

[DESCRIPTION]

[Invention Title]

NOVEL GLYCOSYLTRANSFERASE AND USE THEREOF

[Technical Field]

The present application relates to a novel uridine diphosphate (UDP)–glycosyltransferase B (UGT-B), a polynucleotide encoding the uridine diphosphate (UDP)–glycosyltransferase B (UGT-B), an expression vector containing the polynucleotide, a microorganism including the uridine diphosphate (UDP)–glycosyltransferase B (UGT-B) or a polynucleotide encoding the uridine diphosphate (UDP)–glycosyltransferase B (UGT-B), and a method for producing rebaudioside D and rebaudioside M using the microorganism.

[Background Art]

As the World Health Organization (WHO) recommends lowering the amount of daily sugar intake due to concerns about disease (obesity) caused by sugar consumption, various policies aimed at reducing the amount of sugar intake are actively being discussed by the governments of developed countries. Therefore, as the need for developing various alternative sweeteners in place of sugar and high-fructose sweeteners is increasing in the market, alternative sweeteners are continuously being developed and commercialized.

Such alternative sweeteners are the subject of continuous variation in the form of synthetic high-intensity sweeteners (*e.g.*, saccharin, aspartame, sucralose, *etc.*), synthetic sugar alcohols (*e.g.*, maltitol and xylitol), and high-intensity sweeteners (*e.g.*, rebaudioside A and liquorice). Nevertheless, due to concerns over the safety of synthetic sweeteners, customers' need for natural sweeteners has been steadily increasing; however, because of limitations with peculiar flavor properties of natural sweeteners (*i.e.*, off-odor and off-flavor), natural sweeteners cannot fully replace existing low-calorie and zero-calorie products based on synthetic sweeteners.

A natural high-intensity sweetener that has received considerable attention in recent years is stevia extracted from the leaves of *Stevia*. Stevia has a potential use

as an alternative sweetener because it has been reported that it does not generate calories, it is positive for blood glucose and insulin levels, and has no side effects on the human body; however, stevia has a limitation in use for reducing the amount of sugar because it has a bitter taste.

Stevia is a perennial plant in the Asteraceae family native to Paraguay in South America, and its scientific name is *Stevia rebaudiana Bertonii*. The leaves of *Stevia* contain sweetening components which have 200 to 300 times the sweetness of sugar, and the sweetening components are extracted and used as a natural sweetener. The sweetening components of the *Stevia* extracts contain various steviol glycosides such as stevioside, rebaudioside A, rebaudioside C, rebaudioside D, rebaudioside M, rebaudioside I, rebaudioside E, etc.

The *Stevia* leaves contain relatively high contents of stevioside (STV), rebaudioside A (Reb A), and rebaudioside C (Reb C) among the sweetening components of the *Stevia* extract, and thus, high-purity extracted and purified industrial products have been launched, but they have a limitation in use for reducing the amount of sugar because of bitter taste.

Meanwhile, rebaudioside D (Reb D) and rebaudioside M (Reb M) have less bitter taste than STV, rebaudioside A, and rebaudioside C, and have excellent sweetening qualities, and thus are highly valuable as alternative sweeteners. However, rebaudioside D and rebaudioside M are present only in very small amounts in the *Stevia* leaves, so there is a disadvantage in that a method of extracting and purifying rebaudioside D and rebaudioside M from the leaves and producing the same requires a high cost.

Accordingly, there is a need for research on novel enzymes required for mass production of rebaudioside D and rebaudioside M and a production method using the same.

[Prior Art Literature]

[Patent Literature]

(Patent Literature 0001) Korean Patent No. 1404728

[Disclosure]**[Technical Problem]**

The present inventors have made extensive efforts to develop an enzyme having an activity to convert rebaudioside A to rebaudioside D, and as a result, it has been newly found that a polypeptide sequence whose function was not previously revealed has a glycosyltransferase activity, and that the polypeptide has a glycosyltransferase activity of converting rebaudioside A into rebaudioside D, thereby completing the present application.

[Technical Solution]

One object of the present application is to provide uridine diphosphate (UDP)–glycosyltransferase B (UGT-B) consisting of an amino acid sequence of SEQ ID NO: 1.

Another object of the present application is to provide a polynucleotide encoding the uridine diphosphate (UDP)–glycosyltransferase B (UGT-B) of the present application.

Still another object of the present application is to provide an expression vector containing the polynucleotide.

Yet another object of the present application is to provide a microorganism including the uridine diphosphate (UDP)–glycosyltransferase B (UGT-B) of the present application or a polynucleotide encoding the uridine diphosphate (UDP)–glycosyltransferase B (UGT-B).

Even another object of the present application is to provide a method for producing rebaudioside D, including: reacting nucleotide diphosphate to which glucose is bonded with rebaudioside A in the presence of uridine diphosphate (UDP)–glycosyltransferase B (UGT-B) to prepare rebaudioside D, wherein the uridine diphosphate (UDP)–glycosyltransferase B (UGT-B) is a protein consisting of an amino acid sequence of SEQ ID NO: 1.

Further another object of the present application is to provide a method for producing rebaudioside M, including: reacting nucleotide diphosphate to which glucose is bonded with rebaudioside A in the presence of uridine diphosphate (UDP)–glycosyltransferase B (UGT-B) to prepare rebaudioside D; and reacting the

rebaudioside D with nucleotide diphosphate to which glucose is bonded in the presence of uridine diphosphate (UDP)–glycosyltransferase A (UGT-A) to prepare rebaudioside M, wherein the uridine diphosphate (UDP)–glycosyltransferase B (UGT-B) is a protein consisting of an amino acid sequence of SEQ ID NO: 1.

Still further another object of the present application is to provide a method for producing rebaudioside D from rebaudioside A, including: reacting sucrose, nucleotide diphosphate, rebaudioside A, sucrose synthase and uridine diphosphate (UDP)–glycosyltransferase B (UGT-B) *in situ* to prepare rebaudioside D, wherein the uridine diphosphate (UDP)–glycosyltransferase B (UGT-B) is a protein consisting of an amino acid sequence of SEQ ID NO: 1.

Still further another object of the present application is to provide a method for producing rebaudioside M from rebaudioside A, including: reacting sucrose, nucleotide diphosphate, rebaudioside A, rebaudioside D, sucrose synthase, uridine diphosphate (UDP)–glycosyltransferase A (UGT-A) and uridine diphosphate (UDP)–glycosyltransferase B (UGT-B) *in situ* to prepare rebaudioside M, wherein the uridine diphosphate (UDP)–glycosyltransferase B (UGT-B) is a protein consisting of an amino acid sequence of SEQ ID NO: 1.

Still further another object of the present application is to provide a composition for producing rebaudioside D including the uridine diphosphate (UDP)–glycosyltransferase B (UGT-B).

Still further another object of the present application is to provide a composition for producing rebaudioside M including the uridine diphosphate (UDP)–glycosyltransferase A (UGT-A).

Still further another object of the present application is to provide the use of uridine diphosphate (UDP)–glycosyltransferase B (UGT-B) consisting of an amino acid sequence of SEQ ID NO: 1 as a glycosyltransferase that converts rebaudioside A to rebaudioside D.

[Advantageous Effects]

The uridine diphosphate (UDP)–glycosyltransferase B (UGT-B) of the present application can be used to produce rebaudioside D and/or rebaudioside M, thereby

providing rebaudioside D and rebaudioside M in high purity and high yield, with almost no by-products, and thus can be effectively used for mass production of rebaudioside D and rebaudioside M because it is economical due to the use of inexpensive raw materials, and the procedure is simple and less time-consuming.

[Brief Description of Drawings]

Fig. 1 is a HPLC analysis result showing that rebaudioside A is converted to rebaudioside D by uridine diphosphate (UDP)–glycosyltransferase B (UGT-B).

Fig. 2 is a HPLC analysis result showing that rebaudioside D, rebaudioside M, and rebaudioside I are produced from stevioside and rebaudioside A by uridine diphosphate (UDP)–glycosyltransferase B (UGT-B), uridine diphosphate (UDP)–glycosyltransferase A (UGT-A), and sucrose synthase.

Fig. 3 is a HPLC analysis result showing that rebaudioside D is converted to rebaudioside M by uridine diphosphate (UDP)–glycosyltransferase A (UGT-A).

