ENGINEERED GLYCOSYLTRANSFERASES AND STEVIOL GLYCOSIDE GLUCOSYLATION METHODS

[0001] The present application claims priority to US Prov. Pat. Appln. Ser. No. 62/712,199, filed July 30, 2018, and US Prov. Pat. Appln. Ser. No. 62/712,327, filed July 31, 2018, both of which are hereby incorporated by reference in their entireties, for all purposes.

FIELD OF THE INVENTION

[0002] The present invention provides engineered glycosyltransferase (GT) enzymes, polypeptides having GT activity, and polynucleotides encoding these enzymes, as well as vectors and host cells comprising these polynucleotides and polypeptides. The present invention provides engineered sucrose synthase (SuS) enzymes, polypeptides having SuS activity, and polynucleotides encoding these enzymes, as well as vectors and host cells comprising these polynucleotides and polypeptides. The present invention also provides compositions comprising the GT enzymes and methods of using the engineered GT enzymes to make products with β-glucose linkages. The present invention further provides compositions and methods for the production of rebaudiosides (e.g., rebaudioside M, rebaudioside A, rebaudioside I, and rebaudioside D). The present invention also provides compositions comprising the SuS enzymes and methods of using them. Methods for producing GT and SuS enzymes are also provided.

REFERENCE TO SEQUENCE LISTING, TABLE OR COMPUTER PROGRAM

[0003] The official copy of the Sequence Listing is submitted concurrently with the specification as an ASCII formatted text file via EFS-Web, with a file name of "CX8-180USP1A_ST25.txt", a creation date of July 31, 2018, and a size of 8,929 kilobytes. The Sequence Listing filed via EFS-Web is part of the specification and is incorporated in its entirety by reference herein.

BACKGROUND OF THE INVENTION

[0004] Glycosyltransferases (GT) are enzymes that post-translationally transfer glycosyl residues from an activated nucleoside sugar to monomeric and polymeric acceptor molecules (e.g., other sugars, proteins, lipids, and other organic substrates). Thus, these enzymes utilize an activated donor sugar substrate that contains a substituted phosphate leaving group. Donor sugar substrates (i.e., the "glycosyl donor") are commonly activated as nucleoside diphosphate sugars. However, other sugars, such as nucleoside monophosphate sugars, lipid phosphates and unsubstituted phosphates are also used (See e.g., Lairson et al., Ann. Rev. Biochem., 77:25.1-25.35 [2008]). GTs are classified as either retaining or inverting enzymes, based on the stereochemistry of the substrates and reaction products. In reactions where the stereochemistry of the donor's anomeric bond is retained (e.g., alpha to alpha), the GT is a retaining enzyme. In reactions where the stereochemistry is inverted (e.g., alpha to beta),

the GT is an inverting enzyme. These glycosylated products are involved in various metabolic pathways and processes. Indeed, the biosynthesis of numerous disaccharides, oligosaccharides, and polysaccharides involve the action of various glycosyltransferases. The transfer of a glucosyl moiety can alter the acceptor's bioactivity, solubility, and transport properties within cells. GTs have found use in the targeted synthesis of specific compounds (e.g., glycoconjugates and glycosides), as well as the production of differentially glycosylated drug, biological probes or natural product libraries. In some methods, the large scale use of GTs for glycoconjugate synthesis requires large quantities of glycosyl donors, adding to the cost of such approaches. Nucleotide recycling systems have been developed to allow the resynthesis of glycosyl donors from the released nucleotide. These recycling systems also reduce the amount of nucleotide by-product formed during the reaction, thereby reducing inhibition caused by the GT. Nonetheless, the need remains for improved methods suitable for large-scale production of glycoconjugates by GTs.

SUMMARY OF THE INVENTION

[0005] The present invention provides engineered glycosyltransferase (GT) enzymes, polypeptides having GT activity, and polynucleotides encoding these enzymes, as well as vectors and host cells comprising these polynucleotides and polypeptides. The present invention provides engineered sucrose synthase (SuS) enzymes, polypeptides having SuS activity, and polynucleotides encoding these enzymes, as well as vectors and host cells comprising these polynucleotides and polypeptides. The present invention also provides compositions comprising the GT enzymes and methods of using the engineered GT enzymes to make products with β-glucose linkages. The present invention further provides compositions and methods for the production of rebaudiosides (e.g., rebaudioside M, rebaudioside A, rebaudioside I, and rebaudioside D). The present invention also provides compositions comprising the SuS enzymes and methods of using them. Methods for producing GT and SuS enzymes are also provided.

[0006] The present invention provide engineered glycosyltransferase variants comprising polypeptide sequences that have at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or more sequence identity to SEQ ID NO: 2. In some embodiments, the engineered glycosyltransferase comprises a polypeptide that is at least 95%, 96%, 97%, 98%, 99%, or more sequence identity to SEQ ID NO: 20. In some additional embodiments, the polypeptide sequence of the engineered glycosyltransferase comprises at least one mutation or mutation set at one or more positions selected from 41/72/233/338, 41/72/338, 41/144/233, 41/233, 41/233/338, 61, 61/87/91/107, 61/87/91/259, 61/91/431, 61/107, 61/259/428, 61/407/428, 61/411, 72, 72/76, 72/76/163/197, 72/76/195/233, 72/76/197/204, 72/76/207/233, 72/76/207/338, 72/81, 72/81/195/233, 72/139/195/204, 72/144/338, 72/200/204/207, 72/207, 76/144/197/200, 76/195/197/204/207/233, 76/197/207/233, 76/233, 81/139/144/195/200/204/207/233, 81/144/233, 81/197/200/207/233/338, 81/233/338, 81/338, 107, 107/259, 139/144/233, 144/233, 144/233/338, 156/407, 163/233/338, 200/204/207/233, 233/338, and

259, wherein the positions are numbered with reference to SEQ ID NO: 20. In some additional embodiments, the polypeptide sequence of the engineered glycosyltransferase comprises at least one mutation or mutation set selected from 41E/72P/233Q/338A, 41E/72P/338A, 41E/144Q/233T, 41E/233Q, 41E/233Q/338V, 41E/233T, 41E/233T/338V, 61D, 61D/87K/91L/107L, 61D/87K/91L/259T, 61D/107V, 61D/259T/428I, 61D/407T/428I, 61E/87K/91L/107V, 61E/91L/431M, 61E/411T, 72P, 72P/76S, 72P/76S/163A/197K, 72P/76S/207V/338V, 72P/76T, 72P/76T/195Q/233T, 72P/76T/197K/204T, 72P/76T/207V/233Q, 72P/81T, 72P/81T/195Q/233Q, 72P/139N/195Q/204T, 72P/144Q/338V, 72P/200R/204T/207V, 72P/207V, 76S/144Q/197K/200R, 76S/195Q/197K/204T/207V/233T, 76S/197K/207V/233Q, 76S/233T, 81T/139N/144Q/195Q/200R/204T/207V/233Q, 81T/144Q/233Q, 81T/197K/200R/207V/233Q/338A, 81T/233Q/338V, 81T/338V, 107V, 107V/259T, 139N/144Q/233Q, 144Q/233T, 144Q/233T/338V, 156S/407T, 163A/233T/338A, 200R/204T/207V/233T, 233Q/338V, and 259T, wherein the positions are numbered with reference to SEQ ID NO: 20. In some additional embodiments, the polypeptide sequence of the engineered glycosyltransferase comprises at least one mutation or mutation set selected from A41E/T72P/W233Q/C338A, A41E/T72P/C338A, A41E/M144Q/W233T, A41E/W233Q, A41E/W233Q/C338V, A41E/W233T, A41E/W233T/C338V, Q61D, Q61D/A87K/Q91L/A107L, Q61D/A87K/Q91L/E259T, Q61D/A107V, Q61D/E259T/K428I, Q61D/I407T/K428I, Q61E/A87K/Q91L/A107V, Q61E/Q91L/D431M, Q61E/R411T, T72P, T72P/R76S, T72P/R76S/L163A/Q197K, T72P/R76S/I207V/C338V, T72P/R76T, T72P/R76T/H195Q/W233T, T72P/R76T/Q197K/D204T, T72P/R76T/I207V/W233Q, T72P/H81T, T72P/H81T/H195Q/W233Q, T72P/K139N/H195Q/D204T, T72P/M144Q/C338V, T72P/K200R/D204T/I207V, T72P/I207V, R76S/M144Q/Q197K/K200R, R76S/H195Q/Q197K/D204T/I207V/W233T, R76S/Q197K/I207V/W233Q, R76S/W233T, H81T/K139N/M144Q/H195Q/K200R/D204T/I207V/W233Q, H81T/M144Q/W233Q, H81T/Q197K/K200R/I207V/W233Q/C338A, H81T/W233Q/C338V, H81T/C338V, A107V, A107V/E259T, K139N/M144Q/W233Q, M144Q/W233T, M144Q/W233T/C338V, C156S/I407T, L163A/W233T/C338A, K200R/D204T/I207V/W233T, W233Q/C338V, and E259T, wherein the positions are numbered with reference to SEQ ID NO: 20.

