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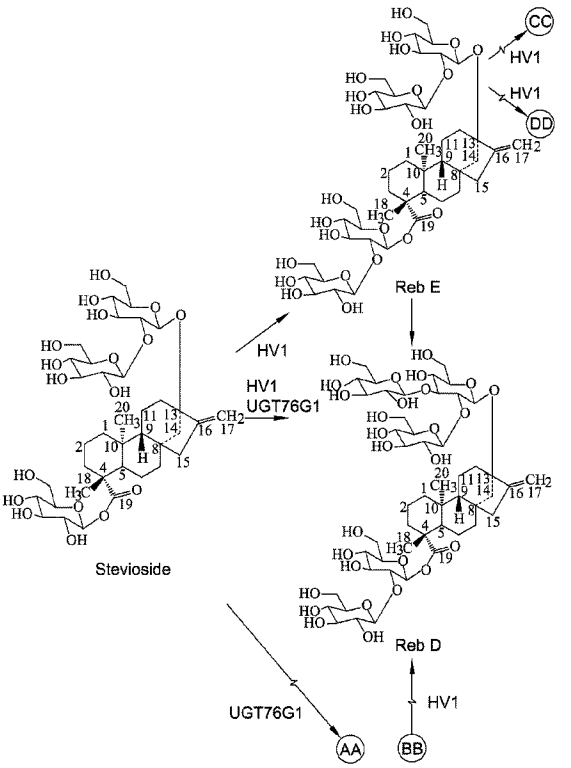
(54) Titre : PRODUCTION PAR RECOMBINAISON DE GLYCOSIDES DE STEVIOL
(54) Title: RECOMBINANT PRODUCTION OF STEVIOL GLYCOSIDES



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Recombinant polypeptides having UDP-glycosyltransferase activities, including a 1,2-19-O-glucose glycosylation activity and a 1,2-13-O-glucose glycosylation activity for synthesizing of steviol glucosides, are provided. A method of producing a steviol glycoside composition using such recombinant polypeptide is also provided. Also disclosed are steviol glycosides referred to as rebaudioside Z1 and rebaudioside Z2.

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(54) **Title**: RECOMBINANT PRODUCTION OF STEVIOL GLYCOSIDES

(57) **Abstract**: Recombinant polypeptides having UDP-glycosyltransferase activities, including a 1,2-19-O-glucose glycosylation activity and a 1,2-13-O-glucose glycosylation activity for synthesizing of steviol glucosides, are provided. A method of producing a steviol glycoside composition using such recombinant polypeptide is also provided. Also disclosed are steviol glycosides referred to as rebaudioside Z1 and rebaudioside Z2.

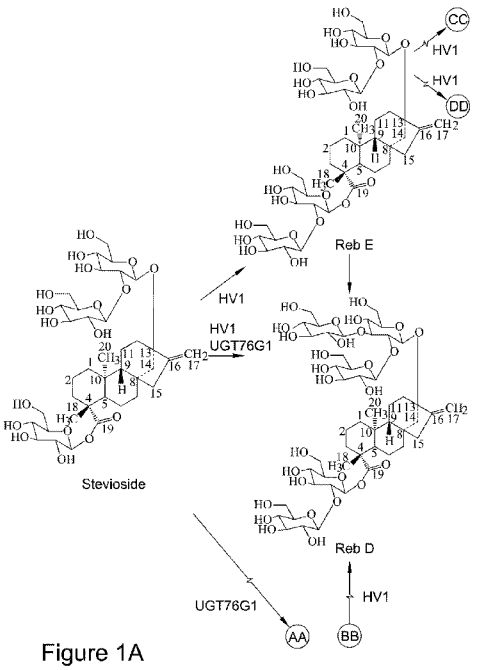

Figure 1A

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RECOMBINANT PRODUCTION OF STEVIOL GLYCOSIDES

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] This application claims priority to U.S. Provisional Patent Application No. 61/898,571, filed on November 1, 2013.

[0002]

BACKGROUND

[0003] The present disclosure relates generally to the biosynthesis of steviol glycosides. In particular, the present disclosure relates to a recombinant polypeptide that catalyzes the production of steviol glycosides such as rebaudioside D, rebaudioside E and a novel rebaudioside (rebaudioside Z).

[0004] Steviol glycosides are natural products isolated from *Stevia rebaudiana* leaves, and are widely used as high intensity, low-calorie sweeteners. Naturally occurring steviol glycosides have the same base structure (steviol) and differ in the content of carbohydrate residues (e.g. glucose, rhamnose, and xylose residues) at the C13 and C19 positions. Steviol glycosides with known structure include stevioside, rebaudioside A, rebaudioside B, rebaudioside C, rebaudioside D, rebaudioside E, rebaudioside F, and dulcoside A.

[0005] On dry weight basis, stevioside, rebaudioside A, rebaudioside C, and dulcoside A, account for 9.1, 3.8, 0.6, and 0.3% of the total weight of the steviol glycosides in the leaves, respectively, while the other steviol glucosides are present in much lower amounts. Extracts from *Stevia rebaudiana* plant are commercially available, which

Date Reçue/Date Received 2021-01-12

typically contain stevioside and rebaudioside A as primary compounds. The other steviol glycosides typically are present in the stevia extract as minor components. For example, the amount of rebaudioside A in commercial preparations can vary from about 20% to more than 90% of the total steviol glycoside content, while the amount of rebaudioside B can be about 1-2%, the amount of rebaudioside C can be about 7-15%, and the amount of rebaudioside D can be about 2% of the total steviol glycosides.

[0006] As natural sweeteners, different steviol glucosides have different degrees of sweetness and after-taste. The sweetness of steviol glycosides is significantly higher than that of sucrose. For example, stevioside is 100-150 times sweeter than sucrose with bitter after-taste, while rebaudioside A and E are 250-450 times sweeter than sucrose and the after-taste is much better than stevioside. Accordingly, the taste profile of any stevia extract is profoundly affected by the relative content of the steviol glycosides in the extract, which in turn may be affected by the source of the plant, the environmental factors (such as soil content and climate), and the extraction process. In particular, variations of the extraction conditions can lead to inconsistent compositions of the steviol glycosides in the stevia extracts, such that the taste profile varies among different batches of extraction products. The taste profile of stevia extracts also can be affected by plant-derived contaminants (such as pigments, lipids, proteins, phenolics, and saccharides) that remain in the product after the extractions process. These contaminants typically have off-flavors undesirable for the use of the stevia extract as a sweetener.

[0007] The majority of the steviol glycosides are formed by several glycosylation reactions of steviol, which typically are catalyzed by the UDP-glycosyltransferases (UGTs) using uridine 5'-diphosphoglucose (UDP-glucose) as a donor of the sugar moiety. In plants, UGTs are a very divergent group of enzymes that transfer a glucose residue from UDP-glucose to steviol. Stevioside is an intermediate in the biosynthesis of rebaudioside compounds. For example, glycosylation of the C-3' of the C-13-O-glucose of stevioside yields rebaudioside A; and glycosylation of the C-2' of the 19-O-glucose of the stevioside yields rebaudioside E. Further glycosylation of rebaudioside A (at 19-O-glucose) or rebaudioside E (at C-13-O-glucose) produces rebaudioside D. (Figures 1A-1C).

[0008] A practical approach to improve the taste quality of stevia extract is to increase the yield of rebaudioside compounds by further glycosylation of stevioside. The UGTs with a 1,2-19-O-glucose glycosylation activity are important enzymes for rebaudioside D and E production.

[0009] Sucrose synthases (SUS) catalyze the conversion of the UDP to UDP-glucose in the presence of sucrose. Thus, for a glycosylation reaction utilizing UDP-glucose (such as those catalyzed by the UGTs), SUS can be used to re-generate UDP-glucose from UDP, enhancing the efficiency of such reaction.

[0010] Accordingly, there is a need for steviol glycosides with consistent taste profile and less off-flavor than the existing commercial products. As described herein, the present disclosure provides a recombinant polypeptide that is useful for preparing steviol glycosides (such as rebaudioside D and rebaudioside E). The present disclosure also provides a method of producing a steviol glycoside (rebaudioside Z) composition using such recombinant polypeptide.

BRIEF DESCRIPTION

[0011] The subject technology generally relates to recombinant polypeptides that have UDP-glycosyltransferase activities. In particular, polypeptides having a 1,2-19-O-glucose glycosylation activity for steviol glycoside compounds are provided. In one aspect, the subject technology relates to a recombinant polypeptide comprising an amino acid sequence having at least 80% identity to SEQ ID NO:6. In an exemplary embodiment, the amino acid sequence of the recombinant polypeptide described herein has at least 85%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or even 100% identity to SEQ ID NO:6.

[0012] In another aspect, the subject technology relates to an isolated nucleic acid comprising a nucleotide sequence encoding the recombinant polypeptide described herein. In another aspect, the subject technology relates to a vector comprising the nucleic acid described herein, and a host cell comprising the vector described herein. In an exemplary embodiment, the host cell of the subject technology is selected from the group consisting of bacteria, yeast, filamentous fungi, cyanobacteria algae and plant cell.

Date Reçue/Date Received 2021-01-12

[0013] In another aspect, the subject technology also relates to a method of producing a steviol glycoside composition, the method comprising incubating a substrate (such as stevioside, rebaudioside A, rebaudioside E or a combination thereof) with a recombinant polypeptide comprising an amino acid sequence having at least 80% identity to SEQ ID NO:6. In an exemplary embodiment, the amino acid sequence of the recombinant polypeptide used in the method described herein has at least 85%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or even 100% identity to SEQ ID NO:6.

[0014] In another aspect, the subject technology also relates to a method of producing a steviol glycoside composition, the method comprising incubating a substrate (such as stevioside and rebaudioside E) with a recombinant polypeptide comprising an amino acid sequence having at least 80% identity to SEQ ID NO:11. In an exemplary embodiment, the amino acid sequence of the recombinant polypeptide used in the method described herein has at least 85%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or even 100% identity to SEQ ID NO:11.

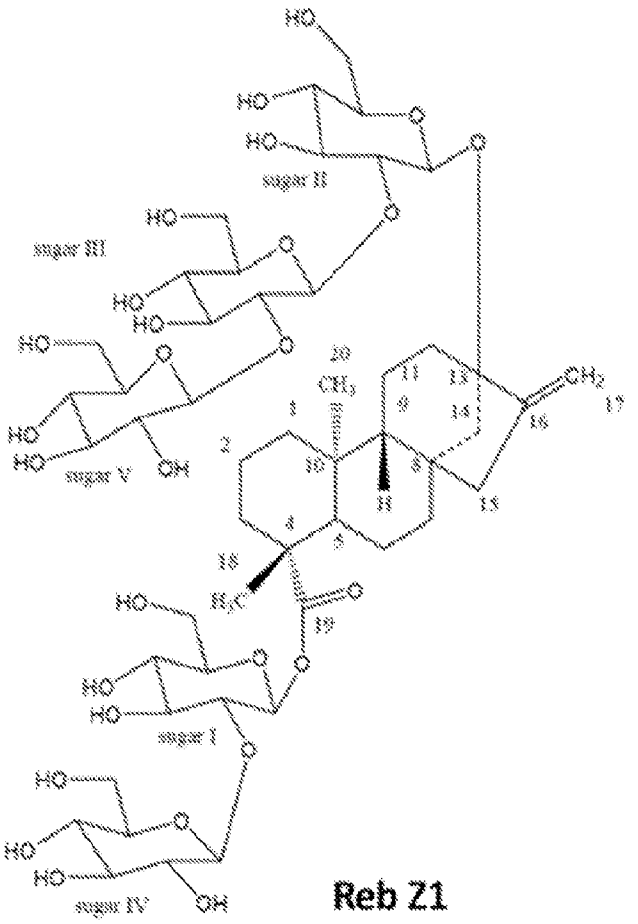
[0015] In one embodiment, the method further comprises incubating a recombinant sucrose synthase (such as one with an amino acid sequence having at least 80% identity to the amino acid sequence of AtSUS1 as set forth in SEQ ID NO:9) with the substrate and the recombinant polypeptide described herein. In another embodiment, the method further comprises incubating a recombinant UDP-glycosyltransferase (such as one with an amino acid sequence having at least 80% identity to the amino acid sequence of UGT76G1 as set forth in SEQ ID NO:11) with the recombinant sucrose synthase, the substrate, and the recombinant polypeptide described herein. In another embodiment, the method described herein comprises incubating the substrate with a host cell expressing the recombinant polypeptide.

[0016] The subject technology also relates to a novel steviol glycoside, termed rebaudioside Z, which is characterized by a retention time of about 6.68 minutes on a HPLC under conditions described herein. The subject technology also relates to a method of producing rebaudioside Z described herein, the method comprising incubating a

substrate with a recombinant polypeptide comprising an amino acid sequence having at least 80% identity to SEQ ID NO:6. As used herein, the terms "rebaudioside Z" or "Reb Z" refer to a mixture of compounds, particularly, a mixture of rebaudioside Z1 ("Reb Z1") and rebaudioside Z2 ("Reb Z2").

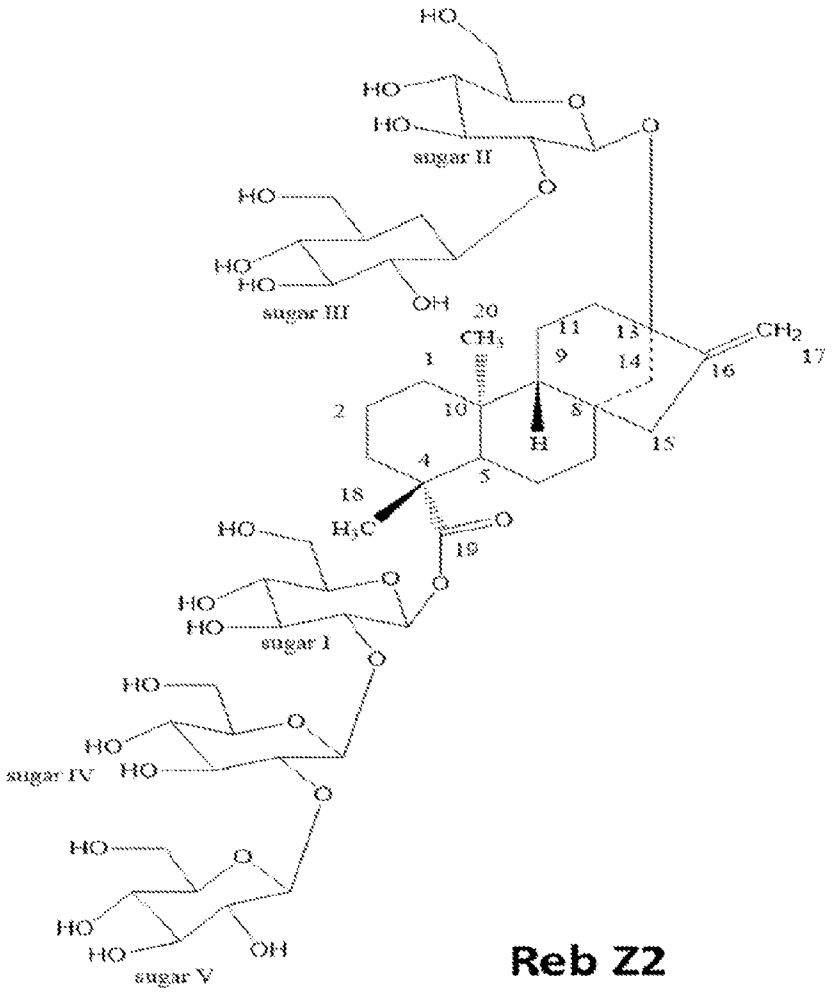
[0017] In one embodiment, the present disclosure is further directed to a method for synthesizing rebaudioside Z from rebaudioside E. The method includes: preparing a reaction mixture comprising rebaudioside E, a substrate selected from the group consisting of sucrose, uridine diphosphate (UDP) and uridine diphosphate-glucose (UDP-glucose), and HV1, and sucrose synthase, incubating the reaction mixture for a sufficient time to produce rebaudioside Z, wherein a glucose is covalently coupled to the rebaudioside E to produce rebaudioside Z, a glucose is covalently coupled to the C2'-13-O-glucose of rebaudioside E to produce rebaudioside Z1, and a glucose is covalently coupled to C2'-19-O-glucose of rebaudioside E to produce rebaudioside Z2.

[0018] In one embodiment, the rebaudioside Z compound is rebaudioside Z1 (Reb Z1) having the structure:



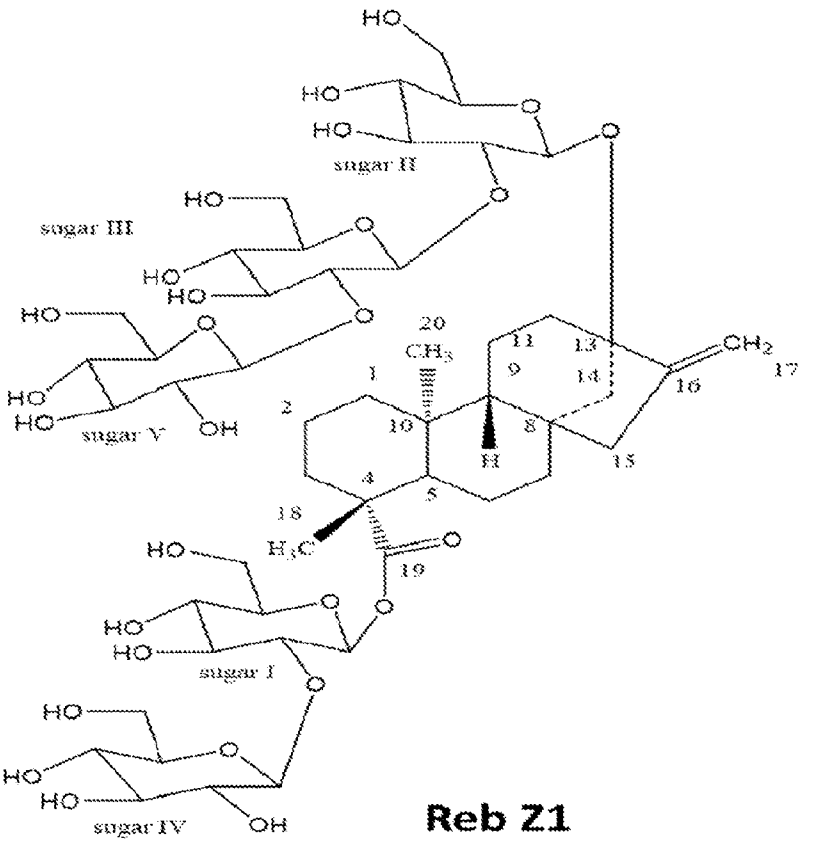
Reb Z1

[0019] In one embodiment, the rebaudioside Z compound is rebaudioside Z2 (Reb Z2) having the structure:

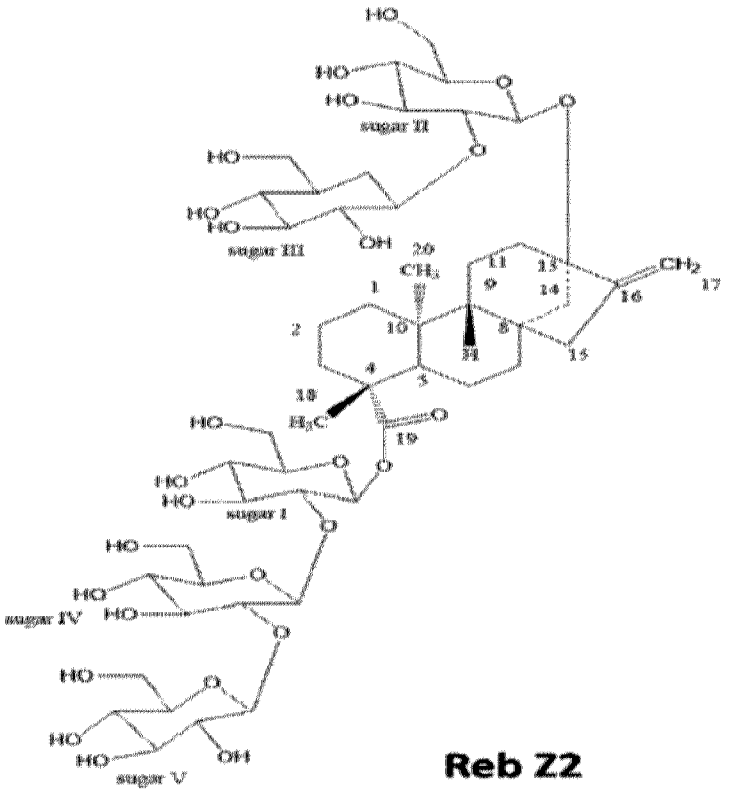


[0020] As described herein, the recombinant polypeptides of the present technology are useful for developing a biosynthetic method for preparing steviol glycosides that are typically of low abundance in natural sources, such as rebaudioside D and rebaudioside E. Accordingly, the present technology also provides for a steviol glycoside composition produced by the biosynthetic method described herein. Such composition can comprise a steviol glycoside compound selected from the group consisting of rebaudioside D, rebaudioside E, a novel rebaudioside (referred to herein as "rebaudioside Z" and "Reb Z") and combinations thereof. Further, a sweetener comprising the steviol glycoside composition described herein is also provided.

