2-hydroxyethylpiperazine-N-ethane-sulphonicacid; **SuSy** , Sucrose synthase; **UDP** , uridine 5'-diphosphate; **UDPG** , uridine 5'-diphosphate glucose; **UGT** , UDP-glycosyltransferase

Abstract

Sucrose synthase (SuSy, EC 2.4.1.13) is a unique glycosyltransferase (GT) for developing cost-effective glycosylation processes. Up to now, some SuSys derived from plants and bacteria have been used to recycle uridine 5'-diphosphate glucose in the reactions catalyzed by Leloir GTs. In this study, after sequence mining and experimental verification, a SuSy from *Micractinium conductrix*(*Mc* SuSy), a single-cell green alga, was identified. In the direction of sucrose cleavage, the optimum temperature and pH of the recombinant *Mc* SuSy were 60 °C and pH 7.0. The mutations of the predicted *N* -terminal phosphorylation site (S31D) and the QN motif (K684T and N685D) significantly stimulated the activity of *Mc* SuSy. When the mutant S31D/684T/685D of *Mc* SuSy, with the highest activity, was applied by coupling the engineered yeast glycosyltransferase UGT51 in a one-pot two-enzyme reaction, 8 mM protopanaxadiol was transformed into 6.02 mM (3.75 g/L) ginsenoside Rh2 within 3 h at 37 °C. The yield was comparable to the control reaction of AtSuSy1 from *Arabidopsis thaliana* . This work reveals the lower eukaryotes as a promising resource for SuSys of industrial interest.

1 INTRODUCTION

Sucrose synthase (SuSy, EC 2.4.1.13) belongs to the glycosyltransferase-4 subfamily (GT-4), which can catalyze the reversible reaction of sucrose synthesis and cleavage.[1] A large number of studies have shown that the activity of SuSy depended on pH value.[2, 3] At pH 7.5–9.5, it displays optimal activity in the direction of sucrose synthesis, while acidic pH promotes the reverse reaction and decomposes sucrose at pH 5.5–7.5 to produce nucleoside diphosphate (NDP) glucose and fructose. Recently, using sucrose to recover the "donor" uridine 5'-diphosphate (UDP) glucose (UDPG) by combining SuSy with Leloir GT (SuSy-GT) has aroused considerable interest in the development of biocatalytic glycosylation process, because the glycosylation of most known conjugates by Leloir GT requires the participation of UDPG.[4-6] In the SuSy-GT cascade reaction (Scheme 1), a UDP cycle is created using sucrose and SuSy which makes UDPG continuously regenerated as an expedient donor for glucoside production, which is the shortest, and probably the most appealing one among the three routes involving synthase, phosphorylase, and kinase for UDPG synthesis.[7]

Scheme 1

It is known that SuSy has a broad substrate spectrum for different NDP "acceptors".[8] In the past five decades, more attention has been focused on plant SuSys with uridine 5'-diphosphate (UDP) preference, which is conducive to the production of UDPG.[4, 9] Prokaryotic SuSys are diversified in nucleotide substrate preference, such as some recently characterized SuSys from *Thermosynechococcus elongatus* (*Te* SuSy),*Nitrosomonas Europaea* (*Ne* SuSy), *Acidithiobacillus caldus* (*Ac* SuSy), and *Denitrovibrio acetiphilus*(*Da* SuSy), which are more inclined to use adenosine 5'-diphosphate (ADP) as nucleotide.[10, 11] However, bacterial SuSys showed better thermostability than plant SuSys, which could be more suitable for application in large-scale industrial production by increasing reaction temperature to avoid microbial contamination.[12, 13] The optimum temperature of plant SuSys is between 40 and 55 °C, but the enzyme stability decreased significantly above 30 °C,[3, 14, 15] while that of bacterial SuSys is between 60 and 80 °C.[10, 11] SuSy from moderately thermophilic *Acidithiobacillus caldus*has the best thermostability reported so far with the optimum temperature at 60 °C and maintains 96% activity after incubating at this temperature for 15 minutes.[11] In 2016, Gutmann et al. used *Ac* SuSy (*A* . caldus) to overcome the limitation of pH and thermodynamics, and 144 g/L UDPG was synthesized with the highest yield of 86%.[3] In this case, biocatalyst production, excessive sucrose, and a pH of 5.0 are crucial for high yield.[16]

The conversion efficiency of the glycosylation reaction is largely due to the removal of UDP, a product inhibitor of Leloir GT, where SuSy plays an indispensable role in the depletion of UDP in the SuSy-GT cascade.[17] To obtain a bacterial SuSy variant suitable for UDPG regeneration during glycosylation reactions, the affinity of *Ac* SuSy for UDP has been significantly improved by introducing plant residues at positions of a putative nucleotide binding motif (QN motif).[13] The comparison was made between the L637M-T640V double mutant of *Ac* SuSy that has a 60-fold decreased Michaelis-Menten constant (K_m) for UDP, and the SuSy from Glycine max (*Gm* SuSy) by coupling them respectively with the glycosyltransferase *Os* CGT in a one-pot reaction for the synthesis of *C* -glucoside nothofagin.[5] Fitness in terms of kinetics, expressed by the relatively low K_m values for UDP and sucrose, superseded enhanced thermostability in bacterial SuSys

as the selection criterion, which made plant SuSys the strongly preferred choice.[5]

