Engineering *Saccharomyces cerevisiae* with the deletion of endogenous glucosidases for the production of flavonoid glucosides

Huimin Wang1†, Yan Yang1†, Lin Lin3, Wenlong Zhou2, Minzhi Liu2, Kedi Cheng2 and Wei Wang1*

**Abstract**

**Background:** Glycosylation of flavonoids is a promising approach to improve the pharmacokinetic properties and biological activities of flavonoids. Recently, many efforts such as enzymatic biocatalysis and the engineered *Escherichia coli* biotransformation have increased the production of flavonoid glucosides. However, the low yield of flavonoid glucosides can not meet the increasing demand for human medical and dietary needs. *Saccharomyces cerevisiae* is a generally regarded as safe (GRAS) organism that has several attractive characteristics as a metabolic engineering platform for the production of flavonoid glucosides. However, endogenous glucosidases of *S. cerevisiae* as a whole-cell biocatalyst reversibly hydrolyse the glucosidic bond and hinder the biosynthesis of the desired products. In this study, a model flavonoid, scutellarein, was used to exploit how to enhance the production of flavonoid glucosides in the engineered *S. cerevisiae*.

**Results:** To produce flavonoid glucosides, three flavonoid glucosyltransferases (SbGTs) from *Scutellaria baicalensis* Georgi were successfully expressed in *E. coli*, and their biochemical characterizations were identified. In addition, to synthesize the flavonoid glucosides in whole-cell *S. cerevisiae*, SbGT34 was selected for constructing the engineering yeast. Three glucosidase genes (*EXG1*, *SPR1*, *YIR007W*) were knocked out using homologous integration, and the *EXG1* gene was determined to be the decisive gene of *S. cerevisiae* in the process of hydrolysing flavonoid glucosides. To further enhance the potential glycosylation activity of *S. cerevisiae*, two genes encoding phosphoglucomutase and UTP-glucose-1-phosphate uridylyltransferase involved in the synthetic system of uridine diphosphate glucose were over-expressed in *S. cerevisiae*. Consequently, approximately 4.8 g (1.2 g/L) of scutellarein 7-O-glucoside (S7G) was produced in 4 L of medium after 54 h of incubation in a 10-L fermenter while being supplied with ~3.5 g of scutellarein.

**Conclusions:** The engineered yeast harbouring SbGT with a deletion of glucosidases produced more flavonoid glucosides than strains without a deletion of glucosidases. This platform without glucosidase activity could be used to modify a wide range of valued plant secondary metabolites and to explore of their biological functions using whole-cell *S. cerevisiae* as a biocatalyst.

**Keywords:** *Saccharomyces cerevisiae*, Glucosidase, Glucosyltransferase, Scutellarein 7-O-glucoside

---

**Background**

Flavonoids are a group of polyphenolic compounds that have various biological activities, such as antioxidant, anti-atherosclerosis and anti-tumour [1–3]. In plants, flavonoids typically exist in the form of glucosides [4], which is beneficial to enhance solubility and stability or to change the pharmacokinetic properties [5]. Generally, glycosylation, which is the final step in the biosynthesis of flavonoid glucosides, is catalysed by glycosyltransferases (GTs) which transfer sugar moieties from the activated donor molecules to specific acceptor molecules [6–8]. GTs are divided into 98 families (CAZy database, Open Access).
March 2016) according to their different donors and acceptors.

To date, with an increasing demand for flavonoid glucosides, natural plant extracts might not satisfy human medical and dietary needs, and chemical synthesis of glucosides requires many protection and deprotection steps, which result in non-sustainable and low yield [9]. Therefore, alternative approaches have been developed for the production of flavonoid glucosides, such as purified GTs in vitro [10] and the engineered Escherichia coli or Saccharomyces cerevisiae in vivo [11, 12]. So far, biocatalytic efforts to synthesize flavonoid glucosides have largely focused on enzymatic synthesis and metabolic engineering of E. coli. The former is typically used to explore novel GTs and to characterize their new functions, and the latter automatically synthesizes sugar donors and directly catalyses the glycosylation of the target substrates supplied in the culture medium [10, 13, 14]. However, there are very few studies on the engineered yeast for the biosynthesis of flavonoid glucosides.

Saccharomyces cerevisiae is an attractive host organism for the production of flavonoid glucosides. Firstly, it is a unicellular eukaryote, which not only has the traits of prokaryotes, such as fast growth and advantages for culture and genetic manipulation, but also has the typical characteristics of eukaryotes, which allows for the modification of proteins after translation. Therefore, S. cerevisiae can be used to express other eukaryotic genes to synthesize the desired products. For example, it can functionally express Arabidopsis thaliana genes UGD1 and UXS3 to generate UDP-xylose [15]. In addition, S. cerevisiae is a generally regarded as safe (GRAS) organism that can be used in the production of pharmaceuticals and dietary supplements. However, whole-cell bioconversion of naringenin to naringenin 7-O-glucoside is impaired by the existence of endogenous glucosidases [16]. Additionally, Sabine Schmidt et al. identified several efficient glucosidases from yeast using an activity analysis of glucosidases in vitro and the corresponding commercial mutant strains [17]. Therefore, these glucosidases, which hydrolyse flavonoid glucosides, impede the use of S. cerevisiae as a host for the biotechnological production of flavonoid glucosides.

In the present work, an engineered S. cerevisiae strain was constructed to improve the production of flavonoid glucosides. Firstly, three glycosyltransferases (SbGTs) were obtained from Scutellaria baicalensis Georgi, and the SbGT34 exhibited the greatest activity toward scutellarein. Then, the glucosidase genes of S. cerevisiae were deleted to remove the hydrolysis of the glucoside products. To enhance the supplementation of the activated sugar donor UDP-glucose (UDP-Glu), two genes (encoding phosphoglucomutase 2 (PGM2) and UDP-glucose-1-phosphate uridylyltransferase 1 (UGP1)) involved in the biosynthesis of UDP-Glu were over-expressed in S. cerevisiae. Finally, an engineered yeast strain was constructed to enhance the production of flavonoid glucosides by combining the expression of SbGT34, PGM2, and UGP1 with the deletion of glucosidases. Additionally, to facilitate the biosynthesis of flavonoid glucosides, assays on the glucose addition and pH were studied in the yeast strain. Bioconversion was further improved in a 10-L fermenter and approximately 1.2 g/L of scutellarein 7-O-glucoside was synthesized in 4 L of medium supplied with ~3.5 g of scutellarein.