[0007] In some further embodiments, the polypeptide sequence of the engineered glycosyltransferase comprises at least one mutation or mutation set at one or more positions selected from 71, 80, 81, 81/270, 83, 85, 97, 124, 263, 286, 334, 402, 420, and 456, wherein the positions are numbered with reference to SEQ ID NO: 20. In some additional embodiments, the polypeptide sequence of the engineered glycosyltransferase comprises at least one mutation or mutation set selected from 71G, 71T, 80D, 80P, 80Q, 81A, 81L, 81M, 81S, 81T, 81V, 81V/270K, 83D, 85T, 85V, 97V, 124P, 263C, 286L, 334V, 402I, 420R, and 456K, wherein the positions are numbered with reference to SEQ ID NO: 20. In some additional embodiments, the polypeptide sequence of the engineered glycosyltransferase comprises at least one mutation or mutation set selected from Q71G, Q71T,

T80D, T80P, T80Q, H81A, H81L, H81M, H81S, H81T, H81V, H81V/Q270K, P83D, A85T, A85V, A97V, A124P, T263C, V286L, I334V, E402I, E420R, and S456K, wherein the positions are numbered with reference to SEQ ID NO: 20.

[0008] In some additional embodiments, the polypeptide sequence of the engineered glycosyltransferase comprises at least one mutation or mutation set at one or more positions selected from 41/72/233/338, 41/72/338, 61, 61/91/431, 61/259/428, 61/407/428, wherein the positions are numbered with reference to SEQ ID NO: 20. In some additional embodiments, the polypeptide sequence of the engineered glycosyltransferase comprises at least one mutation or mutation set selected from 41E/72P/233Q/338A, 41E/72P/338A, 61D, 61D/259T/428I, 61D/407T/428I, 61E/91L/431M, 81T/139N/144Q/195Q/200R/204T/207V/233Q, and 81T/197K/200R/207V/233Q/338A, wherein the positions are numbered with reference to SEQ ID NO: 20. In some additional embodiments, the polypeptide sequence of the engineered glycosyltransferase comprises at least one mutation or mutation set selected from A41E/T72P/W233Q/C338A, A41E/T72P/C338A, Q61D, Q61D/E259T/K428I, Q61D/I407T/K428I, Q61E/Q91L/D431M, H81T/K139N/M144Q/H195Q/K200R/D204T/I207V/W233Q, and H81T/Q197K/K200R/I207V/W233Q/C338A, wherein the positions are numbered with reference to SEQ ID NO: 20.

[0009] In yet some further embodiments, the polypeptide sequence of the engineered glycosyltransferase comprises at least one mutation or mutation set at one or more positions selected from 73/81/144/207/259, 73/81/144/207/285/451, 73/81/259/285/426/451, 73/81/259/451, 73/111/285/451, 73/144/207/252/426/451, 73/144/207/259/285/426/451, 73/144/207/259/285/451, 73/144/207/259/426/451, 73/144/259/285, 73/259/426/451, 73/259/451, 81/111/144/207/285/451, 81/111/259/451, 81/144/207/252/285/426, 81/144/207/259/451, 81/144/252/259/285/451, 81/144/259, 81/144/259/285, 81/144/259/451, 81/207/252/259/451, 81/207/285/426/451, 81/285, 81/285/451, 111/144/207/252/285/426/451, 111/144/252/259/285/451, 111/144/259/285/426/451, 111/207/285, 144/207/252/259, 144/207/259, 144/207/259/285/451, 144/207/259/426/451, 144/207/285/426, 144/207/451, 144/252/259/285, 144/252/259/285/451, 144/252/259/426, 144/252/259/426/451, 144/259/285, 144/285, 144/285/451, 144/451, 207, 207/252/259/451, 207/252/451, 207/259, 207/259/426/451, 207/285/426/451, 207/451, 252/259, 259, 259/285/451, 285, and 285/451, wherein the positions are numbered with reference to SEQ ID NO: 36.

[0010] In some additional embodiments, the polypeptide sequence of the engineered glycosyltransferase comprises at least one mutation or mutation set selected from 73S/81T/144Q/207V/259T, 73S/81T/144Q/207V/285S/451Q, 73S/81T/259T/285S/426I/451Q, 73S/81T/259T/451Q, 73S/111T/285S/451Q, 73S/144Q/207V/252P/426I/451Q, 73S/144Q/207V/259T/285S/426I/451Q, 73S/144Q/207V/259T/285S/451Q, 73S/144Q/207V/259T/426I/451Q, 73S/144Q/259T/285S, 73S/259T/426I/451Q, 73S/259T/451Q, 81T/111T/144Q/207V/285S/451Q, 81T/111T/259T/451Q, 81T/144Q/207V/252P/285S/426I,