[0021] In one embodiment, the present disclosure is directed to a sweetener including a compound having the chemical structure:



[0022] In another embodiment, the sweetener includes a compound having the chemical structure:



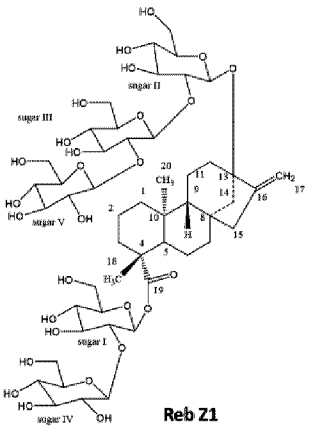
Reb Z2

[0022a] In an embodiment, there is provided a method of producing a steviol glycoside, the method comprising incubating a substrate with a recombinant polypeptide having UDP-glycosyltransferase activity and comprising an amino acid sequence having at least 80% identity to the amino acid sequence as set forth in SEQ ID NO:6 in the presence of UDP-glucose as the glucose donor, wherein the substrate is selected from the group consisting of stevioside, rebaudioside A, rebaudioside E, and combinations thereof.

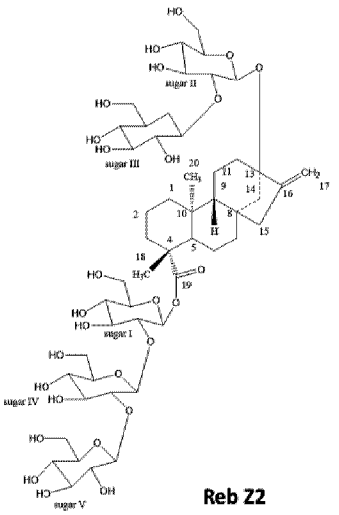
[0022b] In an embodiment, there is provided a method of producing rebaudioside Z1 and/or rebaudioside Z2, the method comprising incubating a substrate with a recombinant polypeptide having UDP-glycosyltransferase activity and comprising an amino acid sequence having at least 80% identity to the amino acid sequence as set forth in SEQ ID NO:6 in the presence of UDP-glucose as the glucose donor, wherein the substrate is selected from the group consisting of rubusoside, stevioside, and combinations thereof.

[0022c] In an embodiment, there is provided a method for synthesizing rebaudioside Z1 and/or rebaudioside Z2 from rebaudioside E, the method comprising: preparing a reaction mixture comprising rebaudioside E and either: (i) a recombinant polypeptide which is a UDP-glycosyltransferase comprising an amino acid sequence having at least 80% identity to the amino acid sequence of the HV1 glycosyltransferase as set forth in SEQ ID NO:6 and UDP-glucose, or (ii) sucrose, UDP and UDP-glucose and a recombinant polypeptide which is a UDP-glycosyltransferase comprising an amino acid sequence having at least 80% identity to the amino acid sequence of the HV1 glycosyltransferase as set forth in SEQ ID NO:6 together with a sucrose synthase, incubating the reaction mixture for a sufficient time whereby a glucose is covalently coupled to the C2' of the 13-O-glucose of rebaudioside E to produce rebaudioside Z1, and a glucose is covalently coupled to C2' of the 19-O-glucose of rebaudioside E to produce rebaudioside Z2.

[0022d] In an embodiment, there is provided a rebaudioside Z1 compound of the structure:

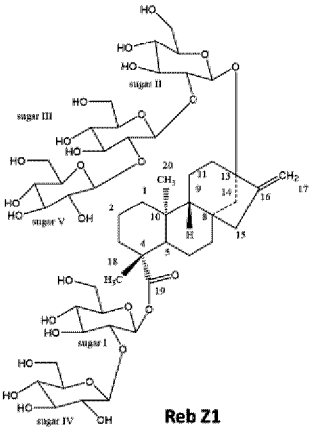


[0022e] In an embodiment, there is provided a rebaudioside Z2 compound of the structure:



Reb Z2

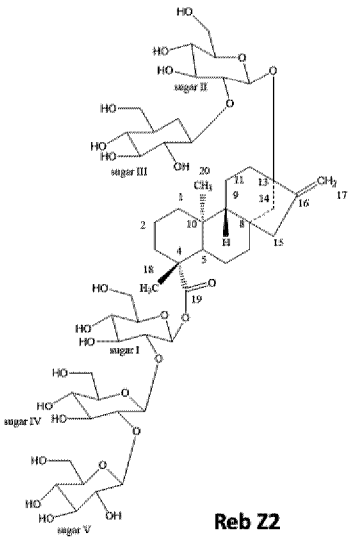
[0022f] In an embodiment, there is provided a sweetener comprising a compound having a chemical structure:



Reb Z1

[0022g] In an embodiment, there is provided a consumable product comprising a sweetening amount of the sweetener as described herein.

[0022h] In an embodiment, there is provided a sweetener comprising a compound having a chemical structure:



Reb Z2

[0022i] In an embodiment, there is provided a consumable product comprising a sweetening amount of the sweetener as described herein.

[0023] The present disclosure is further directed to using the sweeteners in consumable products such as beverages, confectioneries, bakery products, cookies, and chewing gums.

BRIEF DESCRIPTION OF THE DRAWINGS

[0024] The disclosure will be better understood, and features, aspects and advantages other than those set forth above will become apparent when consideration is given to the following detailed description thereof. Such detailed description makes reference to the following drawings, wherein:

[0025] Figures 1A-1C depict a scheme illustrating the pathways of steviol glycoside biosynthesis from stevioside. As described herein, the recombinant HV1

polypeptide ("HV1") contains a 1,2-19-O-glucose glycosylation activity which transfers a second sugar moiety to the C-2' of 19-O-glucose of stevioside to produce rebaudioside E ("Reb E"), or similarly to produce rebaudioside D ("Reb D") from rebaudioside A ("Reb A"). Figures 1A-1C also show that a recombinant UGT76G1 enzyme ("UGT76G1", different from the recombinant HV1 polypeptide) catalyzes the reaction that transfers a sugar moiety to C-3' of the C-13-O-glucose of stevioside to produce rebaudioside A, or similarly to produce rebaudioside D from rebaudioside E.

[0026] Figure 2 shows an exemplary scheme of a coupling reaction system of UDP-glycosyltransferase ("UGT") and sucrose synthase ("SUS"). Reaction 1 shows a UGT catalyzed reaction converting rebaudioside A ("Reb A") to rebaudioside D ("Reb D"), which uses UDP-glucose as a glucose donor and results in the production of UDP. Reaction 2 shows a SUS catalyzed reaction converting UDP to UDP-glucose, which uses sucrose as a glucose donor. Reaction 2 also shows that the SUS catalyzed reaction may be coupled to the UGT catalyzed reaction.

[0027] Figure 3 shows the *in vitro* production of rebaudioside D ("Reb D") from rebaudioside A ("Reb A") catalyzed by a recombinant HV1 polypeptide (SEQ ID NO:6) and a recombinant AtSUS1 (SEQ ID NO:9) in a HV1-AtSUS1 coupling reaction system as described herein. Figure 3A shows the standards of stevioside ("Ste"), Rebaudioside A ("Reb A") and Rebaudioside D ("Reb D"). The results at 6, 9, 12, and 24 hours are shown in Figures 3B-E, respectively. The results from the reaction without the recombinant AtSUS1 (i.e. a non-coupling reaction) at 12 and 24 hours are shown in Figures 3F and 3G, respectively.

[0028] Figure 4 shows the *in vitro* production of rebaudioside E ("Reb E") from stevioside catalyzed by a recombinant HV1 polypeptide (SEQ ID NO:6) and a recombinant AtSUS1 (SEQ ID NO:9) in a HV1-AtSUS1 coupling reaction system as described herein. Figure 4A shows the standards of stevioside ("Ste"), Rebaudioside A ("Reb A") and Rebaudioside D ("Reb D"). The result at 20 hours is shown in Figures 4B, which includes a rebaudioside Z compound ("Reb Z").

[0029] Figure 5 shows the *in vitro* production of rebaudioside D ("Reb D") from stevioside catalyzed by a combination of a recombinant HV1 polypeptide (SEQ ID NO:6), a recombinant UGT76G1 (SEQ ID NO:11), and a recombinant AtSUS1 (SEQ ID NO:9). Figure 5A shows the standards of stevioside ("Ste"), Rebaudioside A ("Reb A") and Rebaudioside D ("Reb D"). The results at 6, 9, 12 and 24 hours are shown in Figures 5B-E, respectively.

[0030] Figure 6 shows the SDS-PAGE analysis of the purified recombinant HV1 polypeptide.

[0031] Figure 7 shows the SDS-PAGE analysis of the purified recombinant AtSUS1 polypeptide.

[0032] Figure 8 shows the *in vitro* production of rebaudioside Z ("Reb Z") from rebaudioside E ("Reb E") catalyzed by a recombinant HV1 polypeptide (SEQ ID NO:6) and a recombinant AtSUS1 (SEQ ID NO:9) in a HV1-AtSUS1 coupling reaction system as described herein. Figure 8A shows the standards of rebaudioside E ("Reb E"). The result at 24 hours is shown in Figures 8B, which includes a rebaudioside Z compound ("Reb Z").

[0033] Figure 9 shows the SDS-PAGE analysis of the purified recombinant UGT76G1 polypeptide.

[0034] Figure 10: Structures of Rebaudioside Z (including Reb Z1 and Reb Z2) and Rebaudioside E.

[0035] Figure 11: Key TOCSY and HMBC correlations of Reb Z1 and Reb Z2.

[0036] Figure 12: shows the *in vitro* production of rebaudioside D ("Reb D") from rebaudioside E ("Reb E") catalyzed by a recombinant UGT76G1 (SEQ ID NO:11). Figure 12A-12B shows the standards of Rebaudioside E ("Reb E"), Rebaudioside D ("Reb D"). The results from the reaction without the recombinant AtSUS1(Figure 12C) (i.e. a non-coupling reaction) and with recombinant AtSUS1 (Figure 12D) (i.e. UGT-SUS coupling reaction) at 6 hours are shown respectively.

[0037] While the disclosure is susceptible to various modifications and alternative forms, specific embodiments thereof have been shown by way of example in the drawings and are herein described below in detail. It should be understood, however, that the description of specific embodiments is not intended to limit the disclosure to cover all modifications, equivalents and alternatives falling within the spirit and scope of the disclosure as defined by the appended claims.

DETAILED DESCRIPTION

[0038] The subject technology provides a recombinant polypeptide that has UDP-glycosyltransferase activities, such as 1,2-19-O-glucose glycosylation activity and 1,2-13-O-glucose glycosylation activity for synthesizing steviol glycosides. The recombinant polypeptide of the subject technology (which can also be referred to as "recombinant HV1 polypeptide" hereinafter) is useful for the biosynthesis of steviol glycoside compounds. In the present disclosure, UDP-glycosyltransferase (UGT) refers to an enzyme that transfers a sugar residue from an activated donor molecule (typically UDP-glucose) to an acceptor molecule. The 1,2-19-O-glucose glycosylation activity refers to an enzymatic activity that transfers a sugar moiety to the C-2' of the 19-O-glucose moiety of stevioside, rebaudioside A or rebaudioside E (Figures 1A-1C and Figure 10). The 1,2-13-O-glucose glycosylation activity refers to an enzymatic activity that transfers a sugar moiety to the C-2' of the 13-O-glucose moiety of rebaudioside E (Figure 10).

[0039] The names of the UGT enzymes used in the present disclosure is consistent with the nomenclature system adopted by the UGT Nomenclature Committee (Mackenzie et al., "The UDP glycosyltransferase gene super family: recommended nomenclature updated based on evolutionary divergence," Pharmacogenetics, 1997, vol. 7, pp. 255-269), which classifies the UGT genes by the combination of a family number, a letter denoting a subfamily, and a number for an individual gene. For example, the name "UGT76G1" refers to a UGT enzyme encoded by a gene belonging to UGT family number 76 (which is of plant origin), subfamily G, and gene number 1.

[0040] There is a large UGTs gene family in plants. However, the biological functions of the majority of these UGTs remain unknown.

Definitions

[0041] As used herein, the singular forms "a," "an" and "the" include plural references unless the content clearly dictates otherwise.

[0042] To the extent that the term "include," "have," or the like is used in the description or the claims, such term is intended to be inclusive in a manner similar to the term "comprise" as "comprise" is interpreted when employed as a transitional word in a claim.

[0043] The word "exemplary" is used herein to mean "serving as an example, instance, or illustration." Any embodiment described herein as "exemplary" is not necessarily to be construed as preferred or advantageous over other embodiments.

[0044] The term "complementary" is to be given its ordinary and customary meaning to a person of ordinary skill in the art, and is used without limitation to describe the relationship between nucleotide bases that are capable to hybridizing to one another. For example, with respect to DNA, adenosine is complementary to thymine and cytosine is complementary to guanine. Accordingly, the subjection technology also includes isolated nucleic acid fragments that are complementary to the complete sequences as reported in the accompanying Sequence Listing as well as those substantially similar nucleic acid sequences.

[0045] The terms "nucleic acid" and "nucleotide" are to be given their respective ordinary and customary meanings to a person of ordinary skill in the art, and are used without limitation to refer to deoxyribonucleotides or ribonucleotides and polymers thereof in either single- or double-stranded form. Unless specifically limited, the term encompasses nucleic acids containing known analogues of natural nucleotides that have similar binding properties as the reference nucleic acid and are metabolized in a manner similar to naturally-occurring nucleotides. Unless otherwise indicated, a particular nucleic acid sequence also implicitly encompasses conservatively modified or degenerate variants thereof (e.g., degenerate codon substitutions) and complementary sequences, as well as the sequence explicitly indicated.

[0046] The term "isolated" is to be given its ordinary and customary meaning to a person of ordinary skill in the art, and when used in the context of an isolated nucleic acid or an isolated polypeptide, is used without limitation to refer to a nucleic acid or polypeptide that, by the hand of man, exists apart from its native environment and is therefore not a product of nature. An isolated nucleic acid or polypeptide can exist in a purified form or can exist in a non-native environment such as, for example, in a transgenic host cell.

[0047] The terms "incubating" and "incubation" as used herein means a process of mixing two or more chemical or biological entities (such as a chemical compound and an enzyme) and allowing them to interact under conditions favorable for producing a steviol glycoside composition.

[0048] The term "degenerate variant" refers to a nucleic acid sequence having a residue sequence that differs from a reference nucleic acid sequence by one or more degenerate codon substitutions. Degenerate codon substitutions can be achieved by generating sequences in which the third position of one or more selected (or all) codons is substituted with mixed base and/or deoxyinosine residues. A nucleic acid sequence and all of its degenerate variants will express the same amino acid or polypeptide.

[0049] The terms "polypeptide," "protein," and "peptide" are to be given their respective ordinary and customary meanings to a person of ordinary skill in the art; the three terms are sometimes used interchangeably, and are used without limitation to refer to a polymer of amino acids, or amino acid analogs, regardless of its size or function. Although "protein" is often used in reference to relatively large polypeptides, and "peptide" is often used in reference to small polypeptides, usage of these terms in the art overlaps and varies. The term "polypeptide" as used herein refers to peptides, polypeptides, and proteins, unless otherwise noted. The terms "protein," "polypeptide," and "peptide" are used interchangeably herein when referring to a polynucleotide product. Thus, exemplary polypeptides include polynucleotide products, naturally occurring proteins, homologs, orthologs, paralogs, fragments and other equivalents, variants, and analogs of the foregoing.

[0050] The terms "polypeptide fragment" and "fragment," when used in reference to a reference polypeptide, are to be given their ordinary and customary meanings to a person of ordinary skill in the art, and are used without limitation to refer to a polypeptide in which amino acid residues are deleted as compared to the reference polypeptide itself, but where the remaining amino acid sequence is usually identical to the corresponding positions in the reference polypeptide. Such deletions can occur at the amino-terminus or carboxy-terminus of the reference polypeptide, or alternatively both.

[0051] The term "functional fragment" of a polypeptide or protein refers to a peptide fragment that is a portion of the full length polypeptide or protein, and has substantially the same biological activity, or carries out substantially the same function as the full length polypeptide or protein (e.g., carrying out the same enzymatic reaction).

[0052] The terms "variant polypeptide," "modified amino acid sequence" or "modified polypeptide," which are used interchangeably, refer to an amino acid sequence that is different from the reference polypeptide by one or more amino acids, e.g., by one or more amino acid substitutions, deletions, and/or additions. In an aspect, a variant is a "functional variant" which retains some or all of the ability of the reference polypeptide.

[0053] The term "functional variant" further includes conservatively substituted variants. The term "conservatively substituted variant" refers to a peptide having an amino acid sequence that differs from a reference peptide by one or more conservative amino acid substitutions, and maintains some or all of the activity of the reference peptide. A "conservative amino acid substitution" is a substitution of an amino acid residue with a functionally similar residue. Examples of conservative substitutions include the substitution of one non-polar (hydrophobic) residue such as isoleucine, valine, leucine or methionine for another; the substitution of one charged or polar (hydrophilic) residue for another such as between arginine and lysine, between glutamine and asparagine, between threonine and serine; the substitution of one basic residue such as lysine or arginine for another; or the substitution of one acidic residue, such as aspartic acid or glutamic acid for another; or the substitution of one aromatic residue, such as phenylalanine, tyrosine, or tryptophan for another. Such substitutions are expected to have little or no effect on the apparent molecular weight or isoelectric point of the protein or polypeptide. The phrase

"conservatively substituted variant" also includes peptides wherein a residue is replaced with a chemically-derivatized residue, provided that the resulting peptide maintains some or all of the activity of the reference peptide as described herein.