Thanks to the ever-increasing numbers of sequences deposited in databases and the rapid development of data mining algorithms,[18-20] more SuSys would be uncovered as competitive substitutes to support the development of efficient SuSy-GT cascades. In the present study, by sequence mining, we focused on SuSys from lower eukaryotes like green algae, and their characters are still poorly understood. A candidate SuSy-encoding sequence derived from *Micractinium conductrix* (*Mc* SuSy) was code-optimized synthesized and heterologous overexpressed in *Escherichia coli*BL21(DE3). The recombinant SuSy was characterized, and the site-directed mutagenesis was conducted at the predicted *N* -terminal phosphorylation site (S31) and the QN motif of *Mc* SuSy. Then, the selected mutant S31D/684T/685D with enhanced activity and the engineered glycosyltransferase UGT51 (UGT51m) from *Saccharomyces cerevisiae*were co-expressed in *E. coli* . A SuSy-GT coupled system was constructed by the recombinant enzymes, to transform protopanaxadiol (PPD) into ginsenoside Rh2, a trace ginseng saponin with diverse pharmacological effects.[21] A control experiment was performed under the same conditions using UGT51m coupling with SuSy from *Arabidopsis thaliana*(*At* SuSy1).[22] This work may provide a biocatalyst with potential advantages for the establishment of cost-effective SuSy-GT cascade biotransformation in biocatalytic glycosylation.

2 MATERIALS AND METHODS

2.1 Sequence mining

Two SuSy sequences from *Anabaena* sp. PCC 7119 (*An* SuSy, CAA09297) and *Melioribacter roseus* (*Mr* SuSy, AFN74551) were used as templates for BLAST search in NCBI (https://www.ncbi.nlm.nih.gov/Blast.cgi). The resulting 20,000 sequences were downloaded for further analysis (in April 2020). Multiple sequence alignment was performed using MAFFT-7.037 or ClustalW (https://www.genome.jp/tools-bin/clustalw) with default parameters.[23] After removing redundancy, three putative SuSys from algae, including *Micractinium conductrix*(*Mc* SuSy, PSC73946) and *Chara braunii* (*Cb* SuSy1, GBG73881; *Cb* SuSy2, GBG70160), were selected from the sequences with conserved residues G302, G303, H438, R580, L581, K585, Q648, N654, E675 and E683,[11, 22, 24] and used as candidates in the subsequent experiments. The residue number refers to the sites of SuSy from *Arabidopsis thaliana* (*At* SuSy1, CAA50317) in the multiple sequence alignment.

The translated protein sequences of *Mc* SuSy, *Cb* SuSy1, and*Cb* SuSy2 were used to construct a phylogenetic tree using MEGA 7.0 with the known SuSys from *G. max* (*Gm* SuSy, AAC39323),*A. thaliana* (*At* SuSy1,CAA50317; *At* SuSy3,CAB80721),*S. tuberosum* (*St* SuSy1, AAA33841), *D. acetiphilus*(*Da* SuSy, ADD69694), *A. caldus* (*Ac* SuSy, AIA55343),*N. europaea* (*Ne* SuSy, CAD85125), *M. roseus* , *T. elongatus* (*Te* SuSy, BAC08600), and *Anabaena* sp. PCC 7119 by using the neighbor-joining method.[25, 26]Motifs were found by MEME according to the result of sequence alignment and displayed by WebLogo (http://weblogo.threeplusone.com/).[27, 28]

To predict the phosphorylation sites of SuSys, protein sequences of*Mc* SuSy, *Gm* SuSy, and the SuSy from *Zea mays*(*Zm* SuSy) were submitted to NetPhos 3.1 Server (http://www.cbs.dtu.dk/services/NetPhos/).[29]

2.2 Structure modelling and molecular docking

The homology model of *Mc* SuSy was constructed using the YASARA program.[30] The structures of UDP and sucrose were obtained from ZINC database (http://zinc.docking.org/).To construct the complex structure for evaluating the interaction between the protein and substrates, we tested molecular docking software such as LeDock and AutoDock Vina to dock the structure of *At* SuSy1 (PDB ID: 3S27, chain A) with its substrates.[31, 32] The docking results obtained by LeDock have a relatively good reproducibility to the crystal structure of *At* SuSy1, therefore, LeDock was further used to obtain the complex of *Mc* SuSy. UDP was first docked into the active site of *Mc* SuSy, resulting in the structure of*Mc* SuSy with UDP, which was then docked with sucrose. PyMOL (Version 2.4.1, Schrodinger LLC) was used to visualize and analyze the model structures generated, as well as to build illustrative figures.

2.3 Plasmid and strain construction

After codon optimization for heterologous expression in *E. coli* , the coding region derived from the putative SuSy mentioned above was synthesized and cloned into pRSFDuet-1 (Novagen) between the restriction endonuclease sites *Nco* I and *Eco* RI by GenScript (Nanjing, China). A 6-histidine tag was added at the *C* -terminus of SuSy. The generated plasmids were named as pRSF-*Mc* SuSy, pRSF-*Cb* SuSy1, and pRSF-*Cb* SuSy2, respectively.

The plasmid pRSF-*Mc* SuSy was used as the template for site-directed mutagenesis by a Mut Express® II Fast Mutagenesis Kit V2 (Vazyme Biotech Co., Ltd, Nanjing, China). The primers used in PCR to produce the plasmid mutants are listed in Table S1.