[Detailed Description of Preferred Embodiments]

The present application will be described in detail as follows. Meanwhile, each description and embodiment disclosed herein can be applied to other descriptions and embodiments, respectively. That is, all combinations of various elements disclosed herein fall within the scope of the present application. Further, the scope of the present application is not limited by the specific description described below. Additionally, a number of papers and patent documents have been cited throughout the present specification. The content of the cited papers and patent documents is incorporated herein by reference in their entirety and the level of technical field to which the present application belongs and the contents of the present application will be described more clearly.

One aspect of the present application provides uridine diphosphate (UDP)–glycosyltransferase B (UGT-B) consisting of an amino acid sequence of SEQ ID NO: 1.

As used herein, the term “uridine diphosphate (UDP)–glycosyltransferase (UGT)”

is an enzyme that catalyzes the transfer of a monosaccharide moiety from a glycosyl donor to a glycosyl acceptor molecule, and in particular, it refers to an enzyme that utilizes UDP-sugar as the glycosyl donor. In the present application, the UDP-glycosyltransferase may be interchangeably used with UGT.

The UDP-glycosyltransferase may be one produced from recombinant *E. coli*, *Bacillus*, yeast, *Corynebacterium* or *Agrobacterium* transformed with a vector containing a glycosyltransferase gene, and the UDP-glycosyltransferase may be further purified after production from *E. coli* or the like, or commercially manufactured products may be purchased and used, but the UDP-glycosyltransferase is not limited thereto. In addition, the UDP-glycosyltransferase is known in the art, and the protein and gene sequence of the UDP-glycosyltransferase can be obtained from a known database, for example, GenBank of NCBI, etc., but is not limited thereto.

In the present application, the novel uridine diphosphate (UDP)-glycosyltransferase B (UGT-B) consisting of an amino acid sequence of SEQ ID NO: 1 having an enzyme activity of converting rebaudioside A to rebaudioside D has been newly discovered.

Specifically, the uridine diphosphate (UDP)-glycosyltransferase B (UGT-B) of the present application may have and/or include an amino acid sequence of SEQ ID NO: 1 or essentially consist or consist of the amino acid sequence.

Additionally, the uridine diphosphate (UDP)-glycosyltransferase B (UGT-B) may include an amino acid sequence having a homology or identity of at least 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99%, 99.5%, 99.7%, or 99.9% or more with the amino acid sequence of SEQ ID NO: 1. In addition, it is apparent that any uridine diphosphate (UDP)-glycosyltransferase B (UGT-B) having an amino acid sequence, in which part of the sequence is deleted, modified, substituted, conservatively substituted, or added, may also fall within the scope of the present application as long as the amino acid sequence has such a homology or identity and exhibits an effect corresponding to that of the uridine diphosphate (UDP)-glycosyltransferase B (UGT-B).

As used herein, although it is described as “a polypeptide or protein including an amino acid sequence described by a specific sequence number”, “a polypeptide or

protein consisting of an amino acid sequence described by a specific sequence number”, or “a polypeptide or protein having an amino acid sequence described by a specific sequence number”, it is apparent that any protein having an amino acid sequence in which part of the sequence is deleted, modified, substituted, conservatively substituted or added can be used in the present application even if it has the same or corresponding activity as the polypeptide consisting of the amino acid sequence of the corresponding sequence number. For example, it may be a case where the N-terminus and/or C-terminus of the amino acid sequence is added with a sequence that does not alter the function of the protein, a naturally occurring mutation, a potential mutation thereof (a silent mutation), or a conservative substitution.

For example, it may be a case where the N-terminus, C-terminus and/or inside of the amino acid sequence is added or deleted with a sequence that does not alter the function of the uridine diphosphate (UDP)–glycosyltransferase B (UGT-B) of the present application, a naturally occurring mutation, a potential mutation thereof (a silent mutation), or a conservative substitution.

As used herein, the term “conservative substitution” refers to substitution of an amino acid with another amino acid having similar structural and/or chemical properties. Such amino acid substitution may generally occur based on similarity of polarity, charge, solubility, hydrophobicity, hydrophilicity, and/or amphipathic nature of a residue. For example, positively charged (basic) amino acids include arginine, lysine, and histidine; negatively charged (acidic) amino acids include glutamic acid and aspartic acid; aromatic amino acids include phenylalanine, tryptophan, and tyrosine; and hydrophobic amino acids include alanine, valine, isoleucine, leucine, methionine, phenylalanine, tyrosine, and tryptophan. In addition, amino acids can be classified into amino acids with electrically charged side chains and amino acids with uncharged side chains, and examples of the amino acids with electrically charged side chains include aspartic acid, glutamic acid, lysine, arginine, and histidine. The amino acids with uncharged side chains can be further classified into nonpolar amino acids or polar amino acids. Examples of the non-polar amino acids are glycine, alanine, valine, leucine, isoleucine, methionine, phenylalanine, tryptophan, proline, and examples of the polar amino acids include serine, threonine, cysteine, tyrosine, asparagine, and glutamine. Typically,

conservative substitutions have little or no effect on the activity of the resulting polypeptide. Typically, conservative substitutions may have little or no effect on the activity of the protein or polypeptide.

Additionally, the uridine diphosphate (UDP)–glycosyltransferase B (UGT-B) may also include deletion or addition of amino acids that have minimal influence on the properties and secondary structure of a polypeptide. For example, the polypeptide may be conjugated with a signal (or leader) sequence at the N-terminal involved in the transfer of proteins co-translationally or post-translationally. Further, the polypeptide may also be conjugated with another sequence or linker to identify, purify, or synthesize the polypeptide.

As used herein, the term “homology” or “identity” refers to a degree of relatedness between two given amino acid sequences or nucleotide sequences, and may be expressed as a percentage. The terms homology and identity may often be used interchangeably with each other.

The sequence homology or identity of conserved polynucleotide or polypeptide sequences may be determined by standard alignment algorithms and can be used with a default gap penalty established by the program being used. Substantially, homologous or identical sequences are generally expected to hybridize to all or part of the sequences under moderate or high stringent conditions. It is apparent that hybridization with polynucleotides containing general codon or degenerate codons in hybridizing polynucleotides is also included.

Whether any two polynucleotide or polypeptide sequences have a homology, similarity, or identity may be, for example, determined by a known computer algorithm such as the “FASTA” program (Pearson *et al.*, (1988) *Proc. Natl. Acad. Sci. USA* 85:2444) using default parameters. Alternatively, it may be determined by the Needleman–Wunsch algorithm (Needleman and Wunsch, 1970, *J. Mol. Biol.* 48:443–453), which is performed using the Needleman program of the EMBOSS package (EMBOSS: The European Molecular Biology Open Software Suite, Rice *et al.*, 2000, *Trends Genet.* 16:276–277) (preferably, version 5.0.0 or later) (GCG program package (Devereux, J. *et al.*, *Nucleic Acids Research* 12:387 (1984)), BLASTP, BLASTN, FASTA

(Atschul, S. F. *et al.*, *J MOLEC BIOL* 215:403 (1990); *Guide to Huge Computers*, Martin J. Bishop, ed., Academic Press, San Diego, 1994, and CARILLO *et al.* (1988) *SIAM J Applied Math* 48:1073). For example, the homology, similarity, or identity may be determined using BLAST or ClustalW of the National Center for Biotechnology Information (NCBI).

The homology, similarity, or identity of polynucleotides or polypeptides may be, for example, determined by comparing sequence information using, for example, the GAP computer program, such as Needleman *et al.* (1970), *J Mol Biol.* 48:443 as disclosed in Smith and Waterman, *Adv. Appl. Math* (1981) 2:482. In summary, the GAP program defines the homology, similarity, or identity as the value obtained by dividing the number of similarly aligned symbols (*i.e.*, nucleotides or amino acids) by the total number of the symbols in the shorter of the two sequences. Default parameters for the GAP program may include (1) a unary comparison matrix (containing a value of 1 for identities and 0 for non-identities) and the weighted comparison matrix of Gribskov *et al.* (1986), *Nucl. Acids Res.* 14:6745, as disclosed in Schwartz and Dayhoff, eds., *Atlas of Protein Sequence and Structure*, National Biomedical Research Foundation, pp. 353–358 (1979) (or EDNAFULL substitution matrix (EMBOSS version of NCBI NUC4.4)); (2) a penalty of 3.0 for each gap and an additional 0.10 penalty for each symbol in each gap (or a gap opening penalty of 10 and a gap extension penalty of 0.5); and (3) no penalty for end gaps.