Results

Biochemical characterization of recombinant SbGTs protein in vitro

Three SbGT genes [SbGT30, SbGT34 and SbGT56 (GenBank No. KU712253, KU712254 and KU712255)] encoding flavonoid glucosyltransferase with strictly regioselectivity of 7-hydroxyl group were identified and heterologously expressed in E. coli. The sugar donor specificity of these recombinant proteins was analyzed using scutellarein as a sugar acceptor and UDP-Glu, UDP-glucuronic acid (UDP-GA) and UDP-galactose (UDP-Gal) as sugar donors. The results showed that each enzyme only selectively accepted UDP-Glu as a sugar donor. These three SbGTs catalysed glycosyl transfer to scutellarein and produced a single glycosylated product, which was clearly identified as scutellarein 7-O-glucoside based on the comparison of its LC spectrum, MS and MS/MS fragments with authentic sample and the NMR spectra (Additional file 1: Supplemental results). The relative activity of each enzyme with the same amount of enzyme, scutellarein (0.2 mM) and UDP-Glu (0.6 mM) was the following: SbGT30, 76.3 %; SbGT34, 100 % and SbGT56, 24.6 %. The optimal pH and temperature for the SbGT34-mediated transfer of the glucosyl moiety to scutellarein were 7.5 and 30 °C, respectively (Additional file 1: Figure S2). A kinetic analysis of the SbGTs revealed that the \( K_m \) value of SbGT30 and SbGT56 for scutellarein were 155 and 183 %, respectively, of the \( K_m \) value of SbGT34 for scutellarein (Fig. 1). In addition, SbGT34 also catalyzed the glycosylation of other flavonoids (data not shown). Therefore, SbGT34 was selected to further catalyze the glucosidation of scutellarein in the engineered yeast.

Deletion of S. cerevisiae glucosidase genes and its effects on the production of scutellarein 7-O-glucoside

In S. cerevisiae, the activity of glucosidases, which hydrolyse flavonoid glucosides, hinders the use of yeast as a host for the biotechnological production of glucosides [16, 17]. A flavonoid glycosyltransferase that glycosylated
naringenin to naringenin 7-O-glucoside in vitro from *Dianthus caryophyllus*, which was expressed in *S. cerevisiae*, demonstrated the existence of endogenous glucosidase activity during whole-cell biocatalysis and was responsible for a greatly diminished product yield [16, 17]. In this study, we further confirmed the hydrolytic activity of the whole-cell yeast using luteolin 7-O-glucoside as a substrate (Additional file 1: Figure S3). The results showed that the yeast did hydrolyze β-glucoside, which was in agreement with previous studies [16, 17]. Therefore, if engineered *S. cerevisiae* was used to produce flavonoid glucosides, a key step in the construction of the engineered yeast is the deletion of the glucosidase genes of host strain. Thus, in this study, site-directed integration plasmids were transformed to the W303-1b strain to disrupt the glucosidase open reading frames. The resulting strains were generated through the targeted DNA integration mediated by homologous recombination. The knockout workflow is schematically shown in Additional file 1: Figure S4.

The integration of plasmids containing the selective markers *TRP1*, *ADE2* and *URA3*, used for the disruption of the *EXG1*, *SPR1* and *YIR007W* genes, respectively, of the W303-1b strain resulted in strains being selected on auxotrophic solid plates. Furthermore, positively integrated yeast strains were verified using DNA sequencing of the PCR-amplified fragments corresponding to the glucosidase locus from the genomic DNAs of the tested transformants. The growth rate of the strains with a deletion of the *EXG1* or *YIR007W* gene was similar to the wild-type strain, whereas the strain with the deletion of *SPR1* gene grew faster than the wild type strain (Fig. 2a).

To identify the glucosidase activity of the knockout strains, a degradation assay of flavonoid glucosides with whole-cell biocatalysis was performed using luteolin 7-O-glucoside as a substrate. When the glucoside was added to the culture medium with each knockout strain and the wild-type strain W303-1b, the remaining rate of the substrate in each catalyzing system was varied. The degradation of luteolin 7-O-glucoside to luteolin is presented in Fig. 2b. The luteolin 7-O-glucoside was dramatically diminished within 6 h in the wild-type strain W303-1b and could not be detected at 24 h. The strain W303-1b/EXG1Δ hardly hydrolyzed glucosides within

---

**Fig. 1** Determination of the kinetic parameters for SbGTs. The apparent $K_m$ values were detected using scutellarein as an acceptor and UDP-Glu as a donor at 30 °C and a pH of 7.5. The apparent $K_m$ values of SbGT30 (a), SbGT34 (b) and SbGT56 (c) were 188, 121 and 222 μM, respectively.
24 h, whereas the strains W303-1b/SPR1Δ and W303-1b/YIR007WΔ still showed high levels of hydrolytic activity. It is worthwhile to note that strain W303-1b/EXG1Δ did not hydrolyze glucosides even through it was incubated for 96 h during the whole-cell biocatalyst assay (data not shown). From these, the deletion of glucosidases was determined to be necessary for the glucosylation of whole-cell *S. cerevisiae* [16, 18].

To study strains harbouring SbGT34 to distinguish the biotransformation levels of substrates from flavonoids to flavonoid glucosides, 0.2 mM scutellarein was fed into the biocatalytic reaction systems. The results showed that in strain W303-1b/SbGT34, the conversion rate of scutellarein into its glucoside was increased slowly within the first 20 h, and glucoside achieved the greatest accumulation at 72 h, whereas the production of glucoside in strain W303-1b/ESΔ/SbGT34 (E and S referred as deletion of genes *EXG1* and *SPR1*, respectively) was as approximately twice the production in strain W303-1b/SbGT34 at 72 h (Fig. 3) (i.e., the titres of product ranged from 33.4 to 68.6 mg/L). Therefore, according to the combination advantages of strain W303-1b/EXG1Δ that has almost no glucosidase activity, and strain W303-1b/SPR1Δ that grows fast, a double knockout strain W303-1b/ESΔ, which had a growth rate similar to strain W303-1b/SPR1Δ, was used for the further production of flavonoid glucosides.