[0054] The term "variant," in connection with the polypeptides of the subject technology, further includes a functionally active polypeptide having an amino acid sequence at least 75%, at least 76%, at least 77%, at least 78%, at least 79%, at least 80%, at least 81%, at least 82%, at least 83%, at least 84%, at least 85%, at least 86%, at least 87%, at least 88%, at least 89%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, and even 100% identical to the amino acid sequence of a reference polypeptide.

[0055] The term "homologous" in all its grammatical forms and spelling variations refers to the relationship between polynucleotides or polypeptides that possess a "common evolutionary origin," including polynucleotides or polypeptides from superfamilies and homologous polynucleotides or proteins from different species (Reeck et al., Cell 50:667, 1987). Such polynucleotides or polypeptides have sequence homology, as reflected by their sequence similarity, whether in terms of percent identity or the presence of specific amino acids or motifs at conserved positions. For example, two homologous polypeptides can have amino acid sequences that are at least 75%, at least 76%, at least 77%, at least 78%, at least 79%, at least 80%, at least 81%, at least 82%, at least 83%, at least 84%, at least 85%, at least 86%, at least 87%, at least 88%, at least 89%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, and even 100% identical.

[0056] "Percent (%) amino acid sequence identity" with respect to the variant polypeptide sequences of the subject technology is defined as the percentage of amino acid residues in a candidate sequence that are identical with the amino acid residues of a reference polypeptide (such as, for example, SEQ ID NO:6), after aligning the sequences and introducing gaps, if necessary, to achieve the maximum percent sequence identity, and not considering any conservative substitutions as part of the sequence identity.

[0057] Alignment for purposes of determining percent amino acid sequence identity can be achieved in various ways that are within the skill in the art, for instance, using publicly available computer software such as BLAST, BLAST-2, ALIGN, ALIGN-2 or Megalign (DNASTAR) software. Those skilled in the art can determine appropriate parameters for measuring alignment, including any algorithms needed to achieve maximal alignment over the full-length of the sequences being compared. For example, the % amino acid sequence identity may be determined using the sequence comparison program NCBI-BLAST2. The NCBI-BLAST2 sequence comparison program may be downloaded from ncbi.nlm.nih.gov. NCBI BLAST2 uses several search parameters, wherein all of those search parameters are set to default values including, for example, unmask yes, strand=all, expected occurrences 10, minimum low complexity length=15/5, multi-pass e-value=0.01, constant for multi-pass=25, dropoff for final gapped alignment=25 and scoring matrix=BLOSUM62. In situations where NCBI-BLAST2 is employed for amino acid sequence comparisons, the % amino acid sequence identity of a given amino acid sequence A to, with, or against a given amino acid sequence B (which can alternatively be phrased as a given amino acid sequence A that has or comprises a certain % amino acid sequence identity to, with, or against a given amino acid sequence B) is calculated as follows: 100 times the fraction X/Y where X is the number of amino acid residues scored as identical matches by the sequence alignment program NCBI-BLAST2 in that program's alignment of A and B, and where Y is the total number of amino acid residues in B. It will be appreciated that where the length of amino acid sequence A is not equal to the length of amino acid sequence B, the % amino acid sequence identity of A to B will not equal the % amino acid sequence identity of B to A.

[0058] In this sense, techniques for determining amino acid sequence "similarity" are well known in the art. In general, "similarity" means the exact amino acid to amino acid comparison of two or more polypeptides at the appropriate place, where amino acids are identical or possess similar chemical and/or physical properties such as charge or hydrophobicity. A so-termed "percent similarity" may then be determined between the compared polypeptide sequences. Techniques for determining nucleic acid and amino acid sequence identity also are well known in the art and include determining the nucleotide sequence of the mRNA for that gene (usually via a cDNA intermediate) and determining

the amino acid sequence encoded therein, and comparing this to a second amino acid sequence. In general, "identity" refers to an exact nucleotide to nucleotide or amino acid to amino acid correspondence of two polynucleotides or polypeptide sequences, respectively. Two or more polynucleotide sequences can be compared by determining their "percent identity", as can two or more amino acid sequences. The programs available in the Wisconsin Sequence Analysis Package, Version 8 (available from Genetics Computer Group, Madison, Wis.), for example, the GAP program, are capable of calculating both the identity between two polynucleotides and the identity and similarity between two polypeptide sequences, respectively. Other programs for calculating identity or similarity between sequences are known by those skilled in the art.

[0059] An amino acid position "corresponding to" a reference position is a position that aligns with a reference sequence, as identified by aligning the amino acid sequences. Such alignments can be done by hand or by using well-known sequence alignment programs such as ClustalW2, Blast 2, etc.

[0060] Unless specified otherwise, the percent identity of two polypeptide or polynucleotide sequences refers to as the percentage of identical amino acid residues or nucleotides across the entire length of the shorter of the two sequences.

[0061] "Coding sequence" is to be given its ordinary and customary meaning to a person of ordinary skill in the art, and is used without limitation to refer to a DNA sequence that encodes for a specific amino acid sequence.

[0062] "Suitable regulatory sequences" is to be given its ordinary and customary meaning to a person of ordinary skill in the art, and is used without limitation to refer to nucleotide sequences located upstream (5' non-coding sequences), within, or downstream (3' non-coding sequences) of a coding sequence, and which influence the transcription, RNA processing or stability, or translation of the associated coding sequence. Regulatory sequences may include promoters, translation leader sequences, introns, and polyadenylation recognition sequences.

[0063] "Promoter" is to be given its ordinary and customary meaning to a person of ordinary skill in the art, and is used without limitation to refer to a DNA sequence

capable of controlling the expression of a coding sequence or functional RNA. In general, a coding sequence is located 3' to a promoter sequence. Promoters may be derived in their entirety from a native gene, or be composed of different elements derived from different promoters found in nature, or even comprise synthetic DNA segments. It is understood by those skilled in the art that different promoters may direct the expression of a gene in different tissues or cell types, or at different stages of development, or in response to different environmental conditions. Promoters, which cause a gene to be expressed in most cell types at most times, are commonly referred to as "constitutive promoters." It is further recognized that since in most cases the exact boundaries of regulatory sequences have not been completely defined, DNA fragments of different lengths may have identical promoter activity.

[0064] The term "operably linked" refers to the association of nucleic acid sequences on a single nucleic acid fragment so that the function of one is affected by the other. For example, a promoter is operably linked with a coding sequence when it is capable of affecting the expression of that coding sequence (i.e., that the coding sequence is under the transcriptional control of the promoter). Coding sequences can be operably linked to regulatory sequences in sense or antisense orientation.

[0065] The term "expression" as used herein, is to be given its ordinary and customary meaning to a person of ordinary skill in the art, and is used without limitation to refer to the transcription and stable accumulation of sense (mRNA) or antisense RNA derived from the nucleic acid fragment of the subject technology. "Over-expression" refers to the production of a gene product in transgenic or recombinant organisms that exceeds levels of production in normal or non-transformed organisms.

[0066] "Transformation" is to be given its ordinary and customary meaning to a person of ordinary skill in the art, and is used without limitation to refer to the transfer of a polynucleotide into a target cell. The transferred polynucleotide can be incorporated into the genome or chromosomal DNA of a target cell, resulting in genetically stable inheritance, or it can replicate independent of the host chromosomal. Host organisms containing the transformed nucleic acid fragments are referred to as "transgenic" or "recombinant" or "transformed" organisms.

[0067] The terms "transformed," "transgenic," and "recombinant," when used herein in connection with host cells, are to be given their respective ordinary and customary meanings to a person of ordinary skill in the art, and are used without limitation to refer to a cell of a host organism, such as a plant or microbial cell, into which a heterologous nucleic acid molecule has been introduced. The nucleic acid molecule can be stably integrated into the genome of the host cell, or the nucleic acid molecule can be present as an extrachromosomal molecule. Such an extrachromosomal molecule can be auto-replicating. Transformed cells, tissues, or subjects are understood to encompass not only the end product of a transformation process, but also transgenic progeny thereof.

[0068] The terms "recombinant," "heterologous," and "exogenous," when used herein in connection with polynucleotides, are to be given their ordinary and customary meanings to a person of ordinary skill in the art, and are used without limitation to refer to a polynucleotide (e.g., a DNA sequence or a gene) that originates from a source foreign to the particular host cell or, if from the same source, is modified from its original form. Thus, a heterologous gene in a host cell includes a gene that is endogenous to the particular host cell but has been modified through, for example, the use of site-directed mutagenesis or other recombinant techniques. The terms also include non-naturally occurring multiple copies of a naturally occurring DNA sequence. Thus, the terms refer to a DNA segment that is foreign or heterologous to the cell, or homologous to the cell but in a position or form within the host cell in which the element is not ordinarily found.

[0069] Similarly, the terms "recombinant," "heterologous," and "exogenous," when used herein in connection with a polypeptide or amino acid sequence, means a polypeptide or amino acid sequence that originates from a source foreign to the particular host cell or, if from the same source, is modified from its original form. Thus, recombinant DNA segments can be expressed in a host cell to produce a recombinant polypeptide.

[0070] The terms "plasmid," "vector," and "cassette" are to be given their respective ordinary and customary meanings to a person of ordinary skill in the art, and are used without limitation to refer to an extra chromosomal element often carrying genes which are not part of the central metabolism of the cell, and usually in the form of circular double-stranded DNA molecules. Such elements may be autonomously replicating

sequences, genome integrating sequences, phage or nucleotide sequences, linear or circular, of a single- or double-stranded DNA or RNA, derived from any source, in which a number of nucleotide sequences have been joined or recombined into a unique construction which is capable of introducing a promoter fragment and DNA sequence for a selected gene product along with appropriate 3' untranslated sequence into a cell. "Transformation cassette" refers to a specific vector containing a foreign gene and having elements in addition to the foreign gene that facilitate transformation of a particular host cell. "Expression cassette" refers to a specific vector containing a foreign gene and having elements in addition to the foreign gene that allow for enhanced expression of that gene in a foreign host.

[0071] Standard recombinant DNA and molecular cloning techniques used here are well known in the art and are described, for example, by Sambrook, J., Fritsch, E. F. and Maniatis, T. Molecular Cloning: A Laboratory Manual, 2nd ed.; Cold Spring Harbor Laboratory: Cold Spring Harbor, N.Y., 1989 (hereinafter "Maniatis"); and by Silhavy, T. J., Bennan, M. L. and Enquist, L. W. Experiments with Gene Fusions; Cold Spring Harbor Laboratory: Cold Spring Harbor, N.Y., 1984; and by Ausubel, F. M. et al., In Current Protocols in Molecular Biology, published by Greene Publishing and Wiley-Interscience, 1987.

[0072] Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which the disclosure belongs. Although any methods and materials similar to or equivalent to those described herein can be used in the practice or testing of the present disclosure, the preferred materials and methods are described below.

[0073] The disclosure will be more fully understood upon consideration of the following non-limiting Examples. It should be understood that these Examples, while indicating preferred embodiments of the subject technology, are given by way of illustration only. From the above discussion and these Examples, one skilled in the art can ascertain the essential characteristics of the subject technology, and without departing from the spirit and scope thereof, can make various changes and modifications of the subject technology to adapt it to various uses and conditions.

Date Reçue/Date Received 2021-01-12

Recombinant Polypeptides

[0074] In one aspect, the present disclosure relates to a recombinant polypeptide having an amino acid sequence having at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, and even 100% identity to the amino acid sequence set forth in SEQ ID NO:6. Suitably, the amino acid sequence of the recombinant polypeptide has at least 80% identity to SEQ ID No:6. More suitably, the amino acid sequence of the recombinant polypeptide has at least 85%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, and even 100% identity to SEQ ID NO:6. In an exemplary embodiment, the amino acid sequence of the recombinant polypeptide consists of SEQ ID NO:6. Accordingly, the recombinant polypeptide described herein includes functional fragments of SEQ ID NO:6, functional variants of SEQ ID NO:6, and other homologous polypeptides that have, for example, at least 80%, at least 85%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, and even 100% sequence identity to SEQ ID NO:6.

[0075] In another aspect, the present disclosure relates to an isolated nucleic acid having a nucleotide sequence encoding the recombinant polypeptide described herein. For example, the isolated nucleic acid can include a nucleotide sequence encoding a polypeptide having an amino acid sequence that has at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, and even 100% identity to the amino acid sequence set forth in SEQ ID NO:6. Suitably, the isolated nucleic acid includes a nucleotide sequence encoding a polypeptide having an amino acid sequence that is at least 80% identity to the amino acid sequence set forth in SEQ ID NO:6. More suitably, the isolated nucleic acid includes a nucleotide sequence encoding a polypeptide having an amino acid sequence that has at least 85%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, and even 100% sequence identity to the amino acid sequence set forth in SEQ ID NO:6. The isolated nucleic acid thus includes those encoding functional fragments of

SEQ ID NO:6, functional variants of SEQ ID NO:6, or other homologous polypeptides that have, for example, at least 80%, at least 85%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, and even 100% sequence identity to SEQ ID NO:6.

[0076] In an embodiment, the present disclosure relates to an isolated nucleic acid having a nucleotide sequence that has at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, and even 100% identity to the nucleotide sequence set forth in SEQ ID NO:7. Suitably, the isolated nucleic acid of includes a nucleotide sequence that has at least 80% identity to the nucleotide sequence set forth in SEQ ID NO:7. More suitably, the isolated nucleic acid includes a nucleotide sequence that has at least 85%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, and even 100% identity to the nucleic acid sequence set forth in SEQ ID NO:7.

[0077] In another aspect, the subject technology relates to a vector having the nucleic acids described herein, and a host cell having the vector described herein. In some embodiments, the present disclosure relates to an expression vector including at least one polynucleotide of the subject technology and wherein the expression vector, upon transfection into a host cell, is capable of expressing at least one recombinant HV1 polypeptide described herein. In an embodiment, the expression vector includes a nucleotide sequence set forth in SEQ ID NO:7 or a variant thereof.

[0078] The design of the expression vector depends on such factors as the choice of the host cell to be transformed, the level of expression of protein desired, and the like. The expression vectors can be introduced into the host cell to thereby produce the recombinant polypeptide of the subject technology, such as the recombinant HV1 polypeptide having an amino acid sequence of SEQ ID NO:6 or a variant thereof.

[0079] Expression of proteins in prokaryotes is most often carried out in a bacterial host cell with vectors containing constitutive or inducible promoters directing the expression of either fusion or non-fusion proteins. Fusion vectors add a number of amino

acids to a protein encoded therein, usually to the amino terminus of the recombinant protein. Such fusion vectors typically serve three purposes: 1) to increase expression of recombinant protein; 2) to increase the solubility of the recombinant protein; and 3) to aid in the purification of the recombinant protein by acting as a ligand in affinity purification. Often, a proteolytic cleavage site is introduced at the junction of the fusion moiety and the recombinant protein to enable separation of the recombinant protein from the fusion moiety subsequent to purification of the fusion protein. Such vectors are within the scope of the present disclosure.

[0080] In an embodiment, the expression vector includes those genetic elements for expression of the recombinant polypeptide in bacterial cells. The elements for transcription and translation in the bacterial cell can include a promoter, a coding region for the protein complex, and a transcriptional terminator.

[0081] In an embodiment, the expression vectors of the subject technology include bacterial expression vectors, for example recombinant bacteriophage DNA, plasmid DNA or cosmid DNA, yeast expression vectors e.g. recombinant yeast expression vectors, vectors for expression in insect cells, e.g., recombinant virus expression vectors, for example baculovirus, or vectors for expression in plant cells, e.g. recombinant virus expression vectors such as cauliflower mosaic virus (CaMV), tobacco mosaic virus (TMV), or recombinant plasmid expression vectors such as Ti plasmids.

[0082] In an embodiment, the vector includes a bacterial expression vector. In another embodiment, the expression vector includes a high-copy-number expression vector; alternatively, the expression vector includes a low-copy-number expression vector, for example, a Mini-F plasmid.

[0083] A person of ordinary skill in the art will be aware of the molecular biology techniques available for the preparation of expression vectors. The polynucleotide used for incorporation into the expression vector of the subject technology, as described above, can be prepared by routine techniques such as polymerase chain reaction (PCR).

[0084] A number of molecular biology techniques have been developed to operably link DNA to vectors via complementary cohesive termini. In one embodiment,

complementary homopolymer tracts can be added to the nucleic acid molecule to be inserted into the vector DNA. The vector and nucleic acid molecule are then joined by hydrogen bonding between the complementary homopolymeric tails to form recombinant DNA molecules.

[0085] In an alternative embodiment, synthetic linkers containing one or more restriction sites provide are used to operably link the polynucleotide of the subject technology to the expression vector. In an embodiment, the polynucleotide is generated by restriction endonuclease digestion. In an embodiment, the nucleic acid molecule is treated with bacteriophage T4 DNA polymerase or *E. coli* DNA polymerase I, enzymes that remove protruding, 3'-single-stranded termini with their 3'-5'-exonucleolytic activities, and fill in recessed 3'-ends with their polymerizing activities, thereby generating blunt-ended DNA segments. The blunt-ended segments are then incubated with a large molar excess of linker molecules in the presence of an enzyme that is able to catalyze the ligation of blunt-ended DNA molecules, such as bacteriophage T4 DNA ligase. Thus, the product of the reaction is a polynucleotide carrying polymeric linker sequences at its ends. These polynucleotides are then cleaved with the appropriate restriction enzyme and ligated to an expression vector that has been cleaved with an enzyme that produces termini compatible with those of the polynucleotide.

[0086] Alternatively, a vector having ligation-independent cloning (LIC) sites can be employed. The required PCR amplified polynucleotide can then be cloned into the LIC vector without restriction digest or ligation (Aslanidis and de Jong, Nucl. Acid. Res. 18, 6069-6074, (1990), Haun, et al, Biotechniques 13, 515-518 (1992)).

[0087] In an embodiment, in order to isolate and/or modify the polynucleotide of interest for insertion into the chosen plasmid, it is suitable to use PCR. Appropriate primers for use in PCR preparation of the sequence can be designed to isolate the required coding region of the nucleic acid molecule, add restriction endonuclease or LIC sites, place the coding region in the desired reading frame.

Date Reçue/Date Received 2021-01-12

[0088] In an embodiment, a polynucleotide for incorporation into an expression vector of the subject technology is prepared by the use of PCR using appropriate oligonucleotide primers. The coding region is amplified, whilst the primers themselves become incorporated into the amplified sequence product. In an embodiment, the amplification primers contain restriction endonuclease recognition sites, which allow the amplified sequence product to be cloned into an appropriate vector.

[0089] In an embodiment, the polynucleotide of SEQ ID NO:7 or a variant thereof is obtained by PCR and introduced into an expression vector using restriction endonuclease digestion and ligation according to techniques that are well known in the art.