In one example of the present application, the uridine diphosphate (UDP)–glycosyltransferase B (UGT-B) may have an activity of converting rebaudioside A to rebaudioside M.

As used herein, the term “corresponding to” refers to an amino acid residue at the position recited in a peptide, or an amino acid residue which is similar, identical, or homologous to the residue recited in a peptide. Identifying an amino acid at a corresponding position may be determining a particular amino acid in a sequence that refers to a particular sequence. As used herein, the “corresponding region” generally refers to a similar or corresponding position in the related protein or reference protein.

For example, any amino acid sequence is aligned with SEQ ID NO: 1, and based on the alignment, each amino acid residue of the amino acid sequence can be

numbered with reference to the numerical position of the amino acid residue corresponding to the amino acid residue of SEQ ID NO: 1. For example, a sequence alignment algorithm such as that described herein can identify the position of an amino acid or a position where modifications such as substitutions, insertions or deletions occur compared to a query sequence (also referred to as a “reference sequence”).

Example of the alignment may be determined using the Needleman–Wunsch algorithm (Needleman and Wunsch, 1970, *J. Mol. Biol.* 48:443–453), which is performed using the Needleman program of the EMBOSS package (EMBOSS: The European Molecular Biology Open Software Suite, Rice *et al.*, 2000, *Trends Genet.* 16:276–277), *etc.*, but is not limited thereto, and sequence alignment programs, such as pairwise sequence comparison algorithms, *etc.*, known in the art may be appropriately used.

Another aspect of the present application provides a polynucleotide encoding the uridine diphosphate (UDP)–glycosyltransferase B (UGT-B) of the present application.

As used herein, the term “polynucleotide”, which is a polymer of nucleotides composed of nucleotide monomers connected in a lengthy chain by a covalently bond, is a DNA or RNA strand having at least a certain length. More specifically, it may refer to a polynucleotide fragment encoding the uridine diphosphate (UDP)–glycosyltransferase B (UGT-B).

The polynucleotide encoding the uridine diphosphate (UDP)–glycosyltransferase B (UGT-B) of the present application may include a nucleotide sequence encoding the amino acid sequence represented by SEQ ID NO: 1. As an example of the present application, the polynucleotide may have or include the sequence of SEQ ID NO: 4. In addition, the polynucleotide may consist or consist essentially of the sequence of SEQ ID NO: 4.

The polynucleotide of the present application may undergo various modifications in the coding region within the scope that does not change the amino acid sequence of the uridine diphosphate (UDP)–glycosyltransferase B (UGT-B) of the present application, due to codon degeneracy or in consideration of the codons preferred in an

organism in which the uridine diphosphate (UDP)–glycosyltransferase B (UGT-B) of the present application is to be expressed. Specifically, the polynucleotide of the present application may have or include a nucleotide sequence having a homology or identity of 70% or more, 75% or more, 80% or more, 85% or more, 90% or more, 95% or more, 96% or more, 97% or more, 98% or more, and 100% or less with the sequence of SEQ ID NO: 4, or may consist or consist essentially of a nucleotide sequence having a homology or identity of 70% or more, 75% or more, 80% or more, 85% or more, 90% or more, 95% or more, 96% or more, 97% or more, 98% or more, and 100% or less with the sequence of SEQ ID NO: 4, but is not limited thereto.

Additionally, the polynucleotide of the present application may include a probe that may be prepared from a known gene sequence, for example, any sequence which can hybridize with a sequence complementary to all or part of the polynucleotide sequence of the present application under stringent conditions without limitation. The “stringent conditions” refers to conditions under which specific hybridization between polynucleotides is allowed. Such conditions are specifically described in the literature (J. Sambrook *et al.*, *Molecular Cloning, A Laboratory Manual*, 2nd Edition, Cold Spring Harbor Laboratory press, Cold Spring Harbor, New York, 1989; F. M. Ausubel *et al.*, *Current Protocols in Molecular Biology*, John Wiley & Sons, Inc., New York, 9.50–9.51, 11.7–11.8). For example, the stringent conditions may include conditions under which genes having a high homology or identity of 70% or more, 75% or more, 80% or more, 85% or more, 90% or more, 95% or more, 96% or more, 97% or more, 98% or more, or 99% or more are hybridized with each other and genes having a homology or identity lower than the above homologies or identities are not hybridized with each other, or washing conditions of Southern hybridization, that is, washing once, specifically twice or three times at a salt concentration and a temperature corresponding to 60°C, 1×SSC, 0.1% SDS, specifically 60°C, 0.1×SSC, 0.1% SDS, and more specifically 68°C, 0.1×SSC, 0.1% SDS.

Hybridization requires that two nucleic acids contain complementary sequences, although mismatches between bases are possible depending on the stringency of the hybridization. The term “complementary” is used to describe the relationship between nucleotide bases that can hybridize with each other. For example, with respect to DNA,

adenine is complementary to thymine, and cytosine is complementary to guanine. Therefore, the polynucleotide of the present application may include isolated nucleotide fragments complementary to the entire sequence as well as nucleic acid sequences substantially similar thereto.

Specifically, polynucleotides having a homology or identity with the polynucleotide of the present application may be detected using the hybridization conditions including a hybridization step at a T_m value of 55°C under the above-described conditions. Further, the T_m value may be 60°C, 63°C, or 65°C, but is not limited thereto, and may be appropriately adjusted by those skilled in the art depending on the purpose thereof.

The appropriate stringency for hybridizing the polynucleotides depends on the length of the polynucleotides and the degree of complementation, and these variables are well known in the art (*e.g.*, Sambrook *et al.*).

Still another aspect of the present application provides a vector containing the polynucleotide of the present application. The vector may be an expression vector for expressing the polynucleotide in a microorganism, but is not limited thereto.

As used herein, the term “vector” may include a DNA construct containing the nucleotide sequence of a polynucleotide encoding the target polypeptide operably linked to a suitable expression regulatory region (expression regulatory sequence) so as to be able to express the target polypeptide in a suitable host cell. The expression regulatory sequence may include a promoter capable of initiating transcription, any operator sequence for regulating the transcription, a sequence encoding a suitable mRNA ribosome-binding site, and a sequence for regulating termination of transcription and translation. Once transformed into a suitable microorganism, the vector may replicate or function independently from the host genome, or may integrate into genome thereof.

The vector used in the present application is not particularly limited, and any vector known in the art may be used. Examples of the vector typically used may include natural or recombinant plasmids, cosmids, viruses, and bacteriophages. For example, as a phage vector or cosmid vector, pWE15, M13, MBL3, MBL4, IXII, ASHII,

APII, t10, t11, Charon4A, and Charon21A, *etc.* may be used; and as a plasmid vector, those based on pDZ, pBR, pUC, pBluescriptII, pGEM, pTZ, pCL and pET, *etc.* may be used. Specifically, pDZ, pDC, pDCM2, pACYC177, pACYC184, pCL, pECCG117, pUC19, pBR322, pMW118, pCC1BAC vector, *etc.* may be used.

In one example, a polynucleotide encoding a target polypeptide may be inserted into the chromosome through a vector for intracellular chromosomal insertion. The insertion of the polynucleotide into the chromosome may be performed by any method known in the art, for example, by homologous recombination, but is not limited thereto. The vector may further include a selection marker to confirm the insertion into the chromosome. The selection marker is for selecting the cells transformed with the vector, that is, for confirming whether the target nucleic acid molecule has been inserted, and markers that provide selectable phenotypes, such as drug resistance, auxotrophy, resistance to cell toxic agents, or expression of surface polypeptides, may be used. Only cells expressing the selection marker are able to survive or to show different phenotypes under the environment treated with the selective agent, and thus the transformed cells may be selected.