**Bioconversion of scutellarein in vivo**

UDP-Glu is a critical endogenous donor in the biosynthesis of flavonoid glucosides in whole-cell *S. cerevisiae*. The biosynthetic pathway of UDP-Glu mainly includes two key enzymes: PGM2, which synthesizes glucose-1-phosphate from glucose-6-phosphate, and UGP1, which converts glucose-1-phosphate and UTP into UDP-Glu. The whole-cell *S. cerevisiae* produces endogenous UDP-Glu in the cytoplasm that could be used for the production of flavonoid glucosides [19–21], but does not comprise a GT for the glycosylation of flavonoids. To enhance the production of flavonoid glucosides in strain W303-1b/ESΔ/PU/SbGT34 (P and U referred to the over-expression of genes *PGM2* and *UGP1*, respectively), SbGT34 was over-expressed under the control of the constitutive promoter along with the up-regulation of genes *PGM2* and *UGP1*. The results showed that strain W303-1b/ESΔ/PU/SbGT34 was more effective (~92 % conversion rate) than strain W303-1b/ESΔ/SbGT34 (~75 % conversion rate) (Fig. 3) (i.e., the titres of product ranged from 68.6 mg/L to 82.5 mg/L). The analysis of the reaction product from the HPLC profiles showed a product peak at a retention time of 14.1 min for scutellarein 7-O-glucoside compared to the standard scutellarein 7-O-glucoside that was detected at 19.6 min at a UV absorbance of 280 nm (Fig. 4). The HPLC–MS/MS spectra showed an ion peak...
of scutellarein 7-0-glucoside at m/z = 471 [M + Na]⁺ (Additional file 1: Supplemental results). The negative experiment was carried out under identical conditions by supplementing scutellarein in the strain harbouring the empty vector, and no bioconversion of scutellarein was observed by analysing the HPLC results.

To explore the substrate inhibition on biotransformation and cell growth, different concentrations (0.2, 0.4, 0.6, 0.8, 1.0 mM) of scutellarein were fed into the biocatalytic reaction systems (strain W303-1b/ESΔ/PU/SbGT34). The results showed that the bioconversion of scutellarein at each concentration gradient into its glucoside was rapidly increased in the first 48 h and then became static for 72 h, and the inhibition of cell growth was elevated with an increase in the concentration of the substrate (Fig. 5).

Optimization of the production of flavonoid glucosides by regulating the concentration of glucose and the pH conditions in the medium

During the fermentation process, glucose as the sole carbon source and as the precursor of the sugar donor together with the pH of medium is important for product synthesis. To further explore the potential of the recombinant strain W303-1b/ESΔ/PU/SbGT34 for the production of scutellarein 7-0-glucoside, four different concentrations (2, 5, 10, 15 %) of glucose were

Fig. 4 HPLC analysis for scutellarein 7-O-glucoside from the whole-cell biocatalyst assay using strain W303-1b/ESΔ/PU/SbGT34. a HPLC profile for standard scutellarein. b HPLC profile for the biocatalysis system. c HPLC profile for standard scutellarein 7-O-glucoside

Fig. 5 The influence of different concentrations of scutellarein on the cell growth and the conversion rate of scutellarein to scutellarein 7-O-glucoside. The concentrations of scutellarein were 0.2, 0.4, 0.6, 0.8 and 1.0 mM. a The conversion rate of scutellarein at different concentrations over time. b Biomass (OD₆₀₀). The values are presented as the means, and the error bars show the SD (n = 3)
supplemented in the synthetic complete (SC) medium with 0.6 mM scutellarein as the substrate. As shown in Fig. 6a, the conversion rate of scutellarein into its glucoside increased with the addition of glucose, and a similar transformation rate was observed at glucose concentrations of 10 and 15 % (i.e., the titres of product with the two concentrations of glucose was 161.4 and 168.9 mg/L, respectively). The supplementation of 10 % glucose concentration and 0.6 mM scutellarein acceptor were selected to further optimize pH.

Four different pH gradients (4.5, 5.0, 5.5 and 6.0) were designed for the biotransformation assay. The concentration of the phosphate buffer solution was 50 mM, and the conversion rate of the substrate was improved by approximately 20 % at pH 5.5 compared with the basic SC medium (Fig. 6b).

Scale up for the production of scutellarein 7-O-glucoside using a fermenter
To verify the application of the engineered strain W303-1b/ESΔ/PU/SbGT34 for the large-scale production of scutellarein 7-O-glucoside, optimized concentrations of scutellarein (1.0 mM at 0 h and 2.0 mM at 12 h) were added to the 4-L fermentation system in a 10-L fermenter. The temperature of fermenter was held at 30 °C and the pH was maintained at 5.5 by dripping of ammonia water during the entire process, and the concentration of glucose in the culture was 10 %. The culture medium was collected at a given time interval of 6 h and was analyzed using HPLC to monitor the conversion of substrate to its glucoside.

After 12 h of fermentation, the supplied scutellarein was quickly begun to convert to scutellarein 7-O-glucoside; thus, additional 2.0 mM of scutellarein was added to the reaction system. Additionally, the fermentation condition was identical to the initial 12 h. Approximately 90 % of the supplemented substrate was converted into flavonoid glucoside after 54 h of fermentation. The overall calculations revealed that the production of scutellarein 7-O-glucoside was ~1.2 g/L (i.e., 2.7 mM or 4.8 g/4 L) from 3.0 mM of supplemented scutellarein (Fig. 7).

Discussion
Dietary flavonoids and especially their glucosides, have attracted increasing attention in the past several decades for their considerable biological activities [4]. The glycosylation of flavonoids using GTs as the biocatalyst is of great general interest due to the synthesis of the desired compounds with high stereo- and regio-selectivity under mild conditions [4]. In recent studies, the exploration of
GTs mainly focused on enzymology and the semi-synthesis of glucosides using engineered E. coli [4, 13, 22]. The in vitro recombinant enzyme provided a good model to study the enzymatic characteristics, and the in vivo biosynthesis of flavonoid glucosides using endogenous sugar donors could reduce the expense in engineered whole-cell E. coli system. Engineered whole-cell S. cerevisiae could also synthesize endogenous sugar donors and produce heterogeneous proteins. These virtues motivated us to investigate the glycosylation of engineered yeast in vivo.

To date, all most of GTs (e.g. UDP-glucose: anthocyanin 3-O-glycosyltransferase) reported are involved in the biosynthesis of anthocyanin, however, only a few studies have reported the isolation and characterization of GTs with strictly regio-selective glycosylation of flavonoid 7-hydroxyl group [23–29]. In this study, three additional flavonoid 7-O-glycosyltransferase genes from S. baicalensis were cloned and successfully expressed in E. coli. These three genes had high identities with the genes of the reported GTs from Scutellaria Species (Labiateae) (GenBank: AB031274, GU339042) [14, 24]. The results of the biochemical studies of SbGTs showed that the three SbGTs could regio-selectively catalyze scutellarein to scutellarein 7-O-glucoside, and only UDP-Glu could be used as a sugar donor. Additionally, SbGT34 had the greatest affinity towards scutellarein (Fig. 1). The amino acid sequence of the three SbGTs shared very high identities (more than 90 %) to the reported UBGT and SbUGT [14, 24], but SbGT34 showed the greatest catalytic efficiency for scutellarein compared with that of the SbGT56, which is most likely the counterpart of UBGT. Based on the alignment of their amino acid sequences and the homology modeling (data not shown), a significant deletion of seven amino acid residues in SbGT30 and SbGT34 was within the calculated loop region between the N- and C-terminal domains with similar Rossmann-like GT-B folds among the plant flavonoid GTs (Additional file 1: Figure S5). Additionally, there are several different amino acid residues between SbGT30 and SbGT34 highlighted (Additional file 1: Figure S5), of which the function of these amino acid residues need to be further tested in detail.