The synthesized code-optimized gene of UGT51m (ONH78233, excluding*N* -terminal 721 amino acids, containing seven mutations S81A/L82A/V84A/K92A/E96K/S129A/N172D) and the mutation S31D/K684T/N685D of *Mc* SuSy were subcloned into the restriction endonuclease sites*Nde* I/*Xho* I and *Nco* I/*Eco* RI of the pRSFDuet-1, respectively.[21] The obtained plasmid was named pRSF-UGT51m-*Mc* SuSym. Then, the coding region of the *Mc* SuSy mutant was replaced by that of *At* SuSy1 in pRSF-UGT51m-*Mc* SuSym, giving another plasmid named pRSF-UGT51m-*At* SuSy1.

The aforementioned plasmids were respectively transformed into *E. coli* BL21 (DE3) competent cells (TransGen Biotech, Beijing, China), resulting in the corresponding recombinant strain.

2.4 Expression and purification of SuSys

The recombined *E. coli* was first incubated in 5-mL Luria-Bertani medium containing 10 g/L tryptone, 10 g/L NaCl, 5 g/L yeast extract and 50 μg/mL kanamycin, and incubated overnight at 37 °C with continuous shaking at 200 rpm. Then, 2% (v/v) of the overnight culture was incubated in shake flasks with 100-mL LB medium containing 50 μg/mL kanamycin to cultivate for about 2 h at 37 °C. Isopropyl-β -thiogalactopyranoside in a final concentration of 0.1 mM was added when the culture turbidity (OD_{600}) reached 0.5–0.6, and then the cultivation was continued at 16 °C for another 24 h. The subsequent steps involving purification were performed at 4 °C. Cells harvested by centrifugation at 5,289 *g* for 5 min, were resuspended in an appropriate lysis buffer (500 mM NaCl and 10% glycerine (v/v) in 20 mM sodium phosphate buffer, pH 8.0) and disrupted by sonication. After centrifuge twice at 6,665 *g* for 15 min, the 6 Histidine-tagged proteins in the supernatant were purified by a high-affinity Ni-charged resin FF prepacked column (GenScript, Nanjing, China). The recombination proteins were eluted from the column by stepwise imidazole gradient. Fractions with SuSy activity were pooled and concentrated in an Amicon® Ultra-15 Centrifugal Filter Unit with an Ultracel-30 membrane (Merck Millipore Ltd., Ireland), and the buffer was exchanged to 50 mM HEPES-NaOH (pH 7.0). The protein expression and the purity of recombinant enzymes were analyzed using SDS-PAGE.

2.5 Enzyme assays of SuSys

The SuSy activity in the sucrose cleavage direction was measured with the standard reaction mixture containing 50 mM HEPES-NaOH pH 7.0, 2 mM UDP, 200 mM sucrose, and appropriate amount of purified enzyme in a final volume of 50 μL. Reactions were carried out at 37 °C for 5 min and stopped by heating at 95 °C for 2 min, and control experiments were performed immediately to check the decomposition of sucrose by the heat treatment. The product fructose was determined by the reduction of NAD^+ at 340 nm following the addition of a 150-μL solution that contained 50 mM HEPPS-NaOH (pH 7.0), 1 mM $MgCl_2$, 1 mM NAD^+, 1 mM ATP, 1 μg hexokinase, 1 μg P-glucose isomerase, and 1 μg glucose-6-P dehydrogenase.[10]One unit of SuSy activity was defined as the amount of enzyme that releases 1 μmol of reducing sugars per minute under the specified conditions.

The pH optimum of SuSy activity in the cleavage direction was determined in the pH ranging from 5.0 to 8.5 at 0.5 pH unit intervals. Buffers used were 50 mM MES-HCl (pH 5.0–7.0) and HEPES-NaOH (pH 7.0–8.5). The initial concentrations of sucrose and UDP in the reaction mixture were 200 mM and 2 mM, respectively.

The temperature profiles were obtained by determining the SuSy activity in the direction of sucrose cleavage from 20 °C to 70 °C. In the evaluation of thermal stability, the enzyme was pre-incubated in 50 mM HEPES

buffer (pH 7.0) for 15 min from 22 °C to 60 °C without any substrates, alternatively, with the addition of 200 mM sucrose. After the incubation, the residual activity in the sucrose cleavage direction was checked with the standard assay described above.

The influence of divalent metal ions on SuSy was investigated by measuring the activity in 50 mM HEPES (pH 7.0), 200 mM sucrose, and 2 mM UDP at 37 °C in the presence of 2 mM of $MgCl_2$, $CaCl_2$, $NiCl_2$, $CuCl_2$ or $ZnCl_2$.

The kinetic parameters for sucrose varying from 50 mM to 600 mM at a constant concentration of 2 mM UDP and for UDP varied from 0.05 mM to 5 mM at a constant concentration of 200 mM sucrose were measured at 37 °C in 50 mM HEPES buffer (pH 7.0). K_{m} and V_{max} values were determined by nonlinear regression analysis using the enzyme kinetics component of Origin 2021 software.

All reactions were conducted in triplicate. The relative activity (%) was calculated in terms of that of the maximum activity (100%). Coupled enzymes used for SuSy activity assays were purchased from Shanghai yuanye Bio-Technology Co., Ltd, and all the other reagents were analytical grade and commercially available.