As used herein, the term “transformation” refers to the introduction of a vector containing a polynucleotide encoding a target polypeptide into a microorganism so that the polypeptide encoded by the polynucleotide can be expressed in the microorganism. As long as the transformed polynucleotide can be expressed in the microorganism, it does not matter whether the transformed polynucleotide is integrated into the chromosome of the microorganism and located therein or located extrachromosomally, and both cases can be included. Further, the polynucleotide may include DNA and/or RNA encoding the target polypeptide. The polynucleotide may be introduced in any form, as long as it can be introduced into the microorganism and expressed therein. For example, the polynucleotide may be introduced into the microorganism in the form of an expression cassette, which is a gene construct including all elements required for its autonomous expression. The expression cassette may commonly include a promoter operably linked to the polynucleotide, a transcription terminator, a ribosome-binding site, or a translation terminator. The expression cassette may be in the form of a self-replicable expression vector. Additionally, the polynucleotide may be introduced

into a microorganism as it is and operably linked to sequences required for expression in the microorganism, but is not limited thereto.

Further, as used herein, the term “operably linked” means that the polynucleotide sequence is functionally linked to a promoter sequence that initiates and mediates transcription of the polynucleotide encoding the target uridine diphosphate (UDP)–glycosyltransferase B (UGT-B) of the present application.

Yet another aspect of the present application provides a microorganism including the uridine diphosphate (UDP)–glycosyltransferase B (UGT-B) of the present application or a polynucleotide encoding the uridine diphosphate (UDP)–glycosyltransferase B (UGT-B).

The microorganism of the present application may include the uridine diphosphate (UDP)–glycosyltransferase B (UGT-B) of the present application, a polynucleotide encoding the uridine diphosphate (UDP)–glycosyltransferase B (UGT-B), or a vector containing the polynucleotide of the present application.

As used herein, the term “microorganism” includes all wild-type microorganisms, or naturally or artificially genetically modified microorganisms, and it may be a microorganism in which a particular mechanism is weakened or enhanced due to insertion of a foreign gene, or enhancement or inactivation of the activity of an endogenous gene, and may be a microorganism including genetic modification to produce a desired polypeptide, protein or product. In the present application, the terms “microorganism” and “strain” have the same meaning and can be used interchangeably without limitation.

Specifically, the microorganism may be a microorganism of the genus *Escherichia* or a microorganism of the genus *Corynebacterium*, more specifically *Escherichia coli* or *Corynebacterium glutamicum*, but is not limited thereto.

The microorganism of the present application may be a strain including any one or more of the uridine diphosphate (UDP)–glycosyltransferase B (UGT-B) of the present application, the polynucleotide of the present application, and a vector containing the polynucleotide of the present application; a strain modified to express the uridine diphosphate (UDP)–glycosyltransferase B (UGT-B) of the present application or the

polynucleotide of the present application; a strain expressing the uridine diphosphate (UDP)–glycosyltransferase B (UGT-B) of the present application or the polynucleotide of the present application (e.g., a recombinant strain); or a strain having the activity of the uridine diphosphate (UDP)–glycosyltransferase B (UGT-B) of the present application (e.g., a recombinant strain), but is not limited thereto.

The strain of the present application may be a microorganism producing rebaudioside D and/or rebaudioside M.

As used herein, the term “microorganism producing rebaudioside D and/or rebaudioside M” refers to a prokaryotic or eukaryotic microorganism strain capable of producing rebaudioside D and/or rebaudioside M in an organism. For the purpose of the present invention, as long as the microorganism can produce rebaudioside D and/or rebaudioside M including the uridine diphosphate (UDP)–glycosyltransferase B (UGT-B), both prokaryotic and eukaryotic cells are possible.

The microorganism producing rebaudioside D and/or rebaudioside M including the uridine diphosphate (UDP)–glycosyltransferase B (UGT-B) may include all microorganisms including the uridine diphosphate (UDP)–glycosyltransferase B (UGT-B) by introducing a microorganism including the sequence encoding the uridine diphosphate (UDP)–glycosyltransferase B (UGT-B) on the chromosome and/or a vector including a polynucleotide encoding the uridine diphosphate (UDP)–glycosyltransferase B (UGT-B), but is not limited thereto.

In one example, the strain of the present application is a cell or microorganism which is transformed with a vector containing the polynucleotide of the present application or a polynucleotide encoding the uridine diphosphate (UDP)–glycosyltransferase B (UGT-B) of the present application to express the uridine diphosphate (UDP)–glycosyltransferase B (UGT-B) of the present application, and for the purpose of the present application, the strain may include all microorganisms capable of producing rebaudioside D and/or rebaudioside M including the uridine diphosphate (UDP)–glycosyltransferase B (UGT-B) of the present application. For example, the strain of the present application may be a recombinant strain in which the production ability of rebaudioside D and/or rebaudioside M has been increased upon expression of the uridine diphosphate (UDP)–glycosyltransferase B (UGT-B) by

introducing the polynucleotide encoding the uridine diphosphate (UDP)–glycosyltransferase B (UGT-B) of the present application into a natural wild-type microorganism or a microorganism for producing rebaudioside D and/or rebaudioside M.

The modification of a part or all of the polynucleotide in the microorganism of the present application may be achieved by (a) homologous recombination using a vector for chromosomal insertion in the microorganism or genome editing using engineered nuclease (e.g., CRISPR-Cas9) and/or (b) may be induced by light, such as ultraviolet rays and radiation, *etc.*, and/or chemical treatments, but is not limited thereto. The method of modifying a part or all of the gene may include a method using DNA recombination technology. For example, a part or all of the gene may be deleted by injecting a nucleotide sequence or a vector containing a nucleotide sequence homologous to the target gene into the microorganism to induce homologous recombination. The injected nucleotide sequence or the vector may include a dominant selection marker, but is not limited thereto.

In the microorganism of the present application, the uridine diphosphate (UDP)–glycosyltransferase B (UGT-B), polynucleotide and rebaudioside D and/or rebaudioside M, *etc.*, are as described in other aspects.

Even another aspect of the present application provides a method for producing rebaudioside D, including:

reacting nucleotide diphosphate to which glucose is bonded with rebaudioside A in the presence of uridine diphosphate (UDP)–glycosyltransferase B (UGT-B) to prepare rebaudioside D,

wherein the uridine diphosphate (UDP)–glycosyltransferase B (UGT-B) is a protein consisting of an amino acid sequence of SEQ ID NO: 1.

Specifically, the method for producing rebaudioside D of the present application may include culturing a microorganism including the uridine diphosphate (UDP)–glycosyltransferase B (UGT-B) of the present application, the polynucleotide of the present application, or the vector of the present application in a medium, but is not limited thereto.

As used herein, the term “cultivation” means that the microorganism of the

present application is grown under appropriately controlled environmental conditions. The cultivation process of the present application may be performed in a suitable culture medium and culture conditions known in the art. Such a cultivation process may be easily adjusted for use by those skilled in the art according to the strain to be selected. Specifically, the cultivation may be a batch culture, a continuous culture, and/or a fed-batch culture, but is not limited thereto.

As used herein, the term “medium” refers to a mixture of materials which contains nutrient materials required for the cultivation of the microorganism of the present application as a main ingredient, and it supplies nutrient materials and growth factors, along with water that is essential for survival and growth. Specifically, the medium and other culture conditions used for culturing the microorganism of the present application may be any medium used for conventional cultivation of microorganisms without any particular limitation. However, the microorganism of the present application may be cultured under aerobic conditions in a conventional medium containing an appropriate carbon source, nitrogen source, phosphorus source, inorganic compounds, amino acids, and/or vitamins, while adjusting temperature, pH, etc.

For example, the culture medium for the microorganism of the genus *Corynebacterium* can be found in the literature (“Manual of Methods for General Bacteriology” by the American Society for Bacteriology (Washington D.C., USA, 1981)).

In the present application, the carbon source may include carbohydrates, such as glucose, saccharose, lactose, fructose, sucrose, maltose, etc.; sugar alcohols, such as mannitol, sorbitol, etc.; organic acids, such as pyruvic acid, lactic acid, citric acid, etc.; amino acids, such as glutamic acid, methionine, lysine, etc. Additionally, the carbon source may include natural organic nutrients such as starch hydrolysate, molasses, blackstrap molasses, rice bran, cassava, sugar cane molasses, corn steep liquor, etc. Specifically, carbohydrates such as glucose and sterilized pretreated molasses (i.e., molasses converted to reducing sugar) may be used, and in addition, various other carbon sources in an appropriate amount may be used without limitation. These carbon sources may be used alone or in a combination of two or more kinds, but are not limited thereto.