81T/144Q/207V/259T/451Q, 81T/144Q/252P/259T/285S/451Q, 81T/144Q/259T, 81T/144Q/259T/285S, 81T/144Q/259T/451Q, 81T/207V/252P/259T/451Q, 81T/207V/285S/426I/451Q, 81T/285S, 81T/285S/451Q, 111T/144Q/207V/252P/285S/426I/451Q, 111T/144Q/252P/259T/285S/451Q, 111T/144Q/259T/285S/426I/451Q, 111T/207V/285S, 144Q/207V/252P/259T, 144Q/207V/259T, 144Q/207V/259T/285S/451Q, 144Q/207V/259T/426I/451Q, 144Q/207V/285S/426I, 144Q/207V/451Q, 144Q/252P/259T/285S, 144Q/252P/259T/285S/451Q, 144Q/252P/259T/426I, 144Q/252P/259T/426I/451Q, 144Q/259T/285S, 144Q/285S, 144Q/285S/451Q, 144Q/451Q, 207V, 207V/252P/259T/451Q, 207V/252P/451Q, 207V/259T, 207V/259T/426I/451Q, 207V/285S/426I/451Q, 207V/451Q, 252P/259T, 259T, 259T/285S/451Q, 285S, and 285S/451Q, wherein the positions are numbered with reference to SEQ ID NO: 36. In some additional embodiments, the polypeptide sequence of the engineered glycosyltransferase comprises at least one mutation or mutation set selected from E73S/H81T/M144Q/I207V/E259T, E73S/H81T/M144Q/I207V/E285S/V451Q, E73S/H81T/E259T/E285S/A426I/V451Q, E73S/H81T/E259T/V451Q, E73S/S111T/E285S/V451Q, E73S/M144Q/I207V/S252P/A426I/V451Q, E73S/M144Q/I207V/E259T/E285S/A426I/V451Q, E73S/M144Q/I207V/E259T/E285S/V451Q, E73S/M144Q/I207V/E259T/A426I/V451Q, E73S/M144Q/E259T/E285S, E73S/E259T/A426I/V451Q, E73S/E259T/V451Q, H81T/S111T/M144Q/I207V/E285S/V451Q, H81T/S111T/E259T/V451Q, H81T/M144Q/I207V/S252P/E285S/A426I, H81T/M144Q/I207V/E259T/V451Q, H81T/M144Q/S252P/E259T/E285S/V451Q, H81T/M144Q/E259T, H81T/M144Q/E259T/E285S, H81T/M144Q/E259T/V451Q, H81T/I207V/S252P/E259T/V451Q, H81T/I207V/E285S/A426I/V451Q, H81T/E285S, H81T/E285S/V451Q, S111T/M144Q/I207V/S252P/E285S/A426I/V451Q, S111T/M144Q/S252P/E259T/E285S/V451Q, S111T/M144Q/E259T/E285S/A426I/V451Q, S111T/I207V/E285S, M144Q/I207V/S252P/E259T, M144Q/I207V/E259T, M144Q/I207V/E259T/E285S/V451Q, M144Q/I207V/E259T/A426I/V451Q, M144Q/I207V/E285S/A426I, M144Q/I207V/V451Q, M144Q/S252P/E259T/E285S, M144Q/S252P/E259T/E285S/V451Q, M144Q/S252P/E259T/A426I, M144Q/S252P/E259T/A426I/V451Q, M144Q/E259T/E285S, M144Q/E285S, M144Q/E285S/V451Q, M144Q/V451Q, I207V, I207V/S252P/E259T/V451Q, I207V/S252P/V451Q, I207V/E259T, I207V/E259T/A426I/V451Q, I207V/E285S/A426I/V451Q, I207V/V451Q, S252P/E259T, E259T, E259T/E285S/V451Q, E285S, and E285S/V451Q, wherein the positions are numbered with reference to SEQ ID NO: 36. In yet some further embodiments, the polypeptide sequence of the engineered glycosyltransferase comprises at least one mutation or mutation set at one or more positions selected from 81/111/144/207/285/451, 144/207/252/259, 144/207/259/285/451, 144/252/259/426, 144/252/259/426/451, and 144/285/451, wherein the positions are numbered with reference to SEQ ID NO: 36. In some additional embodiments, the polypeptide sequence of the engineered glycosyltransferase comprises at least one mutation or mutation set selected from

81T/111T/144Q/207V/285S/451Q, 144Q/207V/252P/259T, 144Q/207V/259T/285S/451Q, 144Q/252P/259T/426I, 144Q/252P/259T/426I/451Q, and 144Q/285S/451Q, wherein the positions are numbered with reference to SEQ ID NO: 36. In some additional embodiments, the polypeptide sequence of the engineered glycosyltransferase comprises at least one mutation or mutation set selected from H81T/S111T/M144Q/I207V/E285S/V451Q, M144Q/I207V/S252P/E259T, M144Q/I207V/E259T/E285S/V451Q, M144Q/S252P/E259T/A426I, M144Q/S252P/E259T/A426I/V451Q, and M144Q/E285S/V451Q, wherein the positions are numbered with reference to SEQ ID NO: 36.

[0011] In yet some further embodiments, the polypeptide sequence of the engineered glycosyltransferase comprises at least one mutation or mutation set at one or more positions selected from 80/81, 80/81/85, 80/81/85/238, 80/81/85/251/259, 80/81/85/252/256/259, 80/81/85/259, 80/81/251/252/259, 80/85, 80/85/238, 80/85/238/251/259, 80/85/251, 80/85/251/252, 80/85/251/252/256/259, 80/85/251/252/259, 80/85/251/259, 80/85/259, 80/259, 81/85, 81/85/251/252, 81/85/259, 85, 85/238, 85/251, 85/251/252/256, 85/251/259, 85/256/259, 175/402, 251/252/259, 259, 270/402, and 420, wherein the positions are numbered with reference to SEQ ID NO: 174. In some additional embodiments, the polypeptide sequence of the engineered glycosyltransferase comprises at least one mutation or mutation set selected from 80D/81A, 80D/81A/85V, 80D/81A/85V/238G, 80D/81A/85V/251M/259T, 80D/81A/85V/252P/256G/259T, 80D/85V, 80D/85V/238G, 80D/85V/238G/251M/259T, 80D/85V/251I/252P/256G/259T, 80D/85V/251I/259T, 80D/85V/251M, 80D/85V/251M/252P, 80D/85V/251M/252P/256G/259T, 80D/85V/259T, 80D/259T, 80P/81A/85V/259T, 80P/81A/251I/252P/259T, 80P/85V, 80P/85V/251M/252P/259T, 81A/85V, 81A/85V/251M/252P, 81A/85V/259T, 85V, 85V/238G, 85V/251F, 85V/251F/259T, 85V/251M/252P/256G, 85V/256G/259T, 175D/402I, 251I/252P/259T, 259T, 270K/402I, and 420R, wherein the positions are numbered with reference to SEQ ID NO: 174. In some additional embodiments, the polypeptide sequence of the engineered glycosyltransferase comprises at least one mutation or mutation set selected from T80D/T81A, T80D/T81A/A85V, T80D/T81A/A85V/A238G, T80D/T81A/A85V/A251M/E259T, T80D/T81A/A85V/S252P/L256G/E259T, T80D/A85V, T80D/A85V/A238G, T80D/A85V/A238G/A251M/E259T, T80D/A85V/A251I/S252P/L256G/E259T, T80D/A85V/A251I/E259T, T80D/A85V/A251M, T80D/A85V/A251M/S252P, T80D/A85V/A251M/S252P/L256G/E259T, T80D/A85V/E259T, T80D/E259T, T80P/T81A/A85V/E259T, T80P/T81A/A251I/S252P/E259T, T80P/A85V, T80P/A85V/A251M/S252P/E259T, T81A/A85V, T81A/A85V/A251M/S252P, T81A/A85V/E259T, A85V, A85V/A238G, A85V/A251F, A85V/A251F/E259T, A85V/A251M/S252P/L256G, A85V/L256G/E259T, E175D/E402I, A251I/S252P/E259T, E259T, Q270K/E402I, and E420R, wherein the positions are numbered with reference to SEQ ID NO: 174. In some embodiments, the polypeptide sequence of the engineered glycosyltransferase comprises at least one mutation or mutation set at one or more positions selected from 3, 5, 7, 99, 153, 232/317, 252, 273, 299, 326, 393,