2.6 Coupling reactions

To explore the application of *Mc* SuSy, we established SuSy-GT reactions to catalyze PPD to produce Rh2. The reaction mixtures (5 mL) contained 6 or 8 mM PPD, 200 mM sucrose, potassium phosphate buffer (50 mM, pH 7.0), and 8 mg/mL of total protein from the crude extract prepared from *E. coli*BL21 (pRSF-UGT51m-*Mc* SuSym) or *E. coli* BL21 (pRSF-UGT51m-*At* SuSy1). Expressions of two recombinant enzymes were under the same conditions as SuSys, except for the induction for 36 h. The reaction was incubated at 37 °C and 200 rpm for 3 h. The GT activity of UGT51m was measured at 37 °C in 0.5 mL reaction mixture containing 0.1 mg of total protein from crude extract, 2 mM PPD, 2 mM UDPG, 2% Tween 80 (v/v), 10% DMSO and 50 mM potassium phosphate buffer (pH7.0). Reactions were terminated by heating for 10 min at 95°C and diluted with methanol. One unit (U) of GT activity was defined as the amount of enzyme that produced 1 μmol of Rh2 from PPD. The concentrations of PPD and Rh2 were determined by UltiMate 3000 using an Agilent C18 column (250x4.6 mm) at UV 203 nm. The flow rate was 1.0 mL/min, and the column temperature was set at 30°C. The mobile phase consisted of water with 0.1% phosphoric acid (A) and acetonitrile (B), and a gradient program of 70–95% B in 0–25 min was applied. The crude enzyme activities of SuSys were measured as described in Supporting Information.

3 RESULTS

3.1 Sequence screening

In the sequence mining, the prokaryote-derived SuSy templates*An* SuSy and *Mr* SuSy were used for sequence collection and the sequences without conservative residues G302, G303, H438, R580, L581, K585, and E675,[22] and residues Q648, N654, and E683 that contribute to UDPG binding were removed (the residue number refers to *At* SuSy1 in the multiple sequence alignment).[11, 24] As a result, only a few sequences from lower eukaryote sources like green algae remained together with a large number of the putative plant SuSys. *Mc* SuSy from *M. conductrix* and *Cb* SuSy1 and *Cb* SuSy2 from*C. braunii* were selected and synthesized after codon optimization for heterologous expression in *E. coli* . Enzyme activities of the crude extracts containing *Mc* SuSy and *Cb* SuSy2 were around 15.5 and 5.5 mU/mg total protein, respectively. However, it is difficult to detect the SuSy activity of *Cb* SuSy1. As well, the obvious band corresponding to *Mc* SuSy (Fig. S1) was found in the soluble fraction prepared from the induced cells, indicating the better soluble expression of recombinant *Mc* SuSy than the other two enzymes. Therefore, *Mc* SuSy was chosen for further study of enzymatic properties.

3.2 Purification and enzymatic properties of *Mc*SuSy

The *Mc* SuSy fused with a *C* -terminal histidine-tag that was overexpressed in *E. coli* BL21(pRSF-*Mc* SuSy), was purified by Ni-NTA affinity chromatography. The specific activity of r*Mc* SuSy is 8.65 U/mg at 37 °C

and pH 7.0. In SDS-PAGE,*Mc* SuSy with a *C* -terminal 6-histidine shows a band of roughly 100 kDa (Fig. S2).

According to the pH profile (Fig. 1A), *Mc* SuSy reached its maximum activity at pH 7.0 and showed high enzymatic activity (>70% of maximum value) between pH 7 and pH 7.5. From pH 6.0–7.5, its activity was still higher than 40% of the maximum value, while the activity was undetectable at pH 8.5. The temperature optima of*Mc* SuSy was 60°C ranging from 20 °C to 70 °C (Fig. 1B). After incubating the enzymes for 15 min between 30 and 60 °C with or without sucrose, the thermostability of *Mc* SuSy was determined by measuring the residual activity. The enzyme remained stable up to 42 °C after 15 min of incubation without substrates, but its activity sharply decayed beyond 50 °C (Fig. 1C). It is worth mentioning that sucrose is known to act as a stabilizing agent, and the result found that sucrose plays a positive role in maintaining enzyme activity. Addition of 200 mM sucrose enhanced enzyme activity by about 2 U/mg at the lower incubation temperature (30, 37, 42 °C) compared to the case without sucrose.

In terms of the effect of divalent metal ions, adding 2 mM of EDTA, Mg^{2+} and Ca^{2+}, Ni^{2+}, Cu^{2+}, and Zn^{2+}, the activities of *Mc* SuSy were observed to decrease (Fig. 1D). The activity was especially strongly inhibited by Ni^{2+}, Cu^{2+}, and Zn^{2+}, resulting in undetectable activity, since these ions may influence the interaction with clusters of histidine on the protein surface.[15] EDTA had the least effect on the enzyme activity, followed by Ca^{2+} and Mg^{2+}.The enzyme activity displayed 35% of the maximum value in the presence of 2 mM Mg^{2+} and Ca^{2+}, while 74% was for EDTA.