The nitrogen source may include inorganic nitrogen sources, such as ammonia, ammonium sulfate, ammonium chloride, ammonium acetate, ammonium phosphate, ammonium carbonate, ammonium nitrate, *etc.*; amino acids, such as glutamic acid, methionine, glutamine, *etc.*; and organic nitrogen sources, such as peptone, NZ-amine, meat extract, yeast extract, malt extract, corn steep liquor, casein hydrolysate, fish or a decomposition product thereof, defatted soybean cake or a decomposition product thereof, *etc.* These nitrogen sources may be used alone or in a combination of two or more kinds, but are not limited thereto.

The phosphorus source may include monopotassium phosphate, dipotassium phosphate, or corresponding sodium-containing salts, *etc.* Examples of the inorganic compound may include sodium chloride, calcium chloride, iron chloride, magnesium sulfate, iron sulfate, manganese sulfate, calcium carbonate, *etc.* Additionally, amino acids, vitamins, and/or appropriate precursors may be included. These constituting ingredients or precursors may be added to a medium in a batch or continuous manner, but these phosphorus sources are not limited thereto.

The pH of a medium may be adjusted during the cultivation of the microorganism of the present application by adding a compound such as ammonium hydroxide, potassium hydroxide, ammonia, phosphoric acid, sulfuric acid, *etc.* to the medium in an appropriate manner. Additionally, during the cultivation, an antifoaming agent such as fatty acid polyglycol ester may be added to prevent foam generation. In addition, oxygen or oxygen-containing gas may be injected into the medium in order to maintain an aerobic state of the medium; or nitrogen, hydrogen, or carbon dioxide gas may be injected without the injection of gas in order to maintain an anaerobic or microaerobic state of the medium, but the gas is not limited thereto.

The medium temperature in the cultivation of the present application may be in a range from 20°C to 45°C, and specifically from 25°C to 40°C, and the cultivation may be carried out for about 10 to 160 hours, but is not limited thereto.

The rebaudioside D produced by the cultivation of the present application may be released into the medium or remain in the cells.

In one embodiment of the present application, the nucleotide diphosphate to

which glucose is bonded may be prepared by reacting sucrose and nucleotide diphosphate in the presence of a sucrose synthase, but is not limited thereto.

As used herein, the term “sucrose synthase” has a role in the production of sucrose by reversibly transferring glucose, which is bonded to nucleotide diphosphate, to fructose in plant metabolism. In the present invention, the sucrose synthase demonstrates an activity to separate nucleotide diphosphate to which glucose is bonded and fructose by reacting sucrose and nucleotide diphosphate in a pH range of 5 to 10.

The sucrose synthase may be those derived from rice, corn, wheat, bamboo, *Arabidopsis thaliana*, grass, barley, sorghum or potato. Preferably, the sucrose synthase is those derived from rice, corn, wheat, or barley, and more preferably from rice, in particular, *Oryza sativa*. The sucrose synthase may be produced from recombinant *Escherichia coli*, *Bacillus*, yeast, *Corynebacterium*, or *Agrobacterium* transformed with a vector containing a sucrose synthase gene, and may be further purified after it is produced from *Escherichia coli* and the like. The sucrose synthase may be those known in the art or may be commercially purchased, but is not limited thereto.

Specifically, the sucrose synthase of the present application may have and/or include an amino acid sequence of SEQ ID NO: 3 or may consist or essentially consist of the amino acid sequence.

The sucrose is not particularly limited so long as it can serve as a substrate for sucrose synthase to provide glucose to nucleotide diphosphate. Examples of sucrose may include raw sugar or sugar without limitation.

In the present application, purine nucleotide or pyrimidine nucleotide may be used as the nucleotide diphosphate. Preferably, uridine diphosphate is used as the nucleotide diphosphate, but the nucleotide diphosphate is not limited thereto.

The nucleotide diphosphate to which glucose is bonded may be reacted with rebaudioside A to prepare rebaudioside D by the uridine diphosphate (UDP)–glycosyltransferase B (UGT-B) of the present application.

In one embodiment of the present application, the rebaudioside A may be prepared by reacting nucleotide diphosphate to which glucose is bonded with stevioside in the presence of uridine diphosphate (UDP)–glycosyltransferase A (UGT-A), but is not limited thereto.

The uridine diphosphate (UDP)–glycosyltransferase A (UGT-A) may produce rebaudioside A by reacting the nucleotide diphosphate to which glucose is bonded with stevioside.

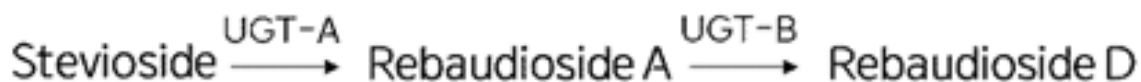
The uridine diphosphate (UDP)–glycosyltransferase A (UGT-A) may be those derived from *Oryza sativa*, *Stevia rebaudiana* Bertoni, *Bambusa oldhamii*, *Brachypodium distachyon*, *Hordeum vulgare*, *Sorghum bicolor*, *Zea mays*, or *Arabidopsis thaliana*. Preferably, the uridine diphosphate (UDP)–glycosyltransferase A (UGT-A) may be those derived from *Oryza sativa*, *Stevia rebaudiana* Bertoni, or *Bambusa oldhamii*. More preferably, it may be those derived from *Stevia rebaudiana* Bertoni. The uridine diphosphate (UDP)–glycosyltransferase A (UGT-A) may be those produced from recombinant *Escherichia coli*, *Bacillus*, yeast, *Corynebacterium* or *Agrobacterium* transformed with a vector containing a glycosyltransferase gene, or may be further purified after it is produced from *Escherichia coli* and the like. The uridine diphosphate (UDP)–glycosyltransferase A (UGT-A) may be those known in the art or may be commercially purchased, but is not limited thereto.

Specifically, the uridine diphosphate (UDP)–glycosyltransferase A (UGT-A) of the present application may have and/or include an amino acid sequence of SEQ ID NO: 2 or may consist or essentially consist of the amino acid sequence.

The stevioside is a hot water or aqueous ethanol solution extract from *Stevia rebaudiana* or purified material thereof, or a by-product after the production of rebaudioside A from the extract. The stevioside may be those having stevioside content of 10 wt% or more, preferably 50 wt% or more, particularly preferably 70 wt% or more, and more particularly preferably 80 wt% or more, based on total weight of steviol glycoside, but is not limited thereto.

In one embodiment of the present application, the rebaudioside D may be produced as illustrated in the Chemical Reaction 1 below, but is not limited thereto.

[Chemical Reaction 1]



Specifically, the production method may be performed consecutively *in situ*.

As used herein, the term “*in situ*” means that a reaction is consecutively performed in a single reaction system.

The production method of the present application provides a consecutive reaction system, wherein one glucose is specifically bonded to the C-3' position of stevioside 13-O-glucose to synthesize rebaudioside A with high yield, and rebaudioside D is synthesized from rebaudioside A in accordance with the Chemical Reaction 1 above.

The method for producing rebaudioside D of the present application may further include a step of preparing the microorganism of the present application, a step of preparing a medium for culturing the strain, or a combination thereof (regardless of the order, in any order), for example, prior to the culturing step.

The method for producing rebaudioside D of the present application may further include a step of recovering rebaudioside D from the culture medium (medium on which the culture was grown) or the microorganism of the present application. The recovering step may be further included after the culturing step.

In the recovering step, desired rebaudioside D may be collected using the method of culturing a microorganism of the present application, for example, using a suitable method known in the art according to a batch culture, continuous culture, or fed-batch culture method. For example, methods such as centrifugation, filtration, treatment with a protein crystallizing precipitant (salting-out method), extraction, ultrasonic disruption, ultrafiltration, dialysis, various kinds of chromatography such as molecular sieve chromatography (gel filtration), adsorption chromatography, ion-exchange chromatography, affinity chromatography, etc., HPLC, or a combination

thereof may be used, and the desired rebaudioside D can be recovered from the medium or the microorganisms using suitable methods known in the art.

Further, the method for producing rebaudioside D of the present application may further include a purification process, which may be performed using an appropriate method known in the art. In one example, when the method for producing rebaudioside D of the present application includes both a recovering step and a purification step, the recovering step and the purification step may be performed continuously or intermittently regardless of the order or simultaneously, or may be integrated into one step, but the method is not limited thereto.

In the method of the present application, the uridine diphosphate (UDP)–glycosyltransferase B (UGT-B), polynucleotides, vectors and microorganism, *etc.* are the same as described in other aspects.