404, 409, 422, 443, 451, and 455, wherein the positions are numbered with reference to SEQ ID NO: 174. In some additional embodiments, the polypeptide sequence of the engineered glycosyltransferase comprises at least one mutation or mutation set selected from 3S, 5A, 5H, 5L, 5T, 7A, 7Q, 99V, 153C, 232I/317L, 252A, 273K, 299M, 326A, 326E, 326N, 326S, 393I, 404M, 404V, 409S, 422I, 443A, 451E, 451V, 455M, and 455R, wherein the positions are numbered with reference to SEQ ID NO: 174. In some additional embodiments, the polypeptide sequence of the engineered glycosyltransferase comprises at least one mutation or mutation set selected from N3S, S5A, S5H, S5L, S5T, T7A, T7Q, E99V, A153C, V232I/Y317L, S252A, S273K, V299M, G326A, G326E, G326N, G326S, V393I, G404M, G404V, A409S, M422I, S443A, Q451E, Q451V, S455M, and S455R, wherein the positions are numbered with reference to SEQ ID NO: 174. In yet some further embodiments, the polypeptide sequence of the engineered glycosyltransferase comprises at least one mutation or mutation set at one or more positions selected from 80/81/85, 80/85, 80/85/251/259, 85, 85/251/252/256, 175/402, and 270/402, wherein the positions are numbered with reference to SEQ ID NO: 174. In some additional embodiments, the polypeptide sequence of the engineered glycosyltransferase comprises at least one mutation or mutation set selected from 80D/81A/85V, 80D/85V/251I/259T, 80P/85V, 85V, 85V/251M/252P/256G, 175D/402I, and 270K/402I, wherein the positions are numbered with reference to SEQ ID NO: 174. In some additional embodiments, the polypeptide sequence of the engineered glycosyltransferase comprises at least one mutation or mutation set selected from T80D/T81A/A85V, T80D/A85V/A251I/E259T, T80P/A85V, A85V, A85V/A251M/S252P/L256G, E175D/E402I, and Q270K/E402I, wherein the positions are numbered with reference to SEQ ID NO: 174.

[0012] In yet some further embodiments, the polypeptide sequence of the engineered glycosyltransferase comprises at least one mutation or mutation set at one or more positions selected from 81/85, 81/85/175/259/402, 81/85/251, 81/85/251/259, 81/85/259, 81/85/259/402, 85, 85/175, 85/175/251, 85/175/251/259/402, 85/175/259, 85/175/259/402, 85/175/402, 85/259, 85/259/402, and 85/402, wherein the positions are numbered with reference to SEQ ID NO: 406. In some additional embodiments, the polypeptide sequence of the engineered glycosyltransferase comprises at least one mutation or mutation set selected from 81A/85V, 81A/85V/175E/259T/402E, 81A/85V/251I, 81A/85V/251I/259T, 81A/85V/259T, 81A/85V/259T/402E, 85V, 85V/175E, 85V/175E/251I, 85V/175E/251I/259T/402E, 85V/175E/259T, 85V/175E/259T/402E, 85V/175E/402E, 85V/259T, 85V/259T/402E, and 85V/402E, wherein the positions are numbered with reference to SEQ ID NO: 406. In some additional embodiments, the polypeptide sequence of the engineered glycosyltransferase comprises at least one mutation or mutation set selected from T81A/A85V, T81A/A85V/D175E/E259T/I402E, T81A/A85V/A251I, T81A/A85V/A251I/E259T, T81A/A85V/E259T, T81A/A85V/E259T/I402E, A85V, A85V/D175E, A85V/D175E/A251I, A85V/D175E/A251I/E259T/I402E, A85V/D175E/E259T, A85V/D175E/E259T/I402E,

A85V/D175E/I402E, A85V/E259T, A85V/E259T/I402E, and A85V/I402E, wherein the positions are numbered with reference to SEQ ID NO: 406.

[0013] In yest some further embodiments, the polypeptide sequence of the engineered glycosyltransferase comprises at least one mutation or mutation set at one or more positions selected from 153, 153/326, 153/326/443, 153/326/443/455, 232, 232/273/299, 232/393/451, 299/451, 326, 404, and 451, wherein the positions are numbered with reference to SEQ ID NO: 408. In some additional embodiments, the polypeptide sequence of the engineered glycosyltransferase comprises at least one mutation or mutation set selected from 153C, 153C/326S, 153C/326S/443A, 153C/326S/443A/455M, 232I, 232I/273K/299M, 232I/393I/451E, 299M/451E, 326E, 326S, 404V, and 451E, wherein the positions are numbered with reference to SEQ ID NO: 408. In some additional embodiments, the polypeptide sequence of the engineered glycosyltransferase comprises at least one mutation or mutation set selected from A153C, A153C/G326S, A153C/G326S/S443A, A153C/G326S/S443A/S455M, V232I, V232I/S273K/V299M, V232I/V393I/Q451E, V299M/Q451E, G326E, G326S, G404V, and Q451E, wherein the positions are numbered with reference to SEQ ID NO: 408. In some further embodiments, the polypeptide sequence of the engineered glycosyltransferase comprises at least one mutation or mutation set at one or more positions selected from 25, 116, 146, 170, 173, 227, 296, 300, 315, 327, 330, 361, 408, 412, 438, 448, and 449, wherein the positions are numbered with reference to SEQ ID NO: 408. In some additional embodiments, the polypeptide sequence of the engineered glycosyltransferase comprises at least one mutation or mutation set selected from 25A, 116D, 116R, 116S, 146G, 170C, 170G, 170P, 170S, 170T, 173S, 227T, 296V, 300S, 315T, 327Y, 330G, 330T, 361C, 408C, 408T, 412A, 412K, 412S, 438Q, 448H, and 449S, wherein the positions are numbered with reference to SEQ ID NO: 408. In some additional embodiments, the polypeptide sequence of the engineered glycosyltransferase comprises at least one mutation or mutation set selected from L25A, E116D, E116R, E116S, S146G, D170C, D170G, D170P, D170S, D170T, R173S, S227T, R296V, A300S, K315T, F327Y, E330G, E330T, T361C, N408C, N408T, R412A, R412K, R412S, A438Q, E448H, and R449S, wherein the positions are numbered with reference to SEQ ID NO: 408. In some additional embodiments, the polypeptide sequence of the engineered glycosyltransferase comprises at least one mutation or mutation set at one or more positions selected from 153, 232, 232/393/451, and 451, wherein the positions are numbered with reference to SEQ ID NO: 408. In some additional embodiments, the polypeptide sequence of the engineered glycosyltransferase comprises at least one mutation or mutation set selected from 153C, 232I, 232I/393I/451E, and 451E, wherein the positions are numbered with reference to SEQ ID NO: 408. In some additional embodiments, the polypeptide sequence of the engineered glycosyltransferase comprises at least one mutation or mutation set selected from A153C, V232I, V232I/V393I/Q451E, and Q451E, wherein the positions are numbered with reference to SEQ ID NO: 408.