Figure 1

3.3 UDP preference of *Mc*SuSy

SuSys, like *St* SuSy (*S. tuberosum* L) and SuSy*Ne*(*N. europaea*) shows high flexibility for nucleoside diphosphates in the cleavage reaction.[11, 15] Plant SuSys preferentially utilizes UDP as an acceptor nucleotide, while bacterial SuSys prefer ADP. By measuring the kinetic parameters of the enzyme on the substrate in the sucrose cleavage direction (Table 1), theK_{m} value of *Mc* SuSy for UDP is 0.13 mM, indicating that *Mc* SuSy has a higher affinity for UDP. And it was difficult to determine the enzyme activity under the same conditions when ADP was the glycosyl receptor. Homology modeling was carried out using the crystal structure of *At* SuSy1 (PDB ID: 3S27) as a template, which has 55.17% sequence identity with *Mc* SuSy, and the complex was obtained by substrate docking using LeDock. The observed secondary structure of *Mc* SuSy is very similar to that of the*At* SuSy1 monomer (Fig. 2A). Two sequence fragments, residues 333 to 345 and residues 683 to 718, were found in the active site of*Mc* SuSy, which are highly conserved in plant SuSys (Fig. 2B). To be specific, the residues 333 to 345 of *Mc* SuSy (light blue), corresponding to the residues 300 to 312 of *At* SuSy1, participate in the binding of fructose and G336 (G303 in *At* SuSy1) also interact with β -phosphate of UDP by forming hydrogen bonds (Figs. 2C and 2D).[22, 24] The residues 683 to 718 of*Mc* SuSy (pink) corresponding to the residues 648 to 683 of*At* SuSy1 belong to the nucleotide-binding domain, which contains the "QN" motif, playing a significant role in the nucleotide preference of SuSy.[13, 24] In particular, the two amino acids Q683 and N689 (Q648 and N654 in *At* SuSy1) are highly conserved in plant SuSys, while in bacteria the residues are highly variable.[13] For example, R636 and A642 in the*N. europaea* create a more spacious binding site for the preference towards the bulkier ADP substrate.[24]As shown in Figs. 2C and 2D, the UDP moieties bind of *Mc* SuSy are the same way as *At* SuSy1, especially in the indicated "QN" motif, which also implies a similar preference for nucleotide bases.[22]

Figure 2

Table 1

3.4 mutation of *Mc*SuSy for enhanced activity

Studies have demonstrated that phosphorylation affected the catalytic activities of SuSys in sucrose cleavage, which may increase the apparent affinity of the enzyme for sucrose and UDP to activate the formation of UDP-glucose and fructose from sucrose plus UDP.[33] Three residues including S7, T22, and S31 at the *N* -terminus of *Mc* SuSy (Table S2), which were predicted reliably as phosphorylation sites by NetPhos

3.1 Server, were mutated into two different acidic amino acid residues Asp (D) or Glu (E), respectively. Unexpectedly, we found that inclusion bodies of the S7D, S7E, and T22D mutants decreased significantly, and the enzyme activity of the crude extracts declined slightly (Fig. S3 and Fig. 3A). Both S31D and S31E mutants were confirmed to have significantly increased enzyme activity in crude extracts (more than 50% compared with wild-type *Mc* SuSy) and had little effect on the soluble expression. After purification, the kinetic parameter of S31D was determined (Table 1). The K_{m} values of S31D were 70.18 mM and 0.09 mM for sucrose and UDP, respectively, indicating a drop of more than 20% and an increased apparent affinity for subtracts compared with the wild type of *Mc* SuSy (Figs. S4 and S5).

Then seven residues surrounding the nucleobase ring of UDP in the "QN" motif,[13, 22] were evaluated by consensus analysis based on sequence alignment of the identified SuSys, to further improve the activity of *Mc* SuSy (Fig. 2B). Only K684 and N685 having the top three or two highest probabilities of alternative residues were chosen for mutagenesis. Other residues in the "QN" motif were very conservative. Three single-site mutants K684M, K684T, and N685D were generated, and the enzyme activities of the crude extracts were measured. The enzyme activities of the mutants K684T and N685D were 126.4% and 149.8% of those of the wild type, respectively (Fig. 4B). Subsequently, the mutant N685D was used as the template to overlie the other two mutations to obtain the multi-site mutants. Compared to the wild type, the crude enzyme activities of three multi-site mutants, that was, K684T/K685D, S31D/K685D, and S31D/684T/685D, were increased by 60%, among which the mutant S31D/684T/685D named *Mc* SuSym was the highest. Interestingly, *Mc* SuSym exhibited comparable activity as the mutant S31D. Moreover, enzymatic kinetic assays (Table 1) showed that *Mc* SuSym is higher than the wild type for sucrose catalytic efficiency ($K_{\mathrm{cat}}/K_{\mathrm{m}}$).

