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(54) Titre : GROUPE DE GLYCOSYLTRANSFERASES ET SON UTILISATION
(54) Title: GROUP OF GLYCOSYLTRANSFERASES AND USE THEREOF

(57) Abrégé/Abstract:
Provided are the use of glycosyltransferases gGT25, gGT13, gGT30, gGT25-1, gGT25-3, gGT25-5, gGT29, gGT29-3, gGT29-4, gGT29-5, gGT29-6, gGT29-7, 3GT1, 3GT2, 3GT3, 3GT4 and derived polypeptides therefrom in the catalyzed glycosylation of terpenoid compounds and the synthesis of new saponins, wherein the glycosyltransferases can specifically and efficiently catalyze tetracyclic triterpenoid compound substrates at positions C-20 and/or C-6 and/or C-3 during hydroxyl glycosylation, and/or transfer the glycosyl from a glycosyl donor to the first glycosyl of the tetracyclic triterpenoid compounds at position C-3, so as to extend the sugar chain. The glycosyltransferases can also be used for constructing man-made synthetic rare ginsenosides and a variety of new ginsenosides and derivatives thereof.

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Abstract

Provided are the use of glycosyltransferases gGT25, gGT13, gGT30, gGT25-1, gGT25-3, gGT25-5, gGT29, gGT29-3, gGT29-4, gGT29-5, gGT29-6, gGT29-7, 3GT1, 3GT2, 3GT3, 3GT4 and derived polypeptides therefrom in the catalyzed glycosylation of terpenoid compounds and the synthesis of new saponins, wherein the glycosyltransferases can specifically and efficiently catalyze tetracyclic triterpenoid compound substrates at positions C-20 and/or C-6 and/or C-3 during hydroxyl glycosylation, and/or transfer the glycosyl from a glycosyl donor to the first glycosyl of the tetracyclic triterpenoid compounds at position C-3, so as to extend the sugar chain. The glycosyltransferases can also be used for constructing man-made synthetic rare ginsenosides and a variety of new ginsenosides and derivatives thereof.

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Group of Glycosyltransferases and Use Thereof

Technical Field

The present invention relates to the biotechnology and the phytobiology field. Specifically, the present invention relates to glycosyltransferases and use thereof.

Background Art

Saponins isolated from *Panax ginseng* and the congener plants thereof (including *Panax. notoginseng and Panax quinguefolium* etc.) are collectively named as ginsenosides. Ginsenosides belong to triterpene saponins and they are the main active ingredient of *Panax.* At present, at least 60 kinds of ginsenosides have been isolated from *Panax*, some of which were proved to have broad physiological functions and pharmaceutical values including anti-cancer, immunoregulation, anti-fatigue, heart protection, hepatoprotection, etc.

Structurally, ginsenosides are small molecules with biological activity formed by the glycosylation of sapogenins. The types of ginsenoside sapogenins are limited, mainly including dammarane-type protopanaxadiol (PPD), protopanaxatriol (PPT), and oleanolic acid. Recently, two new sapogenins, 25-OH-PPD and 25-OCH3-PPD, were isolated from *P. notoginseng.* Both of these new sapogenins present excellent anti-tumor activities.

Upon glycosylation, the water solubility of sapogenins is enhanced and different biological activities are exhibited. The carbohydrate chain of PPD saponin usually binds to C3 and (or) C20 hydroxyl(s) of sapogenin(s). Compared with PPD saponin, PPT saponin has one more hydroxyl at position C6. The glycosylation bindings all occur at C6 (and) or C20 hydroxyl(s) of PPT saponin according to the present findings. Glycosylation binding at C-3 of PPT saponin was not yet reported. The glycosyl can be glucose, rhamnose, xylose or arabinose.

The physiological functions and pharmaceutical values of ginsenosides can dramatically vary with different glycosyl binding sites, and composition and length of carbohydrate chains. For example, ginsenoside Rb1, Rd and Rc are all saponins with PPD as their sapogenins; they only vary in glycosyl modification, but their physiological functions differ a lot. Rb1 possesses the function of stabilizing the central neural system; while the function of Rc is to inhibit the function of the central neural system. Rb1 presents broad physiological functions while the functions of Rd are quite limited.

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Structural diversities of ginsenoside sapogenins and saponins are also embodied in their stereo structures. Despite many chiral carbon atoms on tetracyclic triterpenoids skeleton, C20 is the dominant site for forming stereo structures. C20 epimers exist in almost every kind of ginsenosides and sapogenins. The content of ginsenosides and sapogenins with S-configuration at C20 in ginseng is far above that of R- configuration. Thus, in most cases, ginsenosides and sapogenins generally refer to C20 S- configuration ginsenosides and sapogenins. However, physiological activities of C20 epimers of ginsenosides and sapogenins are distinctly different. For example, the S-type ginsenoside Rh2 (3-*O*-β-(D-glucopyranosyl)-20(*S*)- protopanaxadiol) can significantly inhibit prostate caner cells, while the inhibiting effect of R-type ginsenosides Rh2 (3-*O*-β-(D-glucopyranosyl)-20(*R*)-protopanaxadiol) is quite poor. The R-type ginsenoside Rh2 can selectively inhibit the generation of osteoclasts without any cytotoxicity, while the S-type ginsenoside Rh2 poorly inhibits the osteoclasts generation with strong cytotoxicity to osteoclasts. Besides, the regulatory effects of the S-type and R-type ginsenoside Rh2s on P-glycoprotein are substantially different.

The function of glycosyltransferases is transferring glycosyl(s) from glycosyl donor(s) (nucleotide diphosphate sugar, such as, UDP-glucose) to different glycosyl receptor(s). At present, glycosyltransferases have been classified into 94 families based on different amino acid sequences. More than one hundred different glycosyltransferases were identified among the sequenced plant genomes for now. Glycosyl acceptors for these glycosyltransferases include saccharides, lipids, proteins, nucleic acids, antibiotics, and other small molecules. The function of glycosyltransferases involved in saponin glycosylation in ginseng is transferring glycosyls from glycosyl donors to hydroxyls at position C-3, C-6, or C-20 of sapogenins or aglycones, thereby forming saponins with various pharmaceutical values.

At present, upon analyzing the transcriptome of *P. ginseng, P. quinguefolium* and *P. notoginseng*, researchers have identified huge amounts of glycosyltransferase genes. However, which of them are involved in ginsenosides synthesis remained ambiguous. The studies on isolation and purification of glycosyltransferases are making slow progress due to the numerous kinds of glycosyltransferases in ginseng and the extremely low content thereof.

Rare ginsenosides refer to the saponins with extremely low content in *P. ginseng*. Ginsenoside CK (20-*O*-β-(D-glucopyranosyl)-20(*S*)-protopanaxadiol) belongs to PPD-type

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saponins with a glucosyl group attached to C-20 hydroxyl of sapogenins. The content of ginsenoside CK in *P. ginseng* is extremely low, and it is the main metabolite produced by microbiological hydrolysis of PDD-type saponins in human intestinal tract. Researches indicated that most PDD-type saponins can be absorbed by human body only upon being metabolized into CK. Thus, ginsenosides CK is the real entity which can be directly absorbed by human body and take effects, while other saponins are only prodrugs. Ginsenoside CK has excellent anti-tumor activity. It can induce tumor cell apoptosis and inhibit tumor cell metastasis. The assays using it with combination of radiotherapy or chemotherapy came out to possess the effect of radiotherapy or chemotherapy enhancement. Besides, ginsenoside CK has the activities of anti-allergy, anti-inflammation, neural protection, anti-diabetes, and anti-skin aging. The pharmacological activities of ginsenoside CK are characterized by its multiple-targets, high activity, and low toxicity.

Ginsenoside F1 (20-*O*-β-D-glucopyranosyl-20(*S*)-protopanaxatriol) belonging to PPT saponins also has a very low content in *P. ginseng* and is one of the rare ginsenosides as well. Ginsenoside F1 is quite similar to CK in structure, also having a glucosyl group attached to C-20 hydroxyl of sapogenin. Ginsenoside F1 also possesses unique pharmaceutical values. It has the function of anti-aging and anti-oxidization.

Ginsenoside Rh1 (6-*O*-β-D-glucopyranosyl-20(*S*)-protopanaxatriol) belonging to PPT saponins also has a very low content in *P. ginseng* and is one of the rare ginsenosides as well. Ginsenoside Rh1 is quite similar to F1 in structure, but its glycosylation site is the hydroxyl at the C-6 position. Ginsenoside Rh1 also possesses unique physiological functions, such as anti-allergy and anti-inflammation.

Ginsenoside Rh2 (3-*O*-β-(D-glucopyranosyl)-20(*S*)-protopanaxadiol) with an extremely low content in *P. ginseng* of about 0.01% of ginseng dry weight is one of the rare ginsenosides as well. However, ginsenoside Rh2 has an excellent anti-tumor activity, which enabling it to be one of the most primary anti-tumor active ingredients in ginseng. It can inhibit tumor cell growth, induce tumor cell apoptosis, and inhibit tumor cell metastasis. Researches showed that ginsenoside Rh2 can inhibit the proliferation of lung cancer cells 3LL (mice), Morris liver cancer cells (rats), B-16 melanoma cells (mice), and HeLa cells (human). Clinically, treatments by combing ginsenoside Rh2 with radiotherapy or chemotherapy can improve the effects of theses therapies. Moreover, ginsenoside Rh2 also has the function of anti-allergy, improving

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body immunity, and inhibiting the inflammation produced by NO and PEG.

Ginsenoside Rg3 with a low content in ginseng has a significant anti-tumor effect, and it is complementary to ginsenoside Rh2 in anti-tumor effect. Clinic uses demonstrated that the combination of Rg3 and Rh2 can further enhance their synergetic effect on tumor treatment.

Because of the extremely low content of rare ginsenosides CK, F1, Rh1, Rh2 and Rg3 in *P. ginseng*, the present preparation method is, starting from the large amounts of saponins in *P. ginseng*, extracting and purifying upon conversion by selectively hydrolyzing glycosyls. Total saponins or protopanaxadiol type saponins of panax plants are used as raw materials for converting, isolating, and extracting 20(*S*)-protoginsenoside-Rh2. This preparation method is advantaged in that the huge amounts of diol type saponins are utilized. However, the reaction must be conducted under high temperature and high pressure (Changchun SONG etc. Preparation Method of 20(*S*)-ginsenosides-Rh2, Pharmaceutical Composition and Use Thereof, CN patent No.1225366, 1999). Two methods of preparing 20(R&S)-ginsenosides-Rh2 from ginseng ingredients are disclosed by Korea Ginseng and Tobacco Institution; wherein the PPD saponin ingredients are obtained first, and then subjected to acidic hydrolysis to give 20(R&S)-ginsenosides-Rg3, the ginsenoside Rg3 is then treated to obtain ginsenoside Rh2. The major defect of the above methods is that they need a set of PPD-type saponin monomers as the starting materials for the products, which results in the complicated reaction steps, great loss of raw materials and complicated operations, thereby leading to the increased costs and difficulty in improving the yield. Since the glycosyls at C-20 of CK and F1 can be easily destroyed during the hydrolysis process, chemical methods are unsuitable for CK and F1 production. The yield of Rh1 by hydrolyzing saponins through acid or alkaline method is very low and many by-products are produced as well.

Enzymatic conversion method is characterized with its mild condition, high specificity, and easy isolation and purification of products, and hence it is the major method for CK, F1 and Rh1 production at present. The enzymes used for preparing ginsenosides CK, F1, Rh1 and Rh2 mainly include naringinase, pectinase, cellulase, lactase and the like. Ginsenoside CK can be also obtained by microbiological conversion which mainly utilizes anaerobion originated from intestinal tracts. Although great progresses have been made for preparing rare ginsenosides CK, F1, Rh1 and Rh2 by biological conversion (enzymatic method and microbiological method), the cost for preparing CK1, F1, Rh1 and Rh2 is still high and the yield

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is quite limited due to the fact that these methods use ginsenosides as the raw material (CN patent: CN1105781C; Dongshi JIN, Journal of Dalian Light Industry Academy, 2001).

In view of the important biological activities and tremendous economic values of ginsenoside Rh2, continuous efforts have been made for decades to produce such ginsenoside through chemical synthesis, the basic principle of which is the condensation reaction of PPD and the corresponding glycosyls, namely semi-synthesis (JP patent: JP8-208688, 1996). This method uses PPD as the raw material for semi-synthesizing 20(*S*)-protoginsenoside-Rh2. Its synthesis comprises six steps, and equivalent silver carbonate is used as catalyst in the glycosylation reaction. The high price of the catalyst results in a high cost, and at the same time, the poor stereoselectivity of the catalyst results in a low yield of product. In an alternative method, PPD with its C-12 hydroxyl substituted by aromatic acyl or alkyl is used and glucosyl group donor with activated C1 hydroxyl is added under the protection of organic solvents and inert gas for condensation reaction catalyzed by Lewis acid with the presence of molecular sieve. The resultant product is subjected to column chromatography or recrystallization purification and then the protecting groups are removed, thereby obtaining 20(*S*)-ginsenosides-Rh2 (Yongzheng HUI, A Method for Preparing 20(*S*)-ginsenosides-Rh2, CN patent: CN 1587273A, 2005).

At present, there is no method to effectively prepare rare ginsenosides CK, F1, Rh1, Rh2 and Rg3 in this field. Therefore, there is an urgent need to develop various glycosyltransferases with high specificity and efficiency.

Content of the Invention

The object of the present invention is to provide a group of glycosyltransferases and use thereof.

The first aspect of the present invention is to provide a method for *in vitro* glycosylation, comprising the steps of:

in the presence of a glycosyltransferase, transferring a glycosyl from a glycosyl donor to the following site on tetracyclic triterpenoid compounds:

positions C-20, C-6, C-3 or the first glycosyl at position C-3;

thereby forming glycosylated tetracyclic triterpenoid compounds;

wherein, said glycosyltransferase is selected from the group consisting of:

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a glycosyltransferase as set forth by SEQ ID NOs.: 2, 16, 18, 20, 22, 24, 26, 28, 43, 55, 57, 59 or 61.

The second aspect of the present invention is to provide an isolated polypeptide; said polypeptide is selected from the group consisting of:

(a) a polypeptide having the amino acid sequence as set forth by any one of SEQ ID NOs.: 2, 16, 18, 20, 26, 28, 43, 55, 57, 59 or 61;

(b) a derivative polypeptide, which is derived from a polypeptide having the amino acid sequence as set forth by any one of SEQ ID NOs.: 2, 16, 18, 20, 26, 28, 43, 55, 57, 59 or 61 by substitution, deletion, or addition of one or more amino acid residues, or by addition of a signal peptide sequence, and has the activity of glycosyltransferase;

(c) a derivative polypeptide, which has the polypeptide sequence of (a) or (b) in its sequence;

(d) a derivative polypeptide, which has ≥85% or ≥90% (preferably≥95%) sequence homology with the amino acid sequence as set forth by any one of SEQ ID NOs: 2, 16, 18, 20, 26, 28, 43, 55, 57, 59 or 61 and has the activity of glycosyltransferase.

In another preferred embodiment, said sequence (c) is a fusion protein derived from (a) or (b) by addition of a tag sequence, signal sequence, or secretory signal sequence.

In another preferred embodiment, said polypeptide is set forth by SEQ ID NOs: 2, 16, 18, 20, 26, 28, 3, 55, 57, 59 or 61.

The third aspect of the present invention is to provide an isolated polypeptide; said polypeptide is selected from the group consisting of:

(a1) a polypeptide having the amino acid sequence as set forth by any one of SEQ ID NOs.: 22，24 and 41;

(b1) a polypeptide having the polypeptide sequence of (a1) in its sequence; and/or

said polypeptide is selected from the group consisting of:

(a2) a polypeptide having the amino acid sequence as set forth by any one of SEQ ID NOs.: 4 and 6;

(b2) a derivative polypeptide, which is derived from a polypeptide having the amino acid sequence as set forth by any one of SEQ ID NOs.: 4 and 6 by substitution, deletion, or addition of one or more amino acid residues, or by addition of a signal peptide sequence, and has the activity of glycosyltransferase;

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(c2) a derivative polypeptide, which has the polypeptide sequence of (b2) in its sequence;

(d2) a derivative polypeptide, which has ≥85% or ≥90% (preferably≥95%) sequence homology with the amino acid sequence as set forth by any one of SEQ ID NOs: 4 and 6 and has the activity of glycosyltransferase.

In another preferred embodiment, sequence (c2) is a fusion protein derived from (a2) or (b2) by addition of a tag sequence, signal sequence, or secretory signal sequence.

The fourth aspect of the present invention is to provide an isolated polynucleotide; said polynucleotide is selected from the group consisting of:

(A) a nucleotide sequence encoding the polypeptide of the first or the second aspect;

(B) a nucleotide sequence encoding the polypeptide as set forth by SEQ ID NOs.: 2, 4, 6, 16, 18, 20, 22, 24, 26, 28, 41, 43, 55, 57, 59 or 61;

(C) a nucleotide sequence as set forth by SEQ ID NOs.: 1, 3, 5, 15, 17, 19, 21, 23, 25, 27, 40, 42, 54, 56, 58 or 60;

(D) a nucleotide sequence, which has ≥95% (preferably ≥98%) homology with the sequence as set forth by SEQ ID NOs.: 1, 3, 5, 15, 17, 19, 21, 27, 40, 42, 54, 56, 58 or 60;

(E) a nucleotide sequence derived from the nucleotide sequence as set forth by SEQ ID NOs.: 1, 3, 5, 15, 17, 19, 21, 23, 25, 27, 40, 42, 54, 56, 58 or 60 by deletion or addition of 1-60 (preferably 1-30, more preferably 1-10) nucleotides at its 5' end and/or 3' end;

(F) a nucleotide sequence complementary to (preferably completely complementary to) any one of the nucleotide sequence of (A)-(E).

In another preferred embodiment, said nucleotide sequence is as set forth by SEQ ID NOs.: 1, 3, 5, 15, 17, 19, 21, 23, 25, 27, 40, 42, 54, 56, 58 or 60.

In another preferred embodiment, the polynucleotide with a sequence as set forth by SEQ ID NOs.: 1, 3, 5, 15, 17, 19, 21, 23, 25, 27, 40, 42, 54, 56, 58 or 60 encodes the polypeptide with an amino acid sequence as set forth by SEQ ID NOs.: 2, 4, 6, 16, 18, 20, 22, 24, 26, 28, 41, 43, 55, 57, 59 or 61,respectively.

The fifth aspect of the present invention is to provide a vector; said vector contains the polynucleotide in the third aspect of the present invention. Preferably, said vector includes expression vector, shuttle vector, or integration vector.

The fifth aspect of the present invention is to provide use of said isolated polypeptide in the first or the second aspect for catalyzing one or more of the following reactions, or for

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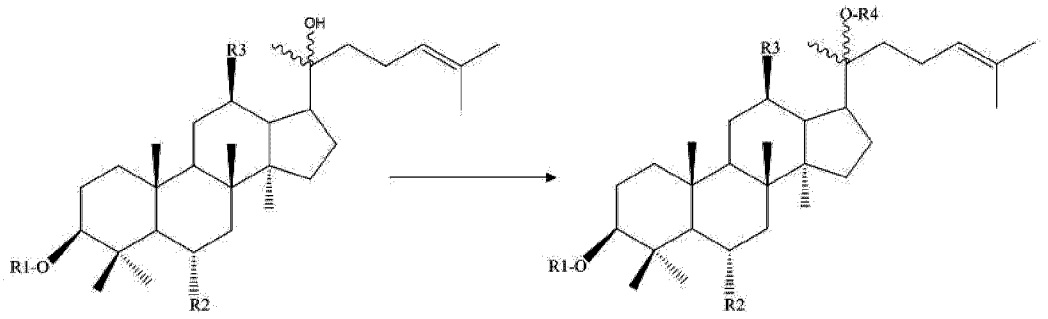
preparing a catalyst preparation used in the catalyzation of one or more of the following reactions: transferring glycosyl(s) from glycosyl donor(s) to hydroxyl(s) at position(s) C-20 and/or C-6 and/or C-3 of tetracyclic triterpenoid compound(s) so as to substitute H in said hydroxyl, and transferring glycosyl(s) from glycosyl donor(s) to the first glycosyl at position C-3 of tetracyclic triterpenoid compound(s) so as to extend carbohydrate chain.

In another preferred embodiment, said glycosyl donor(s) includes a nucleoside diphosphate sugar selected from the group consisting of: UDP- glucose, ADP- glucose, TDP- glucose, CDP- glucose, GDP- glucose, UDP- acetyl glucose, ADP- acetyl glucose, TDP- acetyl glucose, CDP- acetyl glucose, GDP- acetyl glucose, UDP- xylose, ADP- xylose, TDP- xylose, CDP- xylose, GDP- xylose, UDP- galacturonic acid, ADP- galacturonic acid, TDP- galacturonic acid, CDP- galacturonic acid, GDP- galacturonic acid, UDP- galactose, ADP- galactose, TDP- galactose, CDP- galactose, GDP- galactose, UDP- arabinose, ADP- arabinose, TDP- arabinose, CDP- arabinose, GDP- arabinose, UDP- rhamnose, ADP- rhamnose, TDP- rhamnose, CDP- rhamnose, GDP- rhamnose, or other nucleoside diphosphate hexose or nucleoside diphosphate pentose, or the combination thereof.

In another preferred embodiment, said glycosyl donor(s) includes uridine diphosphate (UDP) sugars selected from the group consisting of: UDP- glucose, UDP- galacturonic acid, UDP- galactose, UDP- arabinose, UDP- rhamnose, or other uridine diphosphate hexose or uridine diphosphate pentose, or the combination thereof.

In another preferred embodiment, said isolated polypeptide is used for catalyzing one or more of the following reactions or for preparing a catalyst preparation used in the catalyzation of one or more of the following reactions:

(A)



compound of formula (I) compound of formula (II)

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wherein, R1 is H, monosaccharide glycosyl or polysaccharides glycosyl; R2 or R3 is H or OH; R4 is glycosyl; said polypeptide is selected from SEQ ID NOs.: 2, 16 or 18 or a derivative polypeptide thereof.

In another preferred embodiment, said monosaccharide includes glucose (Glc), rhamnose (Rha), acetyl glucose (Glc (6) Ac), arabinofuranose (Araf), arabopyranose (Arap), and xylose (Xyl), etc.

In another preferred embodiment, said polysaccharide includes polysaccharides composed of 2-4 monosaccharides, such as Glc(2-1)Glc, Glc(6-1)Glc, Glc(6)Ac, Glc(2-1)Rha, Glc(6-1)Arap, Glc(6-1)Xyl, Glc(6-1)Araf, Glc(3-1)Glc(3-1), Glc(2-1) Glu(6)Ac, Glc(6-1)Arap(4-1)Xyl, Glc(6-1)Arap(2-1)Xyl, or Glc(6-1)Arap(3-1)Xyl, etc.

Compounds with R1-R4 substituted are shown in the following table:

substrate	R1	R2	R3	R4	product
PPD	H	H	OH	glycosyl	CK
Rh2	1 glycosyl	H	OH	glycosyl	F2
Rg3	2 glycosyls	H	OH	glycosyl	Rd
PPT	H	OH	OH	glycosyl	F1
DM	H	H	H	glycosyl	20-G-DM

That is, when both of said R1 and R2 are H, and R3 is OH, said compound of formula (I) is protopanaxadiol (PPD);

when R1 is a glucosyl, R2 is H, and R3 is OH, said compound of formula (I) is ginsenoside Rh2;

when R1 is two glucosyls, R2 is H, and R3 is OH, said compound of formula (I) is ginsenoside RG3;

when R1 is H, R2 is OH, and R3 is OH, said compound of formula (I) is protopanaxatriol (PPT);

when R1 is H, R2 is H, and R3 is H, said compound of formula (I) is dammarenediol II (DM).

(B)

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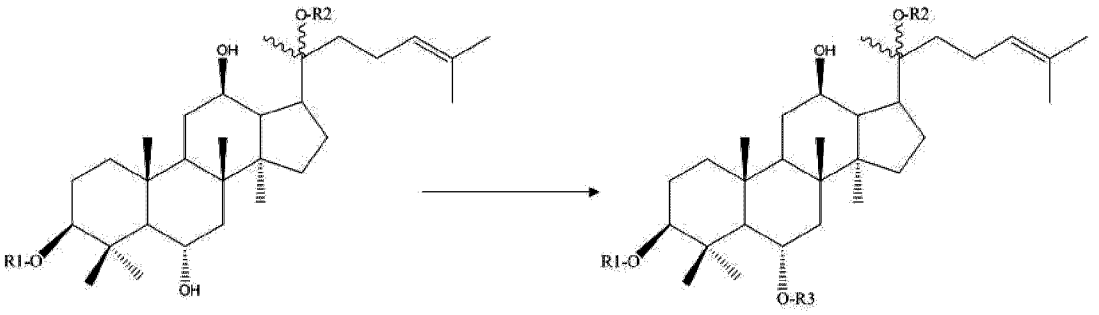


compound of formula (III) compound of formula (IV)

wherein, R1 is H or a glycosyl, R2 is a glycosyl, R3 is a glycosyl, said polypeptide is selected from SEQ ID NOs.: 2, 16 ,18, or 20 or a derivative polypeptide thereof;

or, R1 is H or a glycosyl; R2 is H; R3 is a glycosyl, said polypeptide is selected from SEQ ID NO.: 20 or a derivative polypeptide thereof.

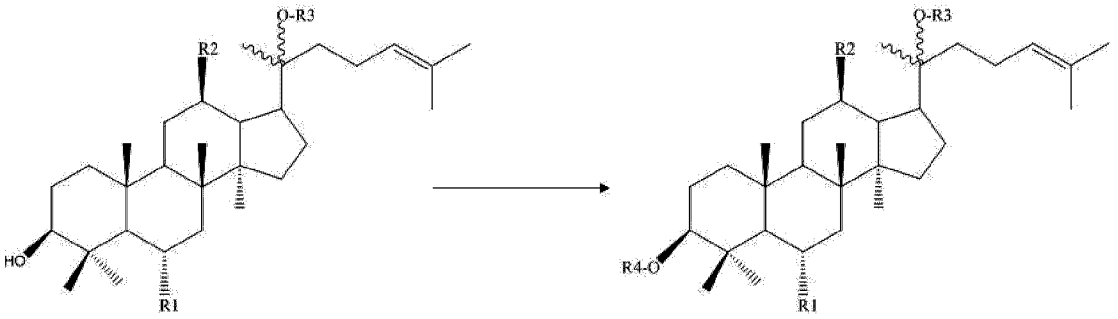
Compounds with R1-R3 substituted are shown in the following table:

substrate	R1	R2	R3	product
F1	H	glycosyl	glycosyl	Rg1
PPT	H	H	glycosyl	Rh1

When both of said R1 and R2 are H, said compound of formula (III) is protopanaxatriol (PPT).

When R1 is H, R2 is a glucosyl, said compound of formula (III) is ginsenoside F1.

(C)



compound of formula (V) compound of formula (VI)

wherein, R1 is H or OH; R2 is H or OH; R3 is H or a glycosyl; R4 is a glycosyl, said polypeptide is selected from SEQ ID NOs.: 22, 24, 41 or 43 or a derivative polypeptide thereof.

Compounds with R1-R4 substituted are shown in the following table:

substrate	R1	R2	R3	R4	product
PPD	H	OH	H	glycosyl	Rh2
CK	H	OH	glycosyl	glycosyl	F2
PPT	OH	OH	H	glycosyl	3-G-PPT
F1	OH	OH	glycosyl	glycosyl	3-G-F1

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DM	H	H	H	glycosyl	3-G-DM

When both of R1 and R3 are H, R2 is OH, said compound of formula (V) is PPD;

R1 is H, R2 is OH, R3 is a glucosyl, said compound of formula (V) is ginsenoside CK;

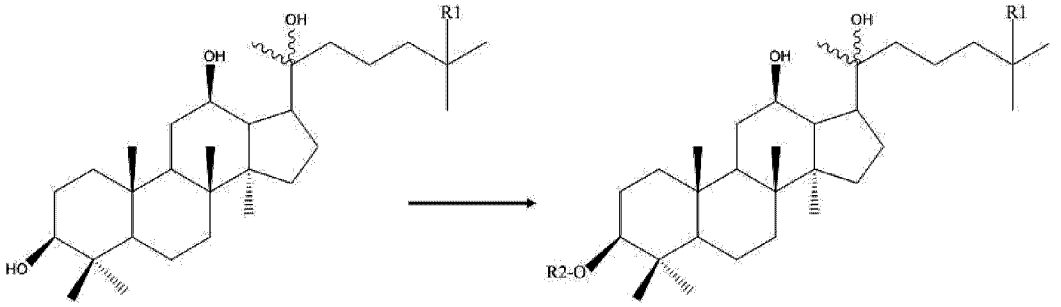
R1 is OH, R2 is OH, R3 is H, said compound of formula (V) is PPT;

R1 is OH, R2 is OH, R3 is a glucosyl, said compound of formula (V) is ginsenoside F1;

R1 is H, R2 is OH, R3 is H, said compound of formula (V) is dammarenediol II (DM).

When the substrate is PPD, said polypeptide is selected from SEQ ID NOs.: 22, 24, 41 or 43 or a derivative polypeptide thereof; when the substrate is CK, said polypeptide is selected from SEQ ID NOs.: 22, 24 or 43 or a derivative polypeptide thereof; when the substrate is PPT, said polypeptide is selected from SEQ ID NOs.: 22, 24 or 41 or a derivative polypeptide thereof; when the substrate is F1 and DM, said polypeptide is selected from SEQ ID NOs.: 22 or 24 or a derivative polypeptide thereof.

(D)



compound of formula (VII) compound of formula (VIII)

wherein, R1 is OH or OCH_3; R2 is glycosyl, said polypeptide is selected from SEQ ID NOs.: 22, 24, 41 or 43 or a derivative polypeptide thereof.

Compounds with R1-R2 substituted are shown in the following table:

substrate	R1	R2	product
25-OH-PPD	OH	glycosyl	3-G-25-OH-PPD
25-OCH_3-PPD	OCH_3	glycosyl	3-G-25-OCH_3-PPD

When R1 is OH, said compound of formula (VII) is 25-OH-PPD;

R1 is OCH, said compound of formula (VII) is 25-OCH_3-PPD.

(E)

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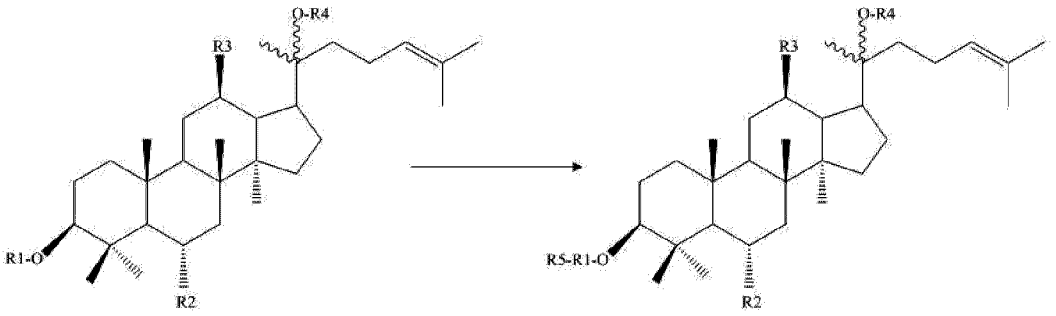


compound of formula (IX) compound of formula (X)

wherein, R1 is glycosyl; R2 or R3 is OH or H; R4 is glycosyl or H; R5 is glycosyl, R5-R1-O is a glycosyl derived from the first glycosyl at C-3, said polypeptide is selected from SEQ ID NOs.: 26, 28, 55, 57, 59 or 61 or a derivative polypeptide thereof.

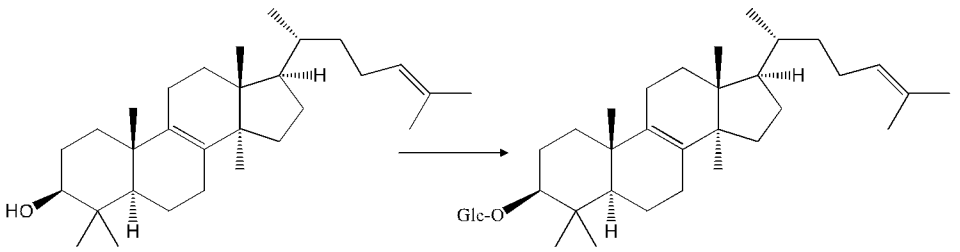
Compounds with R1-R4 substituted are shown in the following table:

substrate	R1	R2	R3	R4	product
Rh2	glycosyl	H	OH	H	Rg3
F2	glycosyl	H	OH	glycosyl	Rd

When R1 is a glucosyl; R2 is H, R3 is OH, R4 is H, compound of formula (IX) is Rh2.

When R1 is a glucosyl; R2 is H, R3 is OH, R4 is a glucosyl, compound of formula (IX) is F2.