[0014] In some embodiments, the polypeptide sequence of the engineered glycosyltransferase comprises at least one mutation or mutation set at one or more positions selected from 146/170/196,

146/170/196/232, 146/170/196/232/423, 146/170/196/232/451/455, 146/170/196/408/451, 146/170/232, 146/170/232/423, 146/170/232/423/448/451/455, 146/170/259, 146/196, 146/196/232, 146/196/232/259/448/451, 146/196/455, 146/232/259/455, 146/232/315, 146/232/315/423/451/455, 146/232/326, 146/232/448, 146/232/451, and 146/413/451/455, wherein the positions are numbered with reference to SEQ ID NO: 440. In some additional embodiments, the polypeptide sequence of the engineered glycosyltransferase comprises at least one mutation or mutation set selected from146G/170S/196I, 146G/170S/232I, 146G/170S/232I/423K/448H/451E/455M, 146G/170T/196I/232I, 146G/170T/196I/232I/423K, 146G/170T/196I/232I/451E/455R, 146G/170T/196I/408T/451E, 146G/170T/232I/423K, 146G/170T/259E, 146G/196I, 146G/196I/232I, 146G/196I/232I/259E/448H/451E, 146G/196I/455R, 146G/232I/259E/455M, 146G/232I/315S, 146G/232I/315S/423K/451E/455M, 146G/232I/326E, 146G/232I/448H, 146G/232I/451E, and 146G/413M/451E/455M, wherein the positions are numbered with reference to SEQ ID NO: 440. In some additional embodiments, the polypeptide sequence of the engineered glycosyltransferase comprises at least one mutation or mutation set selected from S146G/D170S/W196I, S146G/D170S/V232I, S146G/D170S/V232I/R423K/E448H/Q451E/S455M, S146G/D170T/W196I/V232I, S146G/D170T/W196I/V232I/R423K, S146G/D170T/W196I/V232I/Q451E/S455R, S146G/D170T/W196I/N408T/Q451E, S146G/D170T/V232I/R423K, S146G/D170T/T259E, S146G/W196I, S146G/W196I/V232I, S146G/W196I/V232I/T259E/E448H/Q451E, S146G/W196I/S455R, S146G/V232I/T259E/S455M, S146G/V232I/K315S, S146G/V232I/K315S/R423K/Q451E/S455M, S146G/V232I/G326E, S146G/V232I/E448H, S146G/V232I/Q451E, and S146G/V413M/Q451E/S455M, wherein the positions are numbered with reference to SEQ ID NO: 440. In some additional embodiments, the

[0015] polypeptide sequence of the engineered glycosyltransferase comprises at least one mutation or mutation set at one or more positions selected from 9, 12, 107, 131, 156, 161, 169, 199, 204, 209, 233, 262, 289, 337, and 417, wherein the positions are numbered with reference to SEQ ID NO: 440. In some additional embodiments, the polypeptide sequence of the engineered glycosyltransferase comprises at least one mutation or mutation set selected from 9L, 12Q, 107C, 131A, 156S, 161E, 169T, 199S, 204M, 209Y, 233K, 262A, 289A, 337N, 337W, and 417V, wherein the positions are numbered with reference to SEQ ID NO: 440. In some additional embodiments, the polypeptide sequence of the engineered glycosyltransferase comprises at least one mutation or mutation set selected from V9L, R12Q, A107C, P131A, C156S, L161E, E169T, A199S, D204M, E209Y, Q233K, S262A, K289A, S337N, S337W, and P417V, wherein the positions are numbered with reference to SEQ ID NO: 440. In some additional embodiments, the polypeptide sequence of the engineered glycosyltransferase comprises at least one mutation or mutation set at one or more positions selected from 146/170/196, 146/170/196/232, 146/170/196/232/451/455, 146/170/232, 146/170/232/423/448/451/455, 146/196/232, 146/196/232/259/448/451, and 146/232/448, wherein the positions are numbered with reference to SEQ ID NO: 440. In some additional embodiments, the

polypeptide sequence of the engineered glycosyltransferase comprises at least one mutation or mutation set selected from 146G/170S/196I, 146G/170S/232I, 146G/170S/232I/423K/448H/451E/455M, 146G/170T/196I/232I, 146G/170T/196I/232I/451E/455R, 146G/196I/232I, 146G/196I/232I/259E/448H/451E, and 146G/232I/448H, wherein the positions are numbered with reference to SEQ ID NO: 440. In some additional embodiments, the polypeptide sequence of the engineered glycosyltransferase comprises at least one mutation or mutation set selected from S146G/D170S/W196I, S146G/D170S/V232I, S146G/D170S/V232I/R423K/E448H/Q451E/S455M, S146G/D170T/W196I/V232I, S146G/D170T/W196I/V232I/Q451E/S455R, S146G/W196I/V232I, S146G/W196I/V232I/T259E/E448H/Q451E, and S146G/V232I/E448H, wherein the positions are numbered with reference to SEQ ID NO: 440.

In yet some further embodiments, the polypeptide sequence of the engineered glycosyltransferase comprises at least one mutation or mutation set at one or more positions selected from 9/12/107/161/199/209/337, 9/12/161/169/199/209/337, 9/12/199/204/209/337/455, 9/12/199/209/337/451/455, 9/107/131/204/259/417/451, 9/107/156/161/199/204/417/455, 9/107/161/209/259/289/451/455, 9/131/156/209/289/337, 9/131/204/337/451, 9/156/169/204/337, 9/169/204/289/337/451/455, 9/204, 9/204/259/289, 9/337, 12/14/107/204/289/455, 12/107/131/204/289/337/417/451/455, 12/107/156/209/289/455, 107/161/169/199/204/259/451, 107/199/204/289, 107/199/209/259/451/455, 107/204, 156/169/199/204/209/259/289, 161/204/417, 204/451/455, 289, and 451/455, wherein the positions are numbered with reference to SEQ ID NO: 520. In some additional embodiments, the polypeptide sequence of the engineered glycosyltransferase comprises at least one mutation or mutation set selected from 9L/12Q/107C/161E/199S/209Y/337N, 9L/12Q/161E/169T/199S/209Y/337W, 9L/12Q/199S/204M/209Y/337N/455R, 9L/12Q/199S/209Y/337W/451E/455R, 9L/107C/131A/204M/259E/417V/451E, 9L/107C/156S/161E/199S/204M/417V/455R, 9L/107C/161E/209Y/259E/289A/451E/455R, 9L/131A/156S/209Y/289A/337N, 9L/131A/204M/337N/451E, 9L/156S/169T/204M/337N, 9L/169T/204M/289A/337W/451E/455R, 9L/204M, 9L/204M/259E/289A, 9L/337W, 12Q/14I/107C/204M/289A/455R, 12Q/107C/131A/204M/289A/337W/417V/451E/455R, 12Q/107C/156S/209Y/289A/455R, 107C/161E/169T/199S/204M/259E/451E, 107C/199S/204M/289A, 107C/199S/209Y/259E/451E/455R, 107C/204M, 156S/169T/199S/204M/209Y/259E/289A, 161E/204M/417V, 204M/451E/455R, 289A, and 451E/455R, wherein the positions are numbered with reference to SEQ ID NO: 520. In some additional embodiments, the polypeptide sequence of the engineered glycosyltransferase comprises at least one mutation or mutation set selected from V9L/R12Q/A107C/L161E/A199S/E209Y/S337N, V9L/R12Q/L161E/E169T/A199S/E209Y/S337W, V9L/R12Q/A199S/D204M/E209Y/S337N/S455R, V9L/R12Q/A199S/E209Y/S337W/Q451E/S455R, V9L/A107C/P131A/D204M/T259E/P417V/Q451E,