Further aspect of the present application provides a method for producing rebaudioside M, including:

reacting nucleotide diphosphate to which glucose is bonded with rebaudioside A in the presence of uridine diphosphate (UDP)–glycosyltransferase B (UGT-B) to prepare rebaudioside D; and reacting the rebaudioside D with nucleotide diphosphate to which glucose is bonded in the presence of uridine diphosphate (UDP)–glycosyltransferase A (UGT-A) to prepare rebaudioside M, wherein the uridine diphosphate (UDP)–glycosyltransferase B (UGT-B) is a protein consisting of an amino acid sequence of SEQ ID NO: 1.

In one embodiment of the present application, the uridine diphosphate (UDP)–glycosyltransferase A (UGT-A) may be a protein consisting of an amino acid sequence of SEQ ID NO: 2, but is not limited thereto.

In one embodiment of the present application, the nucleotide diphosphate to which glucose is bonded may be prepared by reacting sucrose with nucleotide diphosphate in the presence of a sucrose synthase, but is not limited thereto.

Specifically, the sucrose synthase of the present application may have and/or include an amino acid sequence of SEQ ID NO: 3 or may consist or essentially consist of the amino acid sequence.

In one embodiment of the present application, the rebaudioside A may be prepared by reacting nucleotide diphosphate to which glucose is bonded with stevioside in the presence of uridine diphosphate (UDP)–glycosyltransferase A (UGT-A), but is not limited thereto.

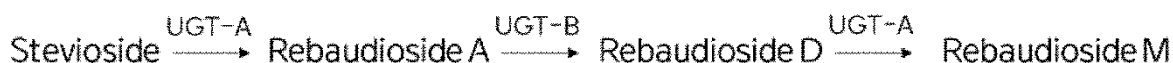
Specifically, the uridine diphosphate (UDP)–glycosyltransferase A (UGT-A) of the present application may have and/or include an amino acid sequence of SEQ ID NO: 2 or may consist or essentially consist of the amino acid sequence.

The uridine diphosphate (UDP)–glycosyltransferase A (UGT-A) of the present application may produce rebaudioside M by reacting the nucleotide diphosphate to which glucose is bonded with rebaudioside D.

Specifically, the uridine diphosphate (UDP)–glycosyltransferase A (UGT-A) of the present application may have and/or include an amino acid sequence of SEQ ID NO: 2 or may consist or essentially consist of the amino acid sequence.

In one embodiment of the present application, the rebaudioside M may be produced as illustrated in the Chemical Reaction 2 below, but is not limited thereto.

[Chemical Reaction 2]



Specifically, the production method may be performed consecutively *in situ*.

The production method of the present application provides a consecutive reaction system, wherein rebaudioside A is synthesized from stevioside with high yield, rebaudioside D is synthesized from rebaudioside A, and rebaudioside M is synthesized from rebaudioside D, in accordance with the Chemical Reaction 2 above.

The method for producing rebaudioside M of the present application may further include a step of preparing the microorganism of the present application, a step of preparing a medium for culturing the strain, or a combination thereof (regardless of the

order, in any order), for example, prior to the culturing step.

The method for producing rebaudioside M of the present application may further include a step of recovering rebaudioside M from the culture medium (medium on which the culture was grown) or the microorganism of the present application. The recovering step may be further included after the culturing step.

In the recovering step, desired rebaudioside M may be collected using the method of culturing a microorganism of the present application, for example, using a suitable method known in the art according to a batch culture, continuous culture, or fed-batch culture method. For example, methods such as centrifugation, filtration, treatment with a protein crystallizing precipitant (salting-out method), extraction, ultrasonic disruption, ultrafiltration, dialysis, various kinds of chromatographies such as molecular sieve chromatography (gel filtration), adsorption chromatography, ion exchange chromatography, affinity chromatography, *etc.*, HPLC or a combination thereof may be used, and the desired rebaudioside M can be recovered from the medium or the microorganisms using suitable methods known in the art.

Further, the method for producing rebaudioside M of the present application may further include a purification process, which may be performed using an appropriate method known in the art. In one example, when the method for producing rebaudioside M of the present application includes both a recovering step and a purification step, the recovering step and the purification step may be performed continuously or intermittently regardless of the order or simultaneously, or may be integrated into one step, but the method is not limited thereto.

In the method of the present application, the uridine diphosphate (UDP)–glycosyltransferase B (UGT-B), polynucleotides, vectors and microorganism are the same as described in other aspects.

Accordingly, the production method of the present application uses raw materials such as stevioside and rebaudioside A, which are inexpensive and can be easily obtained, and can convert stevioside and rebaudioside A, which are components with bitter contained in stevia extract, into rebaudioside D and rebaudioside M, which are components with excellent taste, and thus can be effectively used in the production

of stevia sweetener with excellent sweetening quality.

Still further another aspect of the present application provides a method for producing rebaudioside D from rebaudioside A, including:

reacting sucrose, nucleotide diphosphate, rebaudioside A, sucrose synthase and uridine diphosphate (UDP)–glycosyltransferase B (UGT-B) *in situ* to prepare rebaudioside D, wherein the uridine diphosphate (UDP)–glycosyltransferase B (UGT-B) is a protein consisting of an amino acid sequence of SEQ ID NO: 1.

Still further another aspect of the present application provides a method for producing rebaudioside M from rebaudioside A, including:

reacting sucrose, nucleotide diphosphate, rebaudioside A, rebaudioside D, sucrose synthase, uridine diphosphate (UDP)–glycosyltransferase A (UGT-A) and uridine diphosphate (UDP)–glycosyltransferase B (UGT-B) *in situ* to prepare rebaudioside M, wherein the uridine diphosphate (UDP)–glycosyltransferase B (UGT-B) is a protein consisting of an amino acid sequence of SEQ ID NO: 1.

The sucrose, nucleotide diphosphate, rebaudioside A, rebaudioside D, sucrose synthase, uridine diphosphate (UDP)–glycosyltransferase A (UGT-A), uridine diphosphate (UDP)–glycosyltransferase B (UGT-B), *in situ*, and rebaudioside M, etc., are the same as described in other aspects.

Specifically, the uridine diphosphate (UDP)–glycosyltransferase A (UGT-A) may be a protein consisting of an amino acid sequence of SEQ ID NO: 2, but is not limited thereto.

Still further another aspect of the present application provides a composition for producing rebaudioside D, including the uridine diphosphate (UDP)–glycosyltransferase B (UGT-B) of the present application, a polynucleotide encoding the uridine diphosphate (UDP)–glycosyltransferase B (UGT-B), a vector containing the polynucleotide, or a microorganism including the polynucleotide of the present application; a medium for culturing the microorganism; or a combination of two or more thereof.

Still further another aspect of the present application provides a composition for producing rebaudioside M, including the uridine diphosphate (UDP)–glycosyltransferase

B (UGT-B) and uridine diphosphate (UDP)–glycosyltransferase A (UGT-A) of the present application, a polynucleotide encoding the uridine diphosphate (UDP)–glycosyltransferase B (UGT-B) and a polynucleotide encoding the uridine diphosphate (UDP)–glycosyltransferase A (UGT-A), a vector containing the polynucleotide, or a microorganism including the polynucleotide of the present application; a medium for culturing the microorganism; or a combination of two or more thereof.

The composition of the present application may further include any suitable excipient commonly used in the composition for producing amino acids, and such excipient may be, for example, a preservative, a wetting agent, a dispersing agent, a suspending agent, a buffering agent, a stabilizing agent, or an isotonic agent, but is not limited thereto.

In the composition of the present application, the uridine diphosphate (UDP)–glycosyltransferase B (UGT-B), uridine diphosphate (UDP)–glycosyltransferase A (UGT-A), polynucleotides, vectors, strains, media and L-valine, *etc.* are the same as described in the other aspects.

Still further another aspect of the present application provides the use of uridine diphosphate (UDP)–glycosyltransferase B (UGT-B) consisting of the amino acid sequence of SEQ ID NO: 1 as a glycosyltransferase that converts rebaudioside D from rebaudioside A.

The SEQ ID NO: 1, uridine diphosphate (UDP)–glycosyltransferase B (UGT-B), rebaudioside A, rebaudioside D, *etc.* are the same as described in other aspects.