(F)



compound of formula (XI) compound of formula (XII)

said polypeptide is selected from SEQ ID NO: 22 or SEQ ID NO: 24 or a derivative polypeptide thereof. The compound of formula (XI) is lanosterol, and the compound of formula (XII) is 3-*O*-β-(D- glucopyranosyl)-lanosterol.

In another preferred embodiment, said glycosyl is selected from glucosyl, galacturonic acid radical, galactosyl, arabinosyl, rhamnosyl, and other hexosyls or pentosyls.

In another preferred embodiment, said compounds of formulas (I), (III), (V), (VII), (IX) or (XI) include but are not limited to S- or R- dammarane-type tetracyclic triterpene compounds, lanostane-type typetetracyclic triterpene compounds, tirucallane-type typetetracyclic triterpene

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compounds, cycloartane-type typetetracyclic triterpene compounds, cucurbitane-type typetetracyclic triterpene compounds, or meliacane-type typetetracyclic triterpene compounds.

In another preferred embodiment, said polypeptide is selected from the group consisting of:

(a) a polypeptide having the amino acid sequence as set forth by any one of SEQ ID NOs.: 2, 16, 18, 20, 26, 28, 41, 43, 55, 57, 59 or 61;

(b) a derivative polypeptide, which is derived from a polypeptide having the amino acid sequence as set forth by any one of SEQ ID NOs.: 2, 16, 18, 20, 26, 28, 41, 43, 55, 57, 59 or 61 by substitution, deletion, or addition of one or more amino acid residues, or by addition of a signal peptide sequence, and has the activity of glycosyltransferase;

(c) a derivative polypeptide, which has the polypeptide sequence of (a) or (b) in its sequence;

(d) a derivative polypeptide, which has ≥85% or ≥90% (preferably≥95%)sequence homology with the amino acid sequence as set forth by any one of SEQ ID NOs: 2, 16, 18, 20, 26, 28, 41, 43, 55, 57, 59 or 61 and has the activity of glycosyltransferase.

In another preferred embodiment, said polypeptide is selected from the group consisting of:

(a1) a polypeptide having the amino acid sequence as set forth by any one of SEQ ID NOs.: 22 and 24;

(b1) a polypeptide having the polypeptide sequence of (a1) in its sequence; and/or

said polypeptide is selected from the group consisting of:

(a2) a polypeptide having the amino acid sequence as set forth by any one of SEQ ID NOs.: 4 and 6;

(b2) a derivative polypeptide, which is derived from a polypeptide having the amino acid sequence as set forth by any one of SEQ ID NOs.: 4 and 6 by substitution, deletion, or addition of one or more amino acid residues, or by addition of a signal peptide sequence, and has the activity of glycosyltransferase;

(c2) a derivative polypeptide, which has the polypeptide sequence of (b2) in its sequence;

(d2) a derivative polypeptide, which has ≥85% or ≥90% (preferably≥95%) sequence homology with the amino acid sequence as set forth by any one of SEQ ID NOs: 4 and 6 and has the activity of glycosyltransferase.

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In another embodiment, the polynucleotide encoding said polypeptide is selected from the group consisting of:

(A) a nucleotide sequence encoding the polypeptide of the first or the second aspect;

(B) a nucleotide sequence encoding the polypeptide as set forth by SEQ ID NOs.: 2, 4, 6, 16, 18, 20, 22, 24, 26, 28, 41, 43, 55, 57, 59 or 61;

(C) a nucleotide sequence as set forth by SEQ ID NOs.: 1, 3, 5, 15, 17, 19, 21, 23, 25, 27, 40, 42, 54, 56, 58 or 60;

(D) a nucleotide sequence, which has ≥95% (preferably ≥98%) homology with the sequence as set forth by SEQ ID NOs.: 1, 3, 5, 15, 17, 19, 21, 27, 40, 42, 54, 56, 58 or 60;

(E) a nucleotide sequence derived from the nucleotide sequence as set forth by SEQ ID NOs.: 1, 3, 5, 15, 17, 19, 21, 23, 25, 27, 40, 42, 54, 56, 58 or 60 by deletion or addition of 1-60 (preferably 1-30, more preferably 1-10) nucleotides at its 5' end and/or 3' end;

(F) a nucleotide sequence complementary to (preferably completely complementary to) any one of the nucleotide sequence of (A)-(E).

In another preferred embodiment, said nucleotide sequence is as set forth by SEQ ID NOs.: 1, 3, 5, 15, 17, 19, 21, 23, 25, 27, 40, 42, 54, 56, 58 or 60.

In another preferred embodiment, the polynucleotide with a sequence as set forth by SEQ ID NOs.: 1, 3, 5, 15, 17, 19, 21, 23, 25, 27, 40, 42, 54, 56, 58 or 60 encodes the polypeptide with an amino acid sequence as set forth by SEQ ID NOs.: 2, 4, 6, 16, 18, 20, 22, 24, 26, 28, 41, 43, 55, 57, 59 or 61, respectively.

The sixth aspect of the present invention is to provide a method for conducting catalytic glycosylation, comprising the following steps: in the presence of a polypeptide and a derivative polypeptide according to the second and third aspects of the present invention, conducting the catalytic glycosylation.

In another preferred embodiment, said method further comprises the step of:

In the presence of a glycosyl donor and a polypeptide or a derivative polypeptide according to the second or third aspect of the present invention, transforming said compound of formula (I) into said compound of formula (II), or transforming said compound of formula (III) into said compound of formula (IV), or transforming said compound of formula (V) into said compound of formula (VI), or transforming said compound of formula (VII) into said compound of formula (VIII), or transforming said formula (IX) compound into said compound of formula

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(X), or transforming said compound of formula (XI) into said compound of formula (XII);

In another preferred embodiment, said method further comprises: adding said polypeptide or a derivative polypeptide thereof into the catalytic reaction, respectively; and/or

adding said polypeptide or a derivative polypeptide thereof into the catalytic reaction simultaneously.

In another preferred embodiment, said method further comprises: in the co-presence of a glycosyl donor and at least two of the polypeptide or the derivative polypeptide according to the second and third aspects of the present invention, transforming the compound of formula (I) into the compound of formula (IV), (VI), (VIII), (X), or transforming the compound of formula (III) into the compound of formula (II), (VI), (VIII), (X), or transforming the compound of formula (V) into the compound of formula (II), (IV), (VIII), (X), or transforming the compound of formula (VII) into the compound of formula (II), (IV), (VI), (X), or transforming the compound of formula (IX) into the compound of formula (II), (IV), (VI), (VIII).

In another preferred embodiment, said method further comprises: co-expressing the nucleotide sequence encoding the glycosyltransferase and the key gene(s) in the anabolism pathway of dammarenediol II and/or protopanaxadiol and/or protopanaxatriol in a host cell, thereby obtaining said compound of formula (II), (IV), (VI), (VIII), (X) or (XII).

In another preferred embodiment, said host cell is *saccharomycetes* or *E. coli*.

In another preferred embodiment, said polypeptide is a polypeptide having the amino acid sequence as set forth by SEQ ID NOs.: 2, 4, 6, 16, 18, 20, 22, 24, 26, 28, 41, 43, 55, 57, 59 or 61 and a derivative polypeptide thereof.

In another preferred embodiment, the nucleotide sequence encoding said polypeptide is as set forth by SEQ ID NOs.: 1, 3, 5, 15, 17, 19, 21, 23, 25, 27, 40, 42, 54, 56, 58 or 60.

In another preferred embodiment, said method further comprises: providing additive(s) for modulating enzyme activity to the reaction system.

In another preferred embodiment, said additive(s) for modulating enzyme activity is: additive(s) enhancing enzyme activity or inhibiting enzyme activity.

In another preferred embodiment, said additive(s) for modulating enzyme activity is selected from the group consisting of Ca^{2+}, Co^{2+}, Mn^{2+}, Ba^{2+}, Al^{3+}, Ni^{2+}, Zn^{2+}, and Fe^{2+}.

In another preferred embodiment, said additive(s) for modulating enzyme activity is a material(s) capable of producing Ca^{2+}, Co^{2+}, Mn^{2+}, Ba^{2+}, Al^{3+}, Ni^{2+}, Zn^{2+}, or Fe^{2+}.

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In another preferred embodiment, said glycosyl donor(s) is nucleoside diphosphate sugar(s) selected from the group consisting of: UDP- glucose, ADP- glucose, TDP- glucose, CDP- glucose, GDP- glucose, UDP- acetyl glucose, ADP- acetyl glucose, TDP- acetyl glucose, CDP- acetyl glucose, GDP- acetyl glucose, UDP- xylose, ADP- xylose, TDP- xylose, CDP- xylose, GDP- xylose, UDP- galacturonic acid, ADP- galacturonic acid, TDP- galacturonic acid, CDP- galacturonic acid, GDP- galacturonic acid, UDP- galactose, ADP- galactose, TDP- galactose, CDP- galactose, GDP- galactose, UDP- arabinose, ADP- arabinose, TDP- arabinose, CDP- arabinose, GDP- arabinose, UDP- rhamnose, ADP- rhamnose, TDP- rhamnose, CDP- rhamnose, GDP- rhamnose, or other nucleoside diphosphate hexose or nucleoside diphosphate pentose, or the combination thereof.

In another preferred embodiment, said glycosyl donor(s) is uridine diphosphate (UDP) sugars selected from the group consisting of: UDP- glucose, UDP- galacturonic acid, UDP- galactose, UDP- arabinose, UDP- rhamnose, or other uridine diphosphate hexose or uridine diphosphate pentose, or the combination thereof.

In another preferred embodiment, the pH of the reaction system is: pH4.0-10.0, preferably 5.5-9.0.

In another preferred embodiment, the temperature of the reaction system is: 10℃-105℃, preferably 20℃-50℃.

In another preferred embodiment, the key gene(s) in the anabolism pathway of dammarenediol II includes but are not limited to dammarenediol synthase gene.

In another preferred embodiment, the key gene(s) in the anabolism pathway of PPD includes but is not limited to: dammarenediol synthase gene, cytochrome P450 CYP716A47 gene, and P450 CYP716A47 reductase gene, or the combination thereof.

In another preferred embodiment, the key gene(s) in the anabolism pathway of PPT includes but is not limited to: dammarenediol synthase gene, cytochrome P450 CYP716A47 gene, P450 CYP716A47 reductase gene, cytochrome P450 CYP716A53V2 gene and the reductase gene thereof, or the combination thereof.

In another preferred embodiment, the substrate of the catalytic glycosylation is the compound of formula (I), (III), (V), (VII), (IX) or (XI), and said product is the compound of (II), (IV), (VI), (VIII), (X) or (XII);

In another preferred embodiment, said compound of formula (I) is PPD (Protopanaxadiol),

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and the compound of formula (II) is ginsenoside CK (20-*O*-β-(D-glucopyranosyl)-protopanaxadiol);

or, said compound of formula (I) is ginsenoside Rh2 (3-*O*-β-(D-glucopyranosyl)-protopanaxadiol)), and the compound of formula (II) is ginsenoside F2 (3-*O*-β-(D-glucopyranosyl)-20-*O*- β-(D-glucopyranosyl) -protopanaxadiol);

or, said compound of formula (I) is ginsenoside Rg3, and the compound of formula (II) is ginsenoside Rd;

or, said compound of formula (I) is PPT (Protopanaxatriol), and the compound of formula (II) is ginsenoside F1 (20-*O*-β-(D-glucopyranosyl) -protopanaxatriol);

or, said compound of formula (I) is DM (Dammarenediol II), and the compound of formula (II) is ginsenoside 20-*O*-β-(D-glucopyranosyl)- Dammarenediol II;

or, said compound of formula (III) is PPT, and the compound of formula (IV) is ginsenoside Rh1 (6-*O*-β-(D-glucopyranosyl)-protopanaxatriol);

or, said compound of formula (III) is ginsenoside F1, and the compound of formula (IV) is ginsenoside Rg1 (6-*O*-β-(D-glucopyranosyl) -20-*O*-β- (D-glucopyranosyl)-protopanaxadiol);

or, said compound of formula (V) is PPD, and the compound of formula (VI) is ginsenoside Rh2 (3-*O*-β-(D-glucopyranosyl)-protopanaxadiol);

or, said compound of formula (V) is CK, and the compound of formula (VI) is ginsenoside F2 (3-*O*-β-(D-glucopyranosyl)-20-*O*-β-(D-glucopyranosyl)-protopanaxadiol);

or, said compound of formula (V) is PPT, and the compound of formula (VI) is ginsenoside 3-*O*-β-(D-glucopyranosyl) -protopanaxatriol;

or, said compound of formula (V) is ginsenoside F1, and the compound of formula (VI) is ginsenoside 3-*O*-β-(D-glucopyranosyl)-F1;

or, said compound of formula (V) is DM, and the compound of formula (VI) is ginsenoside 3-*O*-β-(D-glucopyranosyl)-Dammarenediol II;

or, said compound of formula (VII) is 25-OH- PPD(25-OH- protopanaxadiol), and the compound of formula (VIII) is ginsenoside 3-*O*-β-(D-glucopyranosyl)- 25-OH-protopanaxadiol;

or, said compound of formula (VII) is 25-OCH_3-PPD(25-OCH_3-protopanaxadiol), and the compound of formula (VIII) is ginsenoside 3-*O*-β-(D-glucopyranosyl)-25-OCH_3-protopanaxadiol;

or, said compound of formula (IX) is ginsenoside Rh2, and the compound of formula (X) is

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ginsenoside Rg3;

or, said compound of formula (IX) is ginsenoside F2, and the compound of formula (X) is ginsenoside Rd.

Or, said compound of formula (XI) is lanosterol, and the compound of formula (XII) is 3-*O*-β-(D- glucopyranosyl)-lanosterol.

The seventh aspect of the present invention is to provide a genetically engineered host cell; said host cell contains the vector according to the fifth aspect of the present invention, or has a polynucleotide according to the fourth aspect of the present invention integrated in its genome.

In another preferred embodiment, said glycosyltransferase is the polypeptide or the derivative polypeptide according to the second or third aspect of the present invention.

In another preferred embodiment, the nucleotide sequence encoding said glycosyltransferase is as described in the fourth aspect of the present invention.

In another preferred embodiment, said cell is a prokaryocyte or a eukaryocyte.

In another preferred embodiment, said host cell is a eukaryocyte, such as a yeast cell or a plant cell.

In another preferred embodiment, said host cell is a *Saccharomyces cerevisiae* cell.

In another preferred embodiment, said host cell is a prokaryocyte, such as *E. coli*.

In another preferred embodiment, said host cell is a ginseng cell.

In another preferred embodiment, said host cell is not a cell naturally producing the compound of formula (II), (IV), (VI), (VIII), (X) or (XII).

In another preferred embodiment, said host cell is not a cell naturally producing rare ginsenoside CK and/or rare ginsenoside F1 and/or rare ginsenoside Rh2 and/or Rg3 and/or Rh1, and/or novel ginsenoside 20-*O*-β-(D-glucopyranosyl)-dammarendiol II, 3-*O*-β-(D-glucopyranosyl) -PPT, 3-*O*-β-(D-glucopyranosyl)-F1, 3-*O*-β-(D-glucopyranosyl)-DM, 3-*O*-β-D-glucopyranosyl)-25-OH-PPD, 3-*O*-β-(D-glucopyranosyl)-25-OCH_3-PPD, and/or Rh1, F2, Rd and Rg1 etc.

In another preferred embodiment, said key gene(s) in the anabolism pathway of dammarenediol II includes but is not limited to: dammarenediol synthase gene.

In another preferred embodiment, the key gene(s) in the anabolism pathway of PPD contained in said host cell includes but is not limited to dammarenediol synthase gene,

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cytochrome P450 CYP716A47 gene, and P450 CYP716A47 reductase gene, or the combination thereof.

In another preferred embodiment, the key gene(s) in the anabolism pathway of PPT contained in said host cell includes but is not limited to dammarenediol synthase gene, cytochrome P450 CYP716A47 gene, P450 CYP716A47 reductase gene, and cytochrome P450 CYP716A53V2 gene, or the combination thereof.

The eighth aspect of the present invention is to provide use of the host cell according to the seventh aspect, for preparing an enzymatic catalyzation preparation, or for producing a glycosyltransferase, or as a catalytic cell, or for producing the compound of formula (II), (IV), (VI), (VIII), (X) or (XII).

In another preferred embodiment, said host cell is used for producing new saponins 20-*O*-β-(D-glucopyranosyl)-dammarendiol II and/or 3-*O*-β- (D-glucopyranosyl)-dammarendiol II, 3-*O*-β-(D-glucopyranosyl) -protopanaxatriol, 3-*O*-β-(D-glucopyranosyl)-F1 and/or rare ginsenoside CK and/or rare ginsenoside F1 and or rare ginsenoside Rh1 and/or ginsenoside Rh2 and/or rare ginsenoside Rg3 through glycosylation of dammarenediol II (DM) and/or protopanaxadiol (PPD), and/or protopanaxatriol (PPT).

The ninth aspect of the present invention is to provide a method for producing a transgenic plant, comprising the following step: regenerating said genetically engineered host cell according to the seventh aspect of the present invention into a plant, and said genetically engineered host cell is a plant cell.

In another preferred embodiment, said genetically engineered host cell is a ginseng cell.

It should be understood that in the present invention, the technical features specifically described above and below (such as in the Examples) can be combined with each other, thereby constituting a new or preferred technical solution which needs not be described one by one.

Description of Figures

The following figures are used to describe the specific embodiments of the present invention and should not be used as limitation to the scope defined by the claims.

Figure 1 shows the agarose gel electrophoretogram of the PCR products of the genes gGT25, gGT25-1, gGT25-3 and gGT25-5.

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Figure 2 shows the SDS-PAGE detection of gGT25, gGT25-1, gGT25-3 and gGT25-5 gene expression in *Saccharomyces cerevisiae*; lane 1, electrophoresis results of the protein marker(molecular weight from top to bottom: 200,116, 97.2, 66.4 and 44.3kDa); lane 2, lysate supernatant of the GT25-pYES2 recombinant yeast; lane 3, lysate supernatant of the gt25-1-pYES2 recombinant yeast; lane 4, lysate supernatant of the gt25-3-pYES2 recombinant yeast; lane 5, lysate supernatant of the gt25-5-pYES2 recombinant yeast; lane 6, lysate supernatant of the empty vector pYES2 recombinant.

Figure 3 shows the Western Blot detection of gGT25, gGT25-1, gGT25-3 and gGT25-5 gene expression in *S. cerevisiae*; lane 1, lysate supernatant of the recombinant yeast gt25-pYES2; lane 2, lysate supernatant of the recombinant yeast gt25-1-pYES2; lane 4, lysate supernatant of the recombinant yeast gt25-3-pYES2; lane 5, lysate supernatant of the recombinant yeast gt25-5-pYES2; lane 3, lysate supernatant of the empty vector pYES2 recombinant.

Figure 4 shows the SDS-PAGE detection of gGT13 and gGT30 expression in *S. cerevisiae*; lane 1, lysate supernatant of the recombinant yeast gt30-pYES2; lane 2, lysate supernatant of the recombinant yeast gt13-pYES2; lane 3, lysate supernatant of the empty vector pYES2 recombinant.

Figure 5 shows the Western Blot detection of gGT13 and gGT30 expression in *S. cerevisiae*; lane 1, lysate supernatant of the recombinant yeast gt30-pYES2; lane 2, lysate supernatant of the recombinant yeast gt13-pYES2; lane 3, lysate supernatant of the empty vector pYES2 recombinant.

Figure 6 shows the TLC detection of the products obtained by catalyzing protopanaxadiol (PPD) and PPD-type ginsenosides using the glycosyltransferases gGT25, gGT25-1 and gGT25-3. Lane 25, gGT25 crude enzyme (lysate supernatant of the recombinant yeast gt25-pYES2); lane 25-1, gGT25-1 crude enzyme (lysate supernatant of the recombinant yeast gt25-1-pYES2); lane 25-3, gGT25-3 crude enzyme (lysate supernatant of the recombinant yeast gt25-3-pYES2); lane "-", negative control, crude enzyme was substituted by lysate supernatant of the empty vector yeast; lane M, mixed standard samples of PPD and PPD-type ginsenosides.

Figure 7 shows the TLC detection of the products obtained by catalyzing protopanaxatriol (PPT) and PPT-type ginsenosides using the glycosyltransferases gGT25, gGT25-1 and

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gGT25-3. Lane M, mixed standard sample of PPT and PPT-type ginsenosides; lane 25, gGT25 crude enzyme (lysate supernatant of the recombinant yeast gt25-pYES2); lane 25-1, gGT25-1 crude enzyme (lysate supernatant of the recombinant yeast gt25-1-pYES2); lane 25-3, gGT25-3 crude enzyme (lysate supernatant of the recombinant yeast gt25-3-pYES2); lane 25-5, gGT25-5 crude enzyme (lysate supernatant of the recombinant yeast gt25-5-pYES2); lane "-", negative control, crude enzyme was substituted by lysate supernatant of the empty vector yeast.

Figure 8 shows the TLC detection of the products obtained by catalyzing dammarenediol II using the glycosyltransferases gGT25, gGT25-1 and gGT25-3. Lane 25, gGT25 crude enzyme (lysate supernatant of the recombinant yeast gt25-pYES2); lane 25-1, gGT25-1 crude enzyme (lysate supernatant of the recombinant yeast gt25-1-pYES2); lane 25-3, gGT25-3 crude enzyme (lysate supernatant of the recombinant yeast gt25-3-pYES2); lane "-", negative control, crude enzyme was substituted by lysate supernatant of the empty vector yeast; lane M, dammarenediol II (DM) standard sample.

Figure 9 shows the TLC detection ofthe products obtained by catalyzing PPD and PPT using the glycosyltransferases gGT13 and gGT30. Lane M1, mixed standard sample of PPD and PPD-type ginsenosides; lane M2, mixed standard sample of PPT and PPT-type ginsenosides; lane 1, PPD catalyzed by gGT13 crude enzyme; lane 2, PPD catalyzed by gGT30 crude enzyme; lane 3, negative control, crude enzyme was substituted with ddH2O; lane 4, PPT catalyzed by gGT13 crude enzyme; lane 5, PPT catalyzed by gGT30 crude enzyme; lane 6, negative control, crude enzyme was substituted with ddH2O.

Figure 10 shows the HPLC detection of the products obtained by catalyzing PPD using the glycosyltransferase gGT25, the sample of line 2: mixed standard sample of PPD and various ginsenosides (CK, Rh2, F2 and Rg3); the sample of line 1: PPD catalyzed by gGT25 crude enzyme; the sample of the third line: the negative control 1, PPD catalyzed by lysate supernatant of the empty vector recombinant yeast; the sample of the fourth line: negative control 2, dH2O.

Figure 11 shows the HPLC detection of the products obtained by catalyzing PPT using the glycosyltransferase gGT25, the sample of line 2: mixed standard sample of PPT and various PPT-type ginsenosides (F1, Rh1 and Rg1); the sample of line 1: PPT catalyzed by gGT25 crude enzyme; the sample of the third line; the negative control 1, PPT catalyzed by lysate

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supernatant of the empty vector recombinant yeast.

Figure 12 shows the LC/MS detection of the products obtained by catalyzing PPD using the glycosyltransferase gGT25. The mass spectrums of peak 2(product peak) in Figure 10 and the standard CK sample are presented.

Figure 13 shows the LC/MS detection of the products obtained by catalyzing PPT using the glycosyltransferase gGT25. The mass spectrums of peak 1(product peak) in Figure 11 and the standard F1 sample are presented.

Figure 14 shows the Western Blot detection of gGT25-pET28a expression in *E. coli* BL21; lanes 1-3 illustrate the total protein, supernatant and precipitate upon 50μM IPTG induction, respectively.

Figure 15 shows the TLC detection of the products obtained by catalyzing PPD *in vitro* using lysate supernatant of the gGT25-pET28a recombinant *E. coli*; lane 1, mixed standard sample of PPD and CK; lane 2, PPD catalyzed by lysate supernatant of the gGT25-pET28a recombinant *E. coli* upon IPTG induction (50μM IPTG).

Figure 16 shows the HPLC detection of the cell lysate extract of the engineered yeast strain A for CK production, the sample of line 1: mixed standard sample of PPD, dammarenediol II, and CK; the sample of line 2: cell lysate of the engineered yeast A which can produce CK; the sample of line 3: negative control, cell lysate of starting yeast strain.

Figure 17 shows the HPLC detection of the products obtained by catalyzing PPT using the glycosyltransferase gGT25-5, the sample of line 1: mixed standard sample of PPT and PPT-type saponins (F1, Rh1, Rg1 and Re); the sample of line 2: the product obtained by catalyzing PPT using gGT25-5 crude enzyme.

Figure 18 shows the LC/MS detection of the products obtained by catalyzing PPT using the glycosyltransferase gGT25-5. The mass spectrums of peak P1 in Figure 17 (product Rh1 peak) and the standard sample of Rh1 are shown.

Figure 19 shows agarose gel electrophoresis detection of the PCR products of genes (a) 3GT1 and 3GT2, (b) 3GT3 and (c) 3GT4.

Figure 20 shows SDS-PAGE detection of (a)3GT1 and 3GT2, (b)3GT3 and (c)3GT4 expressions in *E. coli*; (a) lane 1, total protein in the lysate of the empty vector pET28a-transformed-*E. coli*; lane 2, lysate supernatant of recombinant *E. coli* 3GT1-pET28a; lane 3, lysate precipitation of the recombinant *E. coli* 3GT1-pET28a; lane 4, total protein in the

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lysate of the recombinant *E. coli* 3GT1-pET28a; lane 5, lysate supernatant of the recombinant *E. coli* 3GT2-pET28a; lane 6, lysate precipitation of the recombinant *E. coli* 3GT2-pET28a; lane 7, total protein of the recombinant *E. coli* 3GT2-pET28a; lane 8, protein molecular-weight Marker. (b) Lane 1, Protein molecular-weight Marker; lane 2, lysate supernatant of the recombinant *E. coli* 3GT3-pET28a; lane 3, lysate precipitation of the recombinant *E. coli* 3GT3-pET28a; lane 4, total protein of the recombinant *E. coli* 3GT3-pET28a lysate. (c) lane 1, lysate supernatant of the recombinant *E. coli* 3GT4-pET28a; lane 2, lysate precipitation of the recombinant *E. coli* 3GT4-pET28a; lane 3, lysate supernatant of the recombinant *E. coli* 3GT4-pET28a; lane 4, lysate of the empty vector pET28a-transformed *E. coli*; lane 5, protein molecular-weight Marker. The target protein is indicated with an arrow.

Figure 21 shows Western Blot detection of (a)3GT1 and 3GT2, (b) 3GT3 and (c)3GT4 expression in *E. coli*; (a) lane 1, total protein in the lysate of the empty vector pET28a-transformed-*E. coli*; lane 2, lysate supernatant of recombinant *E. coli* 3GT1-pET28a; lane 3, lysate precipitation of the recombinant *E. coli* 3GT1-pET28a; lane 4, total protein in the lysate of the recombinant *E. coli* 3GT1-pET28a; lane 5, lysate supernatant of the recombinant *E. coli* 3GT2-pET28a; lane 6, lysate precipitation of the recombinant *E. coli* 3GT2-pET28a; lane 7, total protein of the recombinant *E. coli* 3GT2-pET28a; (b) lane 1, lysate supernatant of the recombinant *E. coli* 3GT3-pET28a; lane 2, lysate precipitation of the recombinant *E. coli* 3GT3-pET28a; lane 3, total protein in the lysate of the recombinant *E. coli* 3GT3-pET28a; (c) lane 1, total protein in the lysate of the recombinant *E. coli* 3GT4-pET28a; lane 2, lysate precipitation of the recombinant *E. coli* 3GT4-pET28a; lane 3, lysate supernatant of the recombinant *E. coli* 3GT4-pET28a; lane 4, lysate of the empty vector pET28a-transformed *E. coli*.

Figure 22 shows the TLC detection of the products obtained by catalyzing PPD and CK using the glycosyltransferases 3GT1 and 3GT2. Lane 1, standard samples of PPD-type ginsenosides; lane 2, ginsenoside Rh2 produced by catalyzing PPD using the glycosyltransferase 3GT1; lane 3, ginsenoside F2 produced by catalyzing ginsenoside CK using glycosyltransferase 3GT1; lane 4, ginsenoside Rh2 produced by catalyzing PPD using glycosyltransferase 3GT2; lane 5, ginsenoside F2 produced by catalyzing ginsenoside CK using glycosyltransferase 3GT2.

Figure 23 shows the TLC detection of the products obtained by catalyzing DM and

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25-OH-PPD using glycosyltransferases 3GT1 and 3GT2. (A) Catalyzation of DM and 25-OH-PPD by 3GT1 crude enzyme (lysate supernatant of the recombinant *E. coli* 3GT1-pET28a). Lane 1, 25-OH-PPD standard sample; lane 2, 3-*O*-β-(D-glucopyranosyl)-25-OH-protopanaxadiol generated by catalyzing 25-OH-PPD using 3GT1 crude enzyme; lane 3, DM standard sample; lane 4, 3-*O*-β- (D-glucopyranosyl)-dammarenediol II produced by catalyzing DM using 3GT1 crude enzyme; (B) Catalyzation of DM and 25-OH-PPD by 3GT2 crude enzyme (lysate supernatant of the recombinant *E. coli* 3GT2-pET28a). Lane 1, 25-OH-PPD standard sample; lane 2, 3-*O*-β-(D-glucopyranosyl)-25-OH- protopanaxadiol produced by catalyzing 25-OH-PPD using 3GT2; lane 3, DM standard sample; lane 4, 3-*O*-β- (D-glucopyranosyl)- dammarenediol II produced by catalyzing DM using 3GT2.

Figure 24 shows TLC detection of the products obtained by catalyzing PPT and F1 using glycosyltransferases 3GT1 and 3GT2. Lane 1, 3-*O*-β-(D-glucopyranosyl)-protopanaxatriol obtained by catalyzing PPT using 3GT1 crude enzyme (lysate supernatant of the recombinant *E. coli* 3GT1-pET28a); lane 2, 3-*O*-β-(D-glucopyranosyl) -F1 obtained by catalyzing F1 using 3GT1 crude enzyme; lane 3, 3-*O*-β-(D-glucopyranosyl)-PPT obtained by catalyzing PPT using 3GT2 crude enzyme (lysate supernatant of the recombinant *E. coli* 3GT2-pET28a); lane 4, 3-*O*-β-(D-glucopyranosyl)-F1 obtained by catalyzing F1 using 3GT2 crude enzyme.

Figure 25 shows TLC detection of the products obtained by catalyzing 20 (*R*)-PPD using glycosyltransferases 3GT1 and 3GT2. Lane 1, 20 (*R*)-PPD standard sample; lane 2, 20 (*R*)-Rh2 obtained by catalyzing 20 (*R*)-PPD using 3GT1 crude enzyme (lysate supernatant of the recombinant *E. coli* 3GT1-pET28a); lane 3, 20 (*R*)-Rh2 obtained by catalyzing 20 (*R*)-PPD using 3GT2 crude enzyme (lysate supernatant of the recombinant *E. coli* 3GT2-pET28a); lane 4, control, the crude enzyme was substituted by lysate supernatant of the empty vector pET28a-transformed *E. coli*; lane 5, 20 (*R*)-Rh2 standard sample.

Figure 26 shows TLC detection of the products obtained by catalyzing lanosterol using glycosyltransferase 3GT1. Lane 1, lanosterol catalyzed by 3GT1 crude enzyme (lysate supernatant of the recombinant *E. coli* 3GT1-pET28a); lane 2, lanosterol catalyzed by 3GT2 crude enzyme (lysate supernatant of the recombinant *E. coli* 3GT2-pET28a); lane 3, control, the crude enzyme was substituted by lysate supernatant of the empty vector pET28a-transformed *E. coli*.

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Figure 27 shows TLC detection of the products obtained by catalyzing PPD, PPT and 25-OH-PPD using glycosyltransferase 3GT3. (a) ginsenoside Rh2 produced by catalyzing PPD using 3GT3 crude enzyme (lysate supernatant of the recombinant *E. coli* 3GT3-pET28a); (b)3-*O*-β-(D-glucopyranosyl)-PPT (3-G-PPT) produced by catalyzing PPT using 3GT3 crude enzyme; (c)3-*O*-β-(D-glucopyranosyl) -25-OH-PPD (3-G-25-OH-PPD) produced by catalyzing 25-OH-PPD using 3GT3 crude enzyme.

Figure 28 shows TLC detection of the products obtained by catalyzing PPD, CK and 25-OH-PPD using glycosyltransferase 3GT4. (a) ginsenoside Rh2 produced by catalyzing PPD using 3GT4 crude enzyme (lysate supernatant of the recombinant *E. coli* 3GT4-pET28a); M represents the mixed standard sample of PPD-type ginsenosides; "+" represents samples with 3GT4 crude enzyme added, "-" represents control, that is, the crude enzyme being substituted by lysate supernatant of pET28a-transformed *E. coli*; (b) F2 produced by catalyzing ginsenoside CK using 3GT4 crude enzyme; "+" represents samples with 3GT4 crude enzyme added, "-" represents control, that is, the crude enzyme being substituted by lysate supernatant of pET28a-transformed *E. coli*; (c) 3-*O*-β-(D-glucopyranosyl)-25-OH-PPD (3-G-25-OH-PPD) produced by catalyzing 25-OH-PPD using glycosyltransferase 3GT4, "+" represents samples with 3GT4 crude enzyme added, "-" represents control, that is, the crude enzyme being substituted by lysate supernatant of the pET28a-transformed *E. coli*.