Figure 3

3.5 Production of ginsenoside Rh2 by the SuSy-GT reaction

Ginsenoside Rh2, which was an important triterpene saponin and originally isolated from red ginseng, has diverse pharmacological activities, including anti-oxidation, hepatoprotection, anti-diabetes and anti-tumor.[34, 35] The engineered glucosyltransferase UGT51 (UGT51m) from *Saccharomyces cerevisiae* , with a seven-residue mutation (S801A/L802A/V804A/K812A/E816K/S849A/N892D), was previously reported that can efficiently transfer a glucosyl moiety onto the C-3-OH of PPD to produce the ginsenoside Rh2.[21] UDPG was used as the sugar donor for the glycosylation of PPD by UGT51m. In the present study, UGT51m and *Mc* SuSy prepared from *E. coli*BL21 (pRSF-UGT51m-*Mc* SuSym) were coupled to form a SuSy-GT system. The *Arabidopsis* -derived *At* SuSy1 which was widely applied in various glycosylation reactions, was used in the control reactions. The cascade reactions were carried out at pH 7.0 and 37 °C. Samples were taken at 0, 0.5, and 3 h, and the concentration of Rh2 was detected by HPLC. The production of Rh2 was raised with the increase of PPD concentration and accumulated rapidly in 30 min, then grew slowly later. When the initial concentration of PPD was 6 mM, the yield of Rh2 was about 76% in each system, which was the same as that of 8 mM PPD concentration, but the product was 1.3 times lower than that of 8 mM PPD (Fig. 4). By measuring the SuSy activity in both systems, the specific activity of *Mc* SuSym (53.2 mU/mg) was comparable to that of*At* SuSy1 (55.8 mU/mg). Results showed that *Mc* SuSym worked as well as *At* SuSy1 for UDPG regeneration. As a result, 6.02 mM (3.75 g/L) of Rh2 was synthesized from 8 mM PPD by*Mc* SuSym-UGT51m.

Figure 4

4 DISCUSSION

In the present study, the prokaryotic *An* SuSy and *Mr* SuSy were used as templates for sequence collection, and the homology, as well as the active site of the reported SuSys, were also considered in the sequence screening process. Particularly, the sequences that have the conserved residues contributing to UDPG binding, corresponding to Q648, N654, and E683 in *At* SuSy1 remained.[11, 24] The phylogenetic tree shows the classification and evolutionary relationship of three selected sequences (*Mc* SuSy, *Cb* SuSy1, and *Cb* SuSy2) from the algae and several other characterized SuSys from plants and bacteria (Fig. S6). They are close to those from plants, falling in the Eukaryotic group, and share the common conserved active site residues in

retaining GT-B glycosyltransferases (Table S3), which was known from the multiple sequence alignment. What we focused on was *Mc* SuSy, which was heterologously expressed in *E. coli* in a more soluble form and with higher activity than *Cb* SuSy1 and *Cb* SuSy2. Lower pH values are known to promote the cleavage reaction of SuSys, yielding NDP-glucose and fructose, and with the increasing of the pH, NDP-glucose synthesis is disfavored.[2-4] While the*Mc* SuSy displayed the highest activity at pH 7.0 in sucrose degradation (Fig. 1B), which is different from other sources of SuSys preferring to hydrolyze sucrose at acidic pH. And it is suitable to apply in SuSy-GT cascade reactions coupling with Leloir GT having the optimal neutral pH. With the residual activity of above 80% after 15 min of incubation at 42°C with sucrose, the efficient recycling of UDPG may be realized by appropriately increasing the temperature of the catalytic reaction.

In addition, the plant SuSy is a known phosphoserine-containing enzyme.[36] One distinctive characteristic feature of SuSys is that phosphorylation of the *N* -terminus at the major phosphorylation site in plants contributes to the fine-tuning of enzyme activity and may be responsible for changes in membrane binding.[36, 37] In contrast, Interestingly, the*N* -terminal sequence alignment of prokaryotic SuSys shows that a highly conserved motif was found in cyanobacteria SuSys as a putative phosphoacceptor, but for non-cyanobacteria SuSys, there is no definite motif to distinguish.[4] Previous studies have shown that phosphorylation or introducing the negative charge at the *N* -terminal phosphorylation site of plant SuSys, such as at S15 of *Zm* SuSy and S11 of*Gm* SuSy and *St* SuSy1, has affected their catalytic activities in sucrose cleavage.[36-38] In the*N* -terminal sequence alignment of four SuSys involving*Mc* SuSy, *Gm* SuSy, *StS* uSy1, and *Zm* SuSy, the reported phosphorylation site is conserved (Fig. S7) in *Mc* SuSy (S31), which is identical to the predicted results obtained from NetPhos 3.1 Server (Table S2). S31D mutation of *Mc* SuSy showed a nearly 1.2-fold increase in the enzyme activity, which suggest that induction of the negative charge at S31, like phosphorylation, may affect the*N* -terminal conformation and the interactions between adjacent region, thus stimulating the catalytic activity of*Mc* SuSy.[22] Low $K_m$values for UDP are beneficial for in vitro recycling of UDPG in SuSy-GT coupled systems due to favored sucrose cleavage, and the product can be synthesized with endogenesis UDP. The K_m of*Mc* SuSy for UDP (0.13 mM) is almost comparable to that of plant SuSys (Table S4) and 1.5 times higher than the S31D mutation. The affinity for sucrose, indicated by the K_m value, was much worse than that of plants, although improved after mutation, which implies that *Mc* SuSy would not be inhibited by high concentrations of sucrose. When the residues in the "QN" motif were mutated, the affinity of *Mc* SuSym to UDP reduced, compared to those of the wild type and S31D mutant of *Mc* SuSy (Table 1), which may be caused by changing the interaction of residues binding with UDP.