The biocatalytic production of flavonoid glucosides using yeast as a biocatalyst is impaired by the metabolic activity of yeast glucosidases, which hydrolyze the glucosidic bond between aglycone and glucosyl. A GT cloned from D. caryophyllus that could glucosylate naringenin to naringenin 7-O-glucoside was expressed in whole-cell S. cerevisiae. However, the endogenous glucosidasomes reversely hydrolyzed naringenin 7-O-glucoside during whole-cell biocatalysis, resulting in a low final glucoside titre [16]. Sabine Schmidt et al. had identified the three yeast β-glucosidases in yeast: EXG1, SPR1 and YIR007W, which played significant roles in the hydrolysis of flavonoid glucosides [17]. In this work, these three glucosidase genes were disrupted in the engineered yeast. The EXG1 gene was identified in vivo as the decisive gene for the degradation of flavonoid glucosides. Strains with the deletion of the EXG1 gene did not hydrolyze the flavonoid glucosides (Fig. 2b), and the growth rate of the strain with the deletion of the SPR1 gene was greater than the wild strain and other recombinant strains (Fig. 2a). The SPR1 gene encodes a sporulation-specific glucanase and is transcribed only during the late stages of sporulation [30]. Deletion of the SPR1 gene contributes to change the proceeding of sporulation and promotes cell growth. The high growth rate of the strain with the deletion SPR1 gene could shorten the process of fermentation. The conversion rate of flavonoid in strain W303-1b/ESΔ/SbGT34 increased twofold compared with the rate in strain W303-1b/SbGT34 (Fig. 3). Therefore, the strain with the disruption of the EXG1 and SPR1 genes is proposed as an option to develop a platform for the expression of GTs and for the production of flavonoid glucosides in yeast transformants.

The combined strategy of deletion of glucosidases and the introduction of GTs plays pivotal roles in generating flavonoid glucosides. Plant GTs expressed in E. coli have been reported for the glycosylation of flavonoids [13, 22]. Based on the expression of the GTs in E. coli, the titre of the flavonoid glucosides was less than 100 mg/L in the culture broth [25, 26]. The two main factors for the biosynthesis of the desired products might be related to the availability of intracellular UDP-sugars and low activity of the GTs. Recently, a number of studies have primarily focused on enhancing endogenous UDP-sugars by over-expressing genes that are part of the biosynthetic pathway of UDP-sugars and on exploring efficient GTs [22, 31–33]. In this study, the successful deletion of glucosidases and the expression of the S. baicalensis flavonoid GT in the engineered yeast for the whole-cell biocatalysis facilitated the bioconversion of aglycones and significantly improved the production of scutellarein 7-O-glucoside. The scale-up feasibility of in vivo glycosylation has been applied for the large-scale production of scutellarein 7-O-glucoside by optimizing the appropriate fermentation conditions. Consequently, up to 1.2 g/L of scutellarein 7-O-glucoside was produced after 54 h of fermentation (Fig. 7). As the titre of the desired products was remarkably high, the engineered strain W303-1b/
expression vector pTWIN1B, a deriv-
using high pressure homogenizer and the cell debris
500 mM NaCl, NaOH solution was added until pH was
in 100 mL of chilled binding buffer (20 mM HEPES,
β-D-thiogalactoside (IPTG) for 20 h at 16 °C with shak-
ing (165 rpm). Cells were harvested by centrifugation
and the regulating of biosynthetic pathway of UDP-sugars, it
is applicable to extend the regio-specific glycosylation to
more secondary metabolites as pharmaceutical ingredi-
ents for future clinical application.

Methods
Strains
Escherichia coli DH5α (TransGen, Beijing, China) was
used for propagation and manipulation of the recom-
binant DNA, and E. coli BL21 (DE3) was employed for
expression of the recombinant enzymes. S. cerevisiae
W303-1b (MATα ade2-1 leu2-3, 112 his3-11, 15 his3-1
trp1-1) was used for construction of engineering strain
as a whole-cell biocatalyst for production of flavonoid
glucosides. E. coli strains were cultured at 37 °C in LB
medium (5 g/L yeast extract, 10 g/L tryptone and 10 g/L
NaCl), and yeast transformants were grown at 30 °C in
YPD medium (10 g/L yeast extract, 20 g/L tryptone and
20 g/L glucose).

Expression and purification of recombinant SbGTs
To prepare the recombinant SbGT enzymes, the veri-
fied cDNA clones corresponding to SbGT30, SbGT34
and SbGT56 were amplified, digested with restriction
enzymes Nco I and BamHI, and then ligated into the
same sites of the expression vector pTWIN1B, a deriva-
tive of the plasmid pTWIN1 (NEB, Biolabs) (Addi-
tional file 1). After the verification of the sequences,
the resulting pTWIN1B-SbGTs plasmids and a control
pTWIN1B were transformed into E. coli BL21 (DE3) for
heterologous expression. Luria–Bertani (LB) medium
(10 mL) containing 100 μg/mL of ampicillin was inocu-
lated with 1 mL of an overnight culture corresponding
to each selected genes and a control vector. Cells were
grown at 37 °C with shaking (200 rpm) until the OD600
achieved 0.4–0.6. The recombinant N-terminal CBD-
tagged SbGTs were induced with 130 μg/mL isopropyl
β-D-thiogalactoside (IPTG) for 20 h at 16 °C with shak-
ing (165 rpm). Cells were harvested by centrifugation
at 8000 g for 5 min at 4 °C. Pellets were resuspended
in 100 mL of chilled binding buffer (20 mM HEPES,
500 mM NaCl, NaOH solution was added until pH was
8.5). Cells were disrupted with 800 bar high pressure
using high pressure homogenizer and the cell debris
was removed by centrifugation at 10,000 g and 4 °C for
30 min.