Figure 29 shows HPLC detection of Rh2 obtained by catalyzing PPD using glycosyltransferases 3GT1, 3GT3 and 3GT4, the sample of line 1: mixed standard sample of CK, Rh2 and F2; the sample of line 2: the product obtained by catalyzing PPD using the glycosyltransferase 3GT1 crude enzyme (lysate supernatant of the recombinant *E. coli* 3GT1-pET28a); the sample of line 3: the product obtained by catalyzing PPD using 3GT3 crude enzyme (lysate supernatant of the recombinant *E. coli* 3GT3-pET28a); the sample of line 4: the product obtained by catalyzing PPD using 3GT4 crude enzyme (lysate supernatant of the recombinant *E. coli* 3GT4-pET28a).

Figure 30 shows LC/MS detection of the products obtained by catalyzing PPD using the glycosyltransferases 3GT1, 3GT3 and 3GT4. The mass spectrum of the standard sample of Rh2, P1 peak of Figure 29 (product peak of 3GT1), P2 peak of Figure 29 (product peak of 3GT2) and P3 peak of Figure 29 (product peak of 3GT4) are shown.

Figure 31 shows agarose gel electrophoresis analysis of the PCR products of (a) gene

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gGT29/gGT29-3 and (b) gene gGT29-4/gGT29-5/gGT29-6 and gGT29-7. (b) Lane 1, nucleic acid Marker; lane 2, PCR product of gene gGT29/gGT29-3; (b) lane 1, PCR product of gene gGT29-4/gGT29-5/gGT29-6; lane 2, PCR product of gene gGT29-7; lane 3, nucleic acid Marker.

Figure 32 shows SDS-PAGE detection of gGT29 and gGT29-3 expression in *S. cerevisiae*; lane 1, lysate supernatant of the pYES2-transformed yeast; lane 2, lysate supernatant of the recombinant yeast gGT29-pYES2; lane 3, lysate supernatant of the recombinant yeast gGT29-3-pYES2.

Figure 33 shows Western Blot detection of gGT29 and gGT29-3 expression in *S. cerevisiae*; lane 1, lysate supernatant of the pYES2-transformed yeast; lane 2, lysate supernatant of the recombinant yeast gGT29-pYES2; lane 3, lysate supernatant of the recombinant yeast gGT29-3-pYES2.

Figure 34 shows TLC detection of the products obtained by catalyzing ginsenoside Rh2 and F2 using glycosyltransferases gGT29 and gGT29-3. Lane 1, mixed standard sample of PPD and PPD-type ginsenosides; lane 2, Rg3 produced by catalyzing Rh2 using gGT29 crude enzyme (lysate supernatant of the recombinant yeast gGT29-pYES2); lane 3, control for catalyzing Rh2 by gGT29 crude enzyme, wherein the crude enzyme was substituted by lysate of the empty vector pYES2-transformed yeast; lane 4, Rd produced by catalyzing F2 using gGT29; lane 5, control for catalyzing F2 by gGT29, wherein the crude enzyme was substituted by lysate of the empty vector pYES2-transformed yeast; lane 6, Rg3 produced by catalyzing Rh2 using gGT29-3 crude enzyme (lysate supernatant of the recombinant yeast gGT29-pYES2); lane 7, Rd produced by catalyzing F2 using gGT29-3 crude enzyme.

Figure 35 shows TLC detection of the products obtained by catalyzing PPD using the combination of glycosyltransferases gGT29 and 3GT1, or the combination of glycosyltransferases gGT29 and 3GT4. (a) catalyzing PPD by using the combination of gGT29 and 3GT1; lane 1, mixed standard sample of PPD and PPD-type ginsenosides; lane 2, Rh2 produced by catalyzing PPD using 3GT1; lane 3, Rg3 produced by catalyzing Rh2 using gGT29; lane 4, Rg3 produced by catalyzing PPD using the combination of 3GT1 and gGT29; (b) PPD is catalyzed by the combination of gGT29 and 3GT4; lane 1, mixed standard sample of PPD and PPD-type ginsenosides; lane 2, Rh2 produced by catalyzing PPD using 3GT1; lane 3, PPD; lane 4, Rg3 produced by catalyzing PPD using the combination of 3GT4 and

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gGT29.

Figure 36 TLC detection of the products obtained by catalyzing 20(*R*)-PPD or 20(*R*)-Rh2 using glycosyltransferase 3GT1 or gGT29 respectively or by catalyzing 20(*R*)-PPD using the combination of these two glycosyltransferases; lane 1, 20(*R*)-Rh2 produced by catalyzing 20(*R*)-PPD using 3GT1; lane 2, 20(*R*)-Rg3 produced by catalyzing 20(*R*)-Rh2 using gGT29; lane 3, 20(*R*)-Rg3 produced by catalyzing 20(*R*)-PPD using the combination of gGT29 and 3GT1.

Figure 37 shows HPLC detection of the products obtained by catalyzing PPD using the combination of glycosyltransferases gGT29 and 3GT1 or gGT29 and 3GT4. Line 1: a mixed standard sample of Rg3, Rh2 and PPD; line 2: PPD is catalyzed by the combination of glycosyltransferases gGT29 and 3GT1; line 3: PPD is catalyzed by the combination of glycosyltransferases gGT29 and 3GT4.

Figure 38 shows LC/MS detection of the products obtained by catalyzing PPD using the combination of glycosyltransferases gGT29 and 3GT1 or gGT29 and 3GT4. The mass spectrum of the standard sample of Rg3, P1 peak (the product obtained by catalyzing PPD using the combination of gGT29 and 3GT1) and P2 peak of figure 37 (the product peak of the product obtained by catalyzing PPD using the combination of gGT29 and 3GT4) are shown.

Figure 39 shows HPLC detection of the cell lysate extracts of the engineered yeast strain A1 for Rh2 production, the sample of line 1: a mixed standard sample of PPD, DM, Rh2 and Rg3; the sample of line 2: cell lysate extracts of the engineered yeast strain A1 which can produce Rh2.

Figure 40 shows HPLC detection of the cell lysate extracts of the engineered yeast strain A2 for Rg3 production, the sample of line 1: a mixed standard sample of PPD, DM, Rh2 and Rg3; the sample of line 2: cell lysate extracts of the engineered yeast strain A2 which can produce Rg3.

Figure 41 shows HPLC detection of the cell lysate extracts of the engineered yeast strain A3 for Rh1 production, the sample of line 1: a mixed standard sample of PPT and ginsenoside Rh1; the sample of line 2: cell lysate extracts of the engineered yeast strain A3 which can produce Rh1.

Figure 42 shows HPLC detection of the cell lysate extracts of the engineered yeast strain A4 for F1 production, the sample of line 1: a mixed standard sample of PPT and ginsenoside

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F1; the sample of line 2: cell lysate extracts of the engineered yeast strain A4 which can produce F1.

Figure 43 shows HPLC detection of the cell lysate extracts of the engineered yeast strain A5 for Rh2 production, the sample of line 1: a mixed standard sample of DM, PPD, ginsenoside Rh2, and ginsenoside Rg3; the sample of line 2: cell lysate extracts of the engineered yeast strain A5 which can produce Rh2.

Figure 44 shows SDS-PAGE detection of the gene gGT29-4, gGT29-5, gGT29-6 and gGT29-7 expression in *E. coli*. Lane 1, total protein in the lysate of the recombinant *E. coli* gGT29-4-pET28a; lane 2, lysate supernatant of the recombinant *E. coli* gGT29-4-pET28a; lane 3, total protein in the lysate of the recombinant *E. coli* gGT29-5-pET28a; lane 4, lysate supernatant of the recombinant *E. coli* gGT29-5-pET28a; lane 5, total protein in the lysate of the recombinant *E. coli* gGT29-6-pET28a; lane 6, lysate supernatant of the recombinant *E. coli* gGT29-6-pET28a; lane 7, total protein in the lysate of the recombinant *E. coli* gGT29-7-pET28a; lane 8, lysate supernatant of the recombinant *E. coli* gGT29-7-pET28a; lane 9, protein molecular-weight Marker.

Figure 45 shows Western Blot detection of the gene gGT29-4, gGT29-5, gGT29-6 and gGT29-7 expression in *E. coli*; lane 1, total protein in the lysate of the gGT29-4-pET28a recombinant *E. coli*; lane 2, lysate supernatant of the recombinant *E. coli* gGT29-4-pET28a; lane 3, total protein in the lysate of the recombinant *E. coli* gGT29-5-pET28a; lane 4, lysate supernatant of the recombinant *E. coli* gGT29-5-pET28a; lane 5, total protein in the lysate of the recombinant *E. coli* gGT29-6-pET28a; lane 6, lysate supernatant of the recombinant *E. coli* gGT29-6-pET28a; lane 7, total protein in the lysate of the recombinant *E. coli* gGT29-7-pET28a; lane 8, lysate supernatant of the recombinant *E. coli* gGT29-7-pET28a.

Figure 46 shows TLC detection of the products obtained by catalyzing Rh2 and F2 using glycosyltransferases gGT29-4, gGT29-5, gGT29-6 and gGT29-7. Lane Rh2, saponin Rh2 is used as substrate; lane F2, saponin F2 is used as substrate. gGT29-4, gGT29-5, gGT29-6 or gGT29-7 represents reactions catalyzed by different enzymes respectively.

Specific Modes for Carrying Out the Invention

Upon extensive and intensive studies, for the first time, the inventors provided use of the glycosyltransferases gGT25(SEQ ID NO.: 2), gGT25-1(SEQ ID NO.: 16), gGT25-3(SEQ ID

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NO.: 18), gGT25-5(SEQ ID NO.: 20), gGT29(SEQ ID NO.: 26), gGT29-3(SEQ ID NO.: 28), gGT29-4 (SEQ ID NO.:55), gGT29-5 (SEQ ID NO.:57), gGT29-6 (SEQ ID NO.:59), gGT29-7 (SEQ ID NO.:61) and 3GT1(SEQ ID NO.: 22), 3GT2(SEQ ID NO.: 24), 3GT3(SEQ ID NO.: 41), 3GT4(SEQ ID NO.: 43), gGT13(SEQ ID NO.: 4), and gGT30(SEQ ID NO.: 6) for the catalytic glycosylation of terpenoids and synthesis of new saponins. Specifically, the glycosyltransferases according to the present invention are capable of specifically and efficiently catalyzing the glycosylation of the hydroxyl group(s) at position(s) C-20 and/or C-6 and/or C3 of a tetracyclic triterpenoid substrate, and/or transferring glycosyl(s) from glycosyl donors to the first glycosyl at position C-3 of a tetracyclic triterpenoid compound to extend the carbohydrate chain. The glycosyltransferases according to the present invention are particularly capable of converting protopanaxadiol into rare ginsenosides CK and Rh2 with anti-tumor activity, converting protopanaxatriol into rare ginsenoside F1 with anti-aging activity and rare ginsenoside Rh1 with anti-allergy activity, converting Rh2 into rare ginsenoside Rg3 with excellent anti-tumor activity. The glycosyltransferases of the present invention can also synthesize unreported novel saponins such as 20-*O*-β-(D-glucopyranosyl)-dammarendiol II, 3-*O*-β-(D-glucopyranosyl)-dammarendiol II, 3-*O*-β-(D-glucopyranosyl)-PPT, 3-*O*-β-(D-glucopyranosyl)-F1, 3-*O*-β-(D-glucopyranosyl)-25-OH-PPD,and 3-*O*-β-(D-glucopyrano-syl)-25-OCH_3-PPD by using dammarendiol, PPT, F1, 25-OH-PPD, or 25-OCH_3-PPD.

The glycosyltransferases according to the present invention can also convert Rh2, CK, or Rg3 into ginsenosides F2, Rd, or Rg1, respectively. The present invention further provides a method for transformation and catalyzation. The glycosyltransferases according to the present invention can also be co-expressed with the key enzymes in the anabolism pathways of dammarenediol II and/or PPD and/or PPT in host cells, or can be used in preparation of the genetically engineered host cells for DM, PPD and PPT, or used in the construction of the metabolic pathways for artificially synthesizing the rare ginsenosides CK, F1, Rh1, Rh2, Rg3, as well as the novel ginsenosides 20-*O*-β-(D-glucopyranosyl)-dammarendiol II, 3-*O*-β-(D-glucopyranosyl) -PPT, 3-*O*-β-(D-glucopyranosyl)-F1, 3-*O*-β-(D-glucopyranosyl)-dammarendiol II, 3-*O*-β-(D-glucopyranosyl)-25-OH-PPD, and 3-*O*-β-(D- glucopyranosyl) -25-OCH_3-PPD, and F2,Rd and Rg1,etc.Based on the above, the present invention was completed.

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Definitions

As used herein, the terms "active peptide(s)", "the polypeptide(s) and derivative polypeptide(s) thereof according to the present invention", "enzyme(s) according to the present invention", "glycosyltransferase(s)", "proteins gGT25, gGT13, gGT30, gGT25-1, gGT25-3, gGT25-5, gGT29, gGT29-3, 3GT1, 3GT2, 3GT3, or 3GT4 according to the present invention" and "glycosyltransferase(s) according to the present invention" all refer to the polypeptides of glycosyltransferases gGT25(SEQ ID NO.: 2), gGT13(SEQ ID NO.: 4), gGT30(SEQ ID NO.: 6), gGT25-1(SEQ ID NO.: 16), gGT25-3(SEQ ID NO.: 18), gGT25-5(SEQ ID NO.: 20), gGT29(SEQ ID NO.: 26), gGT29-3(SEQ ID NO.: 28), gGT29-4 (SEQ ID NO.:55), gGT29-5 (SEQ ID NO.:57), gGT29-6 (SEQ ID NO.:59), gGT29-7 (SEQ ID NO.:61), 3GT1(SEQ ID NO.: 22), 3GT2(SEQ ID NO.: 24), 3GT3(SEQ ID NO.: 41), and 3GT4(SEQ ID NO.: 43), and the derivative polypeptides thereof.

Unless stated otherwise, said ginsenoside and sapogenin according to the present invention refer to the ginsenosides and sapogenins with a C20 of S-configuration.

As used herein, "isolated polypeptide" means that the polypeptides almost has no other proteins, lipids, sugars or other substances that are naturally related to the polypeptide. Said polypeptide(s) can be purified by those skilled in the art using standard protein purification techniques. The substantially purified polypeptide can generate a single main band on nonreductive polyacrylamide gel electrophoresis. The purity of said polypeptide(s) can be further analyzed by using amino acids sequencing.

The active polypeptide(s) according to the present invention can be recombinant polypeptide(s), natural polypeptide(s), or synthetic polypeptide(s). The polypeptide(s) according to the present invention can be a purified natural product or chemically synthesized product, or can be produced from protokaryotic or eukaryotic hosts (e.g. bacteria, yeast, or plant) by recombination techniques. According to the hosts used in the recombinant production procedure, the polypeptide(s) according to the present invention can be glycosylated or non-glycosylated. The polypeptide(s) according to the present invention can or can not include an initiate residue of methionine.

The present invention further includes the fragments, derivatives and analogues of said polypeptides. As used herein, the terms "fragments", "derivatives" and "analogues"

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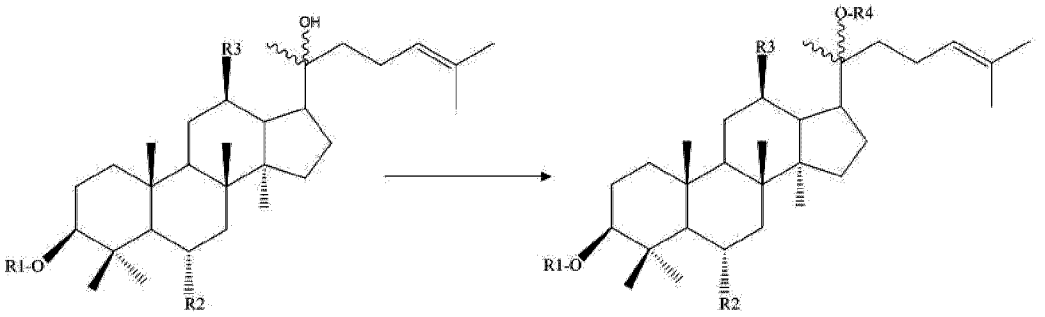
refer to peptides that substantially maintain the same biological function or activity with said polypeptides.

The polypeptide fragments, derivatives or analogs of the present invention could be (i) a polypeptide with one or more conservative or non-conservative amino acid residues (preferably, conservative amino acid residues) being substituted, wherein said amino acid residue substitution can be or not be encoded by genetic code; or (ii) a polypeptide having substitution group(s) in one or more amino acid residues, or (iii) a polypeptide formed by fusion of a mature polypeptide with another compound (such as a compound that prolongs the half life of a polypeptide, such as polyethylene glycol), or (iv) a polypeptide with an additional amino acid sequence fused to said polypeptide sequence (such as a fusion protein formed by fusion with a leader sequence, secretion sequence, a sequence for purifying the peptide, proteinogen sequence, or a fusion protein formed with the IgG fragment of an antigen). According to the teachings of the present application, these fragments, derivatives and analogs are within the scope commonly known by a skilled person.

The active polypeptides of the present invention possess the activity of glycosyltransferases and are able to catalyze one or more of following reaction(s):

(A)



compound of formula (I) compound of formula (II)

wherein, R1 is H, monosaccharide glycosyl or polysaccharides glycosyl; R2 or R3 is H or OH; R4 is glycosyl; said polypeptide is selected from SEQ ID NOs.: 2, 16 or 18 or a derivative polypeptide thereof.

In another embodiment, said monosaccharide includes glucose (Glc), rhamnose (Rha), acetyl glucose (Glc (6)Ac), arabinofuranose (Araf), arabopyranose (Arap), and xylose (Xyl),

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etc.

In another embodiment, said polysaccharides include polysaccharides composed of 2-4 monosaccharides, such as Glc(2-1)Glc, Glc(6-1)Glc, Glc(6)Ac, Glc(2-1)Rha, Glc(6-1)Arap, Glc(6-1)Xyl, Glc(6-1)Araf, Glc(3-1)Glc(3-1), Glc(2-1) Glu(6)Ac, Glc(6-1)Arap(4-1)Xyl, Glc(6-1)Arap(2-1)Xyl, or Glc(6-1)Arap(3-1)Xyl, etc.

Compounds with R1-R4 substituted are shown in the following table:

substrate	R1	R2	R3	R4	product
PPD	H	H	OH	glycosyl	CK
Rh2	1 glycosyl	H	OH	glycosyl	F2
Rg3	2 glycosyls	H	OH	glycosyl	Rd
PPT	H	OH	OH	glycosyl	F1
DM	H	H	H	glycosyl	20-G-DM

When both of said R1 and R2 are H, R3 is OH, said compound of formula (I) is PPD.

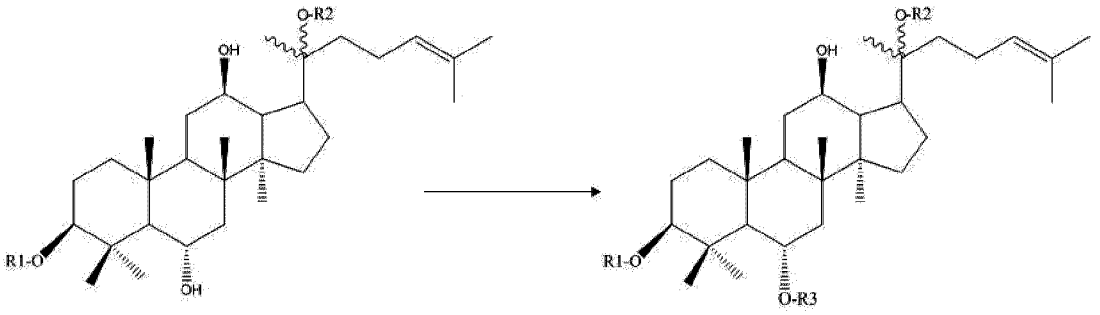
R1 is one glucosyl, R2 is H, R3 is OH, said compound of formula (I) is ginsenoside Rh2.

R1 is two glucosyls, R2 is H, R3 is OH, said compound of formula (I) is ginsenoside Rg3.

R1 is H, R2 is OH, R3 is OH, said compound of formula (I) is PPT.

R1 is H, R2 is H, R3 is H, said compound of formula (I) is dammarenediol II (DM).

(B):



compound of formula (III) formula (IV)compound

wherein, R1 is H or glycosyl, R2 is glycosyl, R3 is glycosyl, said polypeptide is selected from SEQ ID NOs.: 2, 16 ,18 , or 20 or a derivative polypeptide thereof;

or, R1 is H or glycosyl; R2 is H; R3 is glycosyl, said polypeptide is selected from SEQ ID NO.: 20 or a derivative polypeptide thereof.

Compounds with R1-R3 substituted are shown in the following table:

substrate	R1	R2	R3	product
F1	H	glycosyl	glycosyl	Rg1
PPT	H	H	glycosyl	Rh1

When both of said R1 and R2 are H, said compound of formula (III) is PPT.

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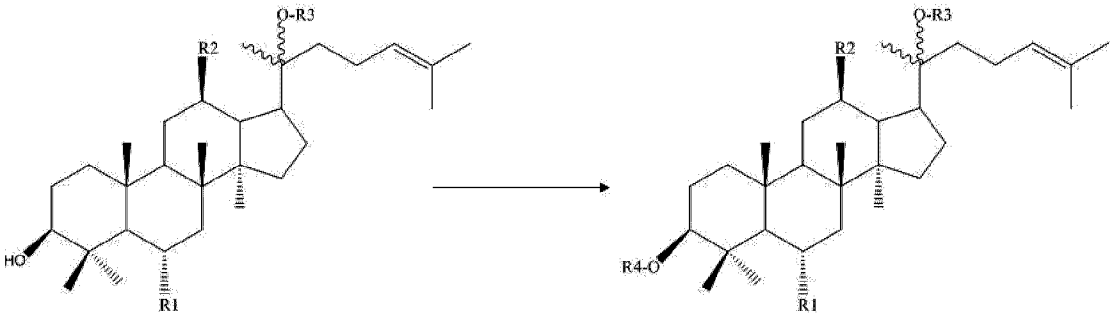
R1 is H, R2 is glucose, said compound of formula (III) is ginsenoside F1.

(C):



compound of formula (V) compound of formula (VI)

wherein, R1 is H or OH; R2 is H or OH; R3 is H or glycosyl; R4 is glycosyl, said polypeptide is selected from SEQ ID NOs.: 22, 24, 41 or 43 or a derivative polypeptide thereof.

Compounds with R1-R4 substituted are shown in the following table:

substrate	R1	R2	R3	R4	product
PPD	H	OH	H	glycosyl	Rh2
CK	H	OH	glycosyl	glycosyl	F2
PPT	OH	OH	H	glycosyl	3-G-PPT
F1	OH	OH	glycosyl	glycosyl	3-G-F1
DM	H	H	H	glycosyl	3-G-DM

When both of R1 and R3 are H, R2 is OH, said compound of formula (V) is PPD; said polypeptide is selected from SEQ ID NOs.: 22, 24, 41 or 43 or a derivative polypeptide thereof;

when R1 is H, R2 is OH, R3 is glucosyl, said compound of formula (V) is ginsenoside CK; said polypeptide is selected from SEQ ID NOs.: 22, 24, or 43 or a derivative polypeptide thereof;

when R1 is OH, R2 is OH, R3 is H, said compound of formula (V) is PPT; said polypeptide is selected from SEQ ID NOs.: 22, 24, or 41 or a derivative polypeptide thereof;

when R1 is OH, R2 is OH, R3 is glucosyl, said compound of formula (V) is ginsenoside F1; said polypeptide is selected from SEQ ID NOs.: 22, or 24 a derivative polypeptide thereof;

when R1 is H, R2 is OH, R3 is H, said compound of formula (V) is dammarenediol II (DM); said polypeptide is selected from SEQ ID NOs.: 22, or 24 a derivative polypeptide thereof;

(D):

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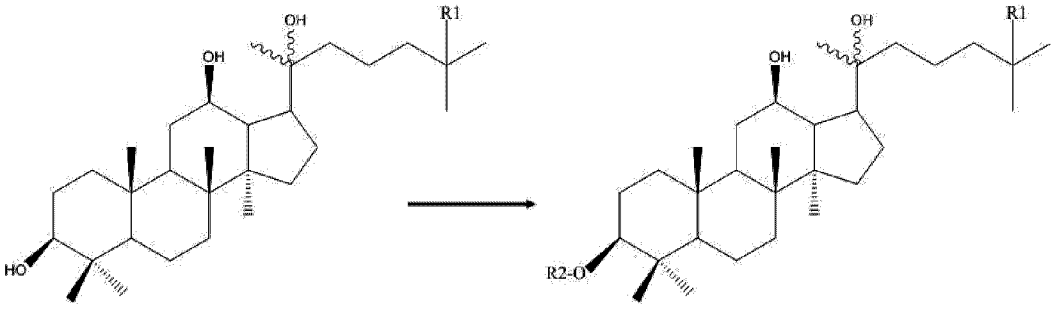


compound of formula (VII) compound of formula (VIII)

wherein, R1 is OH or OCH_3; R2 is glycosyl, said polypeptide is selected from SEQ ID NOs.: 22, 24, 41 or 43 or a derivative polypeptide thereof.

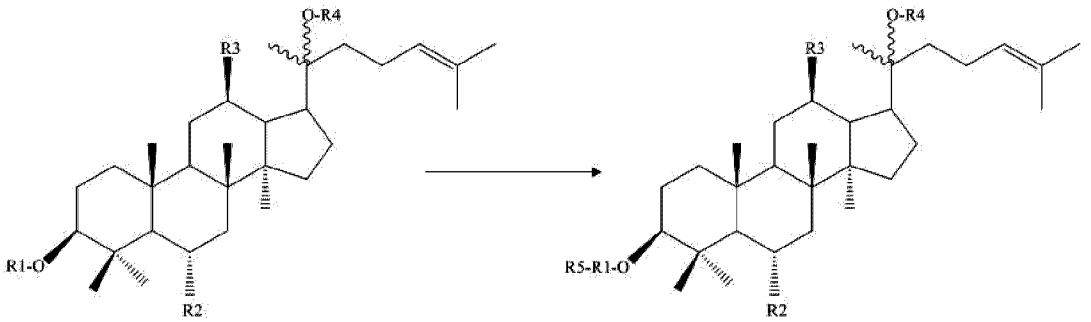
Compounds with R1-R2 substituted are shown in the following table:

substrate	R1	R2	product
25-OH-PPD	OH	glycosyl	3-G-25-OH-PPD
25-OCH_3-PPD	OCH_3	glycosyl	3-G-25-OCH_3-PPD

When R1 is OH, said compound of formula (VII) is 25-OH-PPD;

R1 is OCH, said compound of formula (VII) is 25-OCH_3-PPD.

(E)



compound of formula (IX) compound of formula (X)

wherein, R1 is glycosyl; R2 or R3 is OH or H; R4 is glycosyl or H; R5 is glycosyl, said polypeptide is selected from SEQ ID NOs.: 26, 28, 55, 57, 59 or 61 or a derivative polypeptide thereof.

Compounds with R1-R4 substituted are shown in the following table:

substrate	R1	R2	R3	R4	product
Rh2	glycosyl	H	OH	H	Rg3
F2	glycosyl	H	OH	glycosyl	Rd

When R1 is glucosyl; R2 is H, R3 is OH, R4 is H, the compound of formula (IX) is Rh2.

R1 is glucosyl; R2 is H, R3 is OH, R4 is glucosyl, the compound of formula (IX) is F2.

(F)

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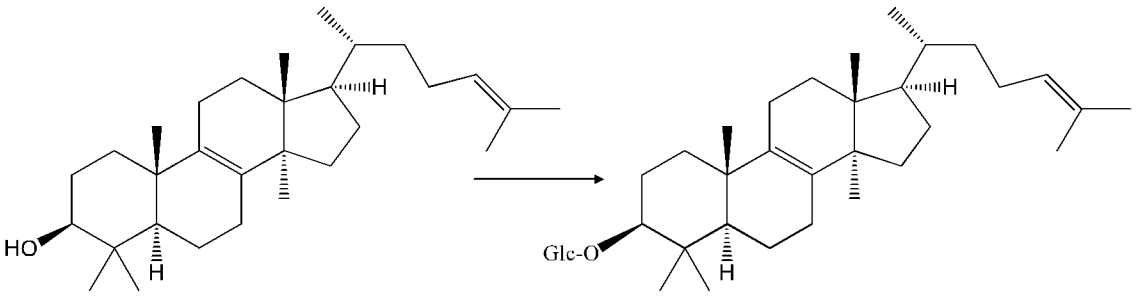

compound of formula (XI) compound of formula (XII)

said polypeptide is selected from SEQ ID NO: 22 or SEQ ID NO: 24 or a derivative polypeptide thereof.

The preferred sequence of said polypeptides is as set forth by SEQ ID NOs.: 2, 16, 18, 20, 22, 24, 41, 26, 28, 43, 55, 57, 59 or 61. The term also comprises variants of the sequences as set forth by SEQ ID NOs.: 2, 16, 18, 20, 22, 24, 41, 26, 28, 43, 55, 57, 59 or 61, which have the same function with said polypeptide, as well as the derivative polypeptide thereof. These variants include but are not limited to, deletions, insertions and/or substitutions of one or more (typically 1-50, preferably 1-30, more preferably 1-20, most preferably 1-10) amino acids, and addition of one or more (typically not more than 20, preferably not more than 10, more preferably not more than 5) amino acids at C-terminus and/or N- terminus. For example, the functions of a protein are usually unchanged when an amino acid is substituted by another amino acid with similar or analogous properties in the art. Further, addition of one or several amino acids at C-terminus and/or N-terminus generally will not change the function of a protein. The terms further includes the active fragment and active derivatives of human EGFRvA protein. The present invention further provides the analogues of said polypeptides. These analogues could differ from the naturally occurring EGFRvA polypeptide either in amino acid sequence or in modifications that do not affect the sequence, or in both. These polypeptides comprise natural or induced genetic variants. These variants can be obtained by various techniques, such as random mutagenesis through radiation or being exposed to mutagenic agents, site directed mutagenesis, or other known molecular biology techniques. Also included are analogues which include residues other than those naturally occurring L-amino acids (e.g., D-amino acids) or which include non-naturally occurring or synthetic amino acids (e.g., beta- or gamma-amino acids). It is understood that the polypeptides of the present invention are not limited to the

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representative polypeptides listed herein above.

Modifications (which do not normally alter the primary sequence) include *in vivo* or *in vitro* chemical derivation of polypeptides, e.g., acelylation, or carboxylation. Glycosylation is also included in modification, e.g., polypeptides that are produced by glycosylation modification during its synthesis and processing or in the further processing steps. These modifications can be achieved by exposing the polypeptide to enzymes for glycosylation (e.g, mammalian glycosylating or deglycosylating enzymes). Also included are sequences that have phosphorylated amino acid residues(e.g.,phosphotyrosine, phosphoserine, phosphothronine), as well as sequences that have been modified to improve their resistance to proteolytic degradation or to optimize solubility properties.

The N-terminal or C-terminal of the proteins gGT25, gGT13, gGT30, gGT25-1, gGT25-3, gGT25-5, gGT29, gGT29-3, gGT29-4, gGT29-5, gGT29-6, gGT29-7 and 3GT1, 3GT2, 3GT3, 3GT4 of the present invention can further comprise one or more polypeptide fragments as a protein tag. Any suitable tag can be used in the present invention. For example, said tag can be FLAG, HA, HA1, c-Myc, Poly -His, Poly-Arg, Strep-TagII, AU1, EE, T7, 4A6, ε, B, gE, or Ty1. These tags can be used for protein purification. Some of the tags and sequences thereof are listed in Table 1.

Table 1

Tag	Residue numbers	Sequence
Poly-Arg	5-6(generally 5)	RRRRR
Poly-His	2-10(generally 6)	HHHHHH
FLAG	8	DYKDDDDK
Strep-TagII	8	WSHPQFEK
C-myc GST	10 220	WQKLISEEDL followed with 6 LVPRGS

In order to achieve the secretory expression of the translated proteins (e.g. secrete out of cells), a signal peptide sequence, such as the signal peptide pelB, can be added to the N-terminal of said amino acids gGT25, gGT13, gGT30, gGT25-1, gGT25-3, gGT25-5, gGT29, gGT29-3, gGT29-4, gGT29-5, gGT29-6, gGT29-7 and 3GT1, 3GT2, 3GT3, 3GT4. The signal peptides can be cut off during the secretion process of the polypeptide from the cells.