V9L/A107C/C156S/L161E/A199S/D204M/P417V/S455R,
V9L/A107C/L161E/E209Y/T259E/K289A/Q451E/S455R,
V9L/P131A/C156S/E209Y/K289A/S337N, V9L/P131A/D204M/S337N/Q451E,
V9L/C156S/E169T/D204M/S337N, V9L/E169T/D204M/K289A/S337W/Q451E/S455R,
V9L/D204M, V9L/D204M/T259E/K289A, V9L/S337W, R12Q/V14I/A107C/D204M/K289A/S455R,
R12Q/A107C/P131A/D204M/K289A/S337W/P417V/Q451E/S455R,
R12Q/A107C/C156S/E209Y/K289A/S455R, A107C/L161E/E169T/A199S/D204M/T259E/Q451E,
A107C/A199S/D204M/K289A, A107C/A199S/E209Y/T259E/Q451E/S455R, A107C/D204M,
C156S/E169T/A199S/D204M/E209Y/T259E/K289A, L161E/D204M/P417V,
D204M/Q451E/S455R, K289A, and Q451E/S455R, wherein the positions are numbered with reference to SEQ ID NO: 520. In some further embodiments, the polypeptide sequence of the engineered glycosyltransferase comprises at least one mutation or mutation set at one or more positions selected from 16, 53, 54, 78, 80, 95, 111, 221, 257, 336, 349, 391, 410, 413, 426, and 430, wherein the positions are numbered with reference to SEQ ID NO: 520. In some additional embodiments, the polypeptide sequence of the engineered glycosyltransferase comprises at least one mutation or mutation set selected from 16M, 53Q, 54P, 78V, 80A, 95W, 111C, 111G, 111S, 221A, 257A, 336R, 349V, 391R, 410V, 413L, 426S, and 430Q, wherein the positions are numbered with reference to SEQ ID NO: 520. In some additional embodiments, the polypeptide sequence of the engineered glycosyltransferase comprises at least one mutation or mutation set selected from L16M, K53Q, T54P, L78V, T80A, H95W, T111C, T111G, T111S, F221A, L257A, K336R, I349V, L391R, I410V, V413L, A426S, and K430Q, wherein the positions are numbered with reference to SEQ ID NO: 520. In some additional embodiments, the polypeptide sequence of the engineered glycosyltransferase comprises at least one mutation or mutation set at one or more positions selected from 9/12/199/204/209/337/455, 9/131/156/209/289/337, 9/204, 12/14/107/204/289/455, 107/161/169/199/204/259/451, 107/199/204/289, and 204/451/455, wherein the positions are numbered with reference to SEQ ID NO: 520. In some additional embodiments, the polypeptide sequence of the engineered glycosyltransferase comprises at least one mutation or mutation set selected from 9L/12Q/199S/204M/209Y/337N/455R, 9L/131A/156S/209Y/289A/337N, 9L/204M, 12Q/14I/107C/204M/289A/455R, 107C/161E/169T/199S/204M/259E/451E, 107C/199S/204M/289A, and 204M/451E/455R, wherein the positions are numbered with reference to SEQ ID NO: 520. In some additional embodiments, the polypeptide sequence of the engineered glycosyltransferase comprises at least one mutation or mutation set selected from
V9L/R12Q/A199S/D204M/E209Y/S337N/S455R, V9L/P131A/C156S/E209Y/K289A/S337N,
V9L/D204M, R12Q/V14I/A107C/D204M/K289A/S455R,
A107C/L161E/E169T/A199S/D204M/T259E/Q451E, A107C/A199S/D204M/K289A, and
D204M/Q451E/S455R, wherein the positions are numbered with reference to SEQ ID NO: 520.
In yet some further embodiments, the polypeptide sequence of the engineered glycosyltransferase

comprises at least one mutation or mutation set at one or more positions selected from 16, 16/59/80/413, 16/80, 16/80/111, 16/80/111/257, 16/80/221/257/336/410, 16/80/221/336/410, 16/80/257, 16/111/221, 16/111/221/257/391, 16/221, 16/221/257, 16/221/257/336/391, 16/221/410, 16/257, 16/257/336/413/420, 80/111/221/257/410, 111/221/257/336/391, 111/221/257/391, 221, and 221/257, wherein the positions are numbered with reference to SEQ ID NO: 626. In some additional embodiments, the polypeptide sequence of the engineered glycosyltransferase comprises at least one mutation or mutation set selected from 16M, 16M/59Q/80A/413L, 16M/80A, 16M/80A/111C, 16M/80A/111S/257A, 16M/80A/221A/257A/336R/410V, 16M/80A/221A/336R/410V, 16M/80A/257A, 16M/111C/221A/257A/391R, 16M/111S/221A, 16M/221A, 16M/221A/257A, 16M/221A/257A/336R/391R, 16M/221A/410V, 16M/257A, 16M/257A/336R/413L/420G, 80A/111S/221A/257A/410V, 111S/221A/257A/336R/391R, 111S/221A/257A/391R, 221A, and 221A/257A, wherein the positions are numbered with reference to SEQ ID NO: 626. In some additional embodiments, the polypeptide sequence of the engineered glycosyltransferase comprises at least one mutation or mutation set selected from L16M, L16M/H59Q/T80A/V413L, L16M/T80A, L16M/T80A/T111C, L16M/T80A/T111S/L257A, L16M/T80A/F221A/L257A/K336R/I410V, L16M/T80A/F221A/K336R/I410V, L16M/T80A/L257A, L16M/T111C/F221A/L257A/L391R, L16M/T111S/F221A, L16M/F221A, L16M/F221A/L257A, L16M/F221A/L257A/K336R/L391R, L16M/F221A/I410V, L16M/L257A, L16M/L257A/K336R/V413L/E420G, T80A/T111S/F221A/L257A/I410V, T111S/F221A/L257A/K336R/L391R, T111S/F221A/L257A/L391R, F221A, and F221A/L257A, wherein the positions are numbered with reference to SEQ ID NO: 626. In some further embodiments, the polypeptide sequence of the engineered glycosyltransferase comprises at least one mutation or mutation set at one or more positions selected from 5, 7, 14, 65, 91, 99, 102, 118, 138, 194, 254, 286, 416, 418, and 420, wherein the positions are numbered with reference to SEQ ID NO: 626. In some additional embodiments, the polypeptide sequence of the engineered glycosyltransferase comprises at least one mutation or mutation set selected from 5L, 5M, 5N, 5Q, 7L, 14L, 65V, 91V, 99L, 99S, 102R, 118S, 118V, 138I, 194P, 254G, 254T, 254V, 286L, 416E, 418D, and 420A, wherein the positions are numbered with reference to SEQ ID NO: 626. In some additional embodiments, the polypeptide sequence of the engineered glycosyltransferase comprises at least one mutation or mutation set selected from S5L, S5M, S5N, S5Q, T7L, V14L, I65V, Q91V, E99L, E99S, K102R, A118S, A118V, L138I, S194P, S254G, S254T, S254V, V286L, D416E, E418D, and E420A, wherein the positions are numbered with reference to SEQ ID NO: 626. In yet some further embodiments, the polypeptide sequence of the engineered glycosyltransferase comprises at least one mutation or mutation set at one or more positions selected from 16/80/221/257/336/410, 16/111/221, 16/221/257, 16/221/410, 16/257, 80/111/221/257/410, and 221, wherein the positions are numbered with reference to SEQ ID NO: 626. In some additional embodiments, the polypeptide sequence of the engineered glycosyltransferase comprises at least one mutation or mutation set selected from 16M/80A/221A/257A/336R/410V,

16M/111S/221A, 16M/221A/257A, 16M/221A/410V, 16M/257A, 80A/111S/221A/257A/410V, and 221A, wherein the positions are numbered with reference to SEQ ID NO: 626. In some additional embodiments, the polypeptide sequence of the engineered glycosyltransferase comprises at least one mutation or mutation set selected from L16M/T80A/F221A/L257A/K336R/I410V, L16M/T111S/F221A, L16M/F221A/L257A, L16M/F221A/I410V, L16M/L257A, T80A/T111S/F221A/L257A/I410V, and F221A, wherein the positions are numbered with reference to SEQ ID NO: 626.