[Mode for Carrying Out the Invention]

Hereinafter, the present application will be described in detail by way of Examples. However, these Examples are merely preferred Examples given for illustrative purposes, and thus, the scope of the present application is not intended to be limited to or by these Examples. Meanwhile, technical features which are not described herein can be sufficiently understood and easily carried out by those skilled in the art in the technical field of the present application or in a similar technical field.

Example 1. Design of Novel Uridine Diphosphate (UDP)–Glycosyltransferase B (UGT-B)

Example 1-1. Design of Novel Uridine Diphosphate (UDP)–Glycosyltransferase B (UGT-B)

In order to develop excellent enzymes for producing rebaudioside D and rebaudioside M, two types of plant-derived enzymes, which are known to recognize stevia and rebaudioside A as acceptors for glycosyl transfer reaction and UDP-glucose as donors, were selected, and the expression thereof in microorganisms and their conversion activity from rebaudioside A to rebaudioside D were evaluated, and the results are shown in Table 1 below.

[Table 1]

Wild-type protein	Origin	Protein ID	Enzyme Expression Level (--, -, +, ++)	Conversion Rate (%) (--, -, +, ++)
W1	<i>Hordeum vulgare</i>	BAJ94055.1	+	++
W2	<i>Brachypodium distachyon</i>	XP_003560669.1	--	+

As shown in Table 1, the two types of plant-derived enzymes showed a low enzyme expression level and conversion rate, which decreased the efficiency for the industrial production of rebaudioside D, and accordingly, new enzymes were designed to improve the efficiency.

Specifically, based on the chemical structure and reaction mechanism of the substrate used in the glycosyl transfer reaction of the enzymes, it could be expected that the tertiary structure of each protein belonged to the GT-B fold. Since the protein with the GT-B fold structure has a characteristic that the N-domain that recognizes the sugar acceptor is clearly distinguished from the C-domain that recognizes the sugar donor, a total of 12 types of improved enzymes were prepared, and their expressions

and activities were evaluated by way of the method of mixing the N-domain and C-domain of each protein to produce a chimera.

As a result, uridine diphosphate (UDP)–glycosyltransferase B (UGT-B), a novel enzyme with smooth protein expression and improved activity in microorganisms compared to the wild-type enzyme, was prepared as shown in the schematic diagram below, and its sequence is represented by SEQ ID NO: 1.

[Schematic Diagram 1]

W1 (N-domain) 256 AA	W2 (C-domain) 207 AA
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Example 1-2. Preparation of Novel Uridine Diphosphate (UDP)–Glycosyltransferase B (UGT-B) and Measurement of Activity Thereof

For the novel enzyme for converting rebaudioside A to rebaudioside D designed in Example 1-1, W1 and W2 enzymes, a recombinant plasmid (vector-pET24a) containing a gene encoding UDP–glycosyltransferase was prepared and cloned into *E. coli* BL21 (DE3) to produce the enzymes on a large scale, and the enzymes were purified and used.

A test tube containing 5 mL of LB medium was inoculated with the recombinant strain BL21(DE3), followed by seed culturing in an incubator at 37°C until the absorbance at 600 nm reached 2.0. The seed cultured-solution was added to a flask containing 500 mL of LB medium and then cultured. Further, 0.1 mM IPTG (isopropyl β -D-1-thiogalacthiopyranoside) was added until the absorbance at 600 nm reached 0.4, thereby inducing mass expression of the enzymes. The culture conditions were adjusted so that the stirring speed was 180 rpm and the culture temperature was 37°C during the procedure, while the stirring speed was 120 rpm and the culture temperature was 16°C after the addition of IPTG. The culture solution of the transformed strain was centrifuged at 6,000 *g* at 4°C for 20 minutes to separate a cell supernatant as an enzyme solution. In order to precisely identify the properties of the enzymes, the enzyme solution was purified using a Ni-NTA superflow column.

Meanwhile, the recombinant *Corynebacteria* were inoculated into a medium (Bacto-Trypton 10 g/L, Bacto-yeast extract 5 g/L, NaCl 5 g/L, Soytone 5 g/L) containing kanamycin at a concentration of 10 µg/mL with an initial concentration of O.D.₆₀₀=0.1, and cultured at 30°C for 24 hours to induce expression of the enzyme. The thus-obtained culture solution was inoculated into a fermenter containing a medium (glucose 80 g/L, soytone 20 g/L, (NH₄)₂SO₄ 10 g/L, KH₂PO₄ 1.2 g/L, MgSO₄ 1.4 g/L) containing kanamycin at a concentration of 10 µg/mL with O.D.₆₀₀=0.6 and cultured at 30°C for 24 hours.

The raw material used in the enzymatic reaction was RebA (Daepyeong), which was dissolved in water to concentrations of 1 mM, 5 mM, 10 mM, and 20 mM, and the enzymes expressed in microorganisms (uridine diphosphate (UDP)–glycosyltransferase B (UGT-B), W1, W2) were used. The reaction was carried out at 37°C for 24 hours and then analyzed by HPLC. UDP-glucose (Carbosynth) was added to the raw material aqueous solution at 2 mM, 10 mM, 20 mM, and 40 mM. As the enzyme, purified enzymes were used, and the reaction was carried out at an enzyme concentration of 0.1 mg/mL.

The measurement results are shown in Tables 2 and 3 below.

[Table 2]

Comparison Evaluation of Conversion Rate from Rebaudioside A to Rebaudioside D according to Substrate Concentration (%)

Substrate Concentration (mM)	1 mM	5 mM	10 mM	20 mM
W1	96	89.1	63.2	41.5
W2	35.2	23.6	14.8	4.1
Uridine diphosphate	98.6	97.2	78.1	56.3

(UDP)– glycosyltransferase B B(UGT-B)				
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[Table 3]

Comparison Evaluation of Protein Expression Level

Wild type protein	Origin	Protein ID	Enzyme Expression Level (--, -, +, ++)
W1	<i>Hordeum vulgare</i>	BAJ94055.1	+
W2	<i>Brachypodium distachyon</i>	XP_003560669.1	--
Uridine diphosphate (UDP)– glycosyltransferase B (UGT-B)	-	-	++

As shown in Tables 2 and 3, it was confirmed that the novel uridine diphosphate (UDP)–glycosyltransferase B (UGT-B) of the present application showed improved conversion yield of rebaudioside D from rebaudioside A and protein expression level.

**Example 2. Measurement of Uridine Diphosphate (UDP)–
Glycosyltransferase B (UGT-B) Activity for Rebaudioside A as Raw Material**

The enzyme activity of uridine diphosphate (UDP)–glycosyltransferase B (UGT-B) for rebaudioside A as a raw material was measured using the uridine diphosphate (UDP)–glycosyltransferase B (UGT-B) prepared in Example 1-2.

Specifically, the raw material used for the enzymatic reaction was RebA (Daepyeong), which was dissolved in water to a concentration of 2 mM, and the enzymes expressed in microorganisms (SEQ ID NO: 4) were used. The reaction was carried out at 37°C for 16 hours and then analyzed by HPLC. The raw material aqueous solution may contain UDP-glucose or UDP (uridine-diphosphate) at a concentration of 2 mM.

The conditions for HPLC Analysis are as follows:

- Detector wavelength: 210 nm
- Flow rate: 1 mL/min
- Sample injection vol.: 10 µL
- Column: Capcell pak C18 MG II (Shiseido, 250 mm × 4.6 mm, particle size: 5 µm)
- Solvent: Acetonitrile 30%

The measurement results are shown in Table 4 and Fig. 1 below.

[Table 4]

Sample	Rebaudioside D Content (%)	Rebaudioside A Content (%)
Raw Material (Reaction Time: 0)	0.3	99.7
Uridine diphosphate (UDP)– glycosyltransferase B	98.6	1.4

(UGT-B)		
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As shown in Table 4 and Fig. 1, it was confirmed that most (98.6%) of rebaudioside A was converted to rebaudioside D by the uridine diphosphate (UDP)–glycosyltransferase B (UGT-B) of the present application.

Example 3. Measurement of Uridine Diphosphate (UDP)–Glycosyltransferase A (UGT-A) and Uridine Diphosphate (UDP)–Glycosyltransferase B (UGT-B) Activities for Stevioside as Raw Material

Uridine diphosphate (UDP)–glycosyltransferase A (UGT-A) and sucrose synthase were purified using the method disclosed in the prior literature (WO 2014/133248), and their sequences were represented by SEQ ID NO: 2 and SEQ ID NO: 3, respectively.