The polynucleotide of the present invention can be in a form of DNA or RNA. The form of

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DNA includes cDNA, genome DNA or artificially synthesized DNA. DNA can be single strand or double strands. DNA can be a coding strand or a non-coding strand. The coding sequence encoding the mature polypeptide can be identical to the coding sequence indicated by SEQ ID NO: 1 or can be a degenerate variant thereof. As used herein, "degenerate variant" of the present invention refers to a nucleic acid sequence which encodes the protein having the amino acid sequence of SEQ ID NOs.: 2, 4, 6, 16, 18, 20, 22, 24, 26, 28, 41, 43, 55, 57, 59 or 61, but is different from the coding sequence indicated by SEQ ID NOs.: 1, 3, 5, 15, 17, 19, 21, 23, 25, 27, 40, 42, 54, 56, 58 or 60.

The polynucleotides encoding the mature polypeptides of SEQ ID NOs.: 2, 4, 6, 16, 18, 20, 22, 24, 26, 28, 41, 43, 55, 57, 59 or 61 include: coding sequences that only encodes mature polypeptides; coding sequences of mature polypeptides and various additional coding sequences; coding sequences of mature polypeptides (and optionally additional coding sequences) and non-coding sequences.

The term "polynucleotide encoding the polypeptide" can be a polynucleotide encoding said polypeptide, and can also be a polynucleotide further including an additional coding sequence and/or non-coding sequence.

The present invention further encompasses variants of the above-noted polynucleotides, which encodes polypeptides that have the same amino acid sequences with that of the present invention, or fragments, analogues and derivatives thereof. The variants of these polynucleotides can be naturally occurred allelic variants or non-naturally occurred variants. These polynucleotides variants include substitution variants, deletion variants and insertion variants. As known in the art, an allelic variant is the substituted form of polynucleotides, and they can be the substitution, deletion, or insertion of one or more polynucleotides, but do not substantially change the function of the encoded polypeptides.

The present invention further relates to polynucleotides that hybridize with the above-noted sequences and have an identity of at least 50%, preferably 70%, more preferably 80% between the two sequences. Particularly, the present invention relates to the polynucleotides capable of hybridizing with the polynucleotides of the present invention under stringent conditions. According to the present invention, "stringent condition" refers to: (1) hybridization and elution under a low ion strength and a high

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temperature, such as 0.2×SSC, 0.1%SDS, 60℃; or (2) hybridization in the presence of an added denaturant, such as 50%(v/v) of formamide, 0.1% of fetal bovine serum/0.1%Ficoll, 42℃, etc; or (3) hybridization only occurring under the condition that the identity between the two sequences is at least more than 90%, preferably more than 95%. Moreover, the polypeptides encoded by the hybridizable polynucleotides have identical biological functions and activities with the mature polypeptide as set forth by SEQ ID NOs.: 2, 4, 6, 16, 18, 20, 22, 24, 26, 28, 41, 43, 55, 57, 59 or 61.

The present invention further relates to nucleic acid fragments which can hybridize with the sequences described above. As used herein, the length of the "nucleic acid fragments" is at least 15 nucleotides, preferably at least 30 nucleotides, more preferably at least 50 nucleotides, most preferably at least 100 nucleic acids. The nucleic acid fragments can be used in the nucleic acid amplification techniques (such as PCR) for determining and/or isolating the polynucleotides encoding the proteins gGT25, gGT13, gGT30, gGT25-1, gGT25-3, gGT25-5, gGT29, gGT29-3, gGT29-4, gGT29-5, gGT29-6, gGT29-7 and 3GT1, 3GT2, 3GT3, 3GT4.

The polypeptides and polynucleotides of the present invention are preferably provided in the isolated form, more preferably are purified to be homogenous.

The full-length nucleotide sequences of gGT25, gGT13, gGT30, gGT25-1, gGT25-3, gGT25-5, gGT29, gGT29-3, gGT29-4, gGT29-5, gGT29-6, gGT29-7 and 3GT1, 3GT2, 3GT3, 3GT4 or the fragments thereof can be obtained by PCR amplification, recombination, or artificial synthesis. For PCR amplification, the primers can be designed according to the relevant nucleotide sequences (especially the Open Reading Frame) disclosed herein, and the commercially available cDNA library or the cDNA library prepared through the conventional methods known by those skilled in the art can be used as the template, thereby amplifying and obtaining the corresponding sequences. Twice or more PCR amplifications are typically needed for a longer sequence, and then, the respectively amplified fragments are spliced together in correct order.

Once the corresponding sequences are obtained, recombination can be used for giving the corresponding sequences massively. Generally, they are cloned into vectors, followed, transformed into cells, and then, the corresponding sequences are isolated from the host cells upon proliferation by conventional methods.

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Furthermore, the corresponding sequences can be synthesized by artificial synthesis, especially when the fragment length is short. Generally, multiple small peptides are synthesized first and then can be connected to obtain fragments with longer sequences.

At present, the DNA sequences encoding the proteins of the present invention (or the fragments or derivatives thereof) can be obtained entirely via chemical synthesis. After that, the DNA sequences can be introduced into the various existing DNA molecules (or, such as, vectors) and cells known in the art. Moreover, mutations can be introduced into the protein sequences of the present invention through chemical synthesis.

Methods for amplifying DNA/RNA by using PCR amplification is preferably used for obtaining the genes of the present invention. A RACE method (RACE-rapid amplification of cDNA end) is preferred when it is difficult to obtain the full length of cDNA from a library. The primers used for PCR can be properly selected according to the sequence information disclosed in the present invention and can be synthesized by conventional methods. The amplified DNA/RNA fragments can be isolated and purified by conventional methods such as gel electrophoresis.

The present invention further relates to the vectors containing the polynucleotides of the present invention, the host cells produced by genetic engineering using the vectors of the present invention or the sequences encoding the proteins gGT25, gGT13, gGT30, gGT25-1, gGT25-3, gGT25-5, gGT29, gGT29-3, gGT29-4, gGT29-5, gGT29-6, gGT29-7 and 3GT1, 3GT2, 3GT3, 3GT4, and the method for producing polypeptides of the present invention by recombination techniques.

The polynucleotides sequences can be used for expressing or producing the recombinant polypeptides of gGT25, gGT13, gGT30, gGT25-1, gGT25-3, gGT25-5, gGT29, gGT29-3, gGT29-4, gGT29-5, gGT29-6, gGT29-7 and 3GT1, 3GT2, 3GT3, 3GT4 by conventional DNA recombination techniques. Generally, the following steps are included:

(1). transforming or transducing suitable host cells by using the polynucleotides (or the variants) encoding the polypeptides gGT25, gGT13, gGT30, gGT25-1, gGT25-3, gGT25-5, gGT29, gGT29-3, gGT29-4, gGT29-5, gGT29-6, gGT29-7 and 3GT1, 3GT2, 3GT3, 3GT4 of the present invention, or by using the recombinant expression vectors containing said polynucleotides;

(2). culturing the host cells in a proper medium;

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(3). isolating and purifying the proteins from the medium or the cells.

In the present invention, the polynucleotides of gGT25, gGT13, gGT30, gGT25-1, gGT25-3, gGT25-5, gGT29, gGT29-3, gGT29-4, gGT29-5, gGT29-6, gGT29-7 and 3GT1, 3GT2, 3GT3 can be inserted into a recombinant expression vector. The term "recombinant expression vector" refers to a bacterial plasmid, phage, yeast plasmid, virus for plant cells, virus for mammal cells such as adenovirus, retrovirus or other vectors well known in the art. Any plasmids or vectors can be used as long as it can replicate and stabilize inside the hosts. A major characteristic of the expression vector is that it generally contains a replication origin, a promoter, a marker gene and a translation control element.

The well-known methods in the art can be use to construct the vectors containing the DNA sequences encoding gGT25, gGT13, gGT30, gGT25-1, gGT25-3, gGT25-5, gGT29, gGT29-3, gGT29-4, gGT29-5, gGT29-6, gGT29-7 and 3GT1, 3GT2, 3GT3, 3GT4 and suitable transcription/translation control signals. These methods include *in vitro* DNA recombination techniques, DNA synthesis techniques, and *in vivo* recombination techniques, etc. Said DNA sequences can be effectively connected to a proper promoter in the expression vector so as to guide the mRNA synthesis. The representative examples of these promoters are: lac or trp promoter of *E. coli*; PL promoter of λ phage; eukaryotic promoters including CMV immediate-early promoter, HSV thymidine kinase promoter, early and late SV40 promoters, LTRs of retrovirus and some other known promoters capable of controlling the gene expression in protokaryocytes or eukaryocytes or the viruses thereof. The expression vectors further comprise ribosome binding sites for initiating translation and transcription terminators.

Furthermore, the expression vectors preferably contain one or more selective marker genes so as to provide the phenotypic characteristics for selecting the transformed host cells, such as dihydrofolate reductase, neomycin resistance and green fluorescent protein (GFP) used for eukaryocytes culturing, or tetracycline or ampicillin resistance used for *E. coli*.

The vectors containing the suitable DNA sequences and suitable promoters or regulating sequences described above can be used for transforming suitable host cells to express proteins.

The host cells can be prokaryocytes, such as bacterial cells; or lower eukaryocytes, such as yeast cells; or higher eukaryocytes, such as mammal cells. The representative examples are: bacterial cells of *E. coli*, *streptomyces*, *salmonella typhimurium*; fungal cells such as yeast;

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plant cells; insect cells of *Drosophila* S2 or Sf9; animal cells, such as CHO, COS, 293 cells, or Bowes melanoma cells.

When the polynucleotides of the present invention are expressed in higher eukaryocytes, the insertion of an enhancer sequence into the vector will enhance the transcription. The enhancer is a cis-acting element of DNA generally containing about 10-300 base-pairs and acting on promoters to enhance gene transcription. The available examples include the SV40 enhancer of 100-270 base-pairs located at the late-stage side of the replication origins, the polyma enhancer located at the late-stage side of the replication origins, and the adenovirus enhancers, etc.

It is all clear for those skilled in the art to choose suitable vectors, promoters, enhancers and host cells.

The transformation of host cells by using DNA recombination can be conducted by conventional techniques well-known to those skilled in the art. When prokaryotes such as *E. coli* are used as host cells, competent cells capable of absorbing DNA can be harvested after the exponential growth phase, and then treated with the $CaCl_2$ method, wherein the steps used are well known in the art. Another method is using $MgCl_2$. The transformation can also be conducted by electroporation if desired. When the host cell is a eucaryote, the following methods for DNA transfection are for selection: calcium phosphate co-precipitation, conventional mechanical methods such as micro-injection, electroporation, and liposome packing, etc.

The obtained transformants can be cultured by conventional methods, thereby expressing the polypeptides encoded by the genes of the present invention. According to the host cells used, the medium for culturing can be selected from various conventional medium. Upon culturing under the condition suitable for host cell growth, proper methods (such as temperature conversion or chemical induction) are used to induce the selected promoters when the host cells grow to a proper cells density, and then the cells are cultured for another period.

The recombinant polypeptide according to the methods above can be intracellular or membrane expression, or secreted out of the cells. The recombinant proteins can be isolated and purified by various isolating methods according to the physical, chemical, and other characteristics. These methods are well known to those skilled in the art. The

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examples of these methods include, but are not limited to: conventional renaturation treatment, treatment with protein precipitant (the salting-out method), centrifugation, bacterial-breaking by permeation, ultra-treatment, ultracentrifugation, molecular sieve chromatography (gel filtration), adsorption chromatography, ion-exchange column chromatography, high performance liquid chromatography (HPLC) and various other liquid chromatography techniques and the combination thereof.

Applications

The use of the active polypeptides or glycosyltransferases gGT25, gGT13, gGT30, gGT25-1, gGT25-3, gGT25-5, gGT29, gGT29-3, gGT29-4, gGT29-5, gGT29-6, gGT29-7 and 3GT1, 3GT2, 3GT3, 3GT4 of the present invention includes, but is not limited to: specifically and efficiently catalyzing glycosylation of the hydroxyl groups at positions C-20 and/or C-6 and/or C-3 of tetracyclic triterpenoid substrates, or transferring glycosyl(s) from glycosyl donor(s) to the first glycosyl at position C-3 of tetracyclic triterpenoid compound(s) so as to extend the carbohydrate chain. Particularly, they can convert PPD into rare ginsenosides CK and Rh2 with anti-tumor activity, convert PPT into rare ginsenoside F1 with anti-aging activity and rare ginsenoside Rh1 with anti-allergy activity, convert Rh2 into rare ginsenoside Rg3 with better anti-tumor activity. The glycosyltransferases of the present invention can further synthesize novel saponins such as 20-*O*-β-(D-glucopyranosyl)-dammarendiol II, 3-*O*-β-(D-glucopyranosyl) -dammarendiol II, 3-*O*-β-(D-glucopyranosyl)-PPT, 3-*O*-β-(D-glucopyrano- syl)-F1, 3-*O*-β-(D-glucopyranosyl)-25-OH-PPD, 3-*O*-β-(D-glucopyrano-syl)-25-OCH_3-PPD by using DM, PPT, F1, 25-OH-PPD, or 25-OCH_3-PPD. The glycosyltransferases of the present invention can further convert Rh2, CK, or Rg3 into ginsenoside F2, Rd, or Rg1.

Said tetracyclic triterpenoid compounds include but are not limited to S- or R-dammarane-type, lanostane-type, tirucallane-type, cycloartane-type, cucurbitane type, or meliacane type typetetracyclic triterpenoid compounds.

The present invention provides a method for industrial catalyzation, comprises: under the condition of provided glycosyl donors, obtaining compound (II), (IV), (VI), (VIII), (X) and (XII)by using the active peptides or glycosyltransferases gGT25, gGT13, gGT30, gGT25-1, gGT25-3, gGT25-5, gGT29, gGT29-3, gGT29-4, gGT29-5, gGT29-6, gGT29-7, 3GT1, 3GT2,

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3GT3 and/or 3GT4 of the present invention. Specifically, said polypeptide used in reaction (a) is selected from SEQ ID NOs.: 2, 16 or 18; said polypeptide used in reaction (b) is selected from SEQ ID NOs.: 20, 2, 16 or 18; said polypeptide used in reaction (c) and (d) is selected from SEQ ID NOs.: 22, 24, 41 and 43; said polypeptide used in reaction (e) is selected from SEQ ID NOs.: 26, 28, 55, 57, 59 or 61; said polypeptide used in reaction (F) is selected from the active polypeptide as set forth by SEQ ID NOs.: 22 or 24.

Said glycosyl donor(s) is nucleoside diphosphate sugar(s) selected from the group consisting of: UDP- glucose, ADP- glucose, TDP- glucose, CDP- glucose, GDP- glucose, UDP- acetyl glucose, ADP- acetyl glucose, TDP- acetyl glucose, CDP- acetyl glucose, GDP- acetyl glucose, UDP- xylose, ADP- xylose, TDP- xylose, CDP- xylose, GDP- xylose, UDP- galacturonic acid, ADP- galacturonic acid, TDP- galacturonic acid, CDP- galacturonic acid, GDP- galacturonic acid, UDP- galactose, ADP- galactose, TDP- galactose, CDP- galactose, GDP- galactose, UDP- arabinose, ADP- arabinose, TDP- arabinose, CDP- arabinose, GDP- arabinose, UDP- rhamnose, ADP- rhamnose, TDP- rhamnose, CDP- rhamnose, GDP- rhamnose, or other nucleoside diphosphate hexose or nucleoside diphosphate pentose, or the combination thereof.

Said glycosyl donor(s) is preferably uridine diphosphate (UDP) sugars selected from the group consisting of: UDP- glucose, UDP- galacturonic acid, UDP- galactose, UDP- arabinose, UDP- rhamnose, or other uridine diphosphate hexose or uridine diphosphate pentose, or the combination thereof.

For said method, additives for modulating enzyme activity (additives enhancing enzyme activity or inhibiting enzyme activity)can be further added. Said additive(s) for modulating enzyme activity can be selected from the group consisting of Ca^{2+}, Co^{2+}, Mn^{2+}, Ba^{2+}, Al^{3+}, Ni^{2+}, Zn^{2+} and Fe^{2+}; or material(s) capable of producing Ca^{2+}, Co^{2+}, Mn^{2+}, Ba^{2+}, Al^{3+}, Ni^{2+}, Zn^{2+}, or Fe^{2+}.

The pH condition for said method is: pH 4.0-10.0, preferably pH 6.0-pH 8.5, more preferably 8.5.

The temperature condition for said method is: 10℃-105℃, preferably 25℃-35℃, more preferably 35℃.

The present invention further provide a composition, which contains an effective amount of the active polypeptide or glycosyltransferases gGT25, gGT13, gGT30, gGT25-1, gGT25-3,

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gGT25-5, gGT29, gGT29-3, gGT29-4, gGT29-5, gGT29-6, gGT29-7, 3GT1, 3GT2, 3GT3 and 3GT4 of the present invention, and a bromatologically or industrially acceptable carrier or excipient. Such carriers include, but are not limited to: water, buffer solution, glucose, water, glycerol, ethanol, and the combination thereof.

Additive(s) for modulating the activity of enzyme gGT25 of the present invention can be further added into said composition. Any additive(s) having the function of enhancing enzyme activity can be used. Preferably, said additive(s) for enhancing the activity of enzyme gGT25 of the present invention is mercaptoethanol. Furthermore, enzyme activity can be inhibited by many substances, which include but are not limited to Ca^{2+}, Co^{2+}, Mn^{2+}, Ba^{2+}, Al^{3+}, Ni^{2+}, Zn^{2+} and Fe^{2+}; or substances capable of producing Ca^{2+}, Co^{2+}, Mn^{2+}, Ba^{2+}, Al^{3+}, Ni^{2+}, Zn^{2+}, or Fe^{2+} by hydrolysis after being added to the substrate.

Upon obtaining gGT25, gGT13, gGT30, gGT25-1, gGT25-3, gGT25-5, gGT29, gGT29-3, gGT29-4, gGT29-5, gGT29-6, gGT29-7 and 3GT1, 3GT2, 3GT3, 3GT of the present invention, these enzymes can be readily used by the skilled in the art for transferring glycosyls, especially for transferring glycosyls by using DM, PPD and PPT as substrates. As a preferred embodiment for the present invention, two methods for generating rare ginsenosides are further provided, the first of said methods comprises: treating the substrate for transglycosylation with the enzymes gGT25, gGT13, gGT30, gGT25-1, gGT25-3, gGT25-5, gGT29, gGT29-3, gGT29-4, gGT29-5, gGT29-6, gGT29-7, 3GT1 and/or 3GT2, 3GT3, 3GT4 of the present invention, wherein said substrate includes tetracyclic triterpenoid compounds such as DM, PPD, PPT, and the derivatives thereof; preferably, under the condition of pH3.5-10, treating the substrate for transglycosylation with the enzymes gGT25, gGT13, gGT30, gGT25-1, gGT25-3, gGT25-5, gGT29, gGT29-3, gGT29-4, gGT29-5, gGT29-6, gGT29-7, 3GT1 and/or 3GT2, 3GT3, 3GT4; preferably, under the condition of a temperature of 30-105℃, treating the substrate for transglycosylation with the enzymes gGT25, gGT13, gGT30, gGT25-1, gGT25-3, gGT25-5, gGT29, gGT29-3, gGT29-4, gGT29-5, gGT29-6, gGT29-7, 3GT1 and/or 3GT2, 3GT3, 3GT4.

The second of said methods comprises: transferring the genes of gGT25, gGT13, gGT30, gGT25-1, gGT25-3, gGT25-5, gGT29, gGT29-3, gGT29-4, gGT29-5, gGT29-6, gGT29-7 and 3GT1, 3GT2, 3GT3, 3GT4 of the present invention into an engineered strain (such as a yeast or *E. coli* engineered strain) capable of synthesizing DM, PPD or PPT, or alternatively

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co-expressing the genes of gGT25, gGT13, gGT30, gGT25-1, gGT25-3, gGT25-5, gGT29, gGT29-3, gGT29-4, gGT29-5, gGT29-6, gGT29-7 and 3GT1, 3GT2, 3GT3, 3GT4 with the key genes in the anabolism pathways of DM, PPD and PPT in a host cell (such as yeast cells or *E. coli*), thereby obtaining the recombinant strains for directly producing rare ginsenosides CK, Rh2, Rg3, Rh1 or F1.

Said key gene(s) in the anabolism pathway of dammarenediol includes but is not limited to dammarenediol synthase gene.

In another preferred embodiment, the key gene(s) in the anabolism pathway of PPD includes but is not limited to dammarenediol synthase gene, cytochrome P450 CYP716A47 gene, cytochrome P450 CYP716A47 reductase gene, or the combination thereof; or the isoenzymes of the above enzymes, and the combination thereof. Wherein, oxidosqualene (produced by *Saccharomyces cerevisiae* itself) is transformed into DM by dammarenediol synthase, and DM is transformed into PPD by cytochrome P450 CYP716A47 and the reductase thereof. (Han et al, plant & cell physiology, 2011, 52.2062-73)

In another preferred embodiment, the key gene(s) in anabolism pathway of PPT includes but is not limited to dammarenediol synthase gene, cytochrome P450 CYP716A47 gene, cytochrome P450 CYP716A47 reductase gene, cytochrome P450 CYP716A53V2 gene, or the combination thereof; or the isoenzymes of the above enzymes, and the combination thereof. Wherein, oxidosqualene (produced by *Saccharomyces cerevisiae* itself) is transformed into DM by dammarenediol synthase, and then DM is transformed into PPD by cytochrome P450 CYP716A47 and the reductase thereof, and PPD is further transformed into PPT by cytochrome P450 CYP716A53V2 (JX036031) and cytochrome P450 CYP716A47 reductase.(Han et al, plant & cell physiology, 2012, 53. 1535-45)

The Major advantages of the Present Invention

(1) Glucosyl(s) can be transferred to the hydroxyl(s) at position(s) C-20 and/or C-6 and/or C-3 of tetracyclic triterpenoid substrates specifically and efficiently by the glycosyltransferases of the present invention.

(2) Glycosyl(s) from glycosyl donor(s) can be transferred to the first glycosyl at position C-3 of tetracyclic triterpenoid compounds for extending the carbohydrate chain by using glycosyltransferases gGT29 and gGT29-3 of the present invention.

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(3) PPD and PPT can be transformed into rare ginsenoside CK, Rh2 or Rg3 with anti-tumor activity, rare ginsenoside F1 with anti-aging activity, and rare ginsenoside Rh1 with anti-allergy activity respectively by the glycosyltransferases of the present invention.

(4) Unreported novel compounds of 20-*O*-β-(D-glucopyranosyl) -dammarendiol II, 3-*O*-β-(D-glucopyranosyl)-dammarendiol II, 3-*O*-β-(D-glucopyranosyl)-PPT, 3-*O*-β-(D-glucopyranosyl)-F1, 3-*O*-β-(D-glucopyranosyl)-25-OH-PPD, and 3-*O*-β-D-glucopyranosyl)-25-OCH_3-PPD, 3-*O*-β-(D-glucopyranosyl)- lanosterol can be synthesized from DM, PPT, F1, 25-OH-PPD, and 25-OCH_3-PPD by using the glycosyltransferases of the present invention.

(5) The catalytic activities of 3GT1, 3GT2, gGT29, gGT29-3 and gGT25-5 are not affected by the steric configuration of the hydroxyl or glycosyl at position 20 of tetracyclic triterpenoid compounds. These enzymes can catalyze the ginsenosides (sapogenins) of 20(*S*)-type as well as the ginsenosides (sapogenins) of 20(*R*)-type.

(6) The synthetic pathway of ginsengenins (DM, PPD, and PPT) are constructed in yeast, thereby realizing the production of novel compounds of 20-*O*-β-(D-glucopyranosyl)-dammarendiol II, 3-*O*-β- (D-glucopyranosyl)- dammarendiol II, 3-*O*-β-(D-glucopyranosyl)-PPT, 3-*O*-β-(D-glucopyranosyl)-F1, and 3-*O*-β-(D-glucopyranosyl)-lanosterol and rare ginsenosides CK, F1, Rh1, Rh2 and Rg3 through yeasts by using monosaccharide (such as glucose, etc) as substrates. Not only the problem of material source for saponin production is solved, but also the production costs of rare saponins CK, F1, Rh1, Rh2 and Rg3 are significantly decreased.

The invention is further illustrated by the following examples. These examples are only intended to illustrate the invention, but not to limit the scope of the invention. For the experimental methods in the following examples the specific conditions of which are not specifically indicated, they are performed under routine conditions, e.g., those described by Sambrook. et al., in Molecular Cloning: A Laboratory Manual, New York: Cold Spring Harbor Laboratory Press, 1989, or as instructed by the manufacturers, unless otherwise specified.

Example 1

1. Isolation of glycosyltransferases and their encoding genes

More than 100 predicted cDNA sequences of glycosyltransferases were extracted

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from the published expression profile data of the *Panax* plant. 60 cDNAs with full length were cloned, expressed, and subjected to the analysis of glycosyltransfering reaction. Wherein, 11 of the expression products showed glycosyltransfering activities on ginsengenins and saponins.

The RNA of *P. ginseng* was extracted and reverse transcribed to obtain the cDNA of *P. ginseng*. PCR amplification was conducted using the cDNA as the template. Wherein, amplification products were all obtained by using primer pair 1 (SEQ ID NOs.: 7, 8), primer pair 2 (SEQ ID NOs.: 9, 10), primer pair 3 (SEQ ID NOs.: 11, 12), primer pair 5 (SEQ ID NOs.: 34, 35), primer pair 7 (SEQ ID NOs.: 46, 47), primer pair 8 (SEQ ID NOs.:62, 63) and primer pair 9 (SEQ ID NOs.:64, 65). The high-fidelity DNA Polymerase KOD purchased from Takara Bio Inc. was used as the DNA polymerase. The PCR products were detected by agarose gel electrophoresis (Figure 1, 19(c) and 31). The target DNA bands were cut out under a UV lamp. Then, the DNA was recovered from the agarose gel using Axygen Gel Extraction Kit (Axygen Inc.) to give the amplified DNA fragments. An adenine was added to the end of the DNA fragments using the rTaq DNA polymerase (Takara Bio Inc.) and then the product was ligated into the commercially available cloning vector pMD18-T. The ligated products were transformed into the commercially available *E. coli* competent cells EPI300. The liquid containing the transformed *E. coli* strains was plated on a LB plate supplemented with 50ug/mL of ampicillin, 0.5mM of IPTG and 25μg/mL of X-Gal. The recombinant clones were verified by PCR and enzyme digestion. Recombinant plasmids extracted from each clone were subjected to sequencing. The Open Reading Frame (ORF) was searched using software BESTORF. Through sequence alignment, the conserved domain of the glycosyltransferases family 1 was encoded by the ORF, indicating that these genes were glycosyltransferase genes.

The genes obtained by primer pair 1 (SEQ ID NOs.: 7, 8) are as set forth by SEQ ID NOs.: 1, 15, 17 and 19, and named as *gGT25*, *gGT25-1*, *gGT25-3* and *gGT25-5*, respectively. The protein coding sequence (CDS) of *gGT25* is shown as the nucleotides of positions 1- 1425 from the 5' end of SEQ ID NO.: 1 according to the sequence listing. The start codon ATG of gene *gGT25* is shown as the nucleotides of positions 1-3 from the 5' end of SEQ ID NO.: 1. The Open Reading Frame (ORF) of *gGT25-1* is shown as the nucleotides of positions 1-1428 from the 5' end of SEQ ID NO.: 15 according to the

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sequence listing. The start codon ATG of *gGT25-1* is shown as the nucleotides of position 1-3 from the 5' end of SEQ ID NO.: 15 and the stop codon TAA of *gGT25-1* is shown as the nucleotides of positions 1426-1428 from the 5' end of SEQ ID NO.: 15. The Open Reading Frame (ORF) of *gGT25-3* is shown as the nucleotides of positions 1-1428 from the 5' end of SEQ ID NO.: 17 according to the sequence listing. The start codon ATG of *gGT25-3* is shown as the nucleotides of position 1-3 from the 5' end of SEQ ID NO.: 17 and the stop codon TAA of *gGT25-3* is shown as the nucleotides of position 1426-1428 from the 5' end of SEQ ID NO.: 17. The Open Reading Frame (ORF) of *gGT25-5* is shown as the nucleotides of position 1- 1419 from the 5' end of SEQ ID NO.: 19 according to the sequence listing. The start codon ATG of *gGT25-5* is shown as the nucleotides of position 1- 3 from the 5' end of SEQ ID NO.: 19 and the stop codon TAA of *gGT25-5* is shown as the nucleotides of position 1426-1428 from the 5' end of SEQ ID NO.: 19.

The gene obtained by primer pair 2 (SEQ ID NOs.: 9, 10) is as set forth by SEQ ID NO.: 3, and named as *gGT13*. The Open Reading Frame (ORF) of *gGT13* is shown as the nucleotides of position 1-1431 from the 5' end of SEQ ID NO.: 3 according to the sequence listing. The start codon ATG of *gGT13* is shown as the nucleotides of position 1-3 from the 5' end of SEQ ID NO.: 3 and the stop codon TAA of *gGT13* is shown as the nucleotides of position 1429-1431 from the 5' end of SEQ ID NO.: 1.

The gene obtained by primer pair 3 (SEQ ID NOs.: 11, 12) is as set forth by SEQ ID NO.: 5, and named as *gGT30*. The Open Reading Frame (ORF) of *gGT30* is shown as the nucleotides of position 1-1353 from the 5' end of SEQ ID NO.: 5 according to the sequence listing. The start codon ATG of *gGT30* is shown as the nucleotides of position 1-3 from the 5' end of SEQ ID NO.: 5 and the stop codon TAA of *gGT30* is shown as the nucleotides of position 1351-1353 from the 5' end of SEQ ID NO.: 5.

The genes obtained by primer pair 5 (SEQ ID NOs.: 34, 35) are as set forth by SEQ ID NOs.: 25 and 27, and named as *gGT29* and *gGT29-3*. The Open Reading Frame (ORF) of *gGT29* is shown as the nucleotides of position 1-1329 from the 5' end of SEQ ID NO.: 25 according to the sequence listing. The start codon ATG of gene *gGT29* is shown as the nucleotides of position 1-3 from the 5' end of SEQ ID NO.: 25 and the stop codon TAG of gene *gGT29* is shown as the nucleotides of position 1327-1329 from the 5' end of SEQ ID NO.: 25. The start codon ATG of gene *gGT29-3* is shown as the nucleotides of position

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1-3 from the 5' end of SEQ ID NO.: 27 and the stop codon TAG of *gGT29* is shown as the nucleotides of position 1327-1329 from the 5' end of SEQ ID NO.: 27.

The gene obtained by primer pair 6 (SEQ ID NOs.: 46, 47) is as set forth by SEQ ID NO.: 42, and named as 3GT4. The Open Reading Frame (ORF) of 3GT4 is shown as the nucleotides of position 1-1374 from the 5' end of SEQ ID NO.: 42 according to the sequence listing. The start codon ATG of gene 3GT4 is shown as the nucleotides of position 1-3 from the 5' end of SEQ ID NO.: 42 and the stop codon TAG of gene 3GT4 is shown as the nucleotides of position 1372-1374 from the 5' end of SEQ ID NO.: 42.

The genes obtained by primer pair 7 (SEQ ID NOs.: 62, 63) are as set forth by SEQ ID NOs.: 54, 56, and 58, and named as *gGT29-4, gGT29-5* and *gGT29-6*. The Open Reading Frame (ORF) of *gGT29-4* is shown as the nucleotides of position 1-1341 from the 5' end of SEQ ID NO.: 54 according to the sequence listing. The start codon ATG of gene *gGT29-4* is shown as the nucleotides of position 1-3 from the 5' end of SEQ ID NO.: 54 and the stop codon TAG of gene *gGT29*-4 is shown as the nucleotides of position 1339-1341 from the 5' end of SEQ ID NO.: 54. The Open Reading Frame (ORF) of *gGT29-5* is shown as the nucleotides of position 1-1341 from the 5' end of SEQ ID NO.: 56 according to the sequence listing. The start codon ATG of gene *gGT29-5* is shown as the nucleotides of position 1-3 from the 5' end of SEQ ID NO.: 56 and the stop codon TAG of gene *gGT29*-5 is shown as the nucleotides of position 1339-1341 from the 5' end of SEQ ID NO.: 56. The Open Reading Frame (ORF) of *gGT29-6* is shown as the nucleotides of position 1-1341 from the 5' end of SEQ ID NO.: 58 according to the sequence listing. The start codon ATG of gene *gGT29-6* is shown as the nucleotides of position 1-3 from the 5' end of SEQ ID NO.: 58 and the stop codon TAG of gene *gGT29*-6 is shown as the nucleotides of position 1339-1341 from the 5' end of SEQ ID NO.: 58.