The cleared supernatant was immediately applied to
10 mL of chitin resin (NEB, Biolabs) loaded in a column
that was pre-equilibrated with binding buffer. The chitin
resin was subsequently washed with 100 mL of the
washing buffer (20 mM HEPES, 500 mM NaCl, pH 8.5).
Then, the washing buffer was replaced by the cleavage
buffer (20 mM HEPES, 50 mM NaCl, pH 6.5) and the
recombinant protein was cleaved at 4 °C for 3 days. Elu-
tion was carried out with 25 mL of the cleavage buffer,
and the recombinant protein was desalted in 10-times
diluted cleavage buffer and was then lyophilized. Protein
purity was confirmed by SDS-PAGE to be >90 % (Addi-
tional file 1: Figure S1), and the protein concentration for
all studies was determined by the protein quantitative kit
(Bradford) (Bio-Rad, USA).

Glycosyltransferase activity assays in vitro
Three SbGT genes, SbGT30, SbGT34 and SbGT56, were
identified and heterologously expressed in E. coli. The
donor specificity of these recombinant proteins was
analyzed using scutellarein as a glucosyl acceptor and
UDP-Glu, UDP-GA and UDP-Gal as sugar donors. The
reaction mixture (200 μL) for the GTs contained 50 mM
citrate buffer (pH 6.5), 0.6 mM UDP-Glu, 0.2 mM sub-
strate dissolved in dimethylsulfoxide (DMSO) and 10 μg
of the purified SbGTs. Activity assays, which were initi-
ated through the addition of enzymes, were incubated at
30 °C for 30 min and were terminated by adding 200 mL
of methanol. The reaction mixture was subjected to
HPLC analysis for the detection of the reaction product.

Effects of pH, temperature and divalent metal ions
on enzyme activity
To test the necessity of divalent metal ions for SbGT34,
CaCl2, CoCl2, CuCl2, MgCl2, FeCl2, MnCl2, ZnCl2 and
EDTA were used individually at a final concentration of
5 mM. To study the optimal pH, the enzymatic reaction
was performed in various reaction buffers with pH val-
ues in the range of 4.0–6.5 (citrate buffer), 6.0–8.0 (phos-
phate buffer) and 7.5–9.0 (Tris–HCl buffer). To assay for
the optimal reaction temperature, the reaction mixtures
were incubated at different temperatures (0–70 °C). The
assays were performed with UDP-Glu as a donor and
scutellarein as an acceptor.

Determination of kinetic parameters
Assays were performed in a final volume of 200 μL con-
sisting of 50 mM Tris–HCl (pH 7.5) at 30 °C, and con-
tained constant concentrations of enzyme (10 μg) and
saturating UDP-Glu (2 mM) while varying the scutel-
larein concentration (0.1–1.0 mM). Reactions were

ESa/PU/SbGT34 is the most efficient strain for the pro-
duction of flavonoid glucosides in yeast ever reported.

Conclusions
The combined strategy of the deletion of endogenous
glucosidases and the introduction of heterogenous GTs
along with the up-regulation of biosynthetic pathway of
UDP-Glu presents higher efficiency of the production of
flavonoid glucosides. Through replacing other GTs and
the regulating of biosynthetic pathway of UDP-sugars, it
is applicable to extend the regio-specific glycosylation to
more secondary metabolites as pharmaceutical ingredi-
ents for future clinical application.
terminated at 10 min (where the rate of product formation was determined to be linear) by adding an equal volume of ice cold methanol. Mixtures were filtered and analyzed using reverse-phase HPLC, and the total percent conversion was calculated as the percent of the total peak area of the substrate and product. All experiments were performed in triplicate. The value of $K_m$ was calculated using a Lineweaver–Burk plot.

Construction of yeast expression plasmids and yeast transformation

All strains and plasmids used in this study are listed in Table 1. The plasmid construction and yeast transformation methods were same as in [34]. All plasmids were constructed using conventional restriction enzyme-mediated cloning methods. Based on the nucleotide sequences of the target genes, development of primer sets were designed and used to amplify gene fragments by PCR (Additional file 1: Tables S1–S6). The δ DNA-mediated integrative expression vector was used for the cloning of the polymerase chain reaction (PCR) products and the expression of the genes [35]. The acquired plasmids were linearized by digestion with the restriction enzyme NotI or HindIII and were transformed into the S. cerevisiae using the lithium acetate method. Transformants were selected using yeast extract peptone dextrose (YPD) agar plates containing the antibiotics Geneticin (G418, 4 mg/mL) or Hygromycin B (HygB, 1 mg/mL), and the double homologous recombination of the target genes was verified by PCR using the corresponding primers and Sanger sequencing using the isolated genomic DNA as a template. The repeated introduction of the marker genes was implemented via a loxP-marker-loxP gene disruption cassette [36].

Deletion of glucosidases in S. cerevisiae

In S. cerevisiae, glucosidases EXG1, SPR1 and YIR007W have previously been identified as the three yeast β-glucosidases previously [17]. To disrupt these glucosidase genes, three homologous integration vectors were constructed (Additional file 1: Methods). Then, 5 μg of the resulting plasmids that were linearized by the digestion with restriction enzyme NotI or HindIII were transformed into S. cerevisiae using the lithium acetate method. Transformants were identified on synthetic selective medium agar plates without tryptophan (for gene EXG1), adenine (for gene SPR1) or uracil (for gene YIR007W), respectively [37]. The double homologous