The enzyme activities of uridine diphosphate (UDP)–glycosyltransferase B (UGT-B) and uridine diphosphate (UDP)–glycosyltransferase A (UGT-A) for stevioside as a raw material were measured using the uridine diphosphate (UDP)–glycosyltransferase A (UGT-A), sucrose synthase, and the uridine diphosphate (UDP)–glycosyltransferase B (UGT-B) prepared in Example 1-2.

Specifically, the raw material used for the enzymatic reaction were 2 mM stevioside (Haigen) and a 50 mM sugar (CJ Cheiljedang) aqueous solution, and the uridine diphosphate (UDP)–glycosyltransferase A (UGT-A), the sucrose synthase, and the uridine diphosphate (UDP)–glycosyltransferase B (UGT-B) prepared in Example 1-2, which were expressed in microorganisms, were used. The reaction was carried out at 37°C for 16 hours and then analyzed by HPLC. The raw material aqueous solution may contain UDP-glucose or UDP (uridine-diphosphate) at a concentration of 2 mM.

The measurement results are shown in Table 5 and Fig. 2 below.

[Table 5]

Sample	Rebaudiosi	Rebaudiosi	Rebaudiosi	Rebaudiosi	Steviosi
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	de D (%)	de M (%)	de I (%)	de A (%)	de (%)
Raw Material (Reaction Time: 0)	0	0	0	4.6	9 5.4
Uridine diphosphate (UDP)– glycosyltransfer ase B (UGT-B), Uridine diphosphate (UDP)– glycosyltransfer ase A (UGT-A), Sucrose Synthase	3.5	91	5.5	0	0

As shown in Table 5 and Fig. 2, it was confirmed that 100% of stevioside and rebaudioside A was converted to rebaudioside D, rebaudioside M, and rebaudioside I relative to the molar concentration.

Example 4. Measurement of Uridine Diphosphate (UDP)–Glycosyltransferase A (UGT-A) Activity for Rebaudioside D as Raw Material

The conversion rate of rebaudioside M from rebaudioside D was measured by the uridine diphosphate (UDP)–glycosyltransferase A (UGT-A) of Example 3.

Specifically, the raw material used for the enzymatic reaction was rebaudioside D (Haigen), which was dissolved in water to a concentration of 1 mM, and the uridine diphosphate (UDP)–glycosyltransferase A (UGT-A) expressed in microorganisms were used. The reaction was carried out at 37°C for 16 hours and then analyzed by HPLC. The raw material aqueous solution may contain UDP-glucose or UDP (uridine-diphosphate) at a concentration of 2 mM.

The measurement results are shown in Fig. 3.

As shown in Fig. 3, it was confirmed that most rebaudioside D was converted to rebaudioside M by the uridine diphosphate (UDP)–glycosyltransferase A (UGT-A) of the present application.

From the foregoing, a skilled person in the art to which the present application pertains will be able to understand that the present application may be embodied in other specific forms without modifying the technical concepts or essential characteristics of the present application. In this regard, the exemplary embodiments disclosed herein are only for illustrative purposes and should not be construed as limiting the scope of the present application. The scope of the present application is therefore indicated by the appended claims rather than by the foregoing description. All changes which come within the meaning and range of equivalency of the claims are to be embraced within the scope of the present application.

[CLAIMS]

[Claim 1]

Uridine diphosphate (UDP)–glycosyltransferase B (UGT-B) consisting of an amino acid sequence of SEQ ID NO: 1.

[Claim 2]

A polynucleotide encoding uridine diphosphate (UDP)–glycosyltransferase B (UGT-B) consisting of an amino acid sequence of SEQ ID NO: 1.

[Claim 3]

An expression vector containing a polynucleotide encoding uridine diphosphate (UDP)–glycosyltransferase B (UGT-B) consisting of an amino acid sequence of SEQ ID NO: 1.

[Claim 4]

A microorganism comprising uridine diphosphate (UDP)–glycosyltransferase B (UGT-B) consisting of an amino acid sequence of SEQ ID NO: 1 or a polynucleotide encoding the uridine diphosphate (UDP)–glycosyltransferase B (UGT-B).

[Claim 5]

A method for producing rebaudioside D, comprising:
reacting nucleotide diphosphate to which glucose is bonded with rebaudioside A in the presence of uridine diphosphate (UDP)–glycosyltransferase B (UGT-B) to prepare rebaudioside D,
wherein the uridine diphosphate (UDP)–glycosyltransferase B (UGT-B) is a protein consisting of an amino acid sequence of SEQ ID NO: 1.

[Claim 6]

The method of claim 5, wherein the nucleotide diphosphate to which glucose is bonded is prepared by reacting sucrose and nucleotide diphosphate in the presence of a sucrose synthase.

[Claim 7]

The method of claim 5, wherein the rebaudioside A is prepared by reacting nucleotide diphosphate to which glucose is bonded with stevioside in the presence of uridine diphosphate (UDP)–glycosyltransferase A (UGT-A).

[Claim 8]

The method of claim 6, wherein the sucrose synthase is a protein consisting of an amino acid sequence of SEQ ID NO: 3.

[Claim 9]

The method of claim 7, wherein the uridine diphosphate (UDP)–glycosyltransferase A (UGT-A) is a protein consisting of an amino acid sequence of SEQ ID NO: 2.

[Claim 10]

The method of any one of claims 5 to 9, wherein the method is performed consecutively *in situ*.

[Claim 11]

A method for producing rebaudioside M, comprising:

reacting nucleotide diphosphate to which glucose is bonded with rebaudioside A in the presence of uridine diphosphate (UDP)–glycosyltransferase B (UGT-B) to prepare rebaudioside D; and

reacting the rebaudioside D with nucleotide diphosphate to which glucose is bonded in the presence of uridine diphosphate (UDP)–glycosyltransferase A (UGT-A) to prepare rebaudioside M,

wherein the uridine diphosphate (UDP)–glycosyltransferase B (UGT-B) is a protein consisting of an amino acid sequence of SEQ ID NO: 1.

[Claim 12]

The method of claim 11, wherein the uridine diphosphate (UDP)–glycosyltransferase A (UGT-A) is a protein consisting of an amino acid sequence of SEQ ID NO: 2.

[Claim 13]

The method of claim 11, wherein the nucleotide diphosphate to which glucose is bonded is prepared by reacting sucrose with nucleotide diphosphate in the presence of a sucrose synthase.

[Claim 14]

The method of claim 11, wherein the rebaudioside A is prepared by reacting nucleotide diphosphate to which glucose is bonded with stevioside in the presence of uridine diphosphate (UDP)–glycosyltransferase A (UGT-A).

[Claim 15]

The method of claim 13, wherein the sucrose synthase is a protein consisting of an amino acid sequence of SEQ ID NO: 3.

[Claim 16]

The method of claim 14, wherein the uridine diphosphate (UDP)–glycosyltransferase A (UGT-A) is a protein consisting of an amino acid sequence of SEQ ID NO: 2.

[Claim 17]

The method of any one of claims 11 to 16, wherein the method is performed consecutively *in situ*.

[Claim 18]

A method for producing rebaudioside D from rebaudioside A, comprising:
reacting sucrose, nucleotide diphosphate, rebaudioside A, sucrose synthase and uridine diphosphate (UDP)–glycosyltransferase B (UGT-B) *in situ* to prepare

rebaudioside D,

wherein the uridine diphosphate (UDP)–glycosyltransferase B (UGT-B) is a protein consisting of an amino acid sequence of SEQ ID NO: 1.

[Claim 19]

A method for producing rebaudioside M from rebaudioside A, comprising:

reacting sucrose, nucleotide diphosphate, rebaudioside A, rebaudioside D, sucrose synthase, uridine diphosphate (UDP)–glycosyltransferase A (UGT-A) and uridine diphosphate (UDP)–glycosyltransferase B (UGT-B) *in situ* to prepare rebaudioside M,

wherein the uridine diphosphate (UDP)–glycosyltransferase B (UGT-B) is a protein consisting of an amino acid sequence of SEQ ID NO: 1.

[Claim 20]

The method of claim 19, wherein the uridine diphosphate (UDP)–glycosyltransferase A (UGT-A) is a protein consisting of an amino acid sequence of SEQ ID NO: 2.