The gene obtained by primer pair 8 (SEQ ID NOs.: 64, 65) is as set forth by SEQ ID NO.: 60, and named as *gGT29-7*. The Open Reading Frame (ORF) of *gGT29-7* is shown as the nucleotides of position 1-1341 from the 5' end of SEQ ID NO.: 60 according to the sequence listing. The start codon ATG of gene *gGT29-7* is shown as the nucleotides of position 1-3 from the 5' end of SEQ ID NO.: 60 and the stop codon TAG of gene *gGT29-7* is shown as the nucleotides of position 1339-1341 from the 5' end of SEQ ID NO.: 60. The nucleotide sequences as set forth by SEQ ID NOs.: 21, 23 and 40 were artificially

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synthesized and named as *3GT1*, 3*GT2* and 3*GT3*, respectively. The Open Reading Frame (ORF) of 3*GT1* is shown as the nucleotides of position 1-1488 from the 5' end of SEQ ID NO.: 21 according to the sequence listing. The start codon ATG of gene 3*GT1* is shown as the nucleotides of position 1-3 from the 5' end of SEQ ID NO.: 21 and the stop codon TAA of gene 3*GT1* is shown as the nucleotides of position 1486-1488 from the 5' end of SEQ ID NO.: 21.The Open Reading Frame (ORF) of 3*GT2* is shown as the nucleotides of position 1-1488 from the 5' end of SEQ ID NO.: 23 according to the sequence listing. The start codon ATG of gene 3*GT2* is shown as the nucleotides of position 1-3 from the 5' end of SEQ ID NO.: 23 and the stop codon TAA of gene 3*GT2* is shown as the nucleotides of position 1486-1488 from the 5' end of SEQ ID NO.: 23.The Open Reading Frame (ORF) of 3*GT3* is shown as the nucleotides of position 1-1494 from the 5' end of SEQ ID NO.: 40 according to the sequence listing. The start codon ATG of gene 3*GT3* is shown as the nucleotides of position 1-3 from the 5' end of SEQ ID NO.: 40 and the stop codon TAA of gene 3*GT3* is shown as the nucleotides of position 1492-1494 from the 5' end of SEQ ID NO.: 40. PCR was employed to amplify two of the synthesized genes as set forth by SEQ ID NO.: 21 and SEQ ID NO.: 23 using primer pair 4 (SEQ ID NOs.: 29, 30), and the obtained PCR products had the nucleotides sequences as set forth by SEQ ID NO.: 21 and SEQ ID NO.: 23 (Figure 19(a)). PCR was employed to amplify another synthesized gene as set forth by SEQ ID NO.: 40 using primer pair 6 (SEQ ID NOs.: 44, 45), and the obtained PCR products had the nucleotides sequences as set forth by SEQ ID NO.: 40 (Figure 19(b)).

The glycosyltransferase gene *gGT25* encodes a protein gGT25 with 475 amino acids having the amino acid sequence as set forth by SEQ ID NO.: 2 of the sequence listing. The theoretical molecular weight of the protein was predicted to be 53 kDa and the isoelectric point (PI) 5.14 by software. Positions 344-387 from the N-terminal of SEQ ID NO.: 2 correspond to the conserved domain of the glycosyltransferase family 1. The amino acid sequence identity between said glycosyltransferase and the amino acid sequence of the predicted glycosyltransferase gene of saponin in *P. ginseng* transcriptome is lower than 52%.

The glycosyltransferase gene *gGT25-1* encodes a protein gGT25-1 with 475 amino acids having the amino acid sequence as set forth by SEQ ID NO.: 16 of the sequence

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listing. The theoretical molecular weight of the protein was predicted to be 53 kDa and the isoelectric point (PI) 4.91 by software. Positions 344-387 from the N-terminal of SEQ ID NO.: 16 correspond to the conserved domain of the glycosyltransferase family 1. The amino acid sequence identity between said glycosyltransferase and the amino acid sequence of the predicted glycosyltransferase gene of saponin in *P. ginseng* transcriptome is lower than 52%.

The glycosyltransferase gene *gGT25-3* encodes a protein gGT25-3 with 475 amino acids having the amino acid sequence as set forth by SEQ ID NO.: 18 of the sequence listing. The theoretical molecular weight of the protein was predicted to be 53 kDa and the isoelectric point (PI) 5.05 by software. Positions 344-387 from the N-terminal of SEQ ID NO.: 18 correspond to the conserved domain of the glycosyltransferase family 1. The amino acid sequence identity between said glycosyltransferase and the amino acid sequence of the predicted glycosyltransferase gene of saponin in *P. ginseng* transcriptome is lower than 52%.

The glycosyltransferase gene *gGT25-5* encodes a protein gGT25-5 with 472 amino acids having the amino acid sequence as set forth by SEQ ID NO.: 20 of the sequence listing. The theoretical molecular weight of the protein was predicted to be 53 kDa and the isoelectric point (PI) 4.98 by software. Positions 343-386 from the N-terminal of SEQ ID NO.: 20 correspond to the conserved domain of the glycosyltransferase family 1. The amino acid sequence identity between said glycosyltransferase and the amino acid sequence of the predicted glycosyltransferase gene of saponin in *P. ginseng* transcriptome is lower than 52%.

The glycosyltransferase gene *gGT13* encodes a protein gGT13 with 476 amino acids having the amino acid sequence as set forth by SEQ ID NO.: 4 of the sequence listing. The theoretical molecular weight of the protein was predicted to be 53 kDa and the isoelectric point (PI) 4.91 by software. Positions 343-386 from the N-terminal of SEQ ID NO.: 4 correspond to the conserved domain of the glycosyltransferase family 1. The highest amino acid sequence identity between said glycosyltransferase and the amino acid sequence of the predicted glycosyltransferase gene of saponin in *P. ginseng* transcriptome is 99.5%.

The glycosyltransferase gene *gGT30* encodes a protein gGT30 with 451 amino acids

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having the amino acid sequence as set forth by SEQ ID NO.: 6 of the sequence listing. The theoretical molecular weight of the protein was predicted to be 51 kDa and the isoelectric point (PI) 6.79 by software. Positions 318-361 from the N-terminal of SEQ ID NO.: 6 correspond to the conserved domain of the glycosyltransferase family 1. This glycosyltransferase has the highest similarity with the glycosyltransferase of *Vitis vinifera* (XP_002271587)(53%), indicating that this glycosyltransferase is a novel enzyme.

The glycosyltransferase gene *3GT1* encodes a protein *3GT1* with 495 amino acids having the amino acid sequence as set forth by SEQ ID NO.: 22 of the sequence listing. The theoretical molecular weight of the protein was predicted to be 56 kDa and the isoelectric point (PI) 5.52 by software. Positions 355-398 from the N-terminal of SEQ ID NO.: 22 correspond to the conserved domain of the glycosyltransferase family 1. The homology between said glycosyltransferase and the glycosyltransferase UGT73C10 originated from *Barbarea vulgaris* is higher than 99%.

The glycosyltransferase gene *3GT2* encodes a protein *3GT2* with 495 amino acids having the amino acid sequence as set forth by SEQ ID NO.: 24 of the sequence listing. The theoretical molecular weight of the protein was predicted to be 56 kDa and the isoelectric point (PI) 5.62 by software. Positions 355-398 from the N-terminal of SEQ ID NO.: 24 correspond to the conserved domain of the glycosyltransferase family 1. The homology between said glycosyltransferase and the glycosyltransferase UGT73C12 originated from *Barbarea vulgaris* is higher than 99%.

The glycosyltransferase gene *gGT29* encodes a protein gGT29 with 442 amino acids having the amino acid sequence as set forth by SEQ ID NO.: 26 of the sequence listing. The theoretical molecular weight of the protein was predicted to be 49 kDa and the isoelectric point (PI) 5.93 by software. Positions 317-360 from the N-terminal of SEQ ID NO.: 26 correspond to the conserved domain of the glycosyltransferase family 1. The sequence similarity between said glycosyltransferase and the glycosyltransferase originated from *Vitis vinifera* is lower than 56%.

The glycosyltransferase gene *gGT29-3* encodes a protein gGT29-3 with 442 amino acids having the amino acid sequence as set forth by SEQ ID NO.: 28 of the sequence listing. The theoretical molecular weight of the protein was predicted to be 49 kDa and the isoelectric point (PI) 5.48 by software. Positions 317-360 from the N-terminal of SEQ ID

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NO.: 26 correspond to the conserved domain of the glycosyltransferase family 1. The sequence similarity between said glycosyltransferase and the glycosyltransferase originated from *Vitis vinifera* is lower than 56%.

The glycosyltransferase gene *3GT3* encodes a protein *3GT3* with 497 amino acids having the amino acid sequence as set forth by SEQ ID NO.: 41 of the sequence listing. The theoretical molecular weight of the protein was predicted to be 55 kDa and the isoelectric point (PI) 5.50 by software. Positions 350-393 from the N-terminal of SEQ ID NO.: 41 correspond to the conserved domain of the glycosyltransferase family 1. The homology between said glycosyltransferase and the glycosyltransferase originated from *Medicago truncatula* is higher than 99%.

The glycosyltransferase gene *3GT4* encodes a protein *3GT4* with 458 amino acids having the amino acid sequence as set forth by SEQ ID NO.: 43 of the sequence listing. The theoretical molecular weight of the protein was predicted to be 51 kDa and the isoelectric point (PI) 5.10 by software. Positions 333-376 from the N-terminal of SEQ ID NO.: 43 correspond to the conserved domain of the glycosyltransferase family 1. The sequence homology between said glycosyltransferase and the glycosyltransferase originated from *Vitis vinifera* is lower than 50%.

The glycosyltransferase gene *gGT29-4* encodes a protein gGT29-4 with 446 amino acids having the amino acid sequence as set forth by SEQ ID NO.: 55 of the sequence listing. The theoretical molecular weight of the protein was predicted to be 50 kDa and the isoelectric point (PI) 5.78 by software. Positions 321-364 from the N-terminal of SEQ ID NO.: 55 correspond to the conserved domain of the glycosyltransferase family 1. The sequence similarity between said glycosyltransferase and the glycosyltransferase originated from *Bupleurum chinense* is lower than 57%.

The glycosyltransferase gene *gGT29-5* encodes a protein gGT29-5 with 446 amino acids having the amino acid sequence as set forth by SEQ ID NO.: 57 of the sequence listing. The theoretical molecular weight of the protein was predicted to be 50 kDa and the isoelectric point (PI) 5.93 by software. Positions 321-364 from the N-terminal of SEQ ID NO.: 57 correspond to the conserved domain of the glycosyltransferase family 1. The sequence similarity between said glycosyltransferase and the glycosyltransferase originated from *Bupleurum chinense* is lower than 58%.

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The glycosyltransferase gene *gGT29-6* encodes a protein gGT29-6 with 446 amino acids having the amino acid sequence as set forth by SEQ ID NO.: 59 of the sequence listing. The theoretical molecular weight of the protein was predicted to be 50 kDa and the isoelectric point (PI) 6.03 by software. Positions 321-364 from the N-terminal of SEQ ID NO.: 59 correspond to the conserved domain of the glycosyltransferase family 1. The sequence similarity between said glycosyltransferase and the glycosyltransferase originated from *Bupleurum chinense* is lower than 59%.

The glycosyltransferase gene *gGT29-7* encodes a protein gGT29-7 with 446 amino acids having the amino acid sequence as set forth by SEQ ID NO.: 61 of the sequence listing. The theoretical molecular weight of the protein was predicted to be 50 kDa and the isoelectric point (PI) 5.80 by software. Positions 321-364 from the N-terminal of SEQ ID NO.: 61 correspond to the conserved domain of the glycosyltransferase family 1. The sequence similarity between said glycosyltransferase and the glycosyltransferase originated from *Bupleurum chinense* is lower than 57%.

Table 2

Glycosyltransferase	C-3	The first glycosyl at C-3	C6	C20
gGT25(SEQ ID NO.: 2)			✓	✓
gGT25-1(SEQ ID NO.: 16)			✓	✓
gGT25-3(SEQ ID NO.: 18)			✓	✓
gGT25-5(SEQ ID NO.: 20)			✓	
gGT29(SEQ ID NO.: 26)		✓		
gGT29-3(SEQ ID NO.: 28)		✓		
gGT29-4(SEQ ID NO.: 55)		✓		
gGT29-5(SEQ ID NO.: 57)		✓		
gGT29-6(SEQ ID NO.: 59)		✓		
gGT29-7(SEQ ID NO.: 61)		✓		
3GT1(SEQ ID NO.: 22)	✓			
3GT2(SEQ ID NO.: 24)	✓			
3GT3(SEQ ID NO.: 40)	✓			
3GT4(SEQ ID NO.: 41)	✓			
gGT13(SEQ ID NO.: 4)	ND	ND	ND	ND
gGT30(SEQ ID NO.: 6)	ND	ND	ND	ND

Example 2

Construction of the recombinant yeast expression vectors for glycosyltransferase genes *gGT25*, *gGT25-1*, *gGT25-3* and *gGT25-5*

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The target genes were amplified using the plasmids gGT25-pMD18T, gGT25-1-pMD18T, gGT25-3-pMD18T and gGT25-5-pMD18T containing genes *gGT25*, *gGT25-1, gGT25-3* and *gGT25-5* constructed in Example 1 as templates.

The collective forward primer is:

5'- GCCGGAGCTCATGAAGTCAGAATTGATATTC - 3'(SEQ ID NO.: 13) with a SacI recognition site added to its 5' end: GAGCTC;

The collective reverse primer is:

5'-GCCGCTCGAGTTAATGATGATGATGATGATGCATAATTTCCTCAAATAGCTTC-3' (SEQ ID NO.: 14) with a XhoI recognition site added to its 5' end: CTCGAG. A 6×His Tag was introduced into the reverse primer for expression detection by Western Blot and purification.

The above primers and templates were used for amplifying genes *gGT25*,*gGT25-1*, *gGT25-3* and *gGT25-5* by PCR method. The high-fidelity DNA polymerase KOD (Toyobo Inc) was selected as DNA polymerase and the PCR program was set according to the instructions: 94℃ 2 min; 94℃ 15s, 58℃ 30s, 68℃ 1.5min for 30 cycles; 68℃ 10min; the temperature was kept at 10℃. The PCR product was detected by agarose gel electrophoresis and the band with a size of the target DNA was cut under a UV lamp. Then, the DNA fragments were recovered from the agarose gel using AxyPrep DNA Gel Extraction Kit (AXYGEN Inc.). The recovered DNA fragments were digested using two Quickcut restricted enzymes Kpn I and Xba I from Takara Inc. for 30 mins. The enzyme-digested products were rinsed and recovered by AxyPrep PCR Cleanup Kit from AXYGEN Inc. The digested products was ligated to the *Saccharomyces cerevisiae* expression plasmid pYES2 (also digested by Kpn I and Xba I and then cut out and recovered) at 25℃ for 2hrs by using T4 DNA ligase (NEB Inc.). The ligated products were transformed into *E. coli* TOP 10 competent cells and coated on a LB plate supplemented with 100μg/mL ampicillin. The positive clones were verified by colony PCR and further verified by sequencing. The results indicated that the expression plasmids gt25-pYES2, gt25-1-pYES2, gt25-3-pYES2 and gt25-5-pYES2 were successfully constructed.

Example 3

Expression of glycosyltransferases *gGT25*, *gGT25-1*, *gGT25-3* and *gGT25-5* genes in

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S. cerevisiae

The constructed expression vectors gt25-pYES2 were transformed into *Saccharomyces cerevisiae through* electroporation and then coated on screening plates SC-Ura (0.67% yeast nitrogen base without amino acids, 2% glucose). The recombinant yeasts were verified by colony PCR. A recombinant yeast colony was inoculated into 10 mL of the SC-Ura (2% glucose) medium and then cultured at 200 rpm under 30℃ for 20 h. The pellets were collected by centrifuge (3500g) at 4℃. The pellets were washed with sterile deionized water for twice, resuspended in the induction medium SC-Ura(2% galactose) and inoculated into 50mL of the induction medium with an OD_{600} of about 0.4 so as to induce the expression at 200 rpm under 30℃. After expression induction for 12 hours, the pellets were collected by centrifugation (3500g) at 4℃, washed with sterile deionized water for twice and then resuspended in the yeast lysis buffer to keep OD_{600} between 50 and 100. The yeast cells were shook and disrupted by the Fastprep cell disruption system. The cell debris was removed by centrifugation (12000g) at 4℃ for 10 mins and the supernatant of the cell lysis was collected. An appropriate amount of supernatant of the cell lysis was subjected to SDS-PAGE electrophoresis detection. Compared with empty vector pYES2 recombinants, no obvious characteristic band was shown for gt25-pYES2，gt25-1-pYES2，gt25-3-pYES2，or gt25-5-pYES2 recombinants, see Figure 2. The expression was detected by using anti-6×His Tag Western Blot. As shown in Figure 3, the *S.cerevisiae* recombinants expressing gGT25，gGT25-1, gGT25-3 or gGT25-5 showed strong Western Blot signals, indicating the soluble expression of gGT25，gGT25-1，gGT25-3 and gGT25-5 in yeasts. In contrast, no anti-6×His Tag Western Blot signal was shown for the recombinants transformed with empty vector pYES2.

Example 4

Glycosyltransfering reaction of the yeast expression products gGT25, gGT25-1, gGT25-3 and gGT25-5 and the product identification

The glycosyltransfering reactions of the substrates PPD, PPT or DM were catalyzed by using lysate supernatant of the recombinant yeasts expressing gGT25，gGT25-1，gGT25-3 or gGT25-5 as crude enzymes. The lysate supernatant of the recombinant yeasts expressing the empty vector was used as control. The 100μL reaction system is

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shown in Table 3:

Table 3

9% Tween 20	11.1 μL
50mM UDP-glucose	10μL
1M Tris-HCl pH8.5	5 μL
100mM substrate (dissolved in ethanol)	0.5μL
crude enzyme	73.4μL

The reaction was conducted under 35℃ for 12hrs, then stopped by adding 100 μL of butanol. The product were extracted, dried in vacuum, and dissolved in methanol.

The reaction products were first detected by thin layer chromatography (TLC). The lysate supernatant (used as the crude enzyme) of the recombinant yeasts expressing gGT25, gGT25-1 or gGT25-3 glycosylated the C20-OH of PPD and PPT, thereby converting them into rare ginsenosides CK and F1 (Figure 6 and Figure 7). PPD-type saponins Rh2 and Rg3 with glycosylated C3-OH were further glycosylated at C20-OH, with the catalyzation by gGT25，gGT25-1 and gGT25-3, to produce F2 and Rd, respectively(Figure 6). Upon the catalyzation of gGT25，gGT25-1 and gGT25-3, not only the C20-OH of PPT could be glycosylated to produce F1, but also C6-OH could be further glycosylated to produce Rg1 (Figure 7). Besides, gGT25，gGT25-1 and gGT25-3 could also glycosylate C20-OH of DM (the precursor of PPD) to produce an unreported saponin 20-*O*-β-(D-glucopyranosyl)-dammarendiol II (Figure 8). However, PPT-type saponins (Rh1, Rg2, and Rf) with a glycosylated C6-OH could not be catalyzed by gGT25，gGT25-1 or gGT25-3 to produce glycosylated C20-OH. Meanwhile, gGT25，gGT25-1 or gGT25-3 could not catalyze the extension of carbohydrate chain. The glycosyltransferase gGT25-5 has different catalytic activities with gGT25，or gGT25-1, or gGT25-3; unlike gGT25，gGT25-1 and gGT25-3, it could not glycosylate the C20-OH of PPD, PPT or DM, but can only glycosylate C6-OH of PPT to produce rare ginsenoside Rh1 (Figure 7).

The converted products of gGT25 were further identified by HPLC (Figure 10 and Figure 11). As shown in Figure 10, there were 3 peaks. The retention time of peak 2 was identical to that of the CK standard sample; the retention time of peak 3 was identical to that of PPD. The small peak 3 indicated that PPD had been substantially transformed into CK. Peak 1, also present in the profile of the negative control, indicated its irrelevance to the conversion of PPD. 3 peaks were shown in Figure 11, the retention time of peak 1 was

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identical to that of F1 standard sample and peak 3 was identical to that of PPT. The small peak 3 indicated that PPT had been substantially transformed into F1. Peak 2, also present in the profile of the negative control, indicated its irrelevance to the conversion of PPT.

Finally, LC/MS was employed to further confirm the products (Figure 12 and Figure 13). Figure 12 showed the mass spectrum of the CK peak from the PPD conversion products (Peak 2 in Figure 10). Its MS was completely identical to that of the CK standard sample. Figure 13 showed the mass spectrum of F1 peak from the PPT conversion products (Peak 1 in Figure 11). Its MS was completely identical to that of the standard sample of F1. These results further confirmed that the conversion product of PPD and PPT by gGT25 is CK and F1, respectively.

Example 5

The cloning and expression of glycosyltransferases *gGT13* and *gGT30*, and the glycosyltransfering reaction of the expression products thereof

Using the same method as in Example 2, clones of *gGT13* and *gGT30* were obtained and recombinant yeast expression vectors were constructed and then transformed into *Saccharomyces cerevisiae*. Glycosyltransferases were induced to express as the steps in Example 3. Although there was no apparent band of target protein on SDS-PAGE (Figure 4), obvious hybridization signals were detected by Western Blot, indicating expressions of gGT13 and gGT30 in the yeasts (Figure 5).

According to the method as in Example 4, the cell lysate of recombinant yeasts expressing gGT13 and gGT30 were used to catalyze PPD and PPT.

Results turned out that the protein expression products of gGT13 and gGT30 neither converted PPD or PPT (Figure 9), nor PPD-type saponins Rh2, CK, F2 or Rg3, or PPT-type saponins F1, Rh1 or Rg1.

The above results indicated that gGT13 and gGT30 exhibited no glycosyltransfering effect on the above substrates in spite of the high identity (99.5%) between gGT13 and amino acid sequence of the predicted ginsenoside glycosyltransferase in *P. ginseng* transcriptome.

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Example 6

The expression of glycosyltransferase *gGT25* in *E. coli* and the glycosyltransfering reaction of the expression product thereof

The target gene *gGT25* was amplified by using the plasmid gGT25-pMD18T containing gene *gGT25* constructed in Example 1 as a template, cloned to the *E. coli* expression vector pet28a (purchased from Merck company) to construct an *E. coli* expression vector *gt25*-pet28a. The product was transformed into the commercial available *E. coli* BL21. The recombinant was inoculated in LB medium and cultured under 30℃ at 200rpm until OD_{600} reached about 0.6-0.8. Then the culture liquid was cooled to 4℃, and IPTG with a final concentration of 50 μM was added for inducing expression under 18℃ at 200rpm for 15hrs. The pellets were collected by centrifugation under 4℃ and then subjected to ultrasonic disruption. The cell lysis supernatant was collected by centrifugation at 12000 g under 4℃ and then an sample was taken for SDS-PAGE electrophoresis.

Western blot (Figure 14) showed that glycosyltransferase gGT25 could also be expressed in *E. coli* under the induction condition of 50μM IPTG. The cell lysis supernatant of the recombinant *E. coli* was used as crude enzyme to conduct the glycosyltransfering reaction, and the reaction condition was identical to that of Example 4.

The reaction was conducted under 35℃ fro 12hrs.100 μL of butanol was added to stop the reaction and the products were extracted. Upon vacuum drying, the product was dissolved by methanol. TLC was first used to detect the reaction product. As shown in Figure 15, the crude enzyme containing gGT25 could transform PPD into CK.

Example 7

Construction of the engineered yeast strain for producing CK and the product charaterization

Dammarenediol synthase (ACZ71036.1) (GAL1/GAL10 GAL10 side promoter, ADH1 terminator), cytochrome P450 *CYP716A47* (AEY75213.1) (FBA1 promoter, CYC1 terminator), and glycosyltransferase gene *GT25* (GAL1/GAL10 GAL1 side promoter, TDH2 terminator) were assembled in the plasmid pESC-HIS (Stratagene, Agilent), thereby constructing an episomal plasmid. The plasmid was used to transform *Saccharomyces cerevisiae* BY4742. Cytochrome P450 reductase gene ATR2-1 (NP_849472.2) from *Arabidopsis thaliana* was

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also integrated to the site of gene *trp1* (GAL1 promoter; using the original terminator of *trp1*)in *the* chromosome of *Saccharomyces cerevisiae* BY4742 so as to construct the recombinant yeast A. Recombinant yeast B was also constructed by the same method except that the reductase gene ATR2-1 from *A. thaliana* was integrated to the recombinant plasmid containing DM synthetase, cytochrome P450 CYP716A47 and glycosyltransferases GT25.The promoter and terminator of ART2-1 were TEF2 promoter and TPI1 terminator, respectively. The promoters or terminators of other 3 genes were identical to the corresponding genes of recombinant strain A.

Recombinant yeast strain C was constructed using the method as for recombinant yeast strain B except the replanned promoter and terminator of each gene as shown in Table 4.

Table 4 Constitution of promoters and terminators of the major enzymes:

Major enzymes	Promoter	Terminator
DM synthetase	GAL1/GAL10 GAL10 side	ADH1
CYP716A47	GAL1/GAL10 GAL1 side	TDH2
ATR2-1	TEF2	TPI1
GT25	FBA1	CYC1

The recombinant yeast strains A, B, C were fermented in SC-Ura culture medium (0.67% yeast nitrogen base without amino acids, and 2% galactose). Additional added amino acids or uracil needed for each recombinant strain was shown in Table 5. 50 mL of the fermentation broth of the recombinant yeast was subjected to centrifugation, and the precipitated pellets were resuspended in 5 mL of yeast lysis buffer (50mM Tris-Hcl, 1mM EDTA, 1mM PMSF, 5% glycerol, pH 7.5). Then the yeasts were shook and disrupted by Fastprep.7-8 times of shaking with the power of 6M/S enabled the complete disruption of the yeast. The lysate was transferred into 2 mL EP tubes with 1 mL for each tube, subjected to extraction by adding *n*-butanol of equivalent volume (1 mL) for about 30 mins, and then centrifuged for 10 mins at 12000g. The supernatant was transferred to a new EP tube. *n*-butanol was evaporated to dry in vacuum under 45℃. Upon being dissolved in methanol (100μL), the product was subjected to HPLC detection.

Upon HPLC analyze, DM, PPD and the ginsenoside active metabolite (CK) were detected in the cell lysate of recombinant yeast A (Figure 16). The yield of CK synthesized by yeast A reached to 0.6mg/L. It could also be concluded from HPLC analyze that there were trace amounts of CK contained in the cell lysate of recombinant yeasts B and C.

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Table 5 The corresponding amino acids or uracil additionally needed for recombinant yeast strains

recombinant yeast strains	Additional amino acids or uracil
A	0.01% of tryptophan, leucine, lysine
B	0.01% of uracil, leucine, lysine
C	0.01% of uracil, leucine, lysine

Example 8

Construction of engineered yeast strain for Rh1 production and the product identification

Dammarenediol synthase (ACZ71036.1) (GAL1/GAL10 GAL10 side promoter, ADH1 terminator), cytochrome P450 *CYP716A47* (AEY75213.1) (FBA1 promoter, CYC1 terminator), cytochrome P450 CYP716A53V2 gene (ENO2 promoter, CYC1 terminator) and glycosyltransferase gene *GT25-5* (GAL1/GAL10 GAL1 side promoter, TDH2 terminator) were assembled in the plasmid pESC-HIS (Stratagene, Agilent), thereby constructing an episomal plasmid. The product was used to transform *S. cerevisiae* BY4742. Cytochrome P450 reductase ATR2-1 (NP_849472.2) from *A. thaliana* was also integrated to the site of gene *trp1* (GAL1 promoter; and the original terminator of *trp1* was used) *in the* chromosome of *S. cerevisiae* BY4742 so as to construct the recombinant yeast A3.Additional added amino acids or uracil needed for each recombinant strain was shown in Table 5.

The lysate of recombinant yeast was transferred into 2 mL EP tubes with 1 mL for each tube, subjected to extraction by adding *n*-butanol of equivalent volume (1 mL) for about 30 mins, and then centrifuged for 10 mins at 12000g. The supernatant was transferred to a new EP tube. *n*-butanol was evaporated to dry in vacuum under 45℃. Upon being dissolved in methanol (100μL), the product was subjected to HPLC detection.

Upon HPLC analysis, PPT and the active metabolite of ginsenoside (Rh1) were detected in the cell lysate of recombinant yeast A3 (Figure 41).

Example 9

The construction of *E. coli* recombinant expression vectors for

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glycosyltransferase genes 3*GT1*, 3*GT2*, 3*GT3* and 3*GT4*

The target genes were amplified using the plasmids 3GT1-pMD18T and 3GT2-pMD18T containing genes 3*GT1* and 3*GT2* constructed in Example 1 as templates.

The collective forward primer of 3*GT1* and 3*GT2* is SEQ ID NO.: 31 with a BamHI recognition site added to its 5' end: GGATCC; the reverse primer of *3GT1* is SEQ ID NO.: 32 with a SalI recognition site added to its 5' end: CTCGAG; the reverse primer of *3GT2* is SEQ ID NO.: 33 with a Sal I recognition site added to its 5' end CTCGAG.

The above primers and templates were used for amplifying genes 3*GT1* and 3*GT2* by PCR. The high-fidelity DNA polymerase KOD (Toyobo Inc) were selected as DNA polymerase and the PCR program was set according to the instructions: 94℃ 2 min; 94℃ 15s, 58℃ 30s, 68℃ 1.5min, for 30 cycles; 68℃ 10min; the temperature was kept at 10℃. The PCR product was detected by agarose gel electrophoresis and the band with a size of the target DNA was cut out under the UV lamp. Then, the DNA fragments were recovered from the agarose gel using AxyPrep DNA Gel Extraction Kit (AXYGEN Inc.). The recovered DNA fragments were digested using two Quickcut restricted enzymes Kpn I and Xba I from Takara Inc. for 30 mins. The enzyme-digested products were washed and recovered by AxyPrep PCR Cleanup Kit from AXYGEN Inc. The digested products were ligated to the *E. coli* expression plasmid pET28a (also digested by BamHI and SalI and then cut and recovered) under 16℃ for 4hrs by using a T4 DNA ligase (NEB Inc.). The ligated products were transformed into *E. coli* EPI300 competent cells and coated on LB plate supplemented with 50μg/mL kanamycin, respectively. The positive clones were verified by colony PCR and the expression plasmids of 3GT1-pET28a and 3GT2-pET28a were further confirmed by sequencing.

The target genes were amplified using the plasmids 3GT3-pMD18T and 3GT4-pMD18T containing genes 3*GT3* and 3*GT4* constructed in Example 1 as templates.

The forward primer of 3*GT3* is SEQ ID NO.: 48 with a sequence homologous with vector pET28a added to its 5' end: ACTTTAAGAAGGAGATATACC; the reverse primer of *3GT3* is SEQ ID NO.: 49 with a sequence homologous with vector pET28a added to its 5' end:CTCGAGTGCGGCCGCAAGCTT.

The forward primer of 3*GT4* is SEQ ID NO.: 50 with a sequence homologous with vector pET28a added to its 5' end: ACTTTAAGAAGGAGATATACC; the reverse primer of *3GT4* is

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SEQ ID NO.: 51 with a sequence of 18 bases homologous with vector pET28a added to its 5' end:CTCGAGTGCGGCCGCAAGCTT.

The above primers were used for amplifying genes 3*GT3* and 3*GT4* by PCR. The high-fidelity DNA polymerase Q5 (NEB Inc) was selected for gene amplification and the PCR program was set according to the instructions: 98℃ 30sec; 98℃ 15s, 58℃ 30s, 72℃ 1min for 35 cycles; 72℃ 2min; the temperature was kept at 10℃.

Further, the vector pET28a was amplified by using SEQ ID NO.: 52 and SEQ ID NO.: 53 as the forward and reverse primer respectively so as to obtain the linearized vector pET28a. The high-fidelity DNA polymerase Q5 (NEB Inc) was also chosen for amplifying the linearized vector pET28a and the PCR program was set according to the instructions: 98℃ 30 sec; 98℃ 15 s, 58℃ 30s, 72℃ 1 min for 35 cycles; 72℃ 2 min; the temperature was kept at 10℃.

The PCR products of the above genes 3GT3 and 3GT4 and the linearized vector pET28a were detected by agarose gel electrophoresis and the bands with size of the target DNAs were cut out under a UV lamp. Then, the DNA fragments were recovered from the agarose gel using AxyPrep DNA Gel Extraction Kit (AXYGEN Inc.). The recovered fragment of the linearized vector pET28a, the recovered gene fragments of 3GT3 and 3GT4 and BGclonart seamless cloning reaction solution (Rockgene Biotech Inc.) were mixed up to 20μl in suitable proportions according to the instructions of BGclonart seamless cloning kit from Rockgene Biotech Inc. Upon mixed to homogenous, the product was incubated under 50℃ for 30 mins and the mixed reacting solution was transferred onto ice. *E. coli* EPI1300 competent cells were transformed by 5 μl of the reacting solution and then coated on a LB plate supplemented with 50 μg/mL of kanamycin. The positive clones were verified by colony PCR and the successful expression plasmids of 3GT3-pET28a and 3GT4-pET28a were further confirmed by sequencing.