### Table 1 Strains and plasmids used in this study

| Strains or plasmids | Relevant properties | Source or reference |
|---------------------|---------------------|---------------------|
| **Strains**         |                     |                     |
| E. coli trans1-T1   | F– φ80lacZΔM15 ΔlacY4 hsdR[K− mK+]ΔrecA1398 endA1 tonA | Our lab |
| L. casei BL21 (DE3) | F− ompT hsdS (rB−mB−s−) gal dcm | Our lab |
| S. cerevisiae W303-1b | MATA ade2-1 leu2-3, 112 his3-11, 15 ura3-1 trp1-1 | Our lab |
| W303-1b/EXG1Δ       | W303-1b derivative with pEZ-EXG36-TRP1, EXG1Δ::Trp1 | This study |
| W303-1b/SPR1Δ       | W303-1b derivative with pEZ-SPR36-ADE2, SPR1Δ::ADE2 | This study |
| W303-1b/YIR007WΔ    | W303-1b derivative with pEZ-YIR36-Ura3, YIR007WΔ::Ura3 | This study |
| W303-1b/ESΔ         | W303-1b derivative with pEZ-EXG36-TRP1 and pEZ-SPR36-ADE2, EXG1Δ::Trp1, SPR1Δ::ADE2 | This study |
| W303-1b/SgbGT34     | W303-1b derivative with pδGAPg-SgGT34 | This study |
| W303-1b/ESΔ/SgbGT34 | W303-1b/ESΔ derivative with pδGAPg-SgGT34 | This study |
| W303-1b/ESΔ/PU/SgbGT34 | W303-1b/ESΔ derivative with pδGAPg-PGM2, pδGAPh-UGP1, and pδGAPg-SgGT34 | This study |
| **Plasmids**        |                     |                     |
| pTWIN1B             | A derivative of pTWIN1, an inducible expression vector with ampicillin resistant. | Our lab |
| pTWIN1B/SgbGT30     | pTWIN1B derivative with the SgbGT30 gene DNA | This study |
| pTWIN1B/SgbGT34     | pTWIN1B derivative with the SgbGT34 gene DNA | This study |
| pTWIN1B/SgbGT56     | pTWIN1B derivative with the SgbGT56 gene DNA | This study |
| pEZ-EXG36-TRP1      | Integration vector with Homologous fragment of EXG1 and selective marker TRP1 | This study |
| pEZ-SPR36-ADE2      | Integration vector with Homologous fragment of SPR1 and selective marker ADE2 | This study |
| pEZ-YIR36-Ura3      | Integration vector with Homologous fragment of YIR007W and selective marker URA3 | This study |
| pδGAPg              | pBluescript II KS(+) derivative with homologous 6 region, $P_{gaps} T_{gaps}$, G418’ | [34] |
| pδGAPh              | pBluescript II KS(+) derivative with homologous 6 region, $P_{gaps} T_{gaps}$, HygB’ | [34] |
| pδGAPg-PGM          | pδGAPg derivative with PGM2 from S. cerevisiae, G418’ | This study |
| pδGAPh-UGP1         | pδGAPh derivative with UGP1 from S. cerevisiae, HygB’ | This study |
| pδGAPg-SgGT34       | pδGAPg derivative with SgGT34 from S. baicalensis, G418’ | This study |
recombination of the glucosidase locus was verified by PCR using the corresponding primers and the corresponding genomic DNA as a template. The amplified DNA fragments of the positive constructs were further verified by DNA sequencing (Taihe, Beijing, China).

**Analysis of the glucosidase activity in the knockout strains**

After each successful deletion, strains W303-1b, W303-1b/EXG1A, W303-1b/SPRIΔ and W303-1b/YIR007WΔ were grown at 30 °C in YPD medium for 24 h, and the culture was then inoculated into fresh SC medium (2 % w/v glucose, 0.67 % w/v yeast nitrogen base without amino acids and 2 g/L complete amino acid mixture) at 1 %, and the cells were grown at 30 °C for approximately 10 h. The initial OD600 of the yeast seeds in the SC media was adjusted to 1.0 and 0.2 mM luteolin 7-O-glucoside was then added to the medium. The 1 mL reaction mixture in 5-mL reaction flasks was incubated at 30 °C for 10, 30 min, 1, 3, 6, 12 and 24 h. After the completion of the reaction, the reaction products of three independent experiments were lyophilized and extracted using 500 μL of methanol three times. The combined extracts were evaporated and then re-dissolved in 1 mL of methanol. The hydrolytic rate of each strain for luteolin 7-O-glucoside was determined using HPLC analysis.

**Analysis of the dynamic effects of glucosidase activity on the production of the desired glucosides**

To further confirm whether the strains harbouring SbGT34 with or without the deletion of glucosidase genes had different substrate conversion rates, strains W303-1b/SbGT34 and W303-1b/ESA/SbGT34 were selected to explore the remaining glucosidase activity. Strains W303-1b/SbGT34 and W303-1b/ESA/SbGT34 were grown at 30 °C in YPD medium for 24 h, and the culture was then inoculated into fresh SC medium at 1 % and the cells were grown at 30 °C for approximately 10 h. The initial OD600 of the yeast seed in the SC media was adjusted to 1.0 and 0.2 mM scutellarein was then added to the medium. The 1-mL reaction mixture at 30 °C was incubated for 3, 6, 12, 24, 48 and 72 h in 5-mL reaction flasks. After the completion of the reactions, the reaction products were processed as mentioned above. The conversion rate of each strain for scutellarein was determined using HPLC analysis.

**Assay on S. cerevisiae whole-cell biocatalyst**

Integrative S. cerevisiae transformants were grown overnight at 30 °C in YPD medium until the OD600 was approximately 3.0 as a seed culture. One hundredth of the volume of the seed culture was inoculated into the SC medium and was grown at 30 °C for approximately 10 h, and the OD600 was adjusted to 1.0 using fresh SC medium. Substrates were added to 1 mL of the regulated cell suspension. All of the results shown in this paper were obtained from at least three independent experiments.

Yeast strain W303-1b/ESA/PU/SbGT34 at the same cell density (initial OD600 1.0) was used for the substrate inhibition tests. Different concentrations of scutellarein (0.2, 0.4, 0.6, 0.8, 1.0 mM) dissolved in DMSO were used to biotransform in a 1-mL culture volume in 5-mL reaction flasks. Three independent experiments were removed from the shaker for each concentration and for each reaction time interval. The reaction solutions were lyophilized, and the samples were then extracted using 500 μL methanol three times. The three methanol fractions were merged and completely volatilized, and then re-suspended into 1 mL of methanol, and subjected to HPLC analysis. The final conversion of substrate was calculated according to a standard curve of scutellarein and scutellarein 7-O-glucoside.

**Glucose supplementation and pH optimization**

The yeast strain W303-1b/ESA/PU/SbGT34 was used for glucose supplementation and pH optimization in a biotransformation reaction. Firstly, four different concentrations of glucose (2, 5, 10, 15 %) were supplemented in the SC medium with 0.6 mM scutellarein as a substrate to determine the optimum concentration of glucose for biotransformation. Secondly, when the optimum concentration of glucose was determined, 50 mM phosphate buffer solution was added to the fermentation medium in four pH gradients (4.5, 5.0, 5.5, 6.0) to ascertain the optimal pH for bioconversion. The original cell density and disposing methods of the samples were same as mentioned above. Finally, HPLC analysis was carried out, and the conversion rate of the substrate into product was determined.

**Whole-cell biocatalyst assay in the fermenter system**

A 10-L glass autoclavable fermenter system (Beauty, Shanghai, China) was used for the large-scale analysis of the strains harbouring SbGT34 as a whole-cell biocatalyst. The strain W303-1b/ESA/PU/SbGT34 that was cultured overnight (200 mL) was transferred into the fermenter system containing 4 L of SC medium, and the original OD600 was adjusted to 1.0. The culture was fed with 1.0 mM scutellarein (dissolved in DMSO) (at 0 h) and 2.0 mM (at 12 h) as a substrate, and the incubation temperature and dissolved O2 were 30 °C and 25 %, respectively. The pH of the medium was regulated to remain at approximately 5.5 through the addition of a 12.5 % ammonia solution. Samples were harvested at 6 h intervals, and the growth of the cells was measured at 600 nm. The resulting solution fractions (culture
medium) were disposed as mentioned above and analysed using HPLC.