Ginsenosides are the major pharmacological active compounds in traditional Chinese medicine ginseng. As a promising candidate drug for cancer prevention and treatment, PPD-type ginsenoside Rh2 has gradually aroused great interest in the medicinal and healthcare industries.[34, 35] However, the content of ginsenoside Rh2 in red ginseng is relatively low (0.0001% – 0.0003% in dried ginseng roots).[39] Due to the long cultivation time of ginseng, the complex extraction and purification process of bioactive compounds, at present, the synthesis of Rh2 mainly depends on biological deglycosylation of PPD-type ginsenosides (such as ginsenoside Rb1, Rb2 and Rc).[40] Moreover, ginsenoside Rh2 also can be obtained by heterologous de-novo synthesis through the construction of a synthesis pathway in a yeast cell factory.[41] However, some issues, such as the toxicity of ginsenosides to host cells, the low content of PPD-type ginsenosides in ginseng, and the poor efficiency of enzymatic hydrolysis, still limit Rh2 production. UDP-glycosyltransferases with regiospecificity, such as PgUGT74AE2, UGTPg45 from *P. ginseng* , and UGT73C5 from *A. thaliana* ,[42, 43] are responsible for the PPD-type and PPT-type ginsenoside (Rh2, CK, Rh2, F2, and Rh1) synthesis, providing diverse options of GTs for constructing the cost-effective SuSy-GT cascade reactions. Up to now, Rh2 has been successfully synthesized from PPD by UGT73C5 from *A. thaliana*coupling *At* SuSy1, and Bs-YjiC from *Bacillus subtilis*coupling *At* SuSy1.[42, 44] In such reactions, a high concentration of DMSO was used as a cosolvent of PPD, the high reaction efficiency was obtained by constantly adding fresh enzyme solutions. Thus, the stability of plant-derived UGT and SuSy has a vital impact on the application and amplification of biotransformation of PPD to produce Rh2. For *Mc* SuSy, the optimum temperature is 60 °C, and its enzyme activity may be well maintained in presence of sucrose, indicating higher thermostability than those of plant origin. At the same time, the increasing temperature usually improves the solubility of substances

and the viscosity of the solution. Therefore, higher temperature conditions are more conducive to promoting the transformation of substrates with high concentrations, especially for those with low solubility.

In brief, benefiting from the UDP preference and the inherently better thermostability, *Mc* SuSy may be able to work as a competitive rival of plant and bacteria SuSys for in situ regeneration of UDPG to promote the glycosylation catalyzed by a variety of Leloir GTs.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are mainly available in the supplementary material of this article. Additional data are available from the corresponding author upon reasonable request.

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AUTHOR CONTRIBUTIONS

Lei Lin: Investigation; Data curation; Writing - review&editing. Ruiqi Ma: Investigation; Writing - original draft. Kai Chen: Investigation; Data curation. Jiejie Ding: Investigation; Visualization. Huayi Pan: Investigation; Writing – review & editing. Yehui Tao: Writing - review & editing. Yan Li: Conceptualization; Methodology; Supervision; Funding acquisition; Writing - review & editing preparation. Huahong Jia: Resources; Project administration; Supervision.

CONFLICT OF INTERESTS

The authors declare no conflict of interest.

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Tables

Table 1 Kinetic parameters for the wild-type and mutants of*Mc* SuSy

*Mc*SuSy	Substrate	Substrate	K_{m} (mM)	$V_{\max}$ (U/mg)	K_{cat} (s^{-1})	$K_{\mathrm{cat}}/K_{\mathrm{m}}$ ($mM^{-1}s$
Wild-type	Wild-type	UDP	0.13±0.02	10.39±0.33	17.32±0.55	133.23
		Sucrose	90.10±19.38	10.56±0.71	17.6±1.18	0.20
S31D	S31D	UDP	0.09±0.02	11.38±0.43	18.97±0.72	206.20
		Sucrose	70.18±19.84	13.44±0.93	22.4±1.55	0.32
S31D/K684T/N685D	S31D/K684T/N685D	UDP	0.49±0.10	10.22±0.62	17.04±1.03	34.77
		Sucrose	59.34±12.88	10.24±0.61	17.07±1.02	0.29

Figure legends

Scheme 1 Regeneration of UDPG catalyzed by SuSy in the SuSy-GT coupling reaction.

Fig. 1 Enzymatic properties of the recombinant *Mc* SuSy. (A) pH profile; (B) Temperature profile; (C) Thermal stability; (D) Effect of metal ions on the activity of *Mc* SuSy. The data are presented as the means ± standard deviation of triplicates. The relative activity (%) was calculated in terms of that of the maximum activity (100%)

Fig. 2 The structure models of *Mc* SuSy and*At* SuSy1. (A) Ribbon drawing of the structure of *Mc* SuSy (blue) aligned with that of *At* SuSy1 (purple). (B) The*Mc* SuSy complex with two conserved sequence fragments: residues 300-312 (light blue) and residues 648-683 (pink). The residue number refers to the sites of *At* SuSy1 in the multiple sequence alignment. (C, D) The UDP binding sites of *Mc* SuSy and*At* SuSy1.

Fig. 3 Relative activities of the crude extracts containing the wild-type and mutants of *Mc* SuSy. (A)The mutations at the*N* -terminal phosphorylation sites; (B) The mutations at the "QN" motif

Fig. 4 Synthesis of ginsenoside Rh2 from PPD in the SuSy-GT reactions. Data are plotted as means ± standard deviation of duplicates.