Example 10

The expression of glycosyltransferase 3*GT1*, 3*GT2*, 3*GT3* and 3*GT4* in *E. coli*

The *E. coli* expression vectors 3GT1-pET28a, 3GT2-pET28a, 3GT3-pET28a and 3GT4-pET28a constructed in Example 9 were transformed into the commercially available *E. coli* BL21. A recombinant was inoculated into LB medium and cultured under 30℃ at 200rpm

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until OD_{600} reached about 0.6-0.8. Then the culture liquid was cooled to 4℃, and IPTG with a final concentration of 50 μM was added for inducing expression under 18℃ at 200rpm for 15hrs. The pellets were collected under 4℃ and then subjected to ultrasionic disruption. The cell lysis supernatant was collected by centrifugation at 12000 g and then a sample was taken for SDS-PAGE electrophoresis (Figure 20). Compared with empty vector pYES2 recombinant, obvious bands (about 55 kDa) representing 3GT1, 3GT2, 3GT3 and 3GT4 were shown for the 3GT1-pET28a, 3GT2-pET28a, 3GT3-pET28a and 3GT4-pET28a recombinants. The results of Western Blot (Figure 21) also indicate that the soluble expression of target proteins 3GT1, 3GT2, 3GT3 and 3GT4 were realized in the hosts.

Example 11

Glycosyltransfering reaction of the *E. coli* expression products of 3GT1, 3GT2, 3GT3 and 3GT4 and product identification

The glycosyltransfering reactions of ginsenosides and ginsengenins were catalyzed by using the lysis supernatant of the recombinant yeasts expressing 3GT1, 3GT2, 3GT3 and 3GT4 as crude enzymes. The lysis supernatant of recombinant *E. coli* expressing empty vectors was used as control. The 100μL reaction system is shown as Table 3. The reaction was conducted under 35℃ for 12hrs, then stopped by adding 100 μL of butanol. The product were extracted, dried in vacuum, and dissolved in methanol.

The reaction products were first detected by thin layer chromatography (TLC) (Figure 22-28). The C3-OH of PPD was glycosylated by the crude enzymes expressing 3GT1, 3GT2, 3GT3 and 3GT4 to respectively produce rare ginsenoside Rh2 (Figure 22, 27(a) and 28(a)). PPD-type saponin CK with a glycosylated C20-OH was catalyzed by 3GT1, 3GT2, 3GT3 and 3GT4 for further glycosylating its C3-OH to respectively produce F2 (Figure 22 and 28(b)).C3-OH of DM could be glycosylated to produce a novel compound 3-*O*-β-(D-glucopyranosyl) -Dammarenediol II (Figure 23) by using 3GT1 and 3GT2 for catalyzation. C3-OH of 25-OH-PPD could be glycosylated to produce a novel compound 3-*O*-β- (D-glucopyranosyl)-25-OH-PPD (Figure 23, Figure 27(c) and Figure 28(c)) by using 3GT1, 3GT2, 3GT3 and 3GT4 for catalyzation. C3-OH of PPT could be glycosylated to produce an unreported novel compound 3-*O*-β- (D-glucopyranosyl)-PPT (Figure 24 and Figure 27(b)) by using 3GT1, 3GT2, and 3GT3 for catalyzation. C3-OH of F1 could be

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glycosylated to produce an unreported novel compound 3-*O*-β-(D-glucopyranosyl)-F1 (Figure 24) by using 3GT1 and 3GT2 for catalyzation. C3-OH of lanosterol could be glycosylated to produce a novel compound 3-*O*-β-(D-glucopyranosyl)-lanosterol (Figure 26) by using 3GT1 and 3GT2 for catalyzation. Moreover, the catalytic activities of 3GT1 and 3GT2 were not affected by the steric configuration of the hydoxyls or glycosyls at C20. For example, these enzymes could catalyze both 20(*S*)-PPD and 20(*R*)-PPD to produce rare ginsenoside 20(*R*)-ginsenoside Rh2 (Figure 25). Although, all of the four glycosyltransferase 3GT1, 3GT2, 3GT3 and 3GT4 could introduce glycosyl into C3 of tetracyclic triterpenoid sapogenins, the substrate spectrums that they could catalyze were remarkably distinct. As shown in Table 6, 3GT1 and 3GT2 could catalyze the most number of substrates; 3GT3 could catalyze the least number of substrates; while 3GT4 showed the best specificity: it could only catalyze PPD and PPD-type saponin (CK).

The products obtained by catalyzing PPD using 3GT1, 3GT2, 3GT3 and 3GT4 were further detected by HPLC (Figure 29). Peaks with the same retention time (P1, P2, and P3) could be observed in products obtained by catalyzing PPD using glycosyltransferases 3GT1, 3GT2, 3GT3 and 3GT4. The retention time of these peaks is identical to that of the spectrum of ginsenoside Rh2 in standard sample, indicating that the product obtained by catalyzing PPD using glycosyltransferases 3GT1, 3GT2, 3GT3 and 3GT4 was Rh2. Finally, the three samples in Figure 29, P1, P2, and P3, were subjected to mass spectrometry characterization by LC/MS (Figure 30). Their spectrums were completely identical to that of ginsenoside Rh2 standard sample, further indicating that the product obtained by catalyzing PPD using glycosyltransferases 3GT1, 3GT2, 3GT3 and 3GT4 was Rh2.

The substrates that could be catalyzed by glycosyltransferases 3GT1, 3GT2, 3GT3 and 3GT4 are compared in Table 6：

Table 6

	PPD	20(*R*)-PPD	CK	PPT	F1	DM	25-OH-PPD	lanosterol
3GT1/3GT2	√	√	√	√	√	√	√	√
3GT3	√	×	×	√	×	×	√	×
3GT4	√	×	√	×	×	×	√	×

Example 12

Construction of the engineered yeast strain for Rh2 production and product

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identification

12.1 Dammarenediol synthase (ACZ71036.1) (GAL1/GAL10 GAL10 side promoter, ADH1 terminator), cytochrome P450 *CYP716A47* (AEY75213.1) (FBA1 promoter, CYC1 terminator), and glycosyltransferase 3GT4 (GAL1/GAL10 GAL1 side promoter, TDH2 terminator) were assembled in the plasmid pESC-HIS (Stratagene, Agilent), thereby constructing an episomal plasmid. The plasmid was used to transform *S. cerevisiae* BY4742. Cytochrome P450 reductase ATR2-1 (NP_849472.2) from *Arabidopsis thaliana* was also integrated to the site of gene *trp1* (GAL1 promoter, and the original terminator of *trp1* was used) *in the* chromosome of *S. cerevisiae* BY4742 so as to construct the recombinant yeast A1. Additional added amino acids or uracil needed for each recombinant strain is shown in Table 5.

The lysate of recombinant yeast A1 was transferred into 2 mL EP tubes with 1 mL for each tube, subjected to extraction by adding *n*-butanol of equivalent volume (1 mL) for about 30 mins, and then centrifuged for 10 mins at 12000g. The supernatant was transferred to a new EP tube. *n*-butanol was evaporated to dry in vacuum under 45℃. Upon dissolved in methanol (100μL), the product was subjected to HPLC detection.

DM, PPD and the active metabolite of ginsenoside (Rh2) were detected in the cell lysate of recombinant yeast A1 according to HPLC analyze (Figure 39).

12.2 The same method as 12.1 was used except that glycosyltransferase 3GT4 was substituted by 3GT1, thereby obtaining recombinant yeast A5.

Results were shown in Figure 43, DM, PPD and the active metabolite of ginsenoside (Rh2) were contained in the cell lysate of recombinant yeast A5 according to the HPLC analyze.

Example 13

Construction of the recombinant yeast expression vectors for glycosyltransferase genes *gGT29* and *gGT29-3*

The target genes were amplified using the plasmids gGT29-pMD18T, gGT29-3-pMD18T containing genes *gGT29* and *gGT29-3* constructed in Example 1 as templates.

The forward primer of gGT29 was SEQ ID NO.: 36 with a Kpn I recognition site added to its 5' end: GGATCC; the reverse primer was SEQ ID NO.: 37 with an XhoI recognition site

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added to its 5' end: CTCGAG; a 6×His Tag was introduced into the reverse primer for expression detection by Western Blot and purification.

The forward primer of gGT29-3 was SEQ ID NO.: 38 with a Kpn I recognition site added to its 5' end: GGATCC; the reverse primer was SEQ ID NO.: 39 with an XhoI recognition site added to its 5' end: CTCGAG; a 6×His Tag was introduced into the reverse primer for expression detection by Western Blot and purification.

By using plasmids gGT29-pMD18T and gGT29-3-pMD18T as templates and the primers above, genes *gGT29* and *gGT29-3* were amplified through PCR method. The high-fidelity DNA polymerase KOD (Toyobo Inc) were selected as DNA polymerase and the PCR program was set according to the instructions: 94℃ 2 min; 94℃ 15s, 58℃ 30s, 68℃ 1.5min, for 30 cycles; 68℃ 10min; the temperature was kept at 10℃.The PCR product was detected by agarose gel electrophoresis and the band with a size of the target DNA was cut out under a UV lamp. Then, the DNA fragments were recovered from the agarose gel using AxyPrep DNA Gel Extraction Kit (AXYGEN Inc.). The recovered DNA fragments were digested using two Quickcut restricted enzymes Kpn I and Xba I from Takara Inc. for 30 mins. The enzyme-digested products were washed and recovered by AxyPrep PCR Cleanup Kit from AXYGEN Inc. The digested products was ligated to the *S. cerevisiae* expression plasmid pYES2 (also digested by Kpn I and Xba I and then cut and recovered) under 25℃ for 2hrs by using a T4 DNA ligase (NEB Inc.). The ligated products were transformed into *E. coli* TOP 10 competent cells and coated on LB plate supplemented with 100μg/mL ampicillin. The positive clones were verified by colony PCR and the expression plasmids of gGT29- pYES2 and gGT29-3-pYES2 were further confirmed by sequencing.

Example 14

The expression of glycosyltransferase genes *gGT29* and *gGT29-3* in *S. cerevisiae*

The constructed expression plasmids gGT29-pYES2 and gGT29-3-pYES2 were transformed into *S. cerevisiae* by electrotransformation. The transformants were plated on a screening plate SC-Ura (0.67% yeast nitrogen base without amino acids, and 2% galactose). The recombinant yeast was verified by colony PCR. The recombinant yeast colony was inoculated into 10 mL of SC-Ura (2% glucose) medium and then cultured at 200 rpm

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under 30℃ for 20 h. The pellets were collected by centrifugation (3500g) at 4℃. The pellets were washed with sterile deionized water for twice and resuspended in the induction medium SC-Ura(2% galactose) and inoculated to the 50mL induction medium with an OD_{600} of about 0.4 so as to induce the expression at 200 rpm under 30℃ for 12 hours. The pellets were collected by centrifugation (3500g) at 4℃, washed with sterile deionized water for twice and then resuspended in the yeast lysis buffer to keep OD_{600} between 50 and 100. The yeast cells were shook and disrupted by a cell disruption system (Fastprep). The cell debris was removed by centrifugation (12000g) at 4℃ for 10 mins and the supernatant of the cell lysis was collected. An appropriate amount of the lysate supernatant was subjected to SDS-PAGE electrophoresis detection. Compared with the empty vector pYES2 recombinant, no obvious characteristic band was shown for gGT29-pYES2 or gGT29-3-pYES2 recombinant (Figure 32). *S. cerevisiae* expressing *gGT29* and *gGT29-3* showed very strong Western Blot signals according to anti-6×His Tag Western Blot detection, indicating the soluble expression of *gGT29* and *gGT29-3* in the yeasts. In contrast, no anti-6×His Tag Western Blot signal was shown for the recombinants transformed with the empty vector pYES2 (Figure 33).

Example 15

Glycosyltransfering reaction of the yeast expression products of gGT29 and gGT29-3 and the product identification

The glycosyltransfering reactions of ginsenoside Rh2 and F2 were catalyzed by using the lysate supernatant of the recombinant yeasts expressing gGT29 and gGT29-3 as crude enzyme. The lysate supernatant of the recombinant yeast expressing empty vectors was used as control. The 100 μL of reaction system is shown in Table 3. The reaction was conducted under 35℃ for 12hrs, and then stopped by adding 100 μL of butanol. The product were extracted, dried in vacuum, and dissolved in methanol.

The reaction products were first detected by thin layer chromatography (TLC). C3 of ginsenosides Rh2 and F2 could be extended by one more glycosyl by using the lysate supernatant of yeast hosts expressing gGT29 and gGT29-3 as crude enzymes so as to produce ginsenosides Rg3 and Rd (Figure 34). The catalytic activities of gGT29 and gGT29-3 were not affected by the steric configuration of hydroxyls or glycosyls at C20. These

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enzymes could convert 20(*R*)-Rh2 to 20(*R*)-Rg3 (Figure 36).

Example 16

Glycosyltransfering reaction by combined use of glycosyltransferases 3GT1/3GT4 and gGT29 and the product identification

PPD was catalyzed by using the combination of the lysate supernatant of *E. coli* host expressing 3GT1 or 3GT4 and the lysate supernatant of yeast host expressing gGT29 as crude enzymes. The 100 μL reaction system is shown in Table 3. In the 73.4 μL enzyme liquid, 40 μL was the supernatant of *E. coli* host expressing 3GT1, the rest 33.4μL was the lysate supernatant of yeast host expressing gGT29. The reaction was conducted under 35℃ for 12hrs, and then stopped by adding 100 μL of butanol. The product were extracted, dried in vacuum, and dissolved in methanol. The reaction products were first detected by thin layer chromatography (TLC) (Figure 35). It could be observed that PPD could be transformed into Rg3 either by the combination of glycosyltransferase 3GT1 and gGT29 or the combination of 3GT4 and gGT29.

20(*R*)-PPD could be transformed into 20(*R*)-Rg3 either by the combination of glycosyltransferase 3GT1 and gGT29 or the combination of 3GT4 and gGT29 (Figure 36).

Example 17

Construction of engineered yeast strains for Rg3 production and product identification

17.1 Dammarenediol synthase (ACZ71036.1) (GAL1/GAL10 GAL10 side promoter, ADH1 terminator), cytochrome P450 *CYP716A47* (AEY75213.1) (FBA1 promoter, CYC1 terminator), glycosyltransferases 3GT4 and gGT29 (GAL1/GAL10 GAL1 side promoter, TDH2 terminator) were assembled in the plasmid pESC-HIS (Stratagene, Agilent), thereby constructing an episomal plasmid. The plasmid was used to transform *S. cerevisiae* BY4742. Cytochrome P450 reductase ATR2-1 (NP_849472.2) from *Arabidopsis thaliana* was also integrated to the site of gene *trp1* (GAL1 promoter, and the original terminator of *trp1* was used) *in the* chromosome of *S. cerevisiae* BY4742 so as to construct the recombinant yeast A2. Additional added amino acids or uracil needed for each recombinant strain is shown in Table 5.

The lysate of recombinant yeast A2 was transferred into 2 mL EP tubes with 1 mL for

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each, subjected to extraction by adding *n*-butanol in equivalent volume (1 mL) for about 30 mins, and then centrifuged for 10 mins at 12000g. The supernatant was transferred to a new EP tube. *n*-butanol was dried in vacuum under 45°C. Upon dissolved in methanol (100μL), the product was subjected to HPLC detection.

DM, PPD and the active metabolite of ginsenoside (Rg3) were contained in the cell lysate of recombinant yeast A2 according to HPLC analyze (Figure 40).

17.2 The same method as 17.1 was used except that glycosyltransferase 3GT4 was substituted by 3GT1, thereby obtaining recombinant yeast A6. DM, PPD and the active metabolite of ginsenoside (Rg3) were also contained in the cell lysate of recombinant yeast A6 according to HPLC analyze.

Example 18

Construction of engineered yeast strains for F1 production and product identification

Dammarenediol synthase (ACZ71036.1) (GAL1/GAL10 GAL10 side promoter, ADH1 terminator), glycosyltransferase gGT25 (GAL1/GAL10 GAL1 side promoter, TDH2 terminator), cytochrome P450 *CYP716A47* (AEY75213.1) (FBA1 promoter, FBA1 terminator), cytochrome P450 CYP716A53V2 (ENO2 promoter, CYC1 erminator) were assembled in the plasmid pESC-HIS (Stratagene, Agilent), thereby constructing an episomal plasmid. The product was used to transform *S. cerevisiae* BY4742. Cytochrome P450 reductase ATR2-1 (NP_849472.2) from *Arabidopsis thaliana* was integrated to the site of gene *trp1* (GAL1 promoter, and the original terminator of *trp1* was used) *in the* chromosome of *S. cerevisiae* BY4742 so as to construct the recombinant yeast A4. Additional added amino acids or uracil needed for each recombinant strain is shown in Table 5.

The lysate of recombinant yeast A4 was transferred into 2 mL EP tubes with 1 mL for each, subjected to extraction by adding *n*-butanol with equivalent volume (1 mL) for about 30 mins, and then centrifuged for 10 mins at 12000g. The supernatant was transferred to a new EP tube. *n*-butanol was dried in vacuum under 45°C. Upon dissolved in methanol (100μL), the product was subjected to HPLC detection.

PPT and the active metabolite of ginsenoside (F1) were contained in the cell lysate of recombinant yeast A4 according to HPLC analyze (Figure 42).

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Example 19

Construction of the *E. coli* recombinant expression vectors for glycosyltransferase genes *gGT29-4*, *gGT29-5*, *gGT29-6* and *gGT29-7*

The target genes were amplified using the plasmids gGT29-4-pMD18T, gGT29-5-pMD18T, gGT29-6-pMD18T and gGT29-7-pMD18T containing genes *gGT29-4*, *gGT29-5*, *gGT29-6* and *gGT29-7* constructed in Example 1 as templates.

The forward primer for *gGT29-5* and *gGT29-6* is as set forth by SEQ ID NO.: 66 with a sequence homologous to vector pET28a added to its 5' end: CTGGTGCCGCGCGGCAGC; the used reverse primer is as set forth by SEQ ID NO.: 68 with a sequence homologous to vector pET28a added to its 5' end: TGCGGCCGCAAGCTTGTC.

The forward primer for *gGT29-4* and *gGT29-7* is as set forth by SEQ ID NO.: 67 with a sequence homologous to vector pET28a added to its 5' end: CTGGTGCCGCGCGGCAGC; the used reverse primer is as set forth by SEQ ID NO.: 68 with a fragment of 18 bases homologous to vector pET28a added to its 5' end: TGCGGCCGCAAGCTTGTC.

The above primers were used to amplify genes *gGT29-4, gGT29-5, gGT29-6* and *gGT29-7* by PCR. The high-fidelity DNA polymerase Q5 (NEB Inc.) was selected for gene amplification. The PCR program was set according to the instructions: 98℃ 30s; 98℃ 15s, 58℃ 30s, 72℃ 1min, for 35 cycles; 72℃ 2min; the temperature was kept at 10℃.

Further, the vector pET28a was amplified by using SEQ ID NO.: 69 and SEQ ID NO.: 70 as forward and reverse primer respectively so as to obtain the linearized vector pET28a. The high-fidelity DNA polymerase Q5 (NEB Inc) was also chosen for amplifying the linearized vector pET28a and the PCR program was set according to the instructions: 98℃ 30 sec; 98℃ 15 s, 58℃ 30s, 72℃ 3 min for 35 cycles; 72℃ 2 min; the temperature was kept at 10℃.

The PCR products of above genes *gGT29-4*, *gGT29-5*, *gGT29-6* and *gGT29-7* and the linearized vector pET28a were detected by agarose gel electrophoresis and the bands with size of the target DNAs were cut out under a UV lamp. Then, the DNA fragments were recovered from the agarose gel using AxyPrep DNA Gel Extraction Kit (AXYGEN Inc.). The recovered fragment of the linearized vector pET28a, the recovered gene fragments of *gGT29-4*, *gGT29-5*, *gGT29-6* and *gGT29-7* and BGclonart seamless cloning

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reaction solution (Rockgene Biotech Inc.) were mixed up to 20μl in suitable proportions according to the instruction of the BGclonart seamless cloning kit from Rockgene Biotech Inc. Upon mixed to homogenous, the product was incubated under 50℃ for 30 mins and the mixed reacting solution was transferred onto ice. *E. coli* EPI300 competent cells were transformed by 5 μl of reacting solution and then coated on the LB plate supplemented with 50 μg/mL of kanamycin. The positive clones were verified by colony PCR and the successful expression plasmids of gGT29-*4*-pET28a, gGT29-5-pET28a, gGT29-6-pET28a and gGT29-7-pET28a were further confirmed by sequencing

Example 20

The expression of glycosyltransferases *gGT29-4*, *gGT29-5*, *gGT29-6* and *gGT29-7* in *E. coli*

The *E. coli* expression vectors gGT29-4-pET28a, gGT29-5-pET28a, gGT29-6-pET28a and gGT29-7-pET28a constructed in Example 19 were transformed into the commercially available *E. coli* BL21. A recombinant was inoculated into LB medium and cultured under 30℃ at 200rpm until OD_{600} reached about 0.6-0.8. Then the culture liquid was cooled to 4℃, and IPTG with a final concentration of 50 μM was added for inducing expression under 18℃ at 200rpm for 15hrs. The pellets were collected under 4℃ and then subjected to ultrasionic cell-break. The cell lysis supernatant was collected by centrifugation at 12000 g and then a sample was taken for SDS-PAGE electrophoresis (Figure 44). Obvious bands (about 50 kD) of target proteins could be observed in the lysate, total protein, and supernatant of the recombinants gGT29-4-pET28a, gGT29-5-pET28a, gGT29-6-pET28a and gGT29-7-pET28a, representing glycosyltransferases gGT29-4, gGT29-5, gGT29-6 and gGT29-7, respectively. According to the Western Blot results (Figure 45), target genes gGT29-4, gGT29-5, gGT29-6 and gGT29-7 achieved soluble expression in the hosts.

Example 21

Glycosyltransfering reaction of the *E. coli* expression products gGT29-4, gGT29-5, gGT29-6 and gGT29-7 and product identification

The glycosyltransfering reactions of ginsenosides Rh2 and F2 were catalyzed by using the lysate supernatant of the recombinant yeasts expressing 3GT1, 3GT2, 3GT3

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and 3GT4 as crude enzymes. The 100 μL reaction system is shown as Table 3. The reaction was conducted under 35℃ for 12hrs, and then stopped by adding 100 μL of butanol. The product were extracted, dried in vacuum, and dissolved in methanol.

The reaction products were detected by thin layer chromatography (TLC). C3 glycosyl of ginsenosides Rh2 and F2 could be extended by one more glycosyl using the crude enzymes of gGT29-6 so as to produce ginsenosides Rg3 and Rd (Figure 46); C3 glycosyl of ginsenoside F2 could be extended by one more glycosyl using the crude enzymes of gGT29-4, gGT29-5 and gGT29-7 so as to produce ginsenoside Rd; however, saponin Rh2 could not be catalyzed by them (Figure 46).

Further, it should be understood that, after reading the above contents, the skilled person can make various modifications or changes to the present invention. All these equivalents also fall into the scope defined by the appending claims of the present application.

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Claims

1. A method for *in vitro* glycosylation, wherein said method comprises:

in the presence of a glycosyltransferase, transferring a glycosyl from a glycosyl donor to the following site on tetracyclic triterpenoid compounds:

position C-3 or the first glycosyl at position C-3;

thereby forming glycosylated tetracyclic triterpenoid compounds, wherein, said glycosyltransferase is selected from the group consisting of: a glycosyltransferase as set forth by SEQ ID NOs: 22, 24, 26, 28, 41, 43, 55, 57, 59 and 61, and a derivative polypeptide thereof, wherein, said derivative polypeptide has ≥95% sequence identity with the amino acid sequence as set forth by any one of SEQ ID NOs: 22, 24, 26, 28, 41, 43, 55, 57, 59 and 61.

2. The method according to claim 1, wherein, said glycosyltransferase is selected from the group consisting of:

a glycosyltransferase as set forth by SEQ ID NOs: 22, 24, 26, 28, 41, 43, 55, 57, 59 and 61.

3. A vector, wherein said vector contains a polynucleotide selected from the group consisting of:

(A) a nucleotide sequence encoding a polypeptide selected from the group consisting of:

(a) a polypeptide consisting of the amino acid sequence as set forth by any one of SEQ ID NOs: 26, 28, 41, 43, 55, 57, 59 and 61;

(b) a derivative polypeptide comprising the amino acid sequence as set forth by any one of SEQ ID NOs: 26, 28, 41, 43, 55, 57, 59 and 61 and comprising a substitution, a deletion, an addition of one or more amino acid residues, or an addition of a signal peptide sequence, which has the activity of glycosyltransferase, wherein the derivative polypeptide has at least 85% sequence identity from any one of SEQ ID NOs: 26, 28, 41, 43, 55, 57, 59 and 61; and

(c) a derivative polypeptide, which has≥95% sequence identity with the amino acid sequence as set forth by any one of SEQ ID NOs: 26, 28, 41, 43, 55, 57, 59 and 61 and has the activity of glycosyltransferase；or

a nucleotide sequence encoding the polypeptide selected from the group consisting of:

(a1) a polypeptide having the amino acid sequence as set forth by any one of SEQ

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ID NOs: 22 and 24;

(b1) a polypeptide comprising the polypeptide sequence of (a1) in its sequence;(B) a nucleotide sequence encoding the polypeptide as set forth by any one of SEQ ID NOs: 22, 24, 26, 28, 41, 43, 55, 57, 59 and 61;

(C) the nucleotide sequence as set forth by any one of SEQ ID NOs: 21, 23, 25, 27, 40, 42, 54, 56, 58 and 60;

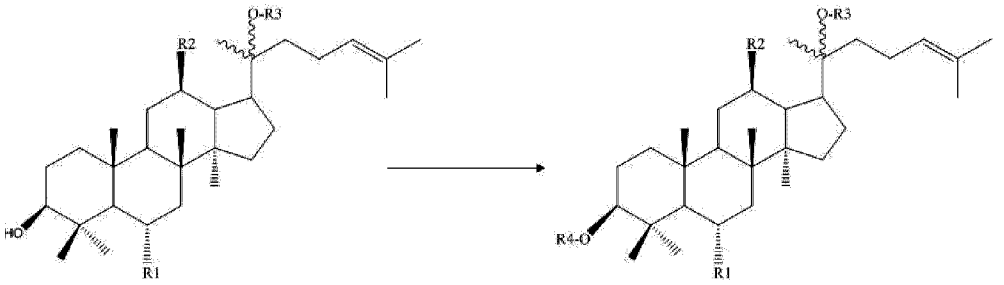
(D) a nucleotide sequence, which has ≥95% identity with the sequence as set forth by any one of SEQ ID NOs: 21, 23, 25, 27, 40, 42, 54, 56, 58 and 60 and encoding a polypeptide having the activity of glycosyltransferase;

(E) a nucleotide sequence derived from the nucleotide sequence as set forth by any one of SEQ ID NOs: 21, 23, 25, 27, 40, 42, 54, 56, 58 and 60 by deletion or addition of 1-10 nucleotides at its 5' end and/or 3' end and encoding a polypeptide having the activity of glycosyltransferase; and

(F) a nucleotide sequence complementary to any one of the nucleotide sequence of (A)-(E)..

4. The method of claim 1, wherein said glycosyltransferase is used for catalyzing one or more of the following reactions, or for preparing a catalyst preparation used in the catalyzation of one or more of the following reactions:

(C)



compound of formula (V) compound of formula (VI)

wherein, R1 is H or OH; R2 is H or OH; R3 is H or a glycosyl; R4 is a glycosyl; said glycosyltransferase is selected from SEQ ID NOs: 22, 24, 41 or 43 or a derivative polypeptide thereof, wherein, said derivative polypeptide has ≥95% sequence identity with the amino acid sequence as set forth by any one of SEQ ID NOs: 22, 24, 41 and 43, and has the activity of

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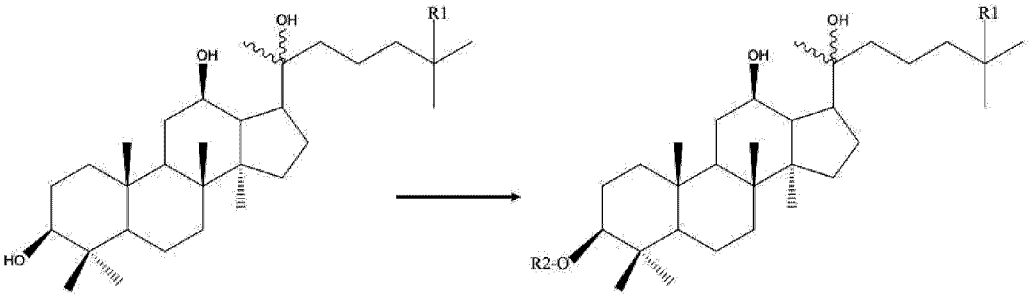
glycosyltransferase;

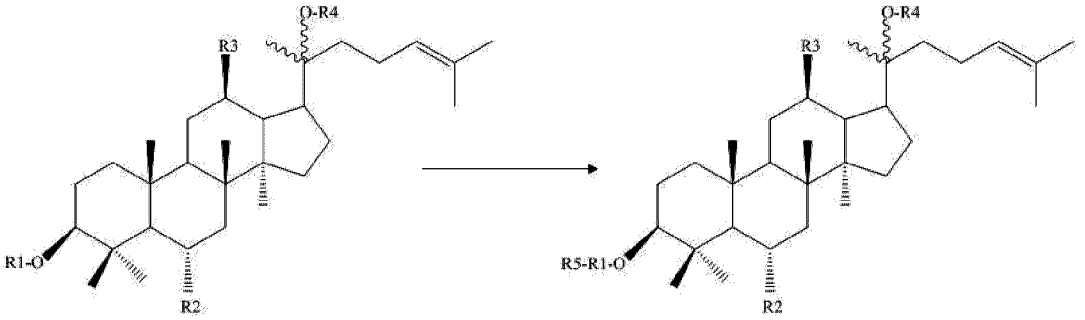
(D)



compound of formula (VII) compound of formula (VIII)

wherein, R1 is OH or OCH3; R2 is a glycosyl; said glycosyltransferase is selected from SEQ ID NOs: 22, 24, 41 or 43 or a derivative polypeptide thereof, wherein, said derivative polypeptide has ≥95% sequence identity with the amino acid sequence as set forth by any one of SEQ ID NOs: 22, 24, 41 and 43, and has the activity of glycosyltransferase;

(E)



compound of formula (IX) compound of formula (X)

wherein, R1 is glycosyl; R2 and R3 are independently H or OH; R4 is glycosyl or H; R5 is glycosyl, R5-R1-O is a glycosyl extended from the first glycosyl at C-3; said glycosyltransferase is selected from SEQ ID NOs: 26, 28, 55, 57, 59 or 61 or a derivative polypeptide thereof, wherein, said derivative polypeptide has ≥95% sequence identity with the amino acid sequence as set forth by any one of SEQ ID NOs: 26, 28, 55, 57, 59 and 61, and has the activity of glycosyltransferase;

(F)

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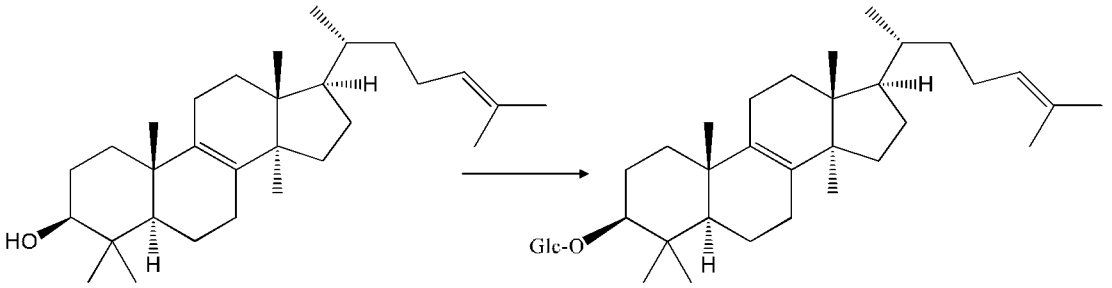

compound of formula (XI) compound of formula (XII)

said glycosyltransferase is selected from SEQ ID NO: 22 or SEQ ID NO: 24 or a derivative polypeptide thereof, wherein, said derivative polypeptide has ≥95% sequence identity with the amino acid sequence as set forth by SEQ ID NO: 22 or SEQ ID NO: 24, and has the activity of glycosyltransferase.

5. A method for conducting catalytic glycosylation *in vitro*, wherein said method comprises the following step: in the presence of a glycosyltransferase, conducting the catalytic glycosylation of tetracyclic triterpenoid as substrates, wherein, said glycosyltransferase is selected from the group consisting of: a glycosyltransferase as set forth by SEQ ID NOs: 21, 23, 25, 27, 40, 42, 54, 56, 58 and 60, and a derivative polypeptide thereof, wherein, said derivative polypeptide has ≥95% sequence identity with the amino acid sequence as set forth by any one of SEQ ID NOs: 21, 23, 25, 27, 40, 42, 54, 56, 58 and 60.