[0090] The present disclosure further relates to a host cell comprising the expression vector described herein. Suitable hosts of the subject technology typically include microbial hosts or plant hosts. For example, the host cell of the subject technology is selected from the group consisting of bacteria, yeast, filamentous fungi, cyanobacteria algae and plant cell.

[0091] The microbial hosts can include any organism capable of expressing the polynucleotide (such as SEQ ID NO:7) to produce the recombinant HV1 polypeptide described herein. Microorganisms useful in the subject technology include bacteria, such as the enteric bacteria (Escherichia and Salmonella for example) as well as Bacillus, Acinetobacter, Actinomycetes such as Streptomyces, Corynebacterium, Methanotrophs such as Methylosinus, Methylomonas, Rhodococcus and Pseudomona; Cyanobacteria, such as Rhodobacterand Synechocystis; yeasts, such as Saccharomyces, Zygosaccharomyces, Kluyveromyces, Candida, Hansenula, Debaryomyces, Mucor, Pichia and Torulopsis; and filamentous fungi such as Aspergillus and Arthrobotrys, and algae, and Escherichia, Klebsiella, Pantoea, Salmonella Corynebacterium, Clostridium, and Clostridium acetobutylicum, for example. Preferably, the microbial host is a bacteria (such as Escherichia) or a yeast (such as Saccharomyces). The expression vectors can be incorporated into these and other microbial hosts to prepare large, commercially useful amounts of steviol glycosides.

[0092] In an embodiment, the recombinant polypeptide can be expressed in a host cell that is a plant cell. As used herein, the term "plant cell" is understood to mean any cell derived from a monocotyledonous or a dicotyledonous plant and capable of constituting undifferentiated tissues such as calli, differentiated tissues such as embryos, portions of monocotyledonous plants, monocotyledonous plants or seed. The term "plant" is understood to mean any differentiated multi-cellular organism capable of photosynthesis, including monocotyledons and dicotyledons. In some embodiments, the plant cell can be an Arabidopsis plant cell, a tobacco plant cell, a soybean plant cell, a petunia plant cell, or a cell from another oilseed crop including, but not limited to, a canola plant cell, a rapeseed plant cell, a palm plant cell, a sunflower plant cell, a cotton plant cell, a corn plant cell, a peanut plant cell, a flax plant cell, and a sesame plant cell.

[0093] Useful plant hosts can include any plant that supports the production of the recombinant polypeptides of the subject technology. Suitable green plants for use as hosts include, but are not limited to, soybean, rapeseed (Brassica napus, B. campestris), sunflower (Helianthus annus), cotton (Gossypium hirsutum), corn, tobacco (Nicotiana tabacum), alfalfa (Medicago sativa), wheat (Triticum sp), barley (Hordeum vulgare), oats (Avena sativa), sorghum (Sorghum bicolor), rice (Oryza sativa), Arabidopsis, cruciferous vegetables (broccoli, cauliflower, cabbage, parsnips, etc.), melons, carrots, celery, parsley, tomatoes, potatoes, strawberries, peanuts, grapes, grass seed crops, sugar beets, sugar cane, beans, peas, rye, flax, hardwood trees, softwood trees, and forage grasses. Algal species include, but are not limited to, commercially significant hosts such as Spirulina, Haemotacoccus, and Dunaliella. Suitable plants for the method of the subject technology also include biofuel, biomass, and bioenergy crop plants. Exemplary plants include Arabidopsis thaliana, rice (Oryza sativa), Hordeum vulgare, switchgrass (Panicum vigratum), Brachypodium spp, Brassica spp., and Crambe abyssinica.

[0094] In some embodiments, the present disclosure includes transgenic host cells or hosts that have been transformed with one or more of the vectors disclosed herein.

[0095] Alternatively, the hosts cells may be those suitable for biosynthesis production including single cell organisms, microorganisms, multicell organisms, plants, fungi, bacteria, algae, cultivated crops, non-cultivated crops, and/or the like.

[0096] The expression vectors can be introduced into plant or microbial host cells by conventional transformation or transfection techniques. Transformation of appropriate cells with an expression vector of the subject technology is accomplished by methods known in the art and typically depends on both the type of vector and cell. Suitable techniques include calcium phosphate or calcium chloride co-precipitation, DEAE-dextran-mediated transfection, lipofection, chemoporation or electroporation.

[0097] Successfully transformed cells, that is, those cells containing the expression vector, can be identified by techniques well known in the art. For example, cells transfected with an expression vector of the subject technology can be cultured to produce polypeptides described herein. Cells can be examined for the presence of the expression vector DNA by techniques well known in the art.

[0098] The host cells can contain a single copy of the expression vector described previously, or alternatively, multiple copies of the expression vector.

[0099] In some embodiments, the transformed cell is an animal cell, an insect cell, a plant cell, an algal cell, a fungal cell, or a yeast cell. In some embodiments, the cell is a plant cell selected from the group consisting of: canola plant cell, a rapeseed plant cell, a palm plant cell, a sunflower plant cell, a cotton plant cell, a corn plant cell, a peanut plant cell, a flax plant cell, a sesame plant cell, a soybean plant cell, and a petunia plant cell.

[0100] Microbial host cell expression systems and expression vectors containing regulatory sequences that direct high level expression of foreign proteins are well known to those skilled in the art. Any of these could be used to construct vectors for expression of the recombinant polypeptide of the subjection technology in a microbial host cell. These vectors could then be introduced into appropriate microorganisms via transformation to allow for high level expression of the recombinant polypeptide of the subject technology.

[0101] Vectors or cassettes useful for the transformation of suitable microbial host cells are well known in the art. Typically the vector or cassette contains sequences directing transcription and translation of the relevant polynucleotide, a selectable marker, and sequences allowing autonomous replication or chromosomal integration. Suitable vectors comprise a region 5' of the polynucleotide which harbors transcriptional initiation

controls and a region 3' of the DNA fragment which controls transcriptional termination. It is preferred for both control regions to be derived from genes homologous to the transformed host cell, although it is to be understood that such control regions need not be derived from the genes native to the specific species chosen as a host.

[0102] Initiation control regions or promoters, which are useful to drive expression of the recombinant polypeptide in the desired microbial host cell are numerous and familiar to those skilled in the art. Virtually any promoter capable of driving these genes is suitable for the subject technology including but not limited to CYC1, HIS3, GAL1, GAL10, ADH1, PGK, PHO5, GAPDH, ADC1, TRP1, URA3, LEU2, ENO, TPI (useful for expression in Saccharomyces); AOX1 (useful for expression in Pichia); and lac, trp, IP_L, IP_R, T7, tac, and trc (useful for expression in Escherichia coli).

[0103] Termination control regions may also be derived from various genes native to the microbial hosts. A termination site optionally may be included for the microbial hosts described herein.

[0104] In plant cells, the expression vectors of the subject technology can include a coding region operably linked to promoters capable of directing expression of the recombinant polypeptide of the subject technology in the desired tissues at the desired stage of development. For reasons of convenience, the polynucleotides to be expressed may comprise promoter sequences and translation leader sequences derived from the same polynucleotide. 3' non-coding sequences encoding transcription termination signals should also be present. The expression vectors may also comprise one or more introns in order to facilitate polynucleotide expression.

[0105] For plant host cells, any combination of any promoter and any terminator capable of inducing expression of a coding region may be used in the vector sequences of the subject technology. Some suitable examples of promoters and terminators include those from nopaline synthase (nos), octopine synthase (ocs) and cauliflower mosaic virus (CaMV) genes. One type of efficient plant promoter that may be used is a high level plant promoter. Such promoters, in operable linkage with an expression vector of the subject technology should be capable of promoting the expression of the vector. High level plant

promoters that may be used in the subject technology include the promoter of the small subunit (ss) of the ribulose-1,5-bisphosphate carboxylase for example from soybean (Berry-Lowe et al., J. Molecular and App. Gen., 1:483 498 (1982)), and the promoter of the chlorophyll a/b binding protein. These two promoters are known to be light-induced in plant cells (see, for example, Genetic Engineering of Plants, an Agricultural Perspective, A. Cashmore, Plenum, N.Y. (1983), pages 29 38; Coruzzi, G. et al., The Journal of Biological Chemistry, 258:1399 (1983), and Dunsmuir, P. et al., Journal of Molecular and Applied Genetics, 2:285 (1983), each of which is hereby incorporated herein by reference to the extent they are consistent herewith).

[0106] The choice of plasmid vector depends upon the method that will be used to transform host plants. The skilled artisan is well aware of the genetic elements that must be present on the plasmid vector in order to successfully transform, select and propagate host cells containing the chimeric polynucleotide. The skilled artisan will also recognize that different independent transformation events will result in different levels and patterns of expression (Jones et al., EMBO J. 4:2411 2418 (1985); De Almeida et al., Mol. Gen. Genetics 218:78 86 (1989)), and thus that multiple events must be screened in order to obtain lines displaying the desired expression level and pattern. Such screening may be accomplished by Southern analysis of DNA blots, Northern analysis of mRNA expression, Western analysis of protein expression, or phenotypic analysis.

[0107] Introduction of the expression vector of the subject technology into a plant cell can be performed by a variety of methods known to those of ordinary skill in the art including insertion of a nucleic acid sequence of interest into an Agrobacterium rhizogenes Ri or Agrobacterium tumefaciens Ti plasmid, microinjection, electroporation, or direct precipitation. By way of providing an example, in some embodiments, transient expression of a polynucleotide of interest can be performed by agro-infiltration methods. In this regard, a suspension of Agrobacterium tumefaciens containing a polynucleotide of interest can be grown in culture and then injected into a plant by placing the tip of a syringe against the underside of a leaf while gentle counter-pressure is applied to the other side of the leaf.

Date Reçue/Date Received 2021-01-12

The Agrobacterium solution is then injected into the airspaces inside the leaf through stomata. Once inside the leaf, the Agrobacterium transforms the gene of interest to a portion of the plant cells where the gene is then transiently expressed.

[0108] As another example, transformation of a plasmid of interest into a plant cell can be performed by particle gun bombardment techniques (i.e., biolistics). In this regard, a suspension of plant embryos can be grown in liquid culture and then bombarded with plasmids or polynucleotides that are attached to gold particles, wherein the gold particles bound to the plasmid or nucleic acid of interest can be propelled through the membranes of the plant tissues, such as embryonic tissue. Following bombardment, the transformed embryos can then be selected using an appropriate antibiotic to generate new, clonally propagated, transformed embryogenic suspension cultures.

[0109] Host cells can be unmodified cells or cell lines, or cell lines that have been genetically modified. In some embodiments, the host cell is a cell line that has been modified to allow for growth under desired conditions, such as at a lower temperature.

[0110] Standard recombinant DNA methodologies may be used to obtain a nucleic acid that encodes a recombinant polypeptide described herein, incorporate the nucleic acid into an expression vector, and introduce the vector into a host cell, such as those described in Sambrook, et al. (eds), Molecular Cloning; A Laboratory Manual, Third Edition, Cold Spring Harbor, (2001); and Ausubel, F. M. et al. (eds.) Current Protocols in Molecular Biology, John Wiley & Sons (1995). A nucleic acid encoding a polypeptide may be inserted into an expression vector or vectors such that the nucleic acids are operably linked to transcriptional and translational control sequences (such as a promoter sequence, a transcription termination sequence, etc.). The expression vector and expression control sequences are generally chosen to be compatible with the expression host cell used.

[0111] The expression of polypeptide in a host described herein can be further improved by codon-optimization. For example, modifying a less-common codon with a more common codon may affect the half-life of the mRNA or alter its structure by introducing a secondary structure that interferes with translation of the message. All or a

portion of a coding region can be optimized. In some cases the desired modulation of expression is achieved by optimizing essentially the entire gene. In other cases, the desired modulation will be achieved by optimizing part of, but not the entire, sequence of the gene.

[0112] The codon usage of any coding sequence can be adjusted to achieve a desired property, for example high levels of expression in a specific cell type. The starting point for such an optimization may be a coding sequence with 100% common codons, or a coding sequence which contains a mixture of common and non-common codons.

[0113] Two or more candidate sequences that differ in their codon usage can be generated and tested to determine if they possess the desired property. Candidate sequences can be evaluated by using a computer to search for the presence of regulatory elements, such as silencers or enhancers, and to search for the presence of regions of coding sequence which could be converted into such regulatory elements by an alteration in codon usage. Additional criteria may include enrichment for particular nucleotides, e.g., A, C, G or U, codon bias for a particular amino acid, or the presence or absence of particular mRNA secondary or tertiary structure. Adjustment to the candidate sequence can be made based on a number of such criteria.

[0114] In certain embodiments, the codon optimized nucleic acid sequence can express its protein, at a level which is about 110%, about 150%, about 200%, about 250%, about 300%, about 350%, about 400%, about 450%, or about 500%, of that expressed by a nucleic acid sequence that has not been codon optimized.

[0115] In addition to the nucleic acid that encodes the recombinant polypeptide of the subject technology, the expression vector of the subject technology may additionally carry regulatory sequences that control the expression of the protein in a host cell, such as promoters, enhancers or other expression control elements that control the transcription or translation of the nucleic acid(s). Such regulatory sequences are known in the art. It will be appreciated by those skilled in the art that the design of the expression vector, including the selection of regulatory sequences may depend on such factors as the choice of the host cell to be transformed, the level of expression of protein desired, etc. In addition, the recombinant expression vectors of the subject technology may carry additional sequences,

such as sequences that regulate replication of the vector in host cells (e.g., origins of replication) and selectable marker genes.

Biosynthesis of Steviol Glycosides

[0116] As described herein, the recombinant polypeptides of the present technology have UDP-glycosyltransferase activities, including more particularly a 1,2-19-O-glucose glycosylation activity, and are useful for developing biosynthetic methods for preparing steviol glycosides that are typically of low abundance in natural sources, such as rebaudioside D and rebaudioside E. The recombinant polypeptides of the present technology have UDP-glycosyltransferase activities, are useful for developing biosynthetic methods for preparing novel steviol glycosides, such as rebaudioside Z1 and rebaudioside Z2.

[0117] Accordingly, in one aspect, the subject technology also relates to a method of producing a steviol glycoside composition, the method including incubating a substrate with a recombinant polypeptide comprising an amino acid sequence having at least 80% identity to SEQ ID NO:6.

[0118] The substrate can be any natural or synthetic compound capable of being converted into a steviol glycoside compound in a reaction catalyzed by one or more UDP-glycosyltransferases. For example, the substrate can be natural stevia extract, steviol, steviol-13-O-glucoside, steviol-19-O-glucoside, steviol-1,2-bioside, rubusoside, stevioside, rebaudioside A, rebaudioside G or rebaudioside E. The substrate can be a pure compound or a mixture of different compounds. Preferably, the substrate includes a compound selected from the group consisting of rubusoside, stevioside, rebaudioside A, rebaudioside E and combinations thereof.

[0119] The method described herein also provides a coupling reaction system in which the recombinant peptides described herein is allowed to function in combination with one or more additional enzymes to improve the efficiency or modify the outcome of the overall biosynthesis of steviol glycoside compounds. For example, the additional enzyme may regenerate the UDP-glucose needed for the glycosylation reaction by converting the UDP produced from the glycosylation reaction back to UDP-glucose (using,

for example, sucrose as a donor of the glucose residue), thus improving the efficiency of the glycosylation reaction. In another example, the recombinant polypeptide of the subject technology may produce an intermediate steviol glycoside product (e.g., rebaudioside E), which is further converted to another steviol glycoside (e.g. rebaudioside D) in a reaction catalyzed by another UDP-glycosyltransferase, such as UGT76G1. In another example, the recombinant polypeptide of the subject technology may produce an intermediate steviol glycoside product (e.g., rebaudioside E), which is further converted to another steviol glycoside (e.g., rebaudioside Z1 and rebaudioside Z2) in a reaction catalyzed by UDP-glycosyltransferase, such as HV1.

[0120] Accordingly, in one embodiment, the method of the subject technology further includes incubating a recombinant sucrose synthase (SUS) with the substrate and the recombinant polypeptide described herein. The recombinant sucrose synthase converts UDP into UDP-glucose using sucrose as a source of glucose. Suitable sucrose synthase includes those derived from *Arabidopsis thaliana* and *Vigna radiate* SUS genes, or from any gene that encodes a functional homolog of the sucrose synthase encoded by the *Arabidopsis thaliana* and *Vigna radiate* SUS1 sequence, or the functional homologs thereof. Suitable sucrose synthases can be for example, an *Arabidopsis* sucrose synthase 1; an *Arabidopsis* sucrose synthase 3; and a *Vigna radiate* sucrose synthase. A particularly suitable sucrose synthase can be, for example, *Arabidopsis* sucrose synthase 1. For example, the recombinant SUS of includes an amino acid sequence that has at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or even 100% identity to the amino acid sequence of AtSUS1 set forth in SEQ ID NO:9. Preferably, the recombinant SUS of the subject technology includes an amino acid sequence that has at least 80% identity to the amino acid sequence of AtSUS1 set forth in SEQ ID NO:9.

[0121] The recombinant sucrose synthase of the subject technology can be obtained by expressing a nucleic acid having a nucleotide sequence encoding an amino acid sequence of interest (e.g. one that has at least 80% identity to the amino acid sequence set forth in SEQ ID NO:9) in a host cell as described above. For example, a vector

including a nucleotide sequence set forth in SEQ ID NO:10 can be introduced into a microbial host (such as E. Coli) by conventional transformation techniques to produce the recombinant sucrose synthase.

[0122] In another embodiment, the method of the subject technology further includes incubating a recombinant UDP-glycosyltransferase with the recombinant sucrose synthase, the substrate, and the recombinant polypeptide described herein. The recombinant UDP-glycosyltransferase can catalyze a different glycosylation reaction than the one catalyzed by the recombinant polypeptide of the subject technology. For example, the recombinant UDP-glycosyltransferase can catalyze the reaction that transfers a sugar moiety to C-3' of the C-13-O-glucose of stevioside to produce rebaudioside A (or similarly to produce rebaudioside D from rebaudioside E), while the recombinant polypeptide of the subject technology transfers a second sugar moiety to the C-2' of 19-O-glucose of stevioside to produce rebaudioside E (or similarly to produce rebaudioside D from rebaudioside A).

[0123] Suitable UDP-glycosyltransferase includes any UGT known in the art as capable of catalyzing one or more reactions in the biosynthesis of steviol glycoside compounds, such as UGT85C2, UGT74G1, UGT76G1, or the functional homologs thereof. For example, the UDP-glycosyltransferase as described herein can include an amino acid sequence that has at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or even 100% identity to the amino acid sequence of UGT76G1 set forth in SEQ ID NO:11. Preferably, the UDP-glycosyltransferase includes an amino acid sequence that has at least 80% identity to the amino acid sequence of UGT76G1 set forth in SEQ ID NO:11.

[0124] The recombinant UDP-glycosyltransferase can be obtained by expressing a nucleic acid having a nucleotide sequence encoding an amino acid sequence of interest (e.g. one that has at least 80% identity to the amino acid sequence set forth in SEQ ID NO:11) in a host cell as described above. For example, a vector including a nucleotide

sequence set forth in SEQ ID NO:12 can be introduced into a microbial host (such as *E. Coli*) by conventional transformation techniques to produce the recombinant UDP-glycosyltransferase of the subject technology.

[0125] Both *in vitro* and *in vivo* production of steviol glycoside compounds are encompassed by the subject technology.