Figures

Scheme 1

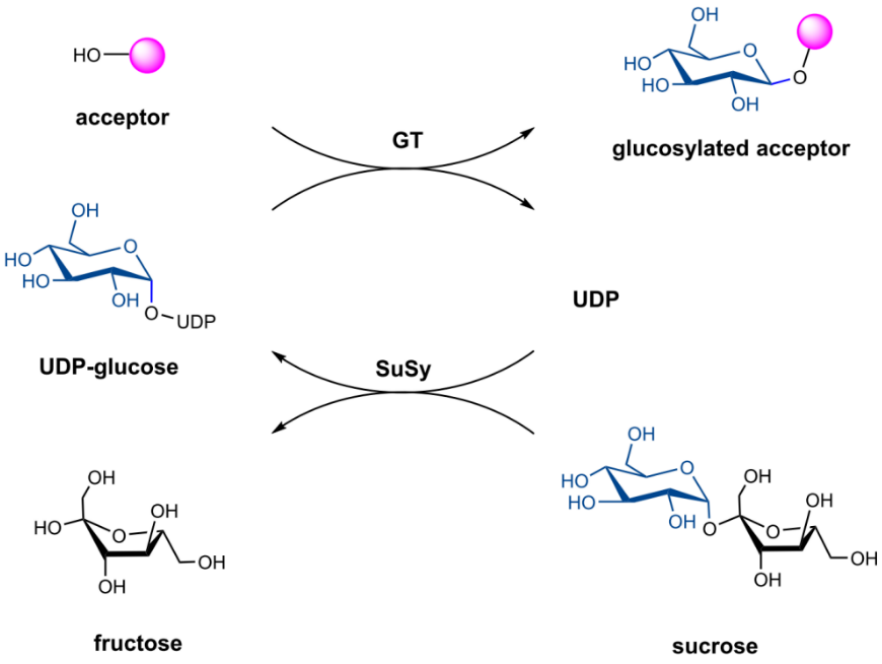

Figure 1

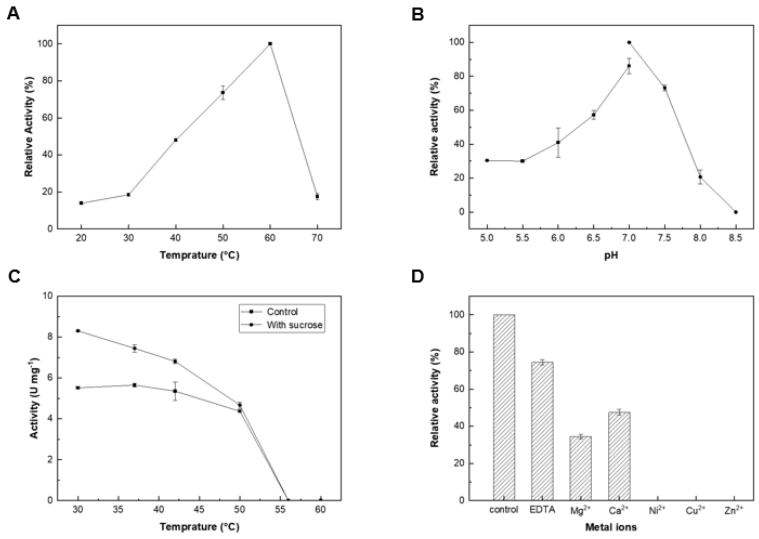

Figure 2

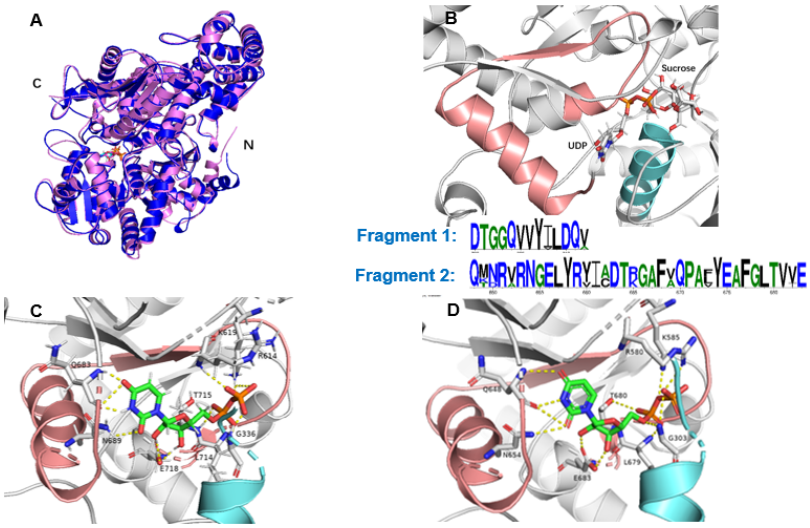

Figure 3

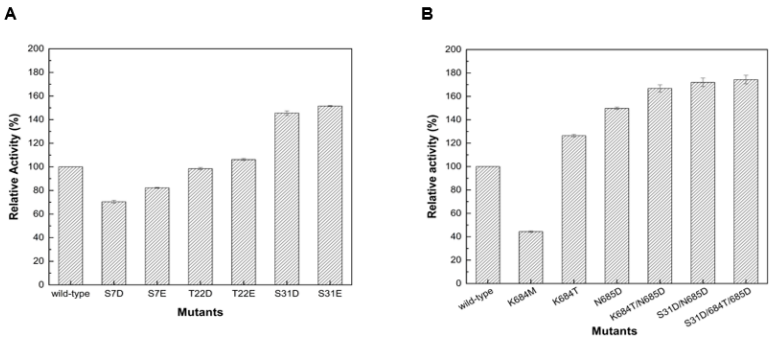

Figure 4