6. The method according to claim 5, wherein the substrate of the catalytic glycosylation is the compound of formula (V), (VII), (IX) or (XI),

wherein

for the compound of formula (V), R1 is H or OH; R2 is H or OH; R3 is H or a glycosyl,

for the compound of formula (VII), R1 is OH or OCH_3; R2 is a glycosyl,

for the compound of formula (IX), R1 is glycosyl; R2 or R3 is OH or H; R4 is glycosyl or H,

and the product of the substrate compound of formula (V) is the compound of formula (VI), the product of the substrate compound of formula (VII) is the compound of formula (VIII), the product of the substrate compound of formula (IX) is the compound of formula (X) and the product of the substrate compound of formula XI) is the compound of formula (XII)

wherein

for the compound of formula (VI), R1 is H or OH; R2 is H or OH; R3 is H or a glycosyl; R4

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is a glycosyl,

for the compound of formula (VIII), R1 is OH or OCH_3; R2 is a glycosyl, and

for the compound of formula (X), R1 is glycosyl; R2 and R3 are independently H or OH; R4 is glycosyl or H; R5 is glycosyl, R5-R1-O is a glycosyl extended from the first glycosyl at C-3.

7. The method according to claim 6, wherein said compound of formula (V) is protopanaxadiol, and the compound of formula (VI) is ginsenoside Rh2 (3-*O*-β-(D-glucopyranosyl)-protopanaxadiol);

or, said compound of formula (V) is CK, and the compound of formula (VI) is ginsenoside F2 (3-*O*-β-(D-glucopyranosyl)-20-*O*-β-(D-glucopyranosyl)-protopanaxadiol);

or, said compound of formula (V) is protopanaxatriol, and the compound of formula (VI) is ginsenoside 3-*O*-β-(D-glucopyranosyl)-protopanaxatriol;

or, said compound of formula (V) is ginsenoside F1, and the compound of formula (VI) is ginsenoside 3-*O*-β-(D-glucopyranosyl)-F1;

or, said compound of formula (V) is DM, and the compound of formula (VI) is ginsenoside 3-*O*-β-(D-glucopyranosyl)-dammarenediol II;

or, said compound of formula (VII) is 25-OH-protopanaxadiol, and the compound of formula (VIII) is ginsenoside 3-*O*-β-(D-glucopyranosyl)- 25-OH-protopanaxadiol;

or, said compound of formula (VII) is 25-OCH_3-protopanaxadiol), and the compound of formula (VIII) is ginsenoside 3-*O*-β-(D-glucopyranosyl)- 25-OCH_3-protopanaxadiol;

or, said compound of formula (IX) is ginsenoside Rh2, and the compound of formula (X) is ginsenoside Rg3;

or, said compound of formula (IX) is ginsenoside F2, and the compound of formula (X) is ginsenoside Rd;

or, said compound of formula (XI) is lanosterol, and the compound of formula (XII) is 3-*O*-β-(D- glucopyranosyl)-lanosterol.

8. A genetically engineered host cell, wherein said host cell contains the vector according to claim 3.

9. A method for producing a transgenic plant, wherein said method comprises the step of: regenerating said genetically engineered host cell according to claim 8 into a plant, and said

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genetically engineered host cell is a plant cell.

10. The method according to claim 1, said tetracyclic triterpenoid compounds are selected from the group consisting of S- or R- dammarane-type, lanostane-type, tirucallane-type, cycloartane-type, cucurbitane-type, and meliacane-type tetracyclic triterpenoid compounds.

11. The method according to claim 5, said tetracyclic triterpenoid compounds are selected from the group consisting of S- or R- dammarane-type, lanostane-type, tirucallane-type, cycloartane-type, cucurbitane-type, and meliacane-type tetracyclic triterpenoid compounds.

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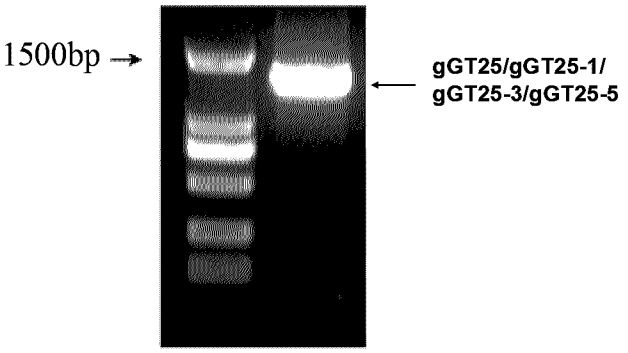

Figure 1

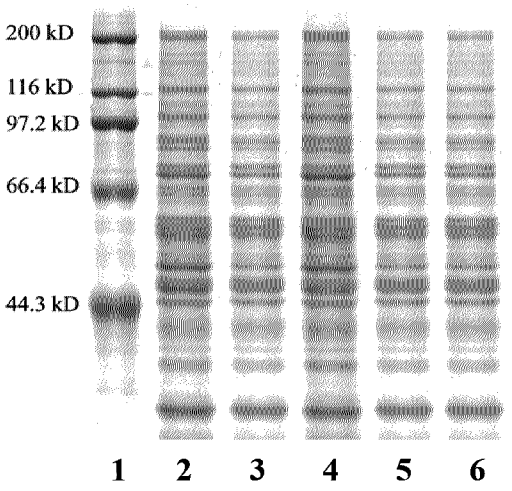

Figure 2

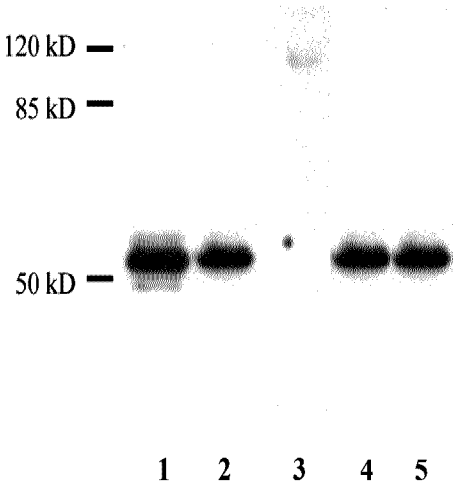

Figure 3

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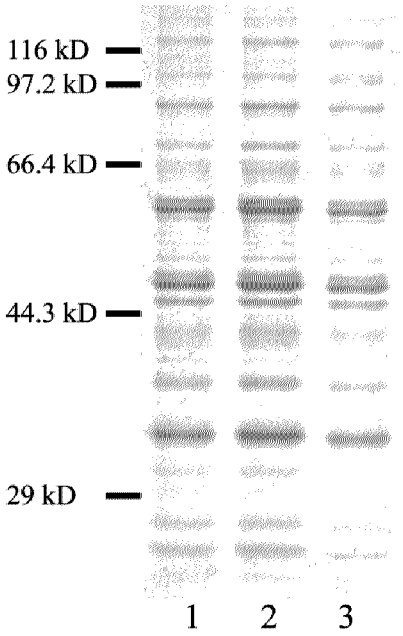

Figure 4

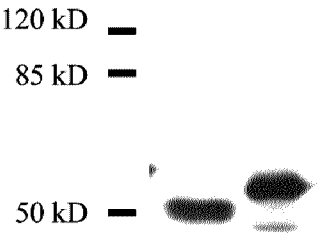

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Figure 5

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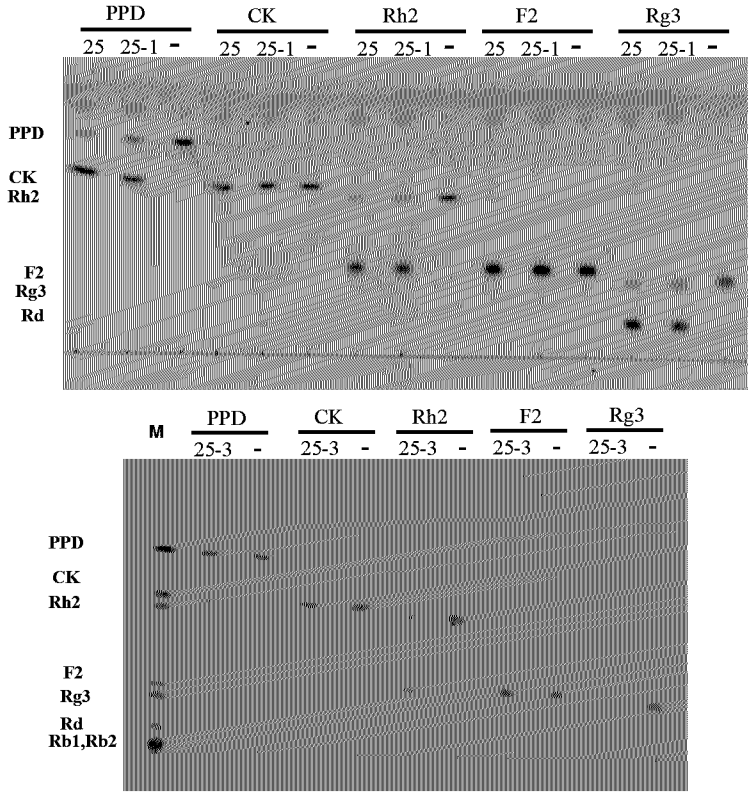

Figure 6

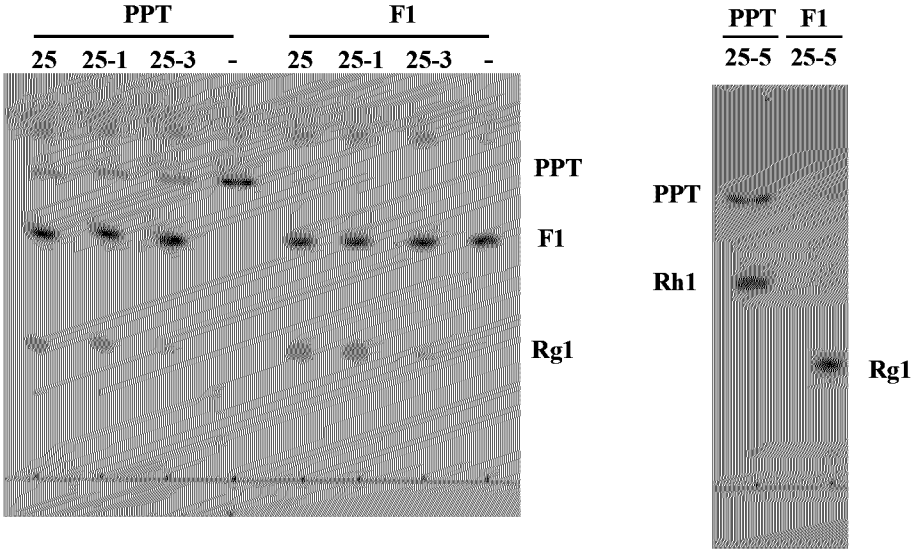

Figure 7

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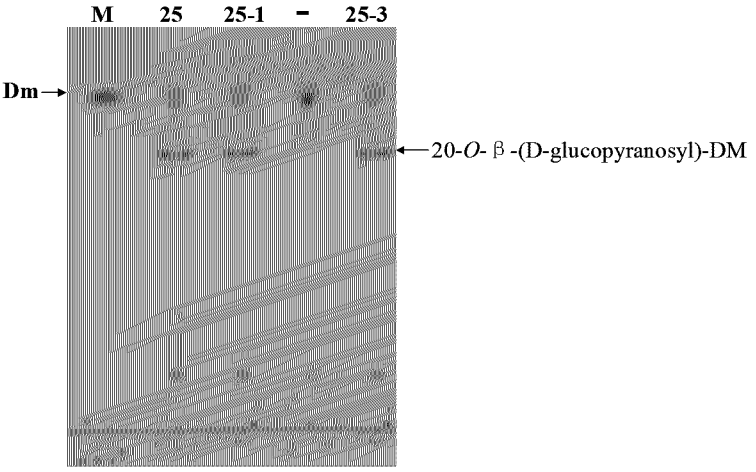

Figure 8

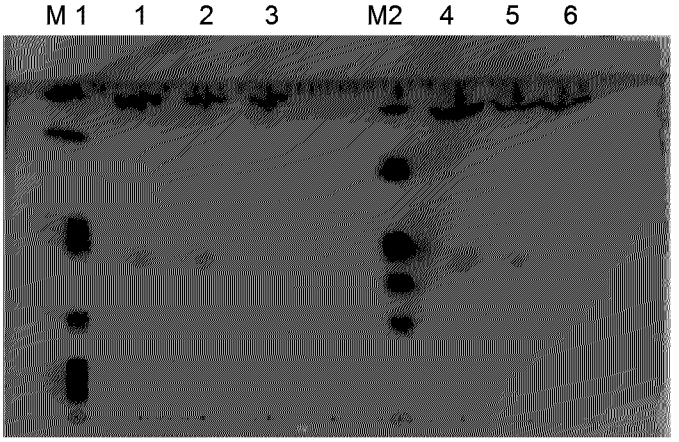

Figure 9

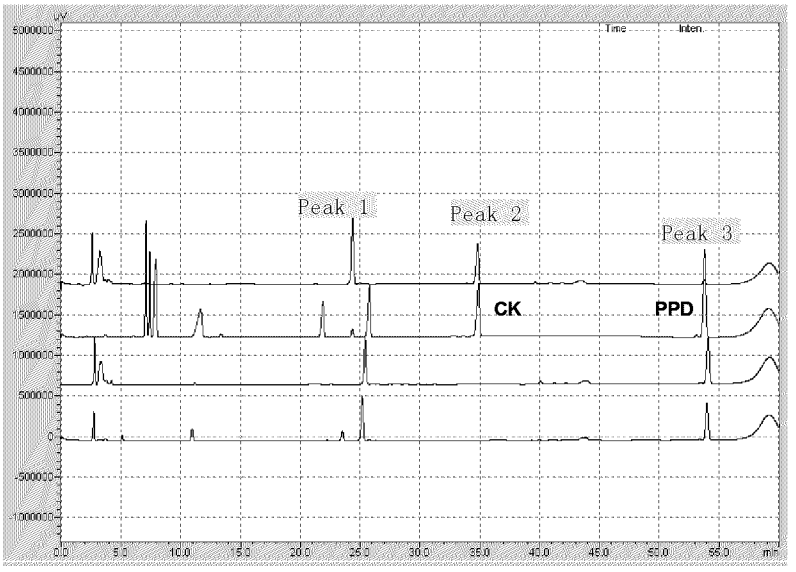

Figure 10

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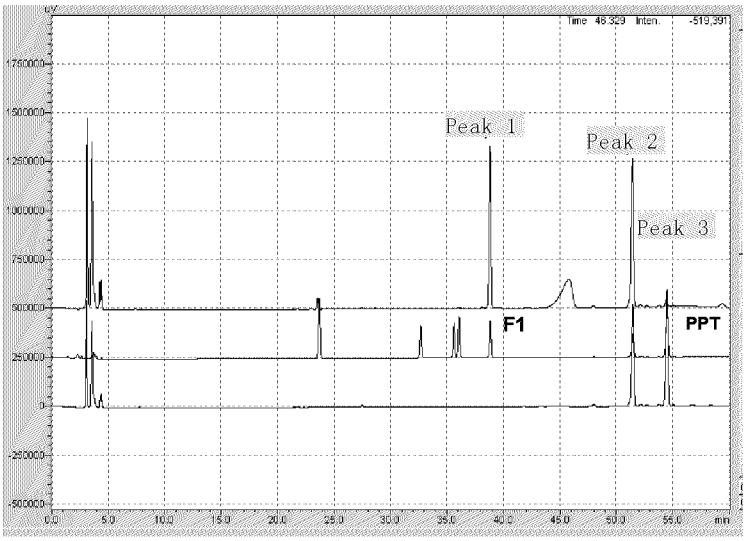

Figure 11

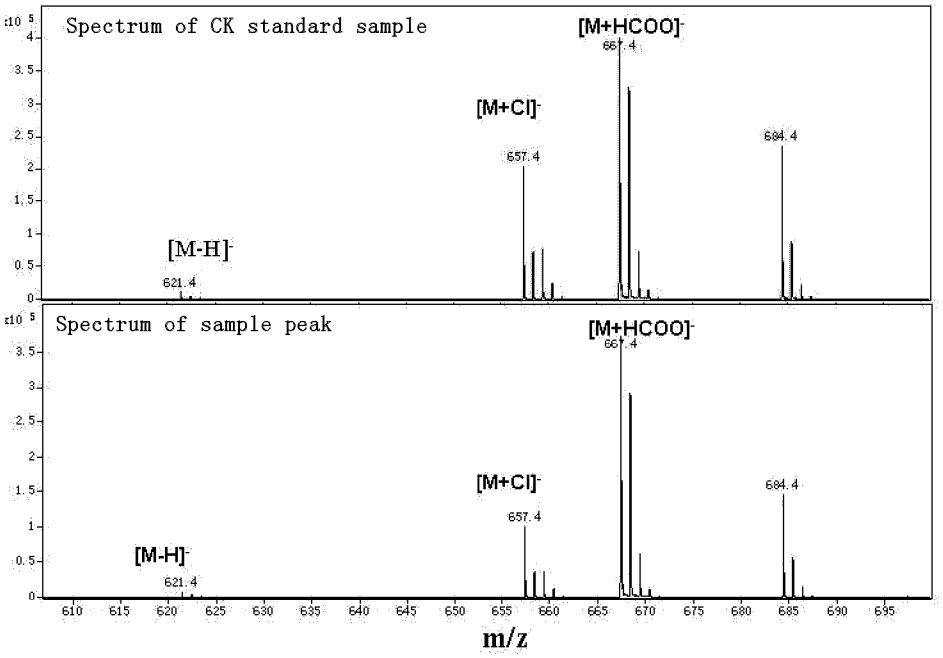

Figure 12

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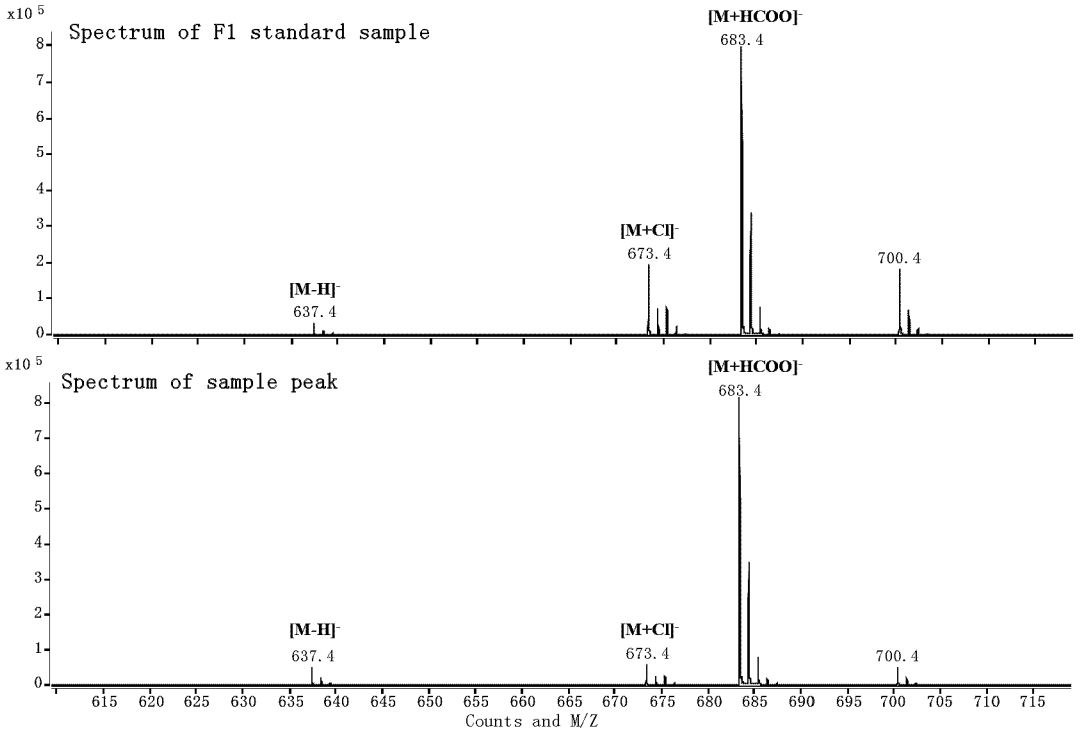

Figure 13

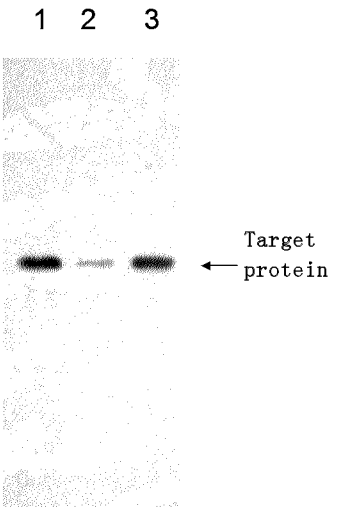

Figure 14

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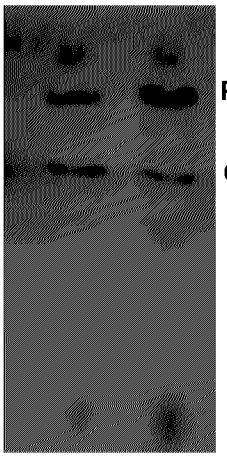

Figure 15

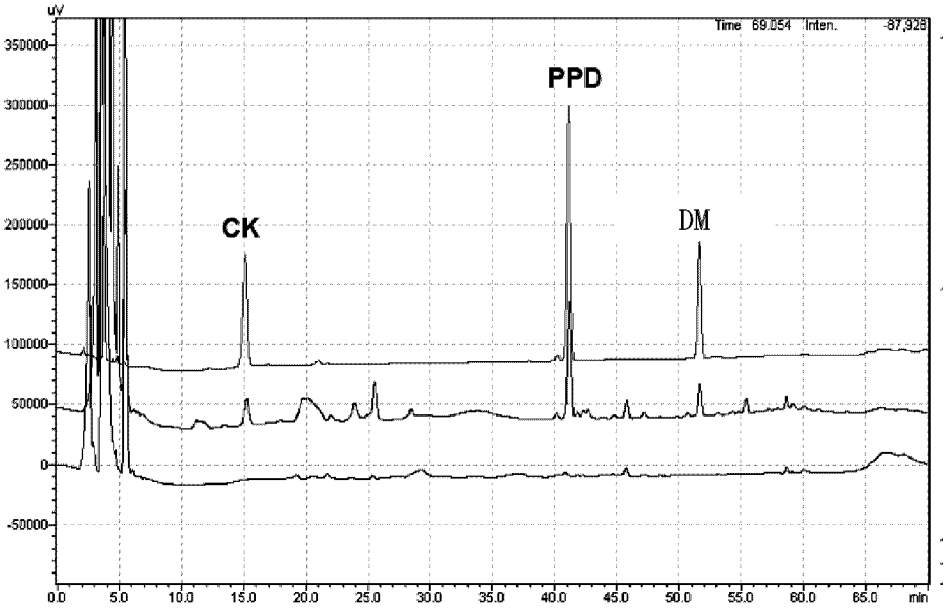

Figure 16

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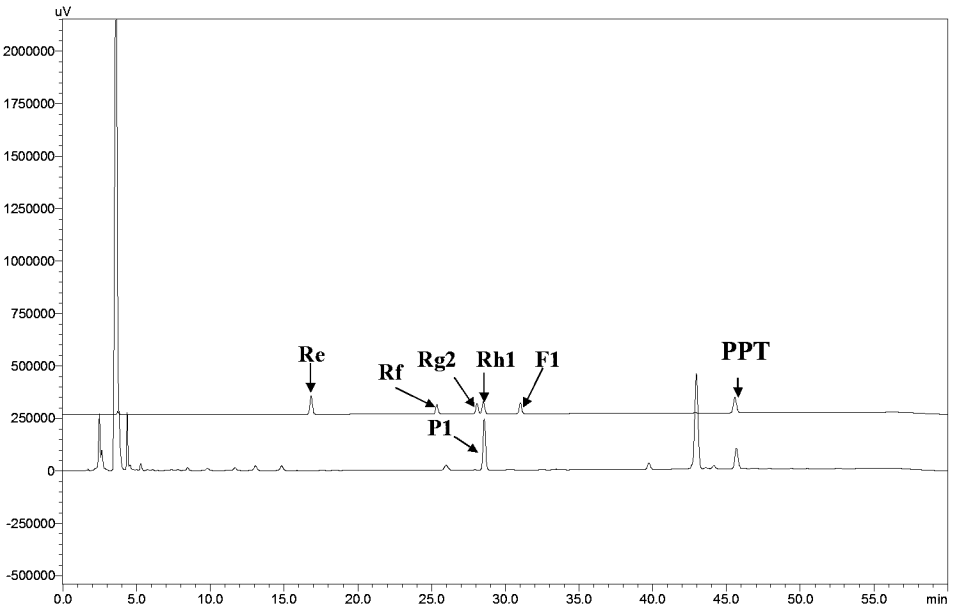

Figure 17

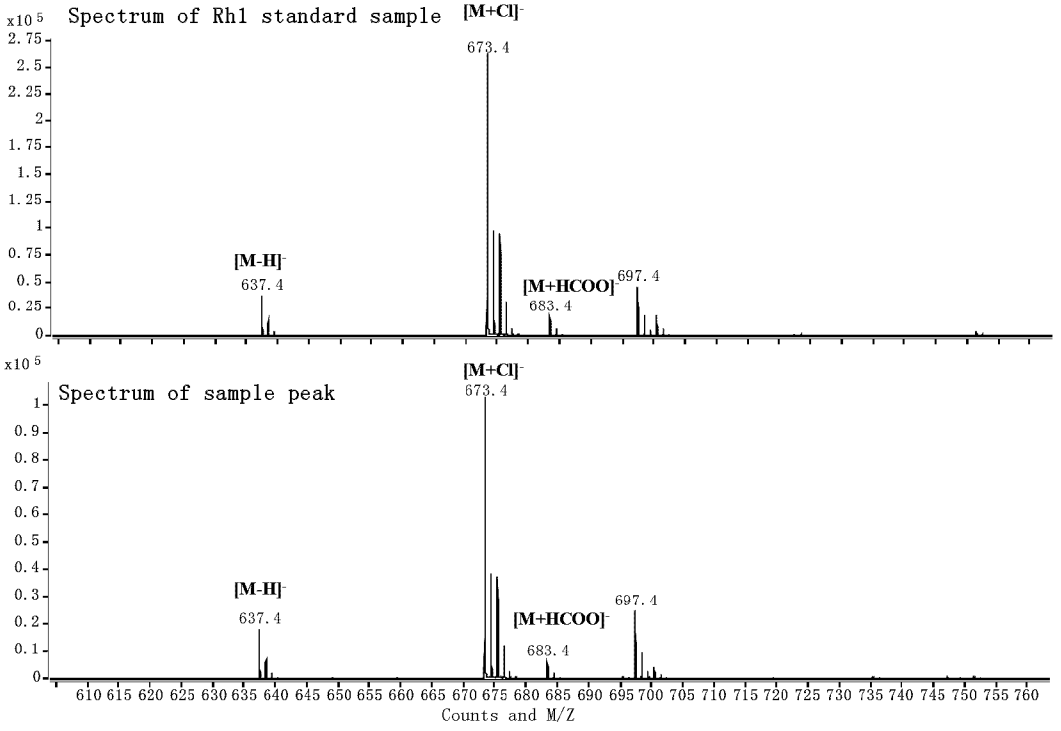

Figure 18

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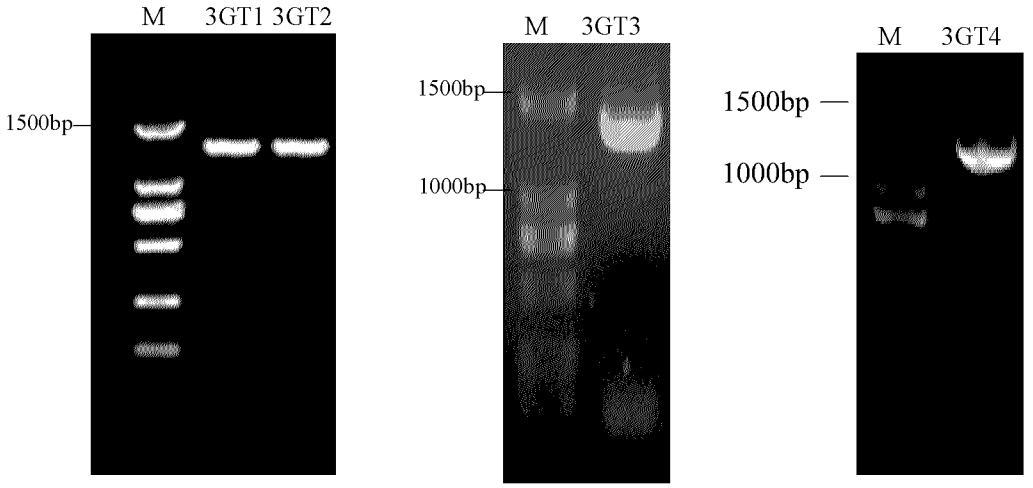
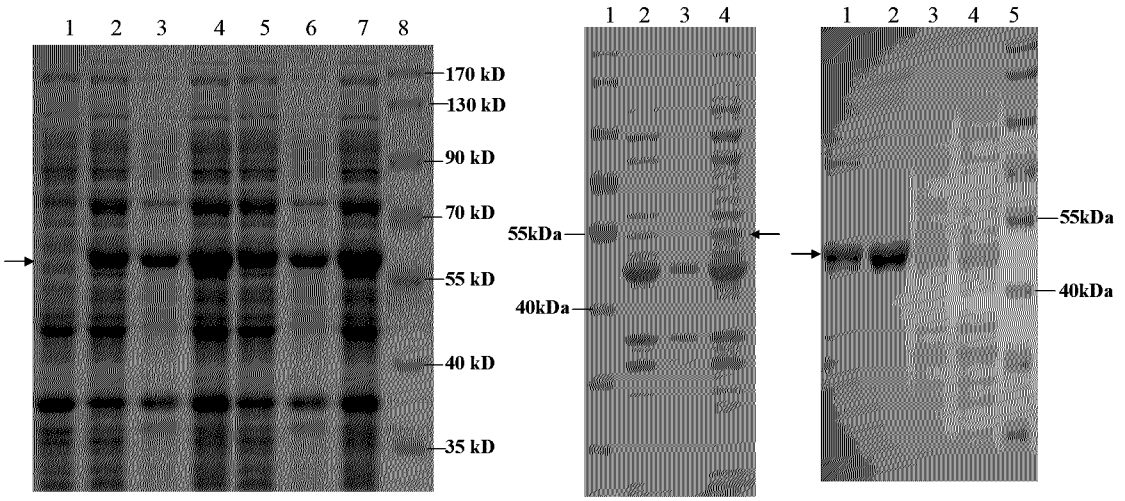


(a) 3GT1 and 3GT2 (b) 3GT3 (c) 3GT4

Figure19



(a) 3GT1 & 3GT2 (b) 3GT3 (c) 3GT4

Figure20

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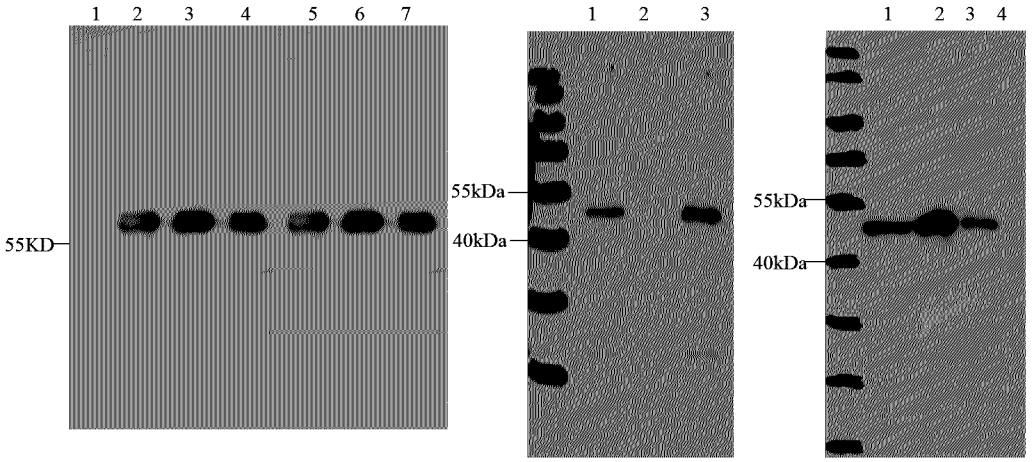