[0126] For example, the incubation can be an *in vitro* process, where a substrate is allowed to interact with a recombinant polypeptide of the subject technology. Preferably, in an *in vitro* process, the recombinant polypeptide is purified before being incubated with the substrate. Conventional polypeptide purification techniques, such as centrifugation, cell lysing, and chromatography, are included in the methods of the subject technology. For example, the nucleic acid encoding the recombinant polypeptide of the subject technology may be cloned into an expressing vector with a Histidine tag, such that the expressed recombinant polypeptide can be purified by affinity column chromatography.

[0127] The *in vitro* method of the subject technology includes any buffer system that is suitable for steviol glycoside production using one or more recombinant polypeptides of the subject technology. Typically, the buffer system is an aqueous solution, such as Tris buffer, HEPES buffer, MOPS buffer, phosphate buffer, with a pH of from about 6.0 to about 8.0. More suitably, the pH is from about 6.5 to about 7.5. Even more suitably, the pH is from about 7.0 to about 7.5.

[0128] Typically, in the *in vitro* method of the subject technology, the substrate is present in the buffer at a concentration of from about 0.2 mg/mL to about 5 mg/mL, preferably from about 0.5 mg/mL to about 2 mg/mL, more preferably from about 0.7 mg/mL to about 1.5 mg/mL.

[0129] Typically, in the *in vitro* method of the subject technology, UDP-Glucose is included in the buffer at a concentration of from about 0.2 mM to about 5 mM, preferably from about 0.5 mM to about 2 mM, more preferably from about 0.7 mM to about 1.5 mM. In an embodiment, when a recombinant sucrose synthase is included in the reaction, sucrose is also included in the buffer at a concentration of from about 100 mM to

about 500 mM, preferably from about 200 mM to about 400 mM, more preferably from about 250 mM to about 350 mM.

[0130] Typically, in the *in vitro* method of the subject technology, the weight ratio of the recombinant polypeptide to the substrate, on a dry weight basis, is from about 1:100 to about 1:5, preferably from about 1:50 to about 1:10, more preferably from about 1:25 to about 1:15.

[0131] Typically, the reaction temperature of the *in vitro* method is from about 20 °C to about 40 °C, suitably from 25 °C to about 37 °C, more suitably from 28 °C to about 32 °C.

[0132] The present disclosure also provides for a steviol glycoside composition produced by the biosynthetic method described herein. The exact nature of the steviol glycoside composition produced using the method described herein, such as types of molecular species and their percentage content in the final product, depends on the substrate used, the incubation conditions, and the enzymatic activities included in the reaction system. For example, when stevioside is used as substrate, the steviol glycoside composition produced can include rebaudioside A, rebaudioside D, rebaudioside E, rebaudioside Z1 and rebaudioside Z2 and combinations thereof.

[0133] The subject technology provides a method for converting the predominant steviol glycoside species (i.e. stevioside and rebaudioside A) in the natural stevia extract into rebaudioside D and rebaudioside E, which are otherwise of low abundance in the natural extract. Accordingly, the subject technology also provides a method of enriching the content of one or more specific steviol glycosides (such as rebaudioside D and rebaudioside E), the method including incubating a substrate (such as a natural stevia extract) with a recombinant polypeptide comprising an amino acid sequence having at least 80% identity to SEQ ID NO:6. For example, when natural stevia extract is used as substrate, the steviol glycoside composition produced can be enriched with rebaudioside D and/or rebaudioside E, which are of low abundance in the natural stevia extract.

[0134] One with skill in the art will recognize that the steviol glycoside composition produced by the method described herein can be further purified and mixed

with other steviol glycosides, flavors, or sweeteners to obtain a desired flavor or sweetener composition. For example, a composition enriched with rebaudioside D produced as described herein can be mixed with a natural stevia extract containing rebaudioside A as the predominant steviol glycoside, or with other synthetic or natural steviol glycoside products to make a desired sweetener composition. Alternatively, a substantially purified steviol glycoside (e.g., rebaudioside D) obtained from the steviol glycoside composition described herein can be combined with other sweeteners, such as sucrose, maltodextrin, aspartame, sucralose, neotame, acesulfame potassium, and saccharin. The amount of steviol glycoside relative to other sweeteners can be adjusted to obtain a desired taste, as known in the art. The steviol glycoside composition described herein (including rebaudioside D, rebaudioside E, rebaudioside Z1, rebaudioside Z2 or a combination thereof) can be included in food products (such as beverages, soft drinks, ice cream, dairy products, confectioneries, cereals, chewing gum, baked goods, etc.), dietary supplements, medical nutrition, as well as pharmaceutical products.

EXAMPLES

EXAMPLE 1: Selection of candidate UGT genes

[0135] Phylogenetic and protein BLAST analysis were used to identify 7 candidate genes belonging to the UGT91 subfamily for 1,2-19-O-glucose glycosylation activity (Table 1).

Table 1: List of UGT candidate genes

Name	Description	Accession	Sequence ID
BD1	PREDICTED: UDP-glycosyltransferase 91C1-like [Brachypodium distachyon]	XP_003560664.1	SEQ ID NO: 1
BD2	PREDICTED: UDP-glycosyltransferase 91C1-like [Brachypodium distachyon]	XP_003560669.1	SEQ ID NO: 2
BD3	PREDICTED: LOW QUALITY PROTEIN: UDP-glycosyltransferase 91C1-like [Brachypodium distachyon]	XP_003581636.1	SEQ ID NO: 3

BD4	PREDICTED: UDP-glycosyltransferase 91C1-like [Brachypodium distachyon]	XP_003580515.1	SEQ ID NO: 4
BD5	PREDICTED: LOW QUALITY PROTEIN: UDP-glycosyltransferase 91B1-like [Brachypodium distachyon]	XP_003559500.1	SEQ ID NO: 5
HV1	predicted protein [Hordeum vulgare subsp. vulgare]	BAJ98242.1	SEQ ID NO: 6
HV2	predicted protein [Hordeum vulgare subsp. vulgare]	BAJ93155.1	SEQ ID NO: 8

Example 2: Enzymatic activity screening of candidate UGT genes

[0136] Full length DNA fragments of all candidate UGT genes were commercially synthesized. Almost all codons of the cDNA were changed to those preferred for *E. coli* (Genscript, NJ). The synthesized DNA was cloned into a bacterial expression vector pETite N-His SUMO Kan Vector (Lucigen).

[0137] Each expression construct was transformed into *E. coli* BL21 (DE3), which was subsequently grown in LB media containing 50 μg/mL kanamycin at 37 °C until reaching an OD600 of 0.8-1.0. Protein expression was induced by addition of 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) and the culture was further grown at 16 °C for 22 hr. Cells were harvested by centrifugation (3,000 x g; 10 min; 4 °C). The cell pellets were collected and were either used immediately or stored at -80 °C.

[0138] The cell pellets typically were re-suspended in lysis buffer (50 mM potassium phosphate buffer, pH 7.2, 25ug/ml lysozyme, 5ug/ml DNase I, 20 mM imidazole, 500 mM NaCl, 10% glycerol, and 0.4% Triton X-100). The cells were disrupted by sonication under 4 °C, and the cell debris was clarified by centrifugation (18,000 x g; 30 min). Supernatant was loaded to a equilibrated (equilibration buffer: 50 mM potassium phosphate buffer, pH 7.2, 20 mM imidazole, 500 mM NaCl, 10% glycerol) Ni-NTA (Qiagen) affinity column. After loading of protein sample, the column was washed with equilibration buffer to remove unbound contaminant proteins. The His-tagged

UGT recombinant polypeptides were eluted by equilibration buffer containing 250mM imidozale.

[0139] The purified candidate UGT recombinant polypeptides were assayed for 1,2-19-O-glucose glycosylation activity by using stevioside or Reb A as the substrate (Figures 1A-1C). Typically, the recombinant polypeptide (10 μg) was tested in a 200 μl *in vitro* reaction system. The reaction system contains 50 mM potassium phosphate buffer, pH 7.2, 3 mM $MgCl_2$, 1 mg/ml stevioside or rebaudioside A, 1 mM UDP-glucose. The reaction was performed at 30 °C and terminated by adding 200 μL 1-butanol. The samples were extracted three times with 200 μL 1-butanol. The pooled fraction was dried and dissolved in 70 μL 80% methanol for high-performance liquid chromatography (HPLC) analysis. Stevia extract (Blue California, CA), containing 95% stevioside, was used as stevioside substrate. Rebaudioside A (purity 99%) was also supplied by Blue California.

[0140] The UGT catalyzed glycosylation reaction was be coupled to a UDP-glucose generating reaction catalyzed by a sucrose synthase (such as AtSUS1 of SEQ ID NO:9). In this method, the UDP-glucose was generated from sucrose and UDP (Figure 2), such that the addition of extra UDP-glucose can be omitted. AtSUS1 sequence (Bieniawska et al., Plant J. 2007, 49: 810-828) was synthesized and inserted into a bacterial expression vector. The recombinant AtSUS1 protein was expressed and purified by affinity chromatography. The purified recombinant AtSUS1 polypeptide was analyzed by SDS-PAGE (molecular weight: 106.3 kD, Figure 7).

[0141] Accordingly, the activities of the recombinant UGT polypeptides were tested without AtSUS1 coupling (50 mM potassium phosphate buffer, pH 7.2, 3mM $MgCl_2$, 1mg/ml stevioside or rebaudioside A, 1mM UDP) or with AtSUS coupling (50 mM potassium phosphate buffer, pH 7.2, 3 mM $MgCl_2$, 1mg/ml stevioside or rebaudioside A, 1mM UDP and 285mM sucrose). Typically, 10 μg of AtSUS1 was used for a 200 μl *in vitro* reaction. The *in vitro* reaction was incubated at 30°C, and was stopped by extraction using 1-butonal.

[0142] HPLC analysis was then performed using a Dionex UPLC ultimate 3000 system (Sunnyvale, CA), including a quaternary pump, a temperature controlled column

compartment, an auto sampler and a UV absorbance detector. Phenomenex™ Luna™ NH2 with guard column was used for the characterization of steviol glycosides. Acetonitrile in water was used for isocratic elution in HPLC analysis. Rebaudioside D, rebaudioside E, rebaudioside Z products were identified by NMR analysis.

[0143] The recombinant polypeptide (SEQ ID NO:6) encoded by SEQ ID NO:7 showed a 1,2-19-O-glucose glycosylation activity, and was subjected to additional analysis. The gene was derived from *Hordeum vulgare subsp. vulgare* (abbreviated as "HV1" herein). The purified recombinant HV1 polypeptide was analyzed by SDS-PAGE (Figure 6). As shown in Figure 6, the recombinant HV1 protein (molecular weight: 61.4kD) was purified by affinity chromatography. The polypeptides encoded by other candidate genes (Table 1) did not show any detectable activity in the assays described herein, even though they share about 62-74% sequence identity with the recombinant HV1 polypeptide.

[0144] As described herein, the recombinant polypeptide of HV1 transferred a sugar moiety to rebaudioside A to produce rebaudioside D in all reaction conditions with or without AtSUS1. Rebaudioside A was completely converted to rebaudioside D by the recombinant HV1 polypeptide in a UGT-SUS coupling reaction system (Figure 3B-E). However, only partial rebaudioside A was converted to rebaudioside D after 24 hours by the recombinant HV1 polypeptide alone without being coupled to AtSUS1 (Figure 3F-G). Thus, the recombinant HV1 polypeptide showed a 1,2-19-O-glucose glycosylation activity to produce rebaudioside D from rebaudioside A and AtSUS1 enhanced the conversion efficiency in the UGT-SUS coupling system.

[0145] In addition, the recombinant HV1 polypeptide coupled with AtSUS1 (SEQ ID NO:9) converted stevioside to rebaudioside E *in vitro* (Figure 4). An unexpected compound ("Reb Z") having a HPLC retention time 6.68 minute (see, Figure 4) that was distinctive from rebaudiosides D and E was produced. This compound represents a novel steviol glycoside, and is termed "rebaudioside Z" ("Reb Z"). To confirm the conversion of Reb E to Reb Z, Reb E substrate (0.5mg/ml) was incubated with the recombinant HV1 polypeptide (20 μg) and AtSUS1 (20 μg) in a UGT-SUS coupling reaction system (200 μL) under conditions similar to those used in the examples above. As shown in Figure 8,

Date Reçue/Date Received 2021-01-12

Reb Z was produced by the combination of the recombinant HV1 polypeptide and AtSUS1. These results indicated that HV1 can transfer glucose moiety to Reb E to form Reb Z.

EXAMPLE 3: Steviol glycoside biosynthesis using the recombinant HV1 polypeptide

[0146] As shown in Figures 1A-1C, rebaudioside D can also be formed by glycosylation of the C-3' of the C-13-O-glucose of rebaudioside E. Thus, rebaudioside D can be produced by different biosynthetic routes (e.g. via rebaudioside A vs. rebaudioside E), depending on the orders in which the glycosylation reactions occur. For example, glycosylation at C-3' of the C-13-O-glucose of stevioside can occur first to produce the intermediate rebaudioside A, followed by glycosylation at C-2' of the 19-O-glucose of rebaudioside A to produce rebaudioside D. So far, UGT76G1 (SEQ ID NO:11) from stevia has been identified as an enzyme that transfers a sugar residue to C-3' of the C-13-O-glucose of stevioside to form rebaudioside A.

[0147] Codon optimized UGT76G1 cDNA was inserted in a bacterial expression vector, and the recombinant UGT76G1 protein was expressed and purified by affinity chromatography. The purified recombinant UGT76G1 polypeptide was analyzed by SDS-PAGE (molecular weight: 65.4 kD, Figure 9). The rebaudioside E substrate was incubated with the recombinant UGT76G1, with or without AtSUS1, under conditions similar to those used in the Examples above. The products were analyzed by HPLC. As shown in Figure 12, rebaudioside D was produced by the recombinant UGT76G1. Addition of recombinant AtSUS in the reaction enhanced the conversion efficiency in UGT-SUS coupling system. Thus, the recombinant UGT76G1 polypeptide showed a 1,3-13-O-glucose glycosylation activity to produce Reb D from Reb E.

[0148] Accordingly, the catalytic activity of the recombinant HV1 polypeptide for steviol glycoside biosynthesis (e.g., production of rebaudioside D) was further determined in combination with UGT76G1. Stevioside substrate was incubated with the recombinant HV1 polypeptide (10 μg), UGT76G1 (10 μg), and AtSUS1 (10 μg) in a UGT-SUS coupling reaction system (200 μL) under conditions similar to those used in the examples above. The products were analyzed by HPLC. As shown in Figure 5, rebaudioside D was produced by the combination of the recombinant HV1 polypeptide, UGT76G1, and

AtSUS1. Thus, the recombinant HV1 polypeptide, which showed at least a 1,2-19-O-glucose glycosylation activity, can be used in combination with other UGT enzymes (such as UGT76G1) for the complex, multi-step biosynthesis of steviol glycosides.

Example 4: NMR analysis the structure of Reb Z

[0149] The material used for the characterization of rebaudioside Z (Reb Z) was produced using enzymatic conversion of rebaudioside E and purified by HPLC.

[0150] HRMS data were generated with a LTQ™ Orbitrap™ Discovery HRMS instrument, with its resolution set to 30k; scanned data from *m/z* 150 to 1500 in positive ion electrospray mode. The needle voltage was set to 4 kV; the other source conditions were sheath gas = 25, aux gas = 0, sweep gas = 5 (all gas flows in arbitrary units), capillary voltage = 30V, capillary temperature = 300°C, and tube lens voltage = 75. The sample was diluted with 2:2:1 acetonitrile:methanol:water (same as infusion eluent) and injected 50 microliters.

[0151] NMR spectra were acquired on Bruker Avance™ DRX 500 MHz or Varian INOVA™ 600 MHz instrument instruments using standard pulse sequences. The 1D (^{1}H and ^{13}C) and 2D (COSY, TOCSY, HMQC, and HMBC) NMR spectra were performed in C_5D_5N.

[0152] Compound Reb Z, shown as a mixture of Reb Z1 and Reb Z2, is shown in Figure 10. The molecular formula of compound Reb Z has been deduced as $C_{50}H_{80}O_{28}$ on the basis of its positive high resolution (HR) mass spectrum, which showed adduct ions corresponding to $[M+ Na]^+$ at *m/z* 1151.4713; this composition was supported by the ^{13}C NMR spectral data. The ^{1}H NMR spectral data of Reb Z showed the presence of a mixture of two compounds (Reb Z1 and Reb Z2) in the ratio between 60:40 to 70:30. Hence the ^{1}H and ^{13}C NMR spectral data of Reb Z showed a sets of peaks for each proton and carbon present in its structure. Acid hydrolysis of Reb Z with 5% H_2SO_4 afforded D-glucose which was identified by direct comparison with authentic sample by TLC. Enzymatic hydrolysis of Reb Z furnished an aglycone, which was identified as steviol by comparison of ^{1}H NMR and co-TLC with standard compound. The ^{1}H and ^{13}C NMR values for

Date Reçue/Date Received 2021-01-12

compound Reb Z were assigned on the basis of TOCSY, HMQC and HMBC data. The large coupling constants observed for the five anomeric protons of the glucose moieties, suggested their β-orientation as reported for steviol glycosides.

Table 2. ^{1}H and ^{13}C NMR spectral data (chemical shifts and coupling constants) for rebaudioside Z ("Reb Z"), and rebaudioside E [a-c].