[0016] In yet some further embodiments, the polypeptide sequence of the engineered glycosyltransferase comprises at least one mutation or mutation set at one or more positions selected from 5, 5/14/99, 5/14/416, 5/91, 5/91/102/118, 5/91/102/254/418, 5/91/106/254/286, 5/91/118/194/254/286, 5/91/118/194/254/418, 5/91/118/194/418, 5/91/118/254/286/418, 5/91/118/286, 5/91/194/254, 5/91/194/418, 5/91/254, 5/91/254/286, 5/102/418, 5/118/194/286/418, 5/118/254, 5/118/286, 5/194, 5/254/286, 5/418, 7/14, 7/14/65/99, 7/14/99/416, 14, 14/65/99/416, 14/99, 14/99/254, 14/99/254/416, 14/99/254/416/455, 14/99/416, 14/167, 14/254, 14/455, 91, 91/102/194, 91/118, 91/118/194, 91/118/194/286, 91/118/194/418, 91/118/286, 91/194/254/286, 91/286, and 118/286/418, wherein the positions are numbered with reference to SEQ ID NO: 678.

[0017] In some additional embodiments, the polypeptide sequence of the engineered glycosyltransferase comprises at least one mutation or mutation set selected from 5L/14L/99L, 5L/14L/416E, 5N, 5N/91V, 5N/91V/102R/118V, 5N/91V/102R/254T/418D, 5N/91V/106I/254T/286L, 5N/91V/118S/194P/254T/418D, 5N/91V/118S/254T/286L/418D, 5N/91V/118S/286L, 5N/91V/118V/194P/254T/286L, 5N/91V/118V/194P/418D, 5N/91V/194P/254T, 5N/91V/194P/418D, 5N/91V/254T, 5N/91V/254T/286L, 5N/102R/418D, 5N/118S/194P/286L/418D, 5N/118S/286L, 5N/118V/254T, 5N/194P, 5N/254T/286L, 5N/418D, 7L/14L, 7L/14L/65V/99L, 7L/14L/99S/416E, 14L, 14L/65V/99L/416E, 14L/99L, 14L/99L/254G, 14L/99L/254G/416E, 14L/99L/254G/416E/455R, 14L/99L/416E, 14L/99S/416E, 14L/167E, 14L/254G, 14L/455R, 91V, 91V/102R/194P, 91V/118V, 91V/118V/194P, 91V/118V/194P/286L, 91V/118V/194P/418D, 91V/118V/286L, 91V/194P/254T/286L, 91V/286L, and 118V/286L/418D, wherein the positions are numbered with reference to SEQ ID NO: 678. In some additional embodiments, the polypeptide sequence of the engineered glycosyltransferase comprises at least one mutation or mutation set selected from S5L/V14L/E99L, S5L/V14L/D416E, S5N, S5N/Q91V, S5N/Q91V/K102R/A118V, S5N/Q91V/K102R/S254T/E418D, S5N/Q91V/S106I/S254T/V286L, S5N/Q91V/A118S/S194P/S254T/E418D, S5N/Q91V/A118S/S254T/V286L/E418D, S5N/Q91V/A118S/V286L, S5N/Q91V/A118V/S194P/S254T/V286L, S5N/Q91V/A118V/S194P/E418D, S5N/Q91V/S194P/S254T, S5N/Q91V/S194P/E418D, S5N/Q91V/S254T, S5N/Q91V/S254T/V286L, S5N/K102R/E418D, S5N/A118S/S194P/V286L/E418D, S5N/A118S/V286L, S5N/A118V/S254T, S5N/S194P, S5N/S254T/V286L, S5N/E418D, T7L/V14L, T7L/V14L/I65V/E99L, T7L/V14L/E99S/D416E,

V14L, V14L/I65V/E99L/D416E, V14L/E99L, V14L/E99L/S254G, V14L/E99L/S254G/D416E, V14L/E99L/S254G/D416E/S455R, V14L/E99L/D416E, V14L/E99S/D416E, V14L/D167E, V14L/S254G, V14L/S455R, Q91V, Q91V/K102R/S194P, Q91V/A118V, Q91V/A118V/S194P, Q91V/A118V/S194P/V286L, Q91V/A118V/S194P/E418D, Q91V/A118V/V286L, Q91V/S194P/S254T/V286L, Q91V/V286L, and A118V/V286L/E418D, wherein the positions are numbered with reference to SEQ ID NO: 678. In some additional embodiments, the polypeptide sequence of the engineered glycosyltransferase comprises at least one mutation or mutation set at one or more positions selected from 5/91/106/254/286, 5/91/118/286, 5/91/254/286, 14, 14/65/99/416, 14/99, and 14/99/254, wherein the positions are numbered with reference to SEQ ID NO: 678. In some additional embodiments, the polypeptide sequence of the engineered glycosyltransferase comprises at least one mutation or mutation set selected from 5N/91V/106I/254T/286L, 5N/91V/118S/286L, 5N/91V/254T/286L, 14L, 14L/65V/99L/416E, 14L/99L, and 14L/99L/254G, wherein the positions are numbered with reference to SEQ ID NO: 678. In some additional embodiments, the polypeptide sequence of the engineered glycosyltransferase comprises at least one mutation or mutation set selected from S5N/Q91V/S106I/S254T/V286L, S5N/Q91V/A118S/V286L, S5N/Q91V/S254T/V286L, V14L, V14L/I65V/E99L/D416E, V14L/E99L, and V14L/E99L/S254G, wherein the positions are numbered with reference to SEQ ID NO: 678.

[0018] The present invention also provides engineered glycosyltransferases comprising polypeptide sequences that have at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or more sequence identity to SEQ ID NO: 12. In some embodiments, the polypeptide sequence of the engineered glycosyltransferase comprises at least one mutation or mutation set at one or more positions selected from 16, 16/127/169, 16/134, 16/143/423, 16/169, 16/169/398/399, 16/169/423, 16/398, 16/398/399, 16/398/427, 16/423, 16/423/427, 134, 143, 399/423, and 423/427, wherein the positions are numbered with reference to SEQ ID NO: 24. In some additional embodiments, the polypeptide sequence of the engineered glycosyltransferase comprises at least one mutation or mutation set selected from 16T, 16T/127V/169E, 16T/134S, 16T/143G/423L, 16T/169E, 16T/169E/398M/399K, 16T/169E/423L, 16T/398M, 16T/398M/399K, 16T/398M/427S, 16T/423L, 16T/423L/427S, 134S, 143G, 399K/423L, and 423L/427S, wherein the positions are numbered with reference to SEQ ID NO: 24. In some additional embodiments, the polypeptide sequence of the engineered glycosyltransferase comprises at least one mutation or mutation set selected from L16T, L16T/Q127V/P169E, L16T/A134S, L16T/P143G/R423L, L16T/P169E, L16T/P169E/A398M/Q399K, L16T/P169E/R423L, L16T/A398M, L16T/A398M/Q399K, L16T/A398M/R427S, L16T/R423L, L16T/R423L/R427S, A134S, P143G, Q399K/R423L, and R423L/R427S, wherein the positions are numbered with reference to SEQ ID NO: 24. In some additional embodiments, the polypeptide sequence of the engineered glycosyltransferase comprises at least one mutation or mutation set at one or more positions selected from 3, 8, 11, 41, 44, 56, 60, 122, 135, 137, 138, 139, 158, 164, 176, 221, 232, 233, 235, 248, 249, 281, 284, 285, 301, 322, 372, 392,