Product analysis and quantification
The culture extracts dissolved in methanol were directly performed on reverse-phase HPLC connected to a C18 column (Mightysil RP-18 GP 4.6 × 250 mm, 5 μm) at 280 nm using binary conditions of H₂O (0.05 % trifluoroacetic acid, mobile phase A) and 100 % acetonitrile (ACN) (mobile phase B) at a flow rate of 1 ml/min for 40 min. The analyses of the substrates and their products were conducted using the following gradient program: 0–25 min (10–35 % B), 25–27 min (35–100 % B), 27–32 min (100 % B), 32–35 min (100–10 % B), 35–40 min (10 % B). To quantify the flavonoid glucosides, a calibration curve of scutellarein 7-O-glucoside was created using 0.05, 0.1, 0.2, 0.5, 0.6, 0.8, 2.0 and 4.0 mM concentrations.

Additional file

Additional file 1. Table S1. Primers used for cloning of S. baccatacensis SbGT cDNAs. Table S2. Primers used for cloning of S. cerevisiae EXGT1 (GenBank No. M34341) homologous DNA fragments. Table S3. Primers used for cloning of S. cerevisiae SPR1 (GenBank No. NW_001183609) homologous DNA fragments. Table S4. Primers used for cloning of S. cerevisiae YIR007W (GenBank No. NW_001179529) homologous DNA fragments. Table S5. Primers used for cloning of S. cerevisiae PGM2 (GenBank No. NW_001182605) gene. Table S6. Primers used for cloning of S. cerevisiae UGP1 (GenBank No. NW_001179601) gene. Figure S1. SDS-PAGE analysis of recombinant SbGTs purified by affinity chromatography. Figure S2. Effects of various divalent metal ions, pH, temperature on enzyme activity of SbGT34. Figure S3. Activity analysis of whole-cell glycosidase in wild-type S. cerevisiae W303-1b using luteolin 7-O-glucoside as a substrate. Figure S4. Diagrammatic sketch of the knockout of glucosidase genes. Figure S5. A multiple alignment of the amino acid sequences of SbGT30, SbGT34, SbGT56, UBG1, SbUGT and UGT71G1. Figure S6. Schematic diagram of biosynthetic pathway of scutellarein 7-O-glucoside.

Abbreviations
UDP: uridine diphosphate; UTP: uridine triphosphate; GT: glucosyltransferase; PGM2: phosphoglucomutase 2; UGP1: UTP-glucose-1-phosphate uridylyltransferase 1.

Authors’ contributions
HW, YY and LL performed the experiments. HW, WZ and ML analyzed the data, and HW drafted the manuscript. KC conceived and coordinated the study and revised the manuscript. All authors read and approved the final manuscript.

Author details
1 State Key Laboratory of Bioactive Substance and Function of Natural Medicines, Institute of Materia Medica, Peking Union Medical College and Chinese Academy of Medical Sciences, 1 Xian Nong Tan St., Beijing 100050, China. 2 Key Laboratory of Biosynthesis of Natural Products of National Health and Family Planning Commission, Institute of Materia Medica, Peking Union Medical College and Chinese Academy of Medical Sciences, 1 Xian Nong Tan St., Beijing 100050, China. 3 College of Life Science, Qufu Normal University, Qufu 273165, Shandong, China.

Acknowledgements
None.

Competing interests
The authors declare that they have no competing interests.

Funding
This work was financially supported by the National Science Foundation of China (No. 30772677, 81072562), the Fundamental Research Funds for the Central Universities (No. 2012N06), and the National Science and Technology Project of China (No. 2012ZX09301002-001). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Received: 23 March 2016   Accepted: 28 July 2016
Published online: 04 August 2016

References
1. Alani L, Miller ND. Antioxidant flavonoids: structure, function and clinical usage. Altern Med Rev. 1996;1:103–11.
2. Salvamani S, Gurusekaran B, Shahruddin NA, Ahmad SA, Shukor MY. Antiatherosclerotic effects of plant flavonoids. BioMed Res Int. 2014;2014:480258.
3. Romano B, Pagano E, Montanaro V, Fortunato AL, Milic N, et al. Novel insights into the pharmacology of flavonoids. Phytother Res. 2013;27:1588–96.
4. Xiao J, Muzashvili TS, Georgiev MI. Advances in the biotechnological glycosylation of valuable flavonoids. Biotechnol Adv. 2014;32:1145–56.
5. Kren V, Martinová L. Glucosides in medicine: “the role of glycosidic residue in biological activity”. Curr Med Chem. 2001;8:1303–28.
6. Jiang Jr, Yuan S, Ding JF, Zhu SC, Xu HD, Chen T, et al. Conversion of paesinarin into its 7-O-glucoside derivatives by Microbacterium oxydans (CGMCC 1788) to improve its water solubility and pharmacokinetic properties. Appl Microbiol Biotechnol. 2008;81:647–57.
7. Jones P, Vogt T. Glycosyltransferases in secondary plant metabolism: tranquillizers and stimulant controllers. Planta. 2001;213:164–74.
8. Williams GJ, Ganit RW, Thorson JS. The impact of enzyme engineering upon natural product glycodiversification. Curr Opin Chem Biol. 2008;12:556–64.
9. Engstrom KM, Daanen JF, Wagaw S, Stewart AO. Gram scale synthesis of the glucuronide metabolite of ABT-724. J Org Chem. 2006;71:8378–83.
10. Ono E, Homma Y, Horikawa M, Kunikane-Doi S, Imai H, Takahashi S, et al. Functional differentiation of the glycosyltransferases that contribute to the chemical diversity of bioactive flavonol glucosides in grapevines (Vitis vinifera). Plant Cell. 2010;22:2856–71.
11. Simkhada D, Kim E, Lee HC, Sohng JK. Metabolic engineering of Escherichia coli for the biological synthesis of 7-O-Xylosyl naringenin. Mol Cells. 2009;28:397–401.
12. Poppenberger B, Berthiller F, Bachmann H, Lucyszyn D, Peterbauer C, Mitterbauer R, et al. Heterologous expression of arabidopsis UDP-glucosyltransferases in Saccharomyces cerevisiae for production of zearalenone-4-O-glucoside. Appl Environ Microbiol. 2006;72:4404–10.
13. Kim BG, Yang SM, Kim SY, Cha MN, Ahn JH. Biosynthesis and production of glycosylated flavonoids in Escherichia coli: current state and perspectives. Appl Microbiol Biotechnol. 2015;99:2979–88.
14. Chiu SJ, Liu WY, Fang CL, Lin TY. Characterization of the Scutellaria barbata glycosyltransferase gene and its promoter. Planta. 2010;232:963–74.
15. Oka T, Igami Y. Reconstruction of the novel pathway for synthesis of UDP-glucuronic acid and UDP-xylose from intrinsic UDP-glucose in Saccharomyces cerevisiae. FEBS J. 2006;273:2645–57.
16. Werner SR, Morgan JA. Expression of a Dianthus caryophyllus flavonoid glucosyltransferase in Saccharomyces cerevisiae for whole-cell biocatalysis. J Biotechnol. 2009;142:233–41.
17. Schmidt S, Rainies S, Witte S, Matern U, Martens S. Identification of a Saccharomyces cerevisiae glucosidase that hydrolyzes flavonoid glucosides. Appl Environ Microbiol. 2011;77:1751–7.
18. McMahon H, Zoecklein BW, Fugelsang K, Jasinski Y. Quantification of glucosidase activities in selected yeasts and lactic acid bacteria. J Ind Microbiol Biotechnol. 1999;23:198–203.