(a) 3GT1 & 3GT2 (b) 3GT3 (c) 3GT4

Figure21

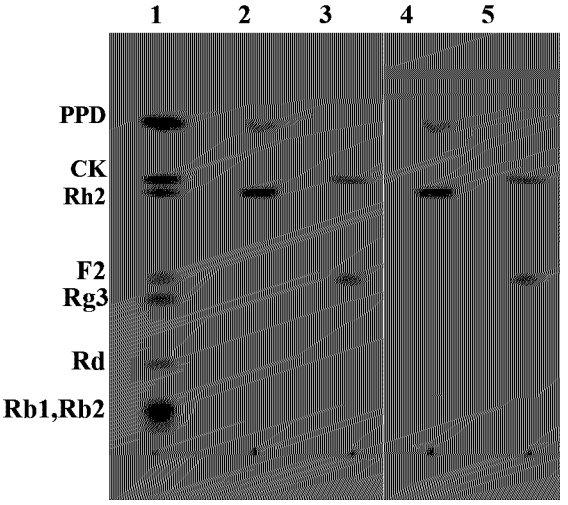


Figure22

Date Reçue/Date Received 2022-02-07

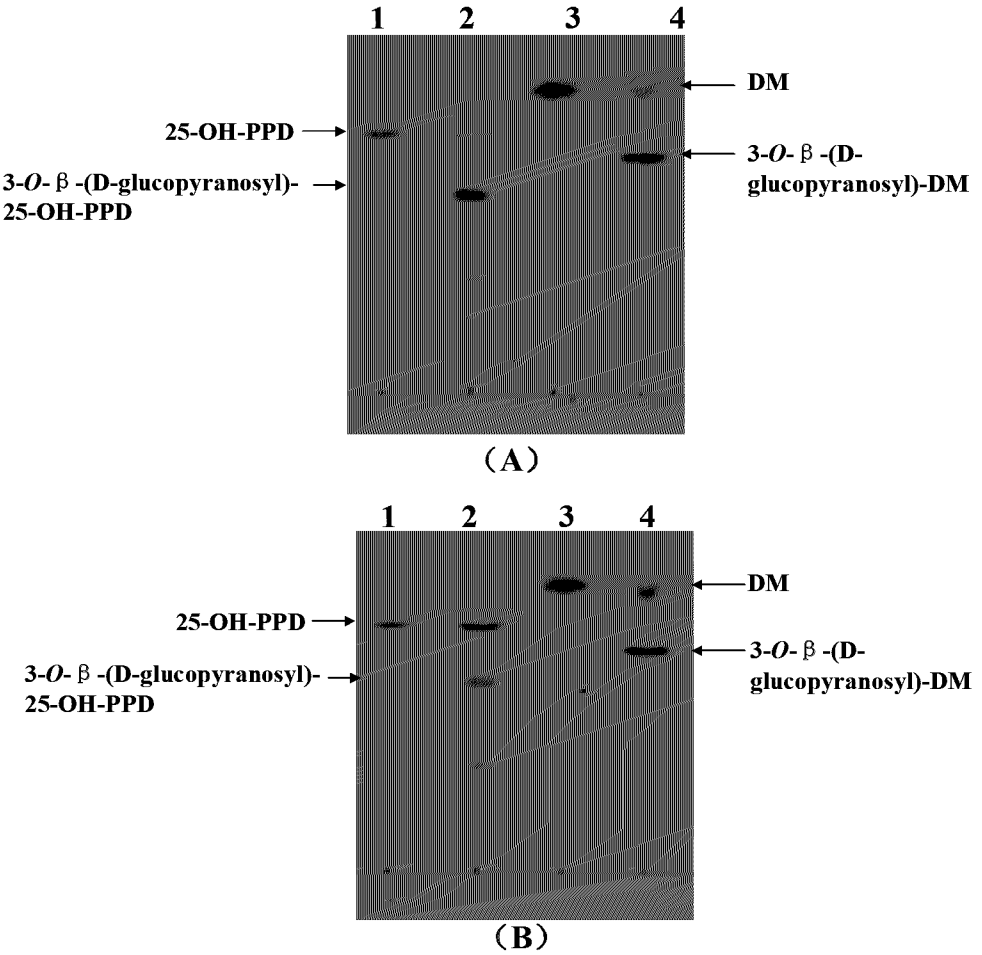


Figure23

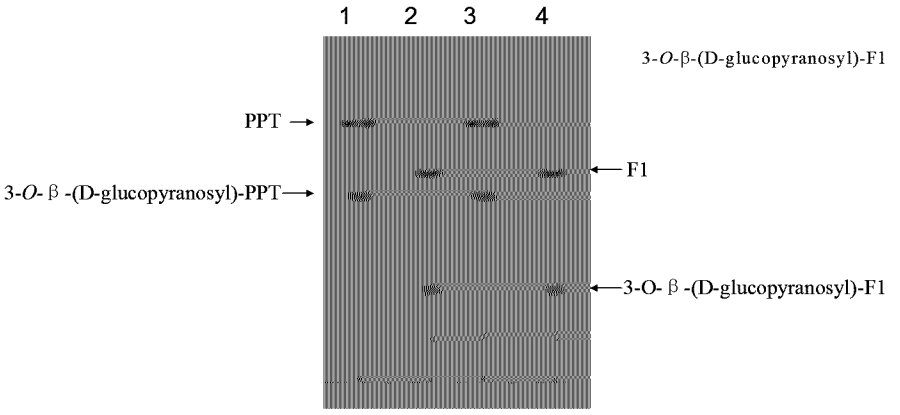


Figure24

Date Reçue/Date Received 2022-02-07

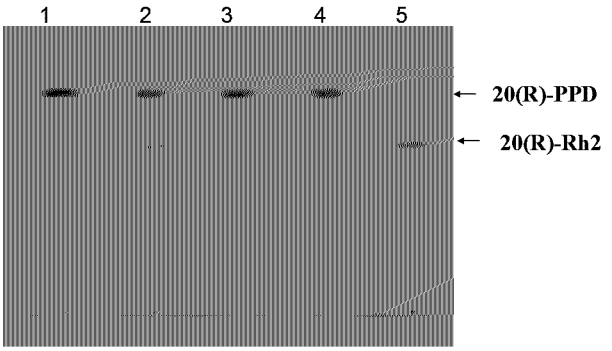


Figure25

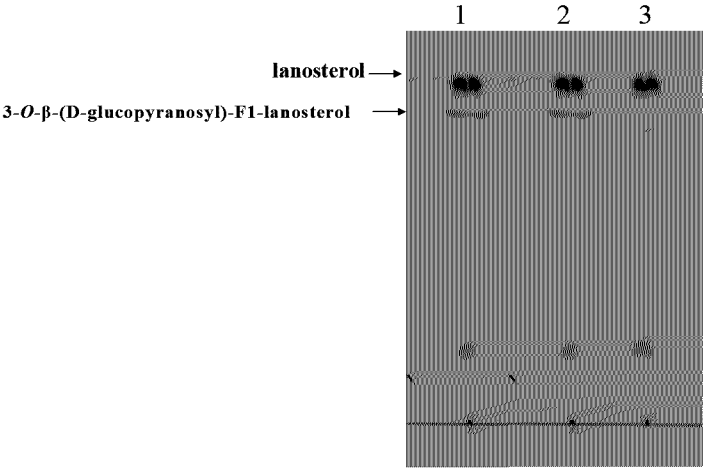


Figure26

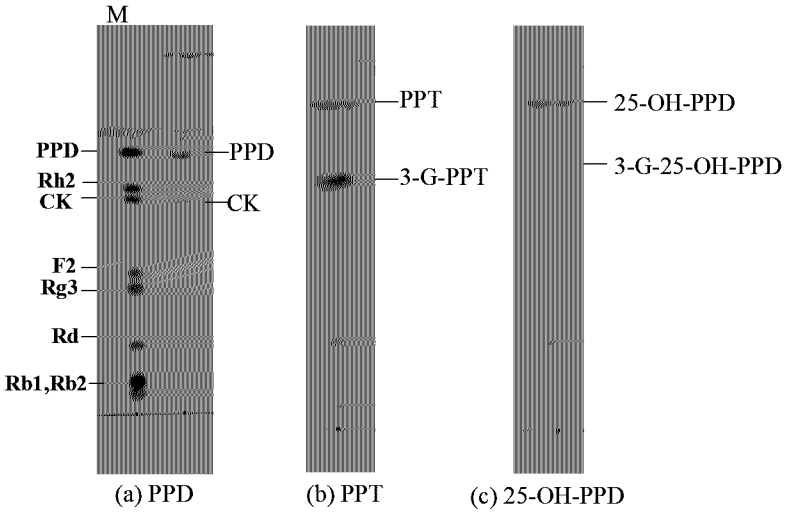
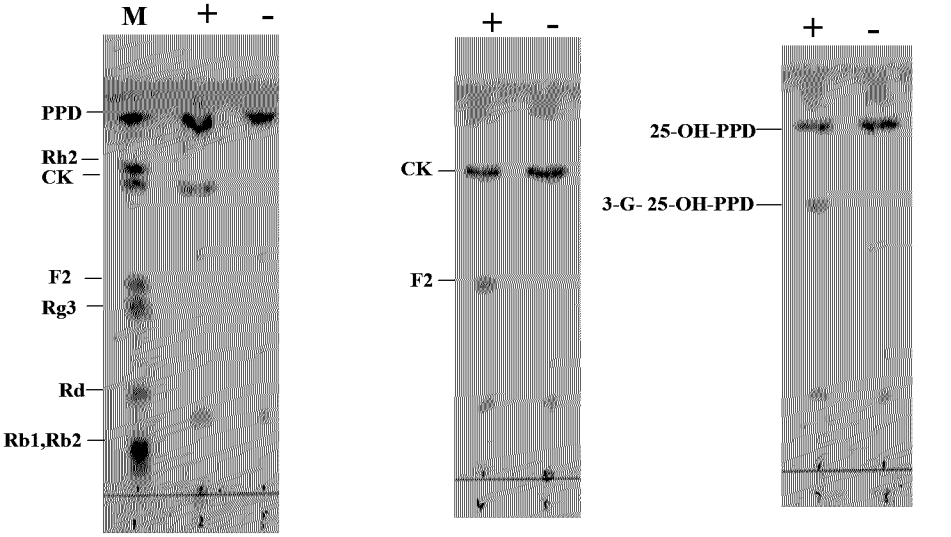


Figure27

Date Reçue/Date Received 2022-02-07



(a) PPD (a) CK (c)25-OH-PPD

Figure28

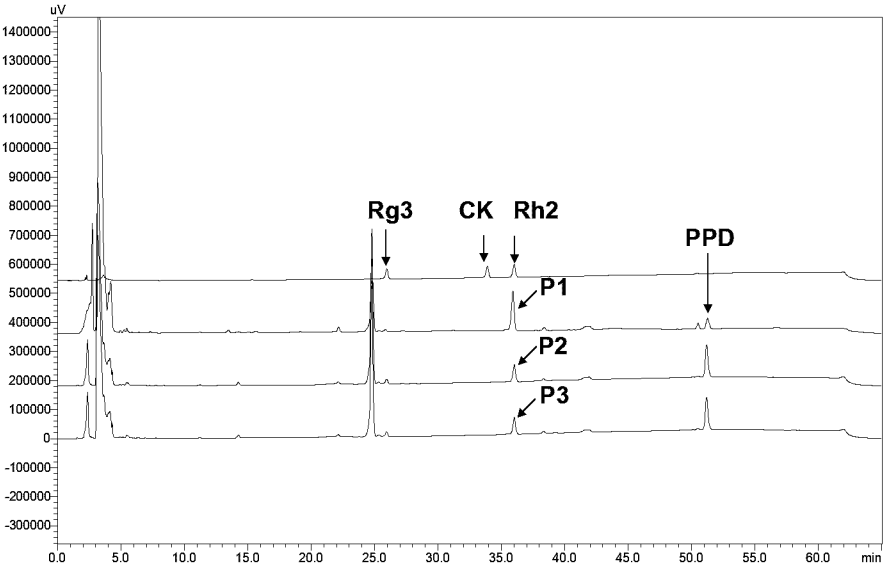


Figure29

Date Reçue/Date Received 2022-02-07

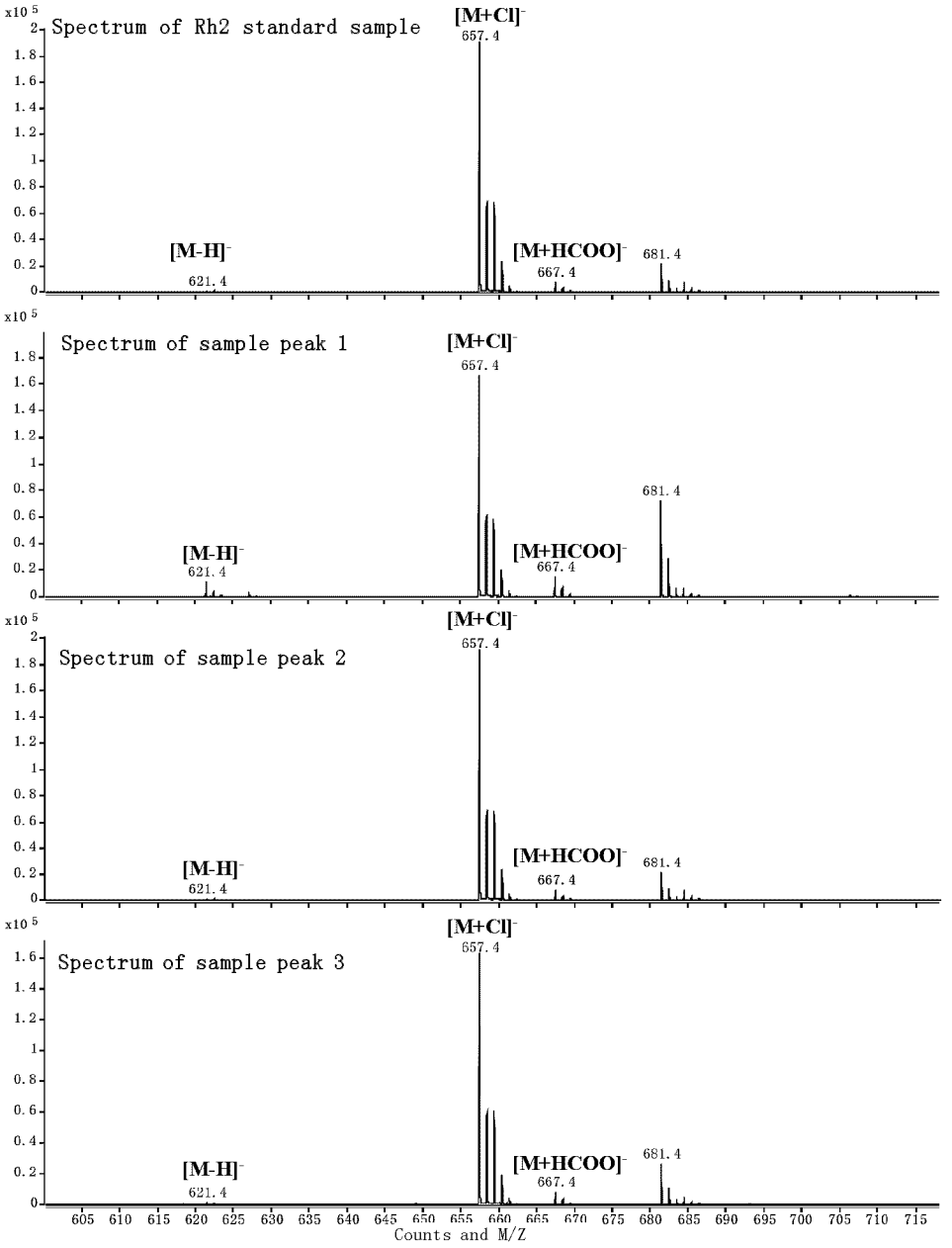


Figure30

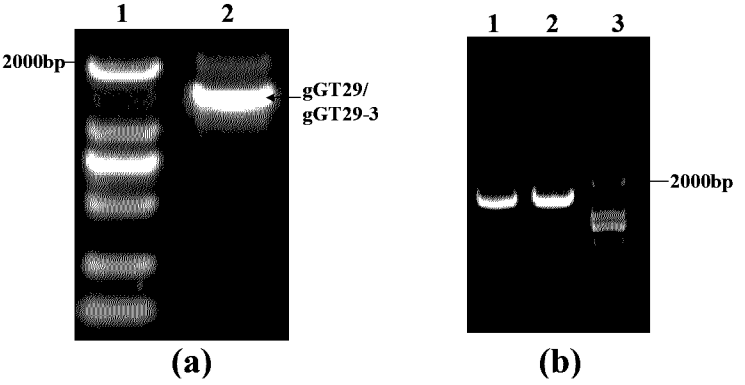


Figure31

Date Reçue/Date Received 2022-02-07

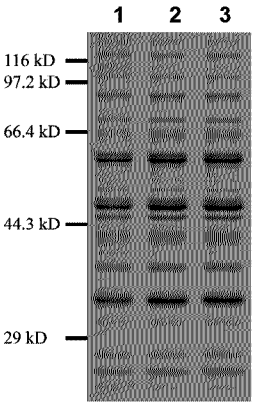


Figure32

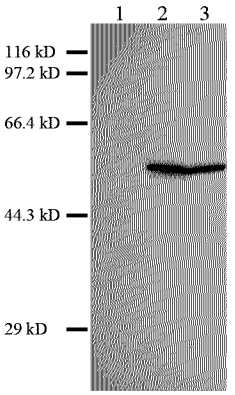


Figure33

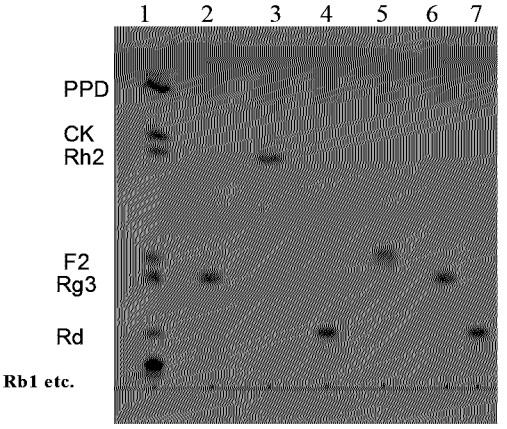
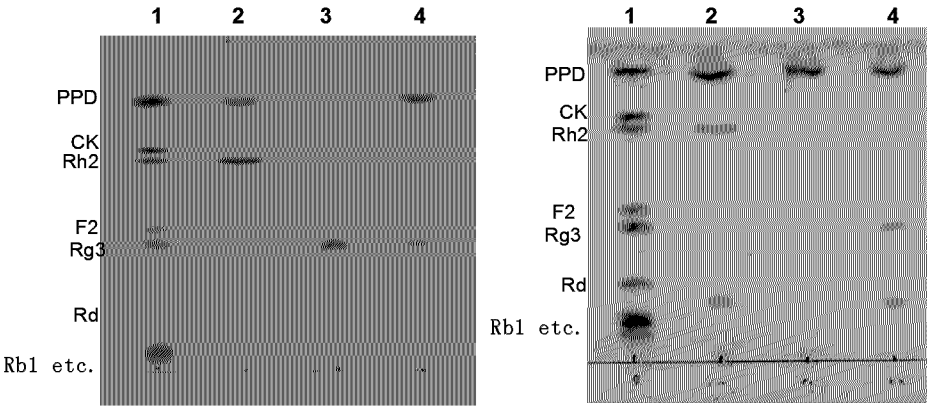


Figure34

Date Reçue/Date Received 2022-02-07



(a) PPD catalyzed by the combination of gGT29 and 3GT1

(b) PPD catalyzed by the combination of gGT29 and 3GT4

Figure35

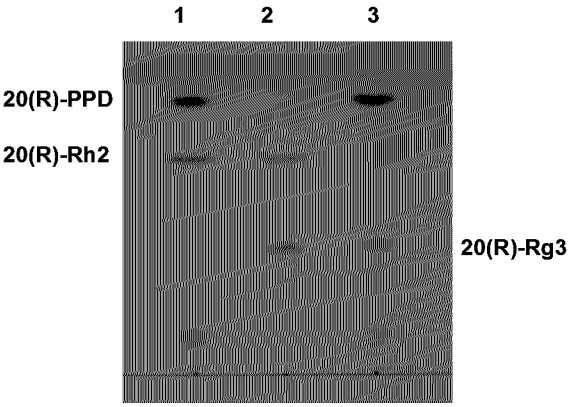


Figure36

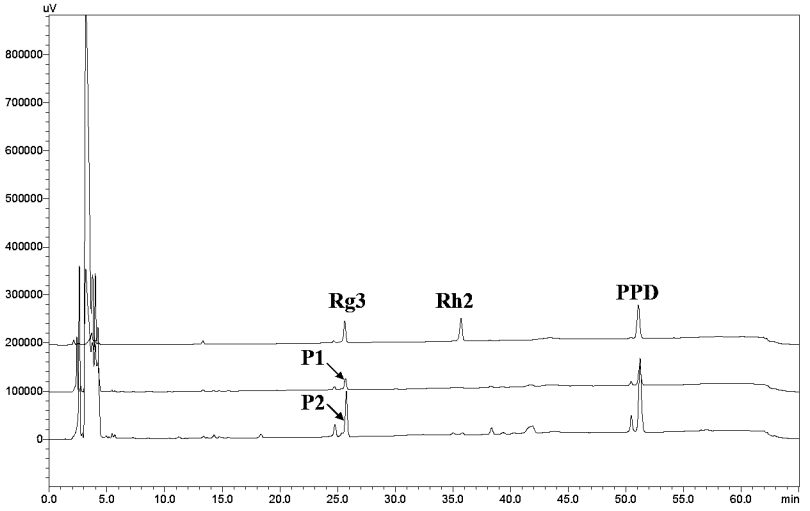


Figure37

Date Reçue/Date Received 2022-02-07

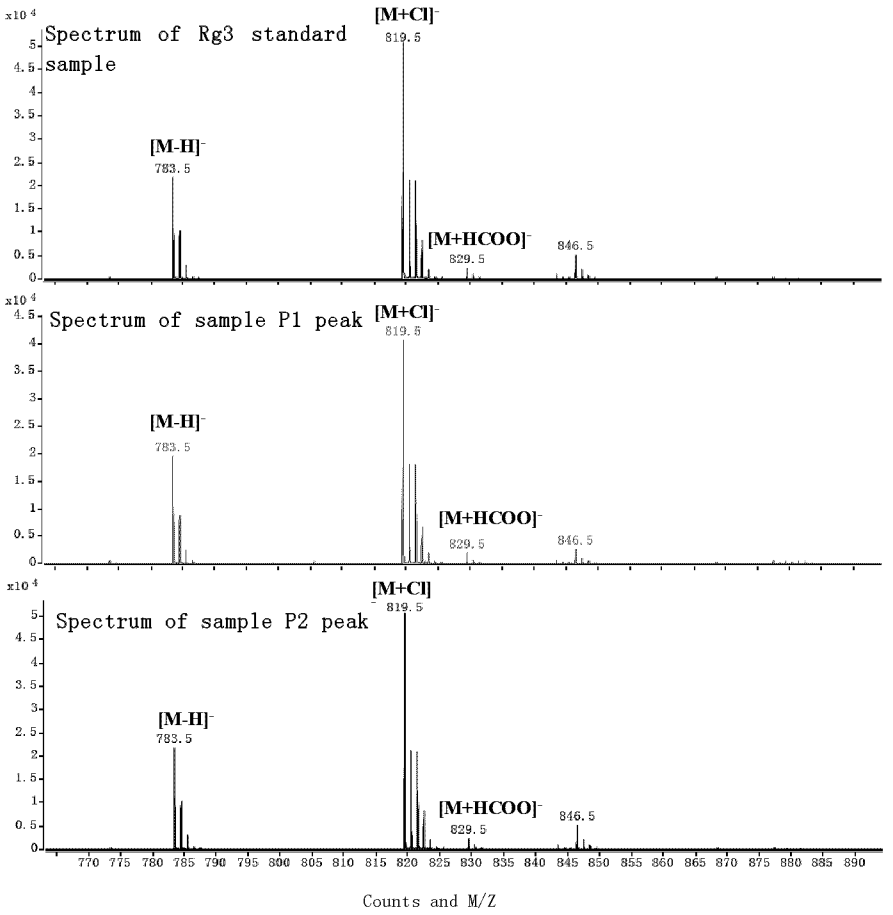


Figure38

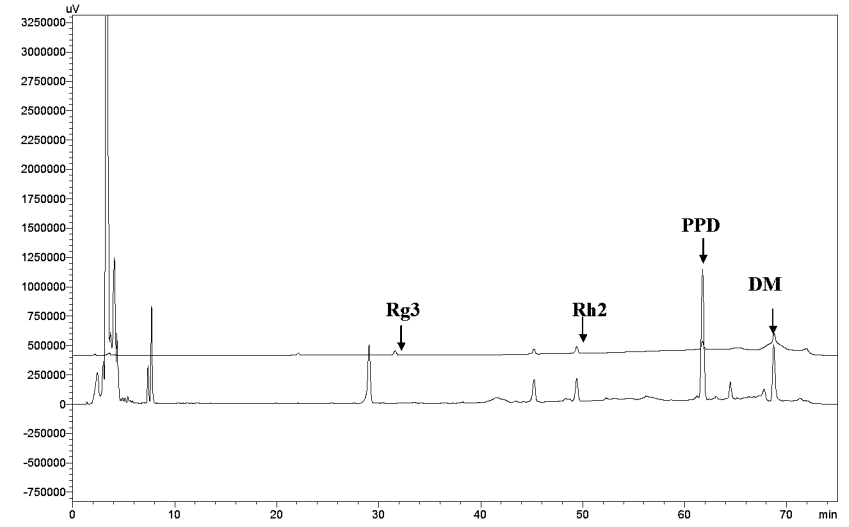


Figure39

Date Reçue/Date Received 2022-02-07

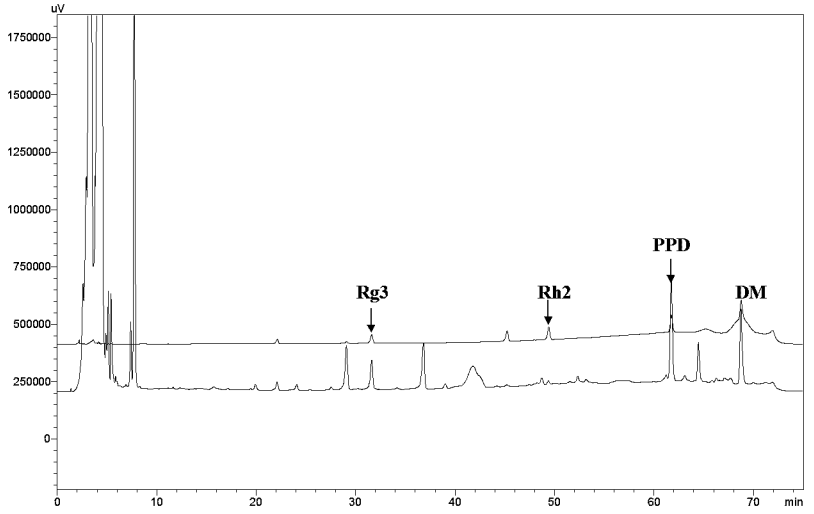


Figure40

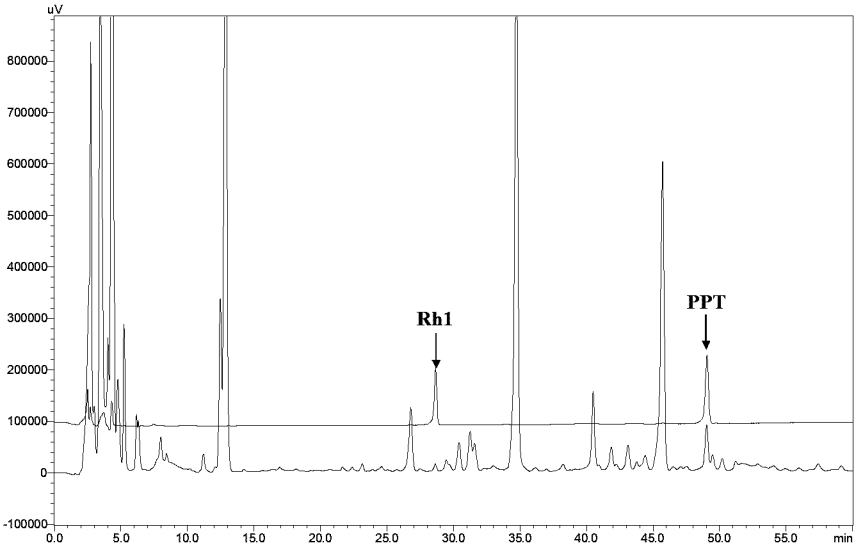


Figure41

Date Reçue/Date Received 2022-02-07

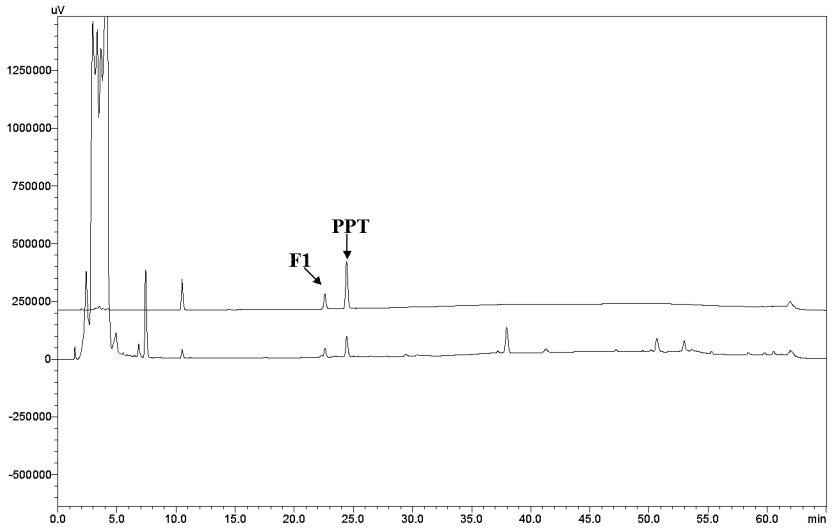


Figure42

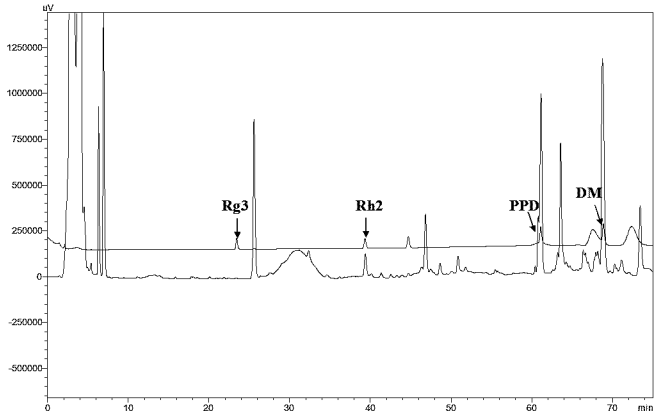


Figure43

Date Reçue/Date Received 2022-02-07

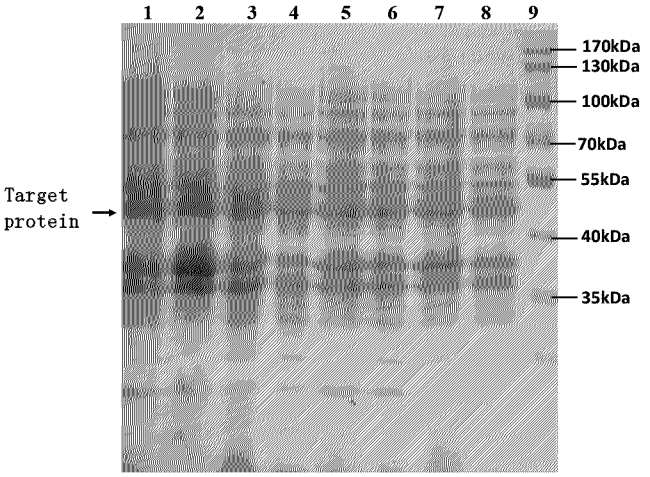


Figure 44

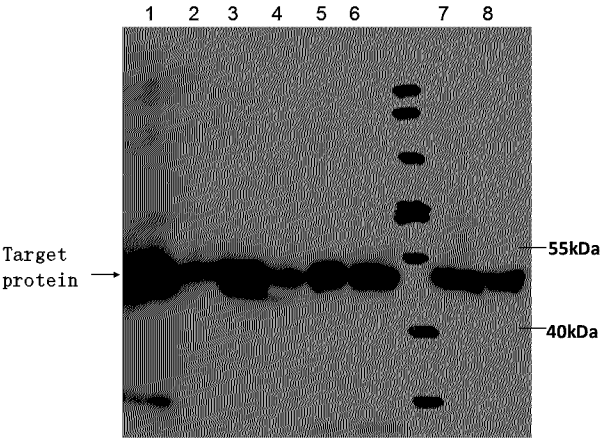


Figure 45

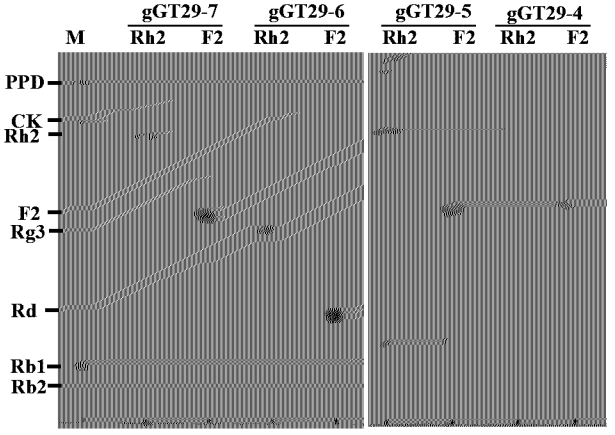


Figure 46

Date Reçue/Date Received 2022-02-07