Position	Reb Z		Rebaudioside E	
	^{1}H NMR	^{13}C NMR	^{1}H NMR	^{13}C NMR
1	0.74 t (12.7), 1.65 m	41.2	0.73 t (13.2), 1.68 m	41.0
2	1.45 m, 2.12 m	20.6	1.46 m, 2.13 m	20.6
3	1.13 m, 2.922 d (13.2)/2.79 d (12.8)	38.2	1.12 m, 2.78 d (12.8)	38.2
4	---	44.9/44.8	---	44.8
5	0.99 m	58.1	0.97 d (11.8)	57.9
6	1.87 m, 2.14 m	22.6	1.85 m, 2.09 m	22.6
7	1.24 m, 1.68 m	42.2	1.27 m, 1.63 m	42.1
8	---	43.2/43.1	---	43.0

9	0.88 br s	54.5	0.88 br s	54.5
10	---	40.2	---	40.2
11	1.68 m	21.1	1.65 m	21.1
12	1.92 m, 2.28 m	37.8	1.96 m, 2.16 m	37.8
13	---	86.6	---	86.6
14	1.72 m, 2.48 d (10.8)	44.9/44.8	1.74 d (11.4), 2.54 d (11.0)	44.8
15	1.92 m, 2.14 m	48.5	2.04 m, 2.12 m	48.5
16	---	155.2/155.0	---	154.9
17	5.09/5.12 s, 5.68/5.74 s	105.4/105.2	5.09 s, 5.76 s	105.4
18	1.43/1.49 s	29.9	1.43 s	29.8
19	---	176.3/176.1	---	176.2
20	1.09 s	17.3	1.10 s	17.2
1′	6.30 d (7.9)/ 6.35 d (7.8)	93.9/93.6	6.30 d (7.9)	93.9

2′	4.38 m	81.8	4.38 m	81.7
3′	4.27 m	78.5	4.26 m	78.4
4′	4.24 m	71.8	4.22 m	71.8
5′	3.94 m	79.6	3.92 m	79.5
6′	4.33 m, 4.46 m	62.6	4.33 m, 4.43 m	62.6
1′′	5.12 d (7.4)/ 5.14 d (7.4)/	98.4/98.2	5.16 d (7.5)	98.4
2′′	4.18 m	84.9	4.17 m	84.9
3′′	4.29 m	78.6	4.32 m	78.5
4′′	4.20 m	72.1	4.22 m	72.1
5′′	3.74 m	78.5	3.72 m	78.2
6′′	4.32 m, 4.38 m	62.8	4.26 m, 4.35 m	62.9
1′′′	5.33 d (7.8)/ 5.46 d (7.8)/	106.7/104.5	5.32 d (7.5)	107.2
2′′′	4.14 t (8.4)	85.7/77.7	4.15 t (8.4)	77.7

3′′′	4.25 m	78.7	4.26 m	78.6
4′′′	4.34 m	72.1/71.5	4.36 m	72.3
5′′′	3.88 m	79.1	3.96 m	79.0
6′′′	4.43 m, 4.56 m	63.1	4.46 m, 4.56 m	63.2
1′′′′	5.48 d (7.9)/ 5.40 d (7.6)	106.3/104.4	5.48 d (7.9)	106.2
2′′′′	4.04 t (7.9)	85.6/77.3	4.06 t (7.9)	76.8
3′′′′	4.22 m	78.8	4.25 m	78.7
4′′′′	4.38 m	71.4/71.0	4.31 m	71.2
5′′′′	3.96 m	79.1	4.02 m	79.1
6′′′′	4.38 m, 4.57 m	63.4	4.42 m, 4.54 m	63.4
1′′′′′	5.29 d (7.5)/ 5.34 d (7.5)/	107.1		
2′′′′′	4.02 m	77.0		

3′ ′ ′ ′ ′	4.21 m	78.6		
4′ ′ ′ ′ ′	4.25 m	71.6/71.2		
5′ ′ ′ ′ ′	3.98 m	79.1		
6′ ′ ′ ′ ′	4.34 m, 4.48 m	63.3		

[a] assignments made on the basis of TOCSY, HMQC and HMBC correlations; [b] Chemical shift values are in δ (ppm); [c] Coupling constants are in Hz.

[0153] Based on the results from NMR spectral data and hydrolysis experiments of Reb Z, and a close comparison of the ^{1}H and ^{13}C NMR values of Reb Z with rebaudioside E suggested the mixture of two compounds produced by the enzymatic conversion were deduced as 13-[(2-O-β-D-glucopyranosyl-2-O-β-D-glucopyranosyl-β-D-glucopyranosyl)oxy] *ent*-kaur-16-en-19-oic acid-2-O-β-D-glucopyranosyl-β-D-glucopyranosyl ester (Reb Z1) or 13-[(2-O-β-D-glucopyranosyl-β-D-glucopyranosyl)oxy] *ent*-kaur-16-en-19-oic acid-[(2-O-β-D-glucopyranosyl-2-O-β-D-glucopyranosyl-β-D-glucopyranosyl) ester (Reb Z2).

[0154] Acid hydrolysis of compound Reb Z. To a solution of compound Reb Z (5 mg) in MeOH (10 ml) was added 3 ml of 5% H_2SO_4 and the mixture was refluxed for 24 hours. The reaction mixture was then neutralized with saturated sodium carbonate and extracted with ethyl acetate (EtOAc) (2 x 25 ml) to give an aqueous fraction containing sugars and an EtOAc fraction containing the aglycone part. The aqueous phase was concentrated and compared with standard sugars using the TLC systems EtOAc/n-butanol/water (2:7:1) and CH_2Cl_2/MeOH/water (10:6:1); the sugars were identified as D-glucose.

[0155] Enzymatic hydrolysis of compound Reb Z. Compound Reb Z (1 mg) was dissolved in 10 ml of 0.1 M sodium acetate buffer, pH 4.5 and crude pectinase from Aspergillus niger (50 uL, Sigma-Aldrich, P2736) was added. The mixture was stirred at

50°C for 96 hr. The product precipitated out during the reaction and was filtered and then crystallized. The resulting product obtained from the hydrolysis of 1 was identified as steviol by comparison of its co-TLC with standard compound and 1H NMR spectral data (Figure 11).

[0156] A mixture of two compounds named Reb Z was produced by the bio-conversion of Rebaudioside E using enzymatic methodology and the structures were characterized as 13-[(2-O-β-D-glucopyranosyl-2-O-β-D-glucopyranosyl-β-D-glucopyranosyl)oxy] *ent*-kaur-16-en-19-oic acid-2-O-β-D-glucopyranosyl-β-D-glucopyranosyl ester (Reb Z1), or 13-[(2-O-β-D-glucopyranosyl-β-D-glucopyranosyl)oxy] *ent*-kaur-16-en-19-oic acid-[(2-O-β-D-glucopyranosyl-2-O-β-D-glucopyranosyl-β-D-glucopyranosyl) ester (Reb Z2), on the basis of extensive 1D and 2D NMR as well as high resolution mass spectral data and hydrolysis studies.

[0157] Thus, the 1,2-19-O-glucose glycosylation activity of the recombinant HV1 polypeptide was confirmed by its ability to transfer a second sugar moiety to the C-2' of a 19-O-glucose of stevioside to produce Rebaudioside E. HV1 recombinant polypeptide also has the activity to transfer the third glucose moiety to the C-2' of a 13-O-glucose or C-2' of a 19-O-glucose of rebaudioside E to produce Reb Z1 or Reb Z2.

CLAIMS:

1. A method of producing a steviol glycoside, the method comprising incubating a substrate with a recombinant polypeptide having UDP-glycosyltransferase activity and comprising an amino acid sequence having at least 80% identity to the amino acid sequence as set forth in SEQ ID NO:6 in the presence of UDP-glucose as the glucose donor, wherein the substrate is selected from the group consisting of stevioside, rebaudioside A, rebaudioside E, and combinations thereof.

2. The method of claim 1, further comprising incubating a recombinant sucrose synthase with the substrate and the recombinant polypeptide.

3. The method of claim 2, wherein the recombinant sucrose synthase comprises an amino acid sequence having at least 80% identity to the amino acid sequence as set forth in SEQ ID NO:9 and has sucrose synthase activity.

4. The method of claim 2, further comprising incubating a recombinant UDP-glycosyltransferase with the sucrose synthase, the substrate, and the recombinant polypeptide.

5. The method of claim 4, wherein the recombinant UDP-glycosyltransferase comprises an amino acid sequence having at least 80% identity to the amino acid sequence as set forth in SEQ ID NO:11 and has UDP-glycosyltransferase activity.

6. A method of producing rebaudioside Z1 and/or rebaudioside Z2, the method comprising incubating a substrate with a recombinant polypeptide having UDP-glycosyltransferase activity and comprising an amino acid sequence having at least 80% identity to the amino acid sequence as set forth in SEQ ID NO:6 in the presence of UDP-glucose as the glucose donor, wherein the substrate is selected from the group consisting of rubusoside, stevioside, and combinations thereof.

7. The method of claim 6, further comprising incubating a recombinant sucrose synthase with the substrate and the recombinant polypeptide.

Date Reçue/Date Received 2021-01-12

8. The method of claim 7, wherein the recombinant sucrose synthase comprises an amino acid sequence having at least 80% identity to the amino acid sequence as set forth in SEQ ID NO:9 and has sucrose synthase activity.

9. The method of claim 7, further comprising incubating a recombinant UDP-glycosyltransferase with the sucrose synthase, the substrate, and the recombinant polypeptide.

10. A method for synthesizing rebaudioside Z1 and/or rebaudioside Z2 from rebaudioside E, the method comprising:

preparing a reaction mixture comprising rebaudioside E and either:

(i) a recombinant polypeptide which is a UDP-glycosyltransferase comprising an amino acid sequence having at least 80% identity to the amino acid sequence of the HV1 glycosyltransferase as set forth in SEQ ID NO:6 and UDP-glucose, or

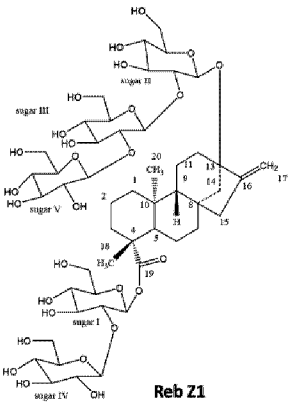
(ii) sucrose, UDP and UDP-glucose and a recombinant polypeptide which is a UDP-glycosyltransferase comprising an amino acid sequence having at least 80% identity to the amino acid sequence of the HV1 glycosyltransferase as set forth in SEQ ID NO:6 together with a sucrose synthase,

incubating the reaction mixture for a sufficient time whereby a glucose is covalently coupled to the C2' of the 13-O-glucose of rebaudioside E to produce rebaudioside Z1, and a glucose is covalently coupled to C2' of the 19-O-glucose of rebaudioside E to produce rebaudioside Z2.

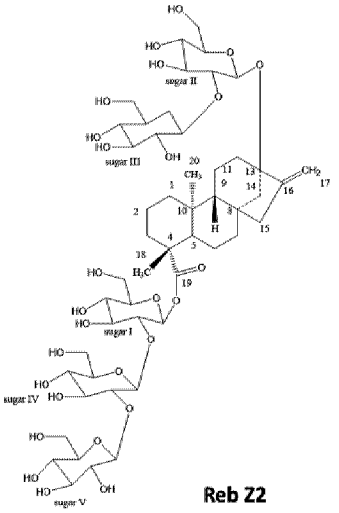
11. The method of claim 10, wherein the sucrose synthase is selected from the group consisting of an *Arabidopsis* sucrose synthase 1, an Arabidopsis sucrose synthase 3 and a *Vigna radiate* sucrose synthase.

12. The method of claim 11, wherein the sucrose synthase is an *Arabidopsis thaliana* sucrose synthase 1.

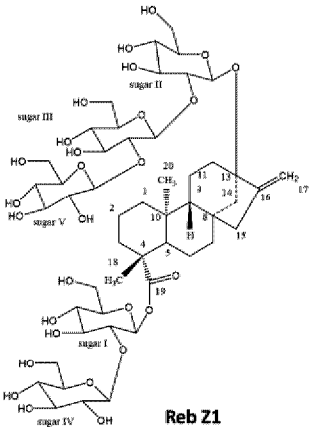
13. A rebaudioside Z1 compound of the structure:



14. A rebaudioside Z2 compound of the structure:



15. A sweetener comprising a compound having a chemical structure:

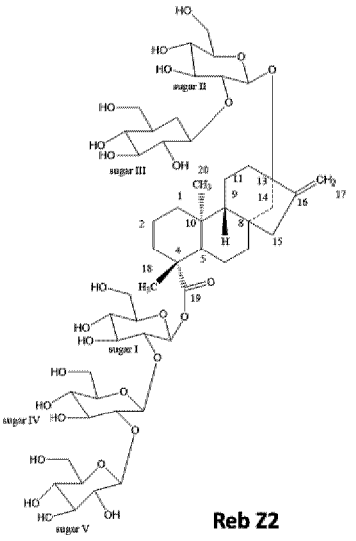


16. The sweetener of claim 15 further comprising at least one of a filler, a bulking agent and an anticaking agent.

17. A consumable product comprising a sweetening amount of the sweetener of claim 15.

18. The consumable product of claim 17 being selected from the group consisting of beverages, confectioneries, bakery products, cookies, and chewing gums.

19. A sweetener comprising a compound having a chemical structure:



Reb Z2

20. The sweetener of claim 19 further comprising at least one of a filler, a bulking agent and an anticaking agent.

21 A consumable product comprising a sweetening amount of the sweetener of claim 19.

22. The consumable product of claim 21 being selected from the group consisting of beverages, confectioneries, bakery products, cookies, and chewing gums.

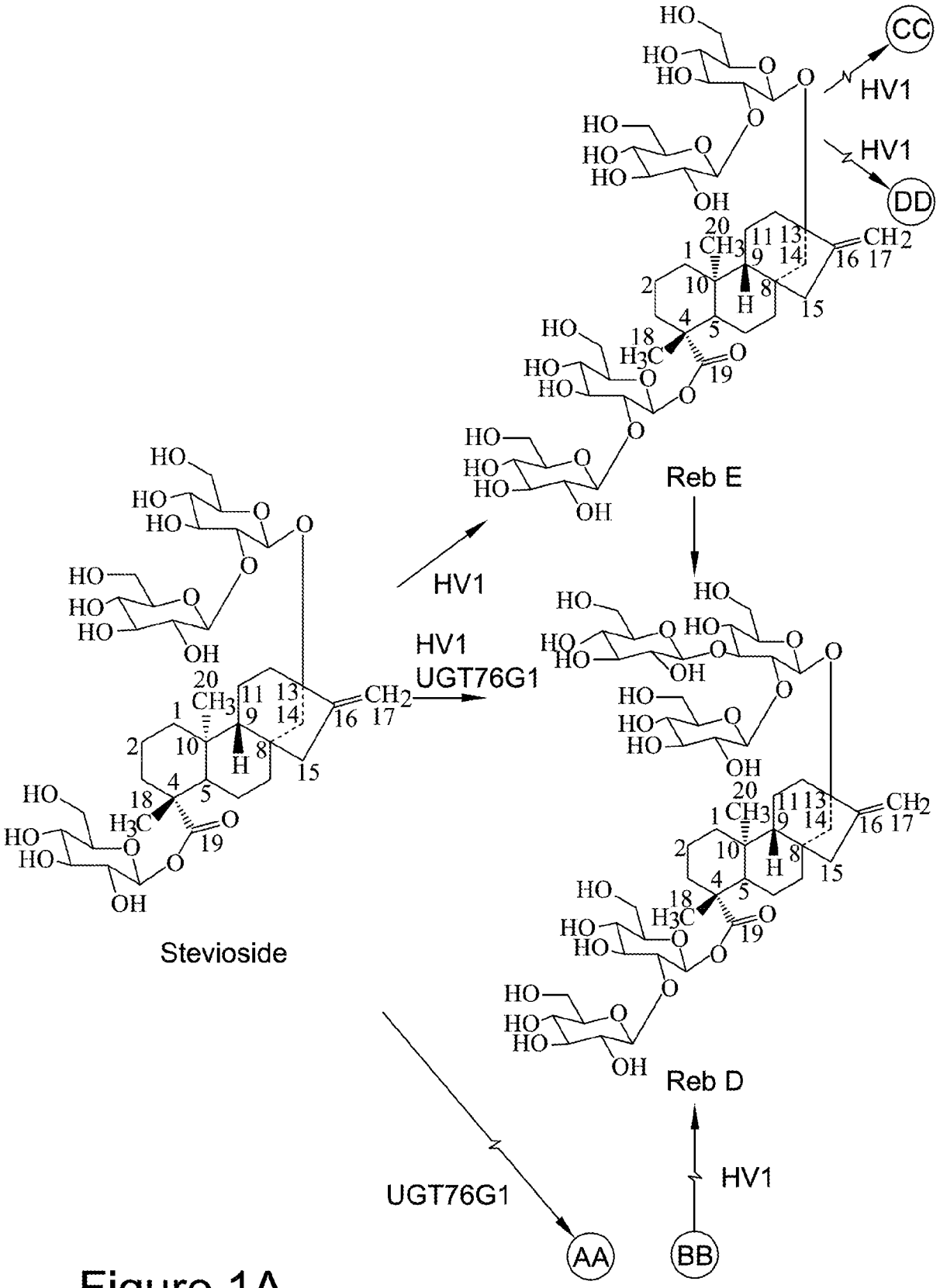

CC
HV1
HV1
DD
Reb E
HV1
HV1
UGT76G1
Stevioside
Reb D
HV1
UGT76G1
AA
BB

Figure 1A

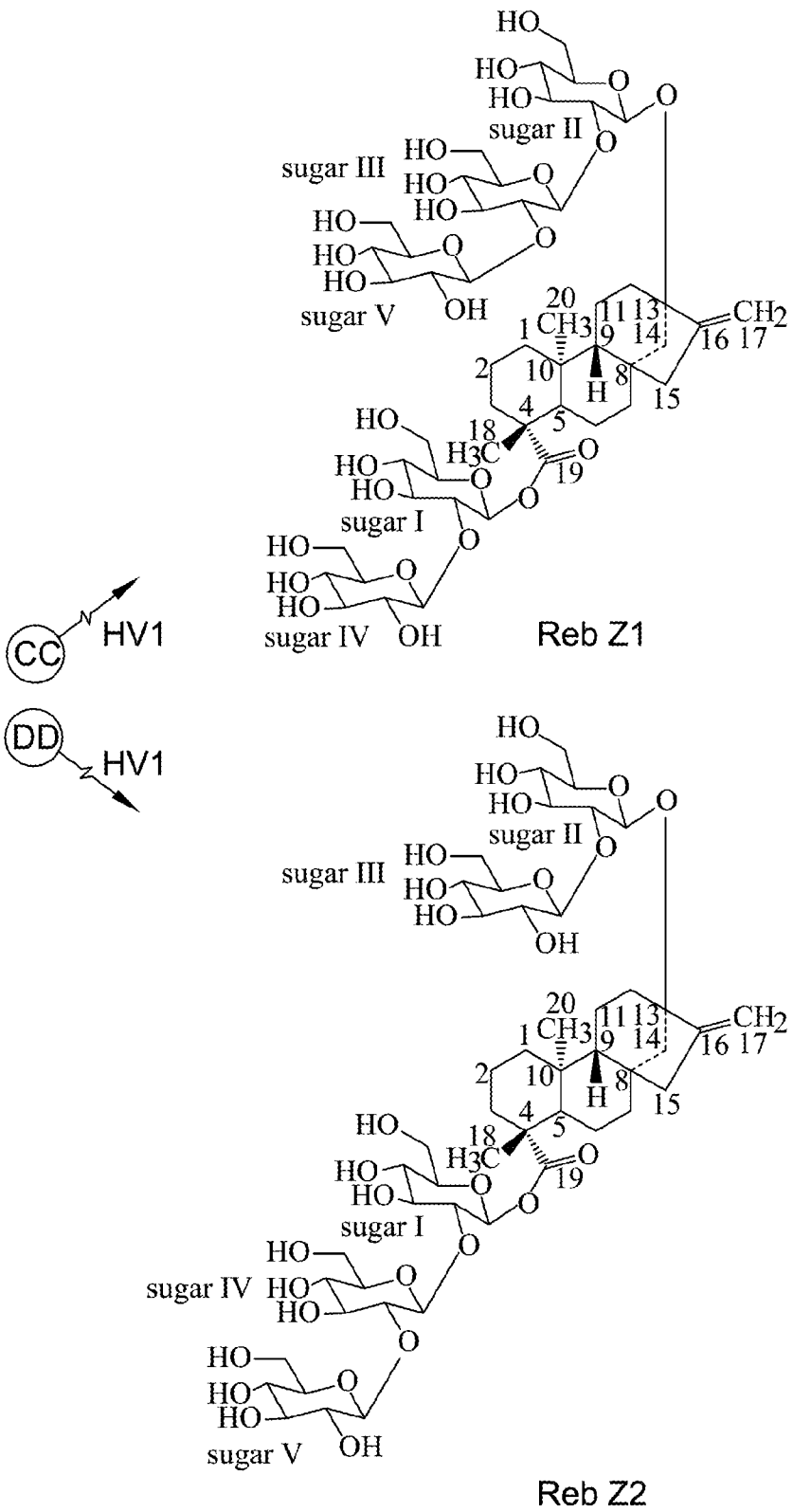

HO
HO
HO
O
O
sugar II
O
sugar III
HO
HO
HO
O
HO
HO
HO
O
O
sugar V
OH
20
1 CH3
11 13
9 14
CH2
16 17
2
10
H
8
15
4
5
18
H3C
O
19
HO
HO
HO
O
O
sugar I
O
HO
HO
HO
O
sugar IV
OH
CC
HV1
Reb Z1
DD
HV1
HO
HO
HO
O
O
sugar II
O
sugar III
HO
HO
HO
O
OH
20
1 CH3
11 13
9 14
CH2
16 17
2
10
H
8
15
4
5
18
H3C
O
19
HO
HO
HO
O
O
sugar I
O
HO
sugar IV HO
HO
O
O
HO
HO
HO
O
sugar V
OH
Reb Z2