19. Douglas CM, Foor F, Manninan JA, Morin N, Nielsen JB, Dahl AM, et al. The \textit{Saccharomyces cerevisiae} FK51 (ETG1) gene encodes an integral membrane protein which is a subunit of 1,3-β-D-glucan synthase. \textit{Proc Natl Acad Sci USA.} 1994;91:12907–11.

20. Mazur P, Morin N, Baginsky W, el-Sherbeini M, Clemas JA, Nielsen JB, et al. Differential expression and function of two homologous subunits of yeast 1,3-β-D-glucan synthase. \textit{Mol Cell Biol.} 1995;15:5671–81.

21. Shahinian S, Bussey H. β-1,6-Glucan synthetase in \textit{Saccharomyces cerevisiae}. \textit{Mol Microbiol.} 2000;35:477–89.

22. de Bruyn F, Van Brempt M, Maertens J, Van Bellegem W, Duchi D, De Mey M. Metabolic engineering of \textit{Escherichia coli} into a versatile glycosylation platform: production of bio-active quercetin glycosides. \textit{Microb Cell Fact.} 2015;14:138.

23. Liu J, Hou J, Li C, Li G, Lu H, Meng F, et al. Deep sequencing of the \textit{Scutellaria baicalensis} georgii transcriptome reveals flavonoid biosynthetic profiling and organ-specific gene expression. \textit{PLoS One.} 2015;10:e0136397.

24. Hirotani M, Kuroda R, Suzuki H, Yoshikawa T. Cloning and expression of UDP-glucose: flavonoid 7-O-glucosyltransferase from hairy root cultures of \textit{Scutellaria baicalensis}. \textit{Planta.} 2000;210:1006–13.

25. Lim EK, Ashford DA, Hou W, Jackson RG, Bowles DJ. Arabidopsis glycosyltransferases as biocatalysts in fermentation for regioselective synthesis of diverse quercetin glycosides. \textit{Biotechnol Bioeng.} 2004;87:623–31.

26. Willis MG, Giovannini M, Prata RT, Kramer CM, De Luca V, Steffens JC, et al. Bio-fermentation of modified flavonoids: an example of in vivo diversification of secondary metabolites. \textit{Phytochemistry.} 2004;65:31–41.

27. Pan H, Fang C, Zhou T, Wang Q, Chen J. Accumulation of calycosin and its 7-O-beta-D-glucoside and related gene expression in seedlings of \textit{Astragalus membranaceus} Bge. var. mongholicus (Bge.) Hisao induced by low temperature stress. \textit{Plant Cell Rep.} 2007;26:1111–20.

28. Noguchi A, Saito A, Homma Y, Nakao M, Sasaki N, Nishino T, et al. A UDP-glucose:isoflavone 7-O-glucosyltransferase from the roots of soybean (glycite max) seedlings. Purification, gene cloning, phylogenetics, and an implication for an alternative strategy of enzyme catalysis. \textit{J Biol Chem.} 2007;282:23581–90.

29. Noguchi A, Horikawa M, Fukui Y, Fukuchi-Mizu. M, Iuchi-Okada A, Ishiguro M, et al. Local differentiation of sugar donor specificity of flavonoid glycosyltransferase in Lamiales. \textit{Plant Cell.} 2009;21:1556–72.

30. Muthukumar G, Suhng SH, Magee PT, Jewell RD, Primerano DA. The \textit{Saccharomyces cerevisiae} SPR1 gene encodes a sporation-specific exo-1,3-β-Glucanase which contributes to ascospore thermostolerance. \textit{J Bacteriol.} 1993;175:386–94.

31. Kim SY, Lee HJ, Park KS, Kim BG, Ahn JH. Metabolic engineering of \textit{Escherichia coli} for the biosynthesis of flavonoid-O-glucuronides and flavonoid-O-galactoside. \textit{Appl Microbiol Biotechnol.} 2015;99:2233–42.

32. Parajuli P, Pandey RP, Trang NT, Chaudhary AK, Suhng JK. Synthetic sugar cassettes for the efficient production of flavonol glucosides in \textit{Escherichia coli}. \textit{Microb Cell Fact.} 2015;14:76.

33. Yoon JA, Kim BG, Lee WJ, Lim Y, Chong Y, Ahn JH. Production of a novel quercetin glucoside through metabolic engineering of \textit{Escherichia coli}. \textit{Appl Environ Microbiol.} 2012;78:4256–62.

34. Tang L, Wang W, Zhou W, Cheng K, Yang Y, Liu M, et al. Three-pathway combination for glutathione biosynthesis in \textit{Saccharomyces cerevisiae}. \textit{Microb Cell Fact.} 2015;14:139.

35. Lee FW, Da Silva NA. Improved efficiency and stability of multiple cloned gene insertions at the δ sequences of \textit{Saccharomyces cerevisiae}. \textit{Appl Microbiol Biotechnol.} 1997;48:339–45.

36. Guldener U, Heck S, Fielder T, Reinhard P, Rothenberg J, Hegemann JH. A new efficient gene disruption cassette for repeated use in budding yeast. \textit{Nucleic Acids Res.} 1996;24:2519–24.

37. Prunk JT. Auxotrophic yeast strains in fundamental and applied research. \textit{Appl Environ Microbiol.} 2002;68:2095–100.