Figure 1B

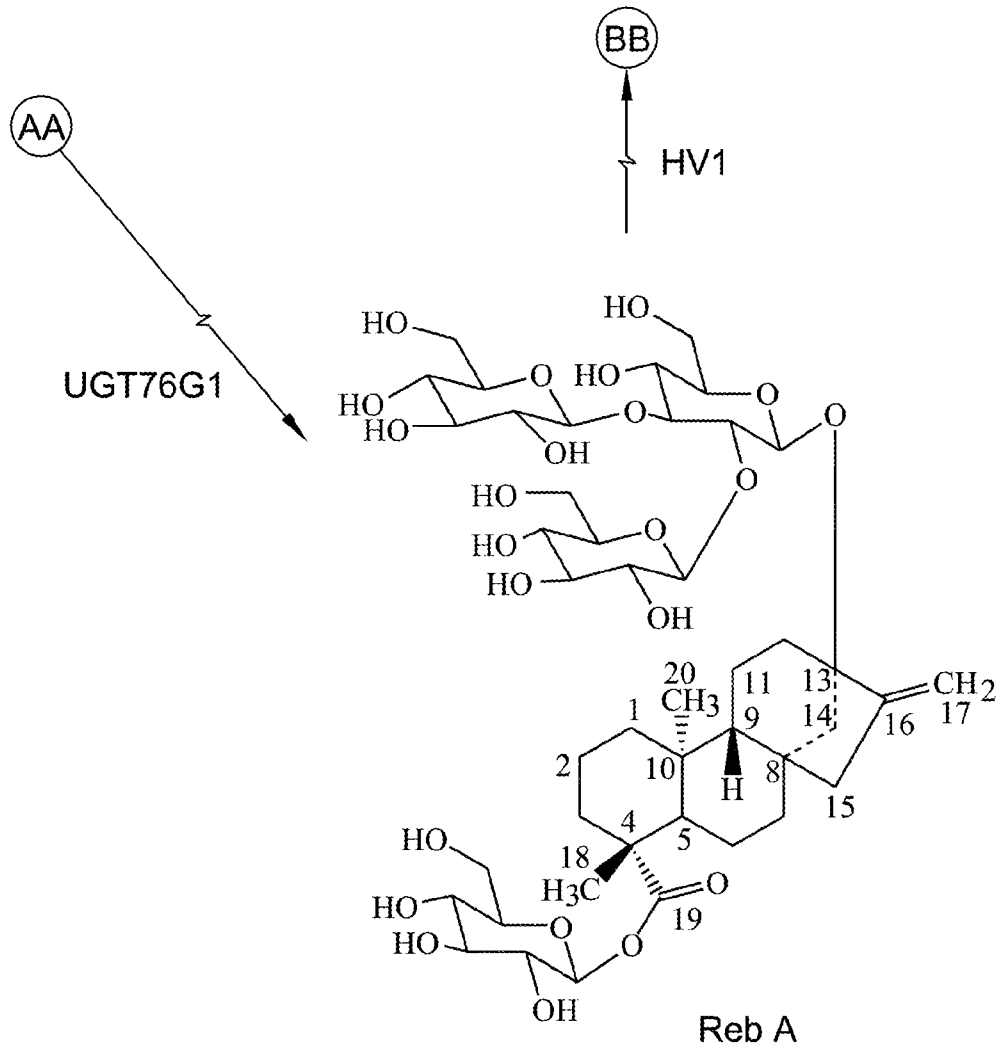

AA
BB
HV1
UGT76G1
HO
HO
HO
OH
O
HO
HO
HO
OH
O
20
CH3
1
2
10
9
H
8
11
13
14
16
CH2
17
15
4
5
18
H3C
O
19
HO
HO
HO
OH
O
Reb A

Figure 1C

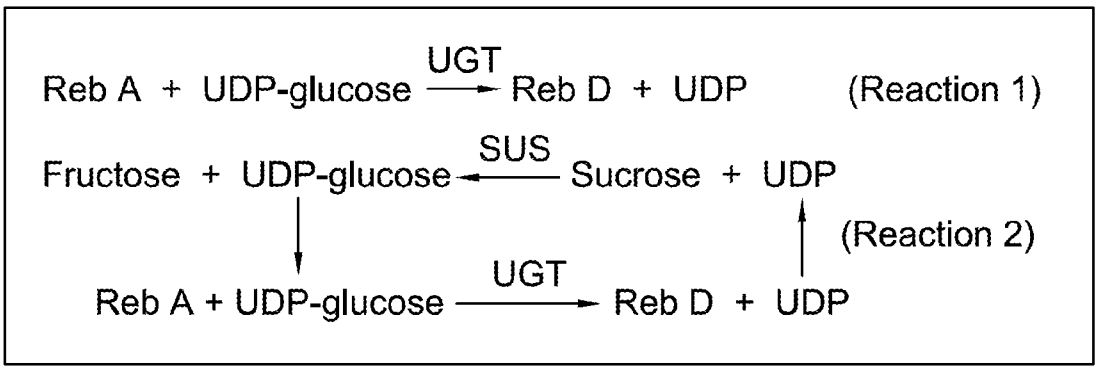

Reb A + UDP-glucose UGT Reb D + UDP (Reaction 1)
Fructose + UDP-glucose SUS Sucrose + UDP
(Reaction 2)
Reb A + UDP-glucose UGT Reb D + UDP

Figure 2

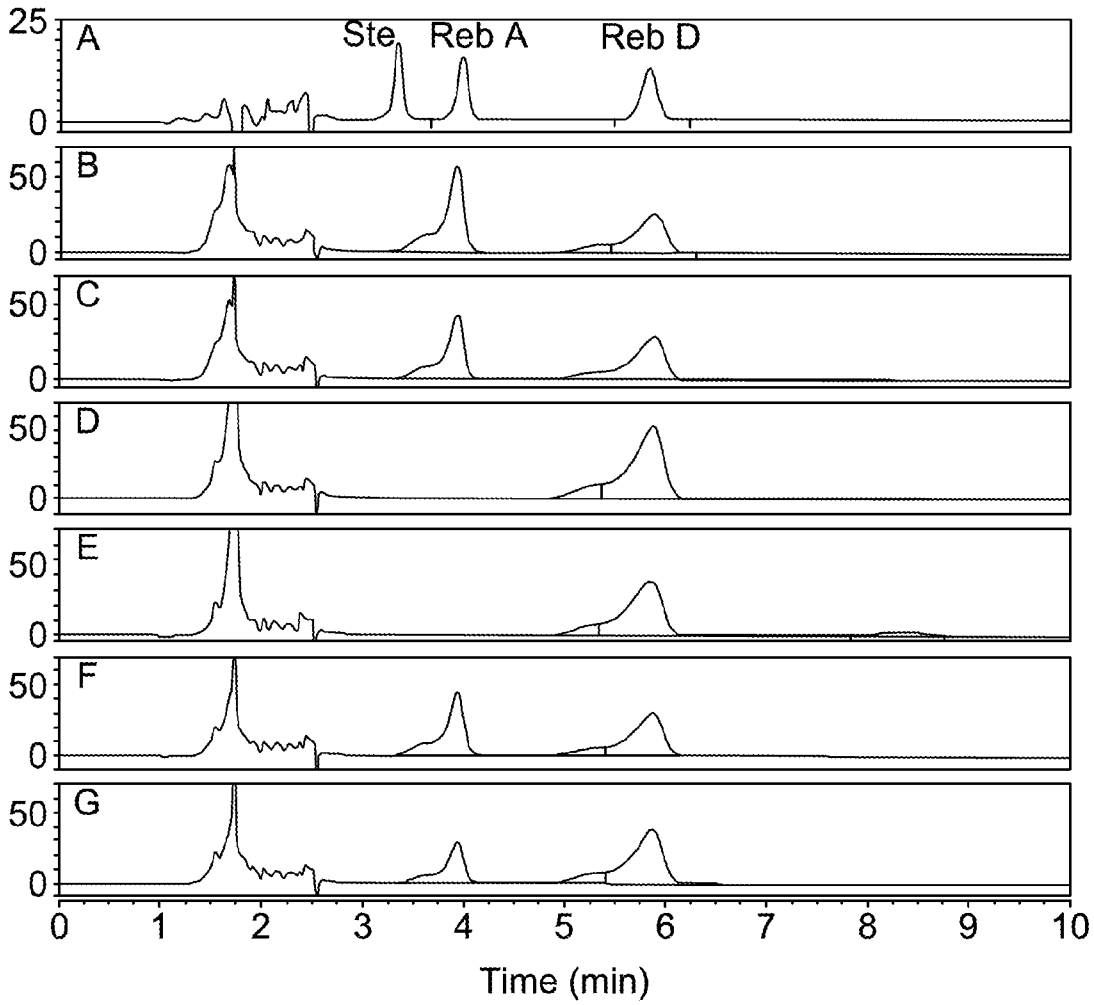

25
A
Ste
Reb A
Reb D
0
50
B
0
50
C
0
50
D
0
50
E
0
50
F
0
50
G
0
0 1 2 3 4 5 6 7 8 9 10
Time (min)

Figure 3

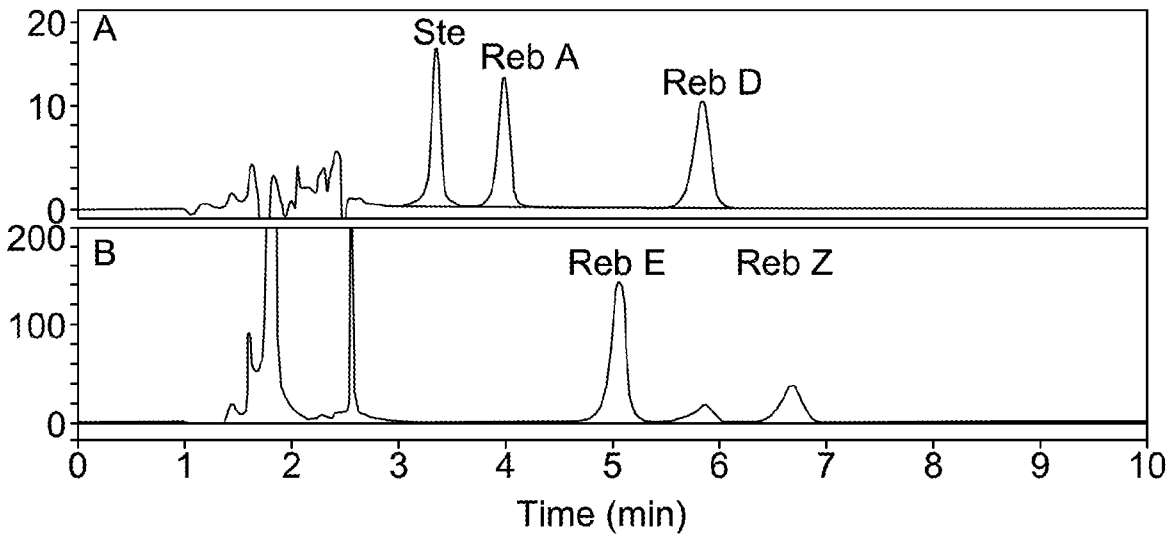

A
Ste
Reb A
Reb D
20
10
0
B
Reb E
Reb Z
200
100
0
0 1 2 3 4 5 6 7 8 9 10
Time (min)

Figure 4

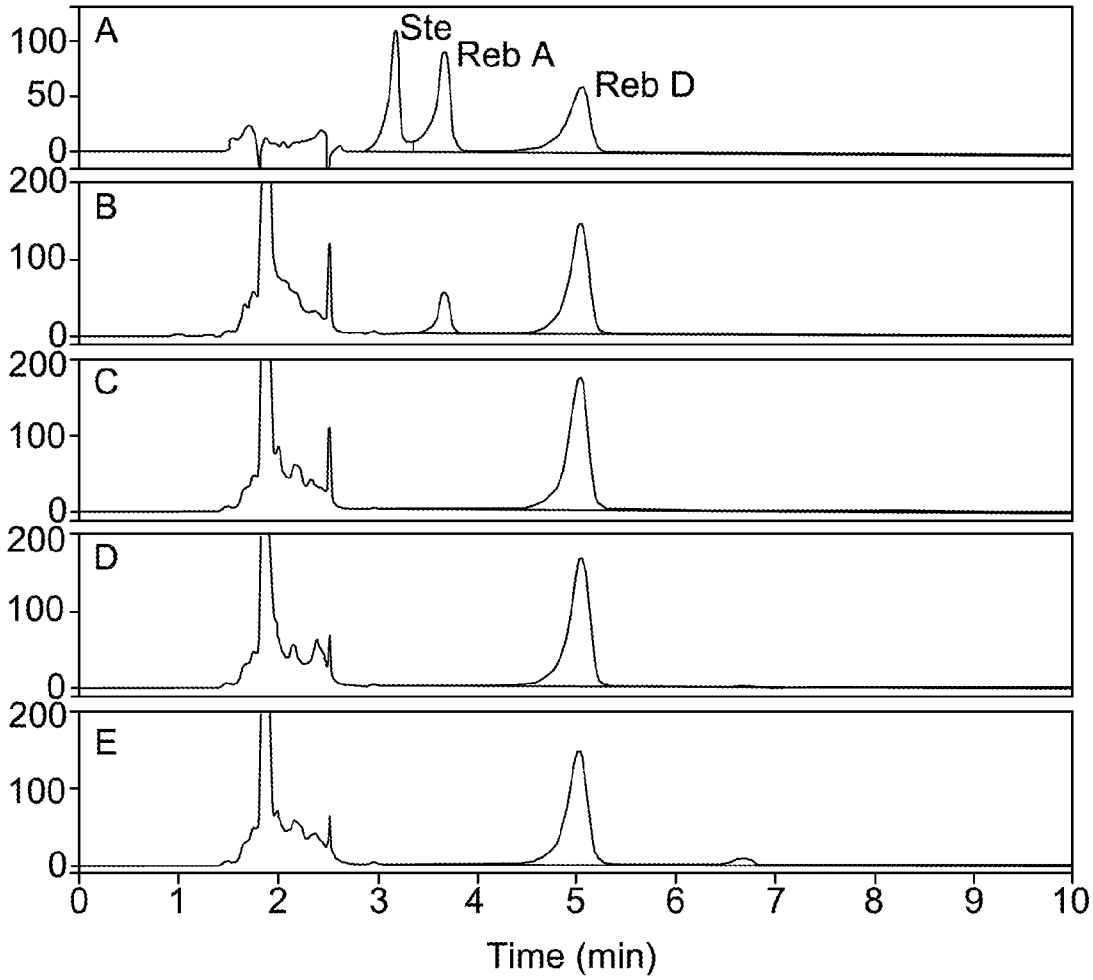

A
Ste
Reb A
Reb D
100
50
0
B
200
100
0
C
200
100
0
D
200
100
0
E
200
100
0
0 1 2 3 4 5 6 7 8 9 10
Time (min)

Figure 5

Figure 6

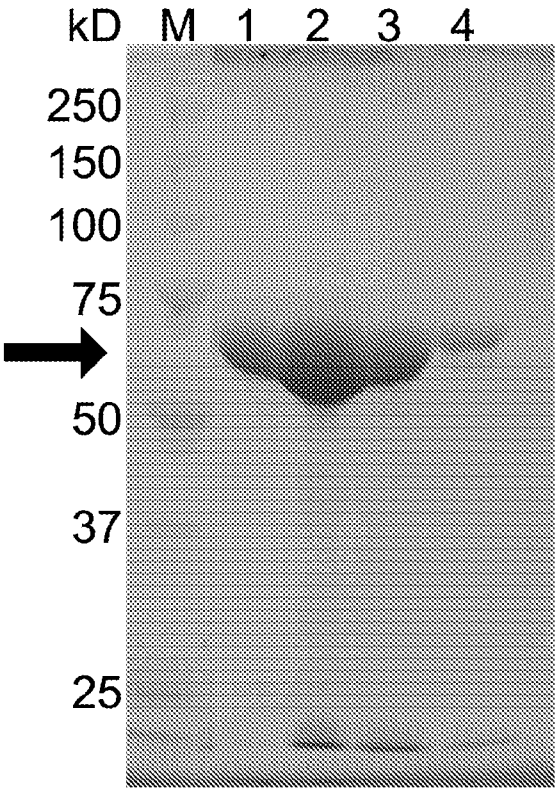
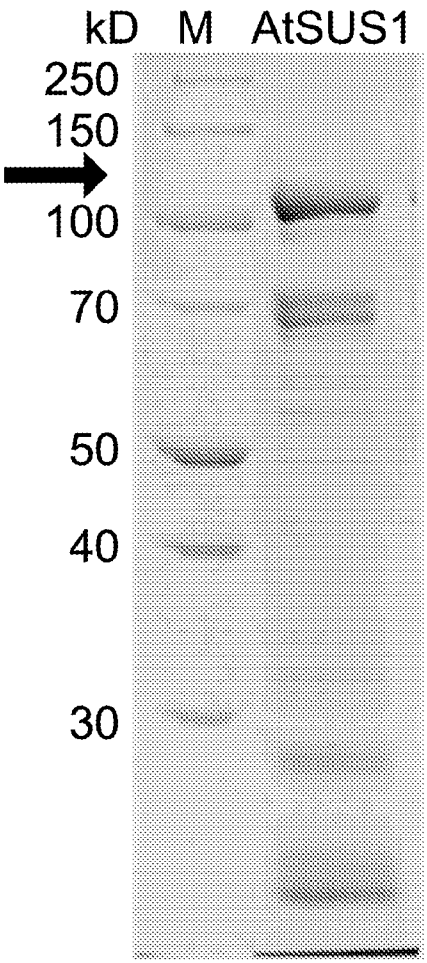


Figure 7



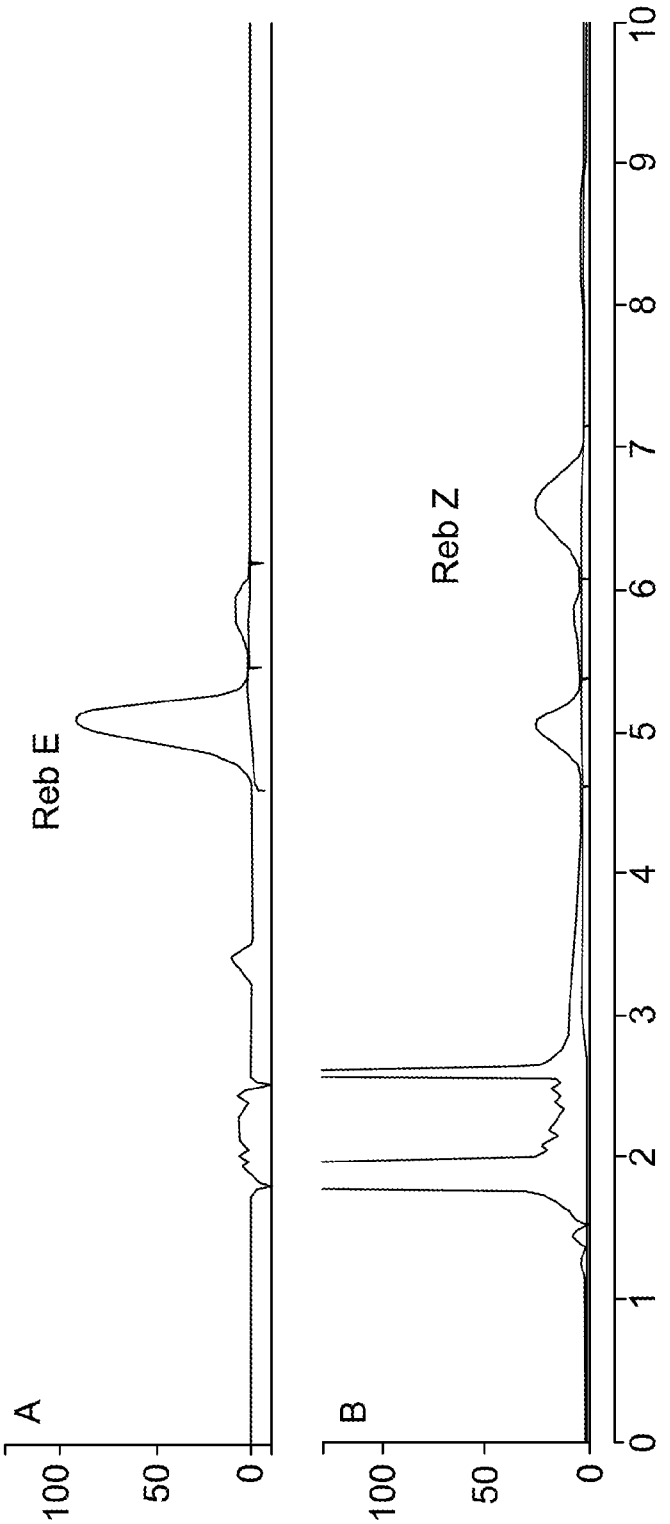
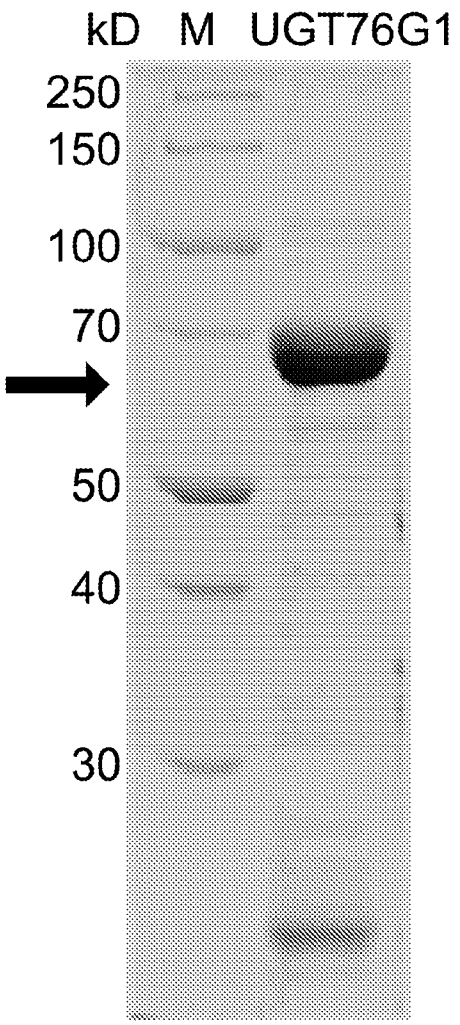


Figure 8

Figure 9



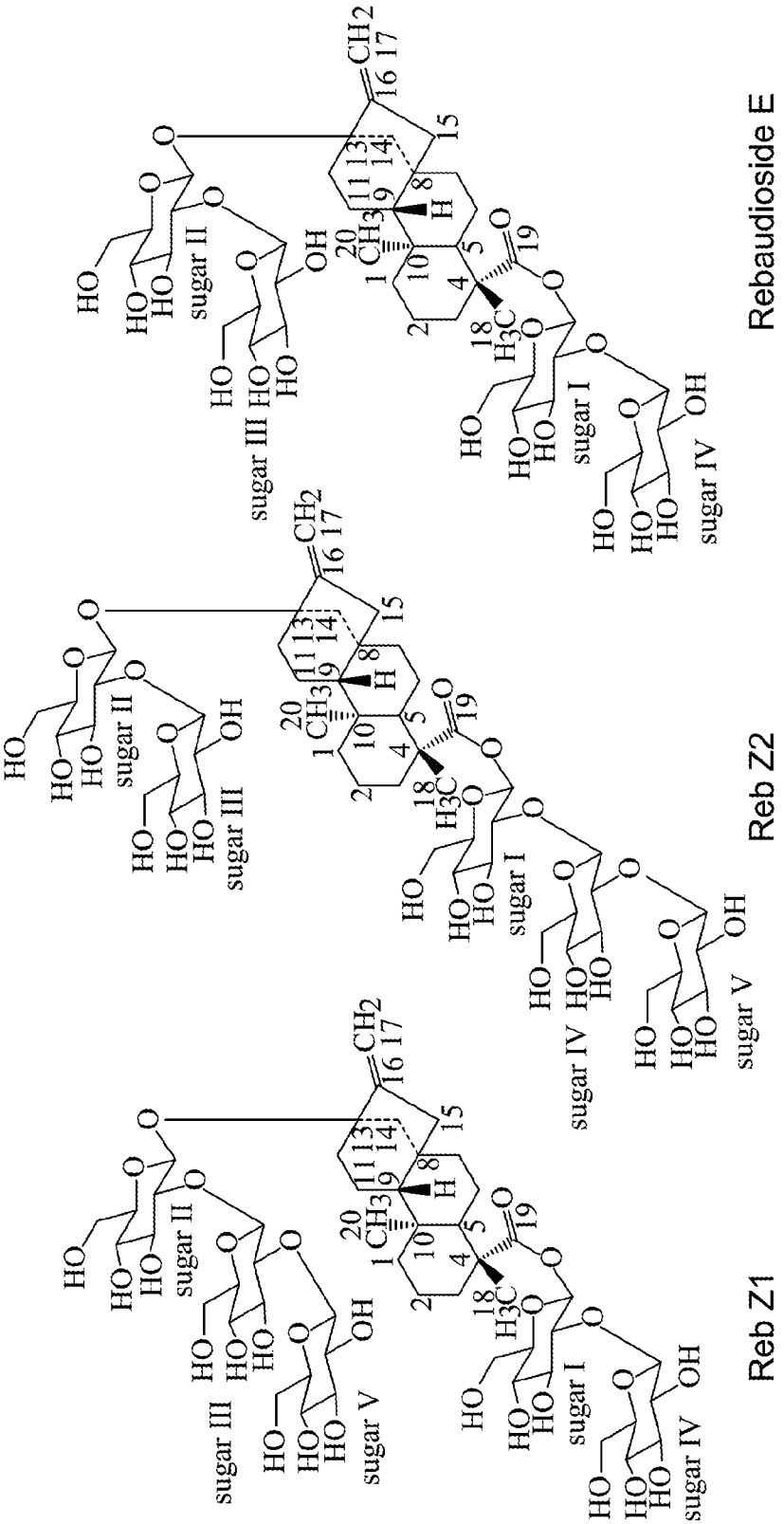

Rebaudioside E
Reb Z2
Reb Z1
sugar I
sugar II
sugar III
sugar IV
sugar V

Figure 10

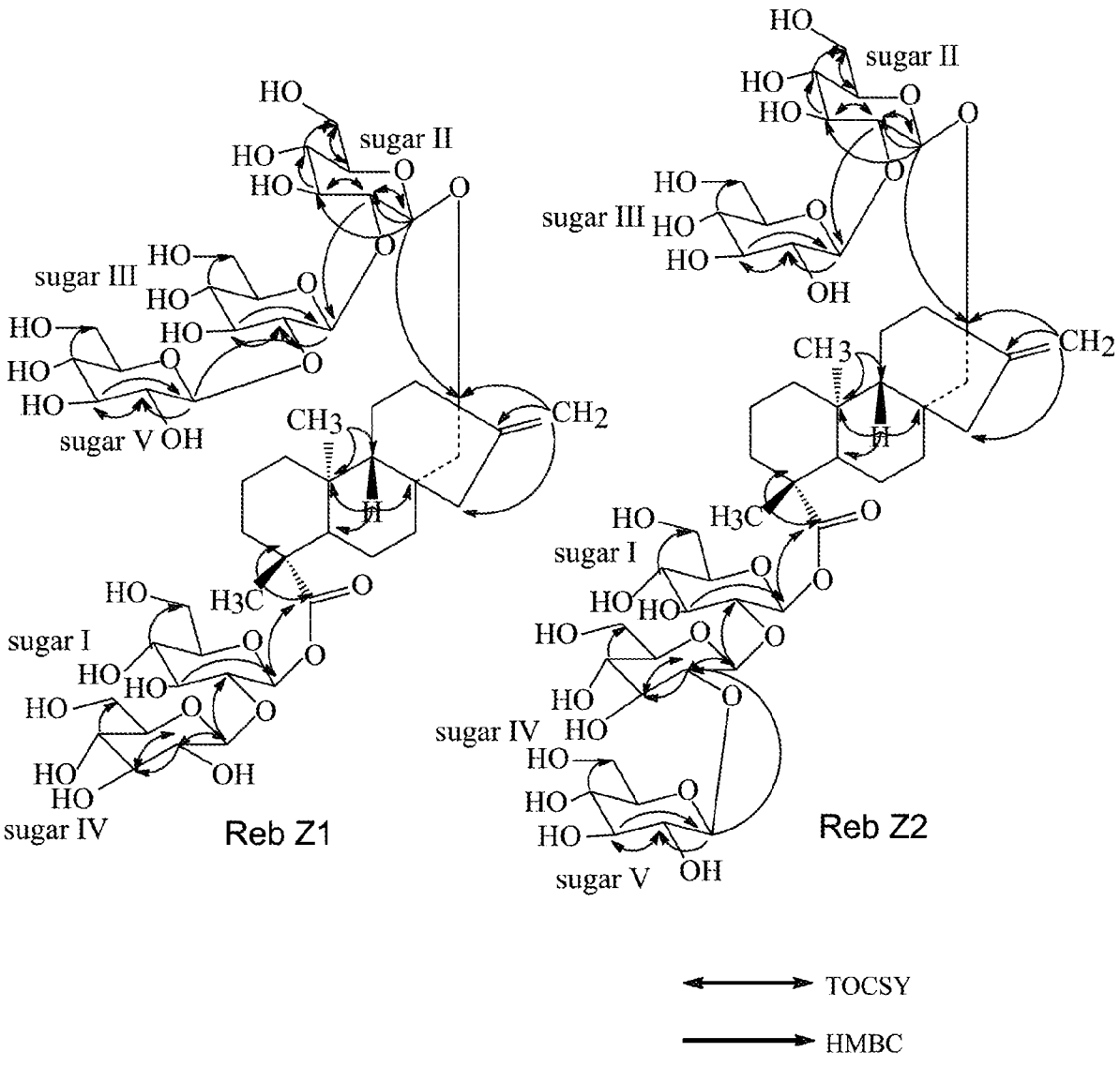

sugar II
sugar III
sugar V
OH
sugar I
sugar IV
Reb Z1
Reb Z2
TOCSY
HMBC

Figure 11

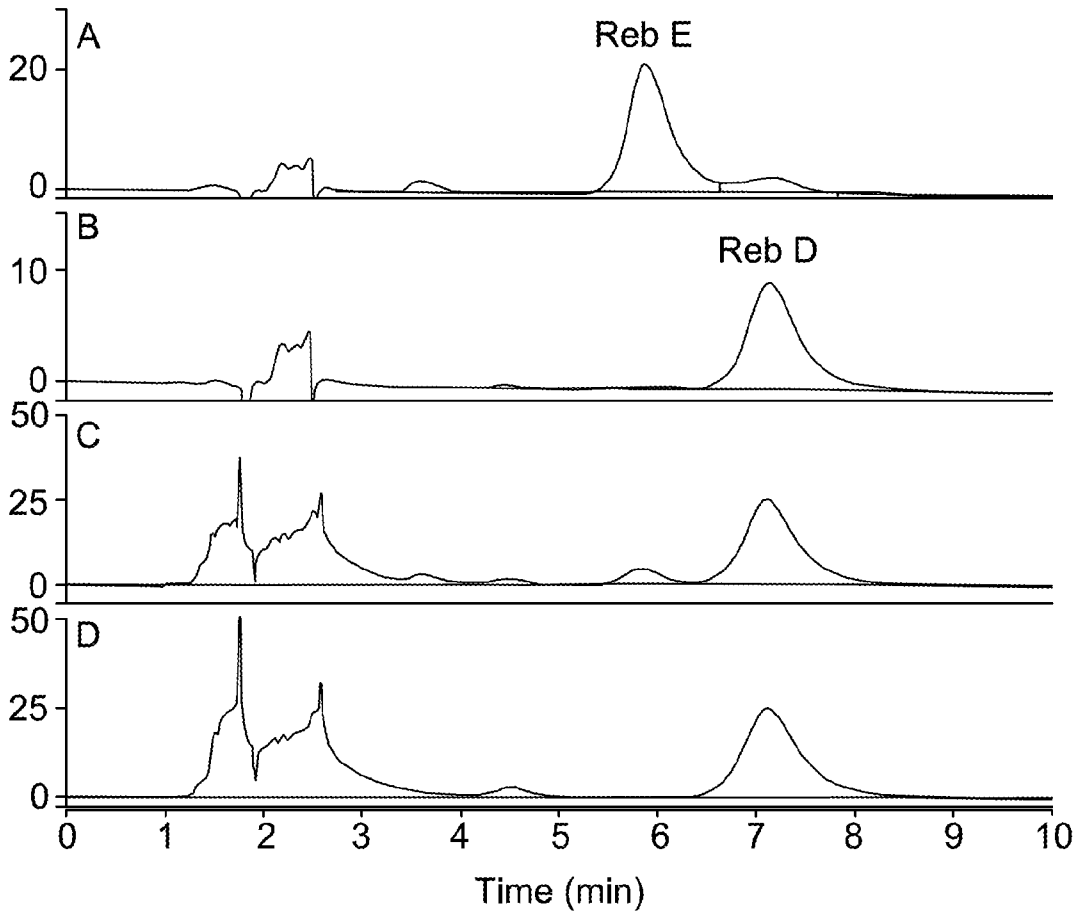
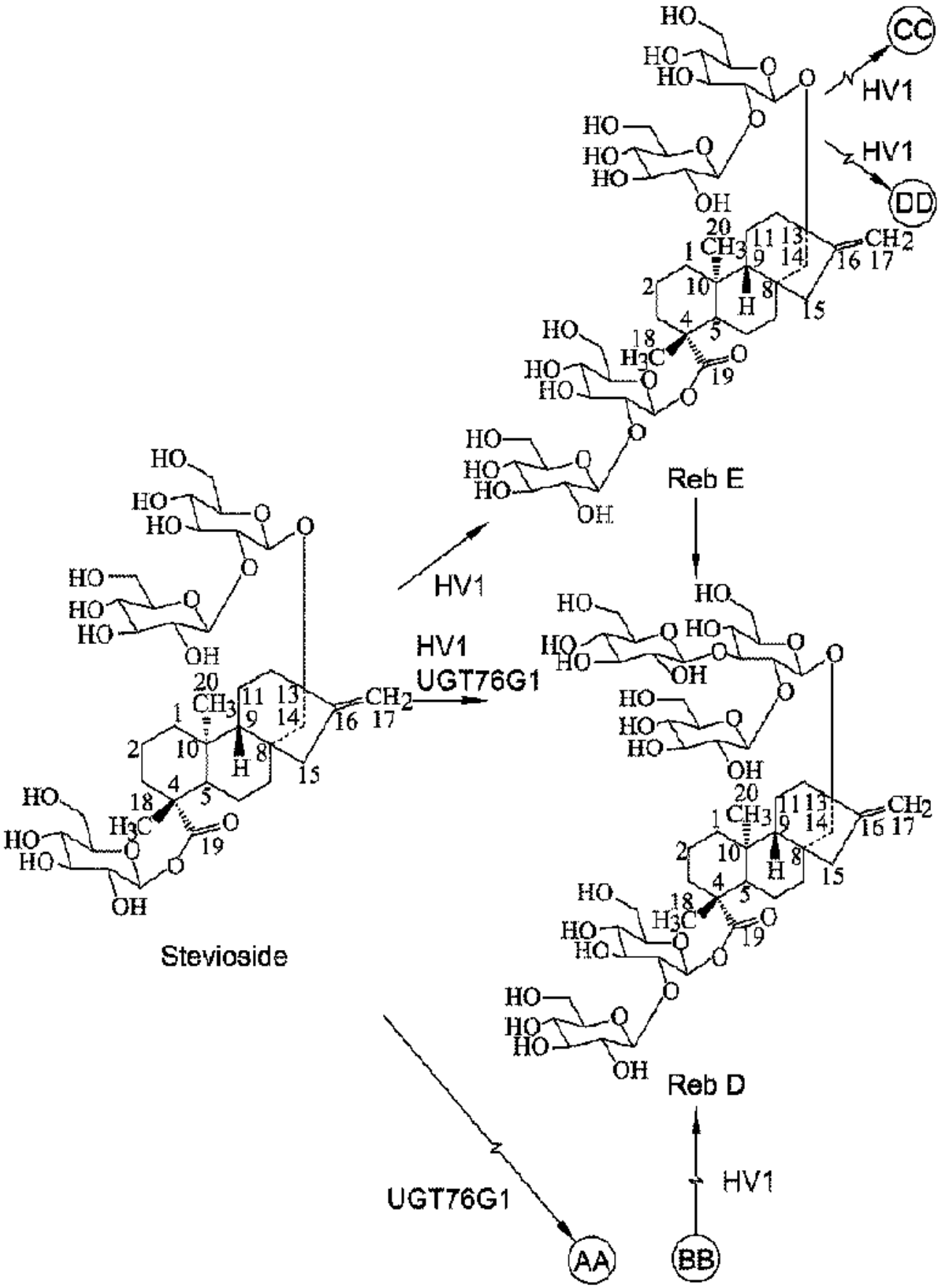


Figure 12


HV1
HV1
CC
DD
HV1
Reb E
HV1
UGT76G1
Stevioside
Reb D
HV1
UGT76G1
AA
BB