400, 421, 426, 427, 433, 440, 443, and 446, wherein the positions are numbered with reference to SEQ ID NO: 24. In some additional embodiments, the polypeptide sequence of the engineered glycosyltransferase comprises at least one mutation or mutation set selected from 3I, 3V, 8R, 11L, 11P, 41M, 44E, 56A, 60T, 122C, 135V, 137T, 138K, 138R, 139T, 158H, 158V, 164A, 164E, 164N, 164Q, 164S, 176S, 221D, 232A, 233S, 233T, 235L, 235M, 248M, 249R, 281S, 284V, 285A, 301G, 322R, 372S, 392V, 400C, 421A, 421G, 426S, 427A, 427W, 433G, 433T, 440L, 440M, 443G, 443H, and 446L, wherein the positions are numbered with reference to SEQ ID NO: 24. In some additional embodiments, the polypeptide sequence of the engineered glycosyltransferase comprises at least one mutation or mutation set selected from H3I, H3V, V8R, Q11L, Q11P, F41M, Y44E, K56A, P60T, L122C, L135V, Q137T, G138K, G138R, I139T, F158H, F158V, H164A, H164E, H164N, H164Q, H164S, R176S, E221D, P232A, P233S, P233T, Q235L, Q235M, I248M, D249R, G281S, L284V, S285A, Q301G, L322R, A372S, I392V, V400C, S421A, S421G, A426S, R427A, R427W, N433G, N433T, I440L, I440M, C443G, C443H, and R446L, wherein the positions are numbered with reference to SEQ ID NO: 24. In some further embodiments, the polypeptide sequence of the engineered glycosyltransferase comprises at least one mutation or mutation set at one or more positions selected from 16, 16/127/169, and 16/169, wherein the positions are numbered with reference to SEQ ID NO: 24. In some additional embodiments, the polypeptide sequence of the engineered glycosyltransferase comprises at least one mutation or mutation set selected from 16T, 16T/127V/169E, and 16T/169E, wherein the positions are numbered with reference to SEQ ID NO: 24. In some additional embodiments, the polypeptide sequence of the engineered glycosyltransferase comprises at least one mutation or mutation set selected from L16T, L16T/Q127V/P169E, and L16T/P169E, wherein the positions are numbered with reference to SEQ ID NO: 24.

[0019] In some further embodiments, the polypeptide sequence of the engineered glycosyltransferase comprises at least one mutation or mutation set at one or more positions selected from 3/22/198/421, 3/22/421, 3/201/248/322, 3/201/248/322/392, 13/233/331/440, 13/440/443, 22/32/56/60/198/248/322/392/421, 22/56, 22/56/137/198/201/248/322/392, 22/56/137/198/248/392/421, 22/56/137/322, 22/56/137/322/392/423, 22/56/198/202/248, 22/56/198/248/322/392/421, 22/56/198/248/421/423, 22/56/421/423, 22/60/392/421, 22/137, 22/137/198/202/248, 22/198/202/248/392, 22/248/392, 22/392, 22/421, 22/421/423, 22/423, 44/164/233, 44/164/233/329/331, 44/331, 125, 125/233/443/446, 164/221/233/331/440/446, 164/233/331/440, 164/233/446, 164/331, 164/440, 164/443, 198/202/392, 221/329/331, 233, 233/446, 329, 421/423, and 443/446, wherein the positions are numbered with reference to SEQ ID NO: 858. In some additional embodiments, the polypeptide sequence of the engineered glycosyltransferase comprises at least one mutation or mutation set selected from 3I/22G/198Q/421G, 3I/22G/421G, 3I/201A/248M/322R, 3I/201A/248M/322R/392V, 13Q/233S/331V/440M, 13Q/440M/443G, 22G/32L/56A/60T/198Q/248M/322R/392V/421G, 22G/56A, 22G/56A/137T/198Q/201A/248M/322R/392V, 22G/56A/137T/198Q/248M/392V/421G,

22G/56A/137T/322R, 22G/56A/137T/322R/392V/423L, 22G/56A/198Q/202Y/248M, 22G/56A/198Q/248M/322R/392V/421G, 22G/56A/198Q/248M/421G/423L, 22G/56A/421G/423L, 22G/60T/392V/421G, 22G/137T, 22G/137T/198Q/202Y/248M, 22G/198Q/202Y/248M/392V, 22G/248M/392V, 22G/392V, 22G/421G, 22G/421G/423L, 22G/423L, 44E/164A/233S, 44E/164E/233S, 44E/164E/233S/329L/331V, 44E/331V, 125L, 125L/233S/443G/446L, 164A/233S/446L, 164A/331V, 164A/440M, 164A/443G, 164E/221D/233S/331V/440M/446L, 164E/233S/331V/440M, 198Q/202Y/392V, 221D/329L/331V, 233S, 233S/446L, 329L, 421G/423L, and 443G/446L, wherein the positions are numbered with reference to SEQ ID NO: 858. In some additional embodiments, the polypeptide sequence of the engineered glycosyltransferase comprises at least one mutation or mutation set selected from H3I/A22G/G198Q/S421G, H3I/A22G/S421G, H3I/G201A/I248M/L322R, H3I/G201A/I248M/L322R/I392V, L13Q/P233S/I331V/I440M, L13Q/I440M/C443G, A22G/I32L/K56A/P60T/G198Q/I248M/L322R/I392V/S421G, A22G/K56A, A22G/K56A/Q137T/G198Q/G201A/I248M/L322R/I392V, A22G/K56A/Q137T/G198Q/I248M/I392V/S421G, A22G/K56A/Q137T/L322R, A22G/K56A/Q137T/L322R/I392V/R423L, A22G/K56A/G198Q/I202Y/I248M, A22G/K56A/G198Q/I248M/L322R/I392V/S421G, A22G/K56A/G198Q/I248M/S421G/R423L, A22G/K56A/S421G/R423L, A22G/P60T/I392V/S421G, A22G/Q137T, A22G/Q137T/G198Q/I202Y/I248M, A22G/G198Q/I202Y/I248M/I392V, A22G/I248M/I392V, A22G/I392V, A22G/S421G, A22G/S421G/R423L, A22G/R423L, Y44E/H164A/P233S, Y44E/H164E/P233S, Y44E/H164E/P233S/A329L/I331V, Y44E/I331V, F125L, F125L/P233S/C443G/R446L, H164A/P233S/R446L, H164A/I331V, H164A/I440M, H164A/C443G, H164E/E221D/P233S/I331V/I440M/R446L, H164E/P233S/I331V/I440M, G198Q/I202Y/I392V, E221D/A329L/I331V, P233S, P233S/R446L, A329L, S421G/R423L, and C443G/R446L, wherein the positions are numbered with reference to SEQ ID NO: 858. In some additional embodiments, the polypeptide sequence of the engineered glycosyltransferase comprises at least one mutation or mutation set at one or more positions selected from 3/22/198/421, 13/440/443, 22/56/198/202/248, 22/137, 22/198/202/248/392, 164/221/233/331/440/446, 164/233/331/440, and 233/446, wherein the positions are numbered with reference to SEQ ID NO: 858. In some additional embodiments, the polypeptide sequence of the engineered glycosyltransferase comprises at least one mutation or mutation set selected from 3I/22G/198Q/421G, 13Q/440M/443G, 22G/56A/198Q/202Y/248M, 22G/137T, 22G/198Q/202Y/248M/392V, 164E/221D/233S/331V/440M/446L, 164E/233S/331V/440M, and 233S/446L, wherein the positions are numbered with reference to SEQ ID NO: 858. In some additional embodiments, the polypeptide sequence of the engineered glycosyltransferase comprises at least one mutation or mutation set selected from H3I/A22G/G198Q/S421G, L13Q/I440M/C443G, A22G/K56A/G198Q/I202Y/I248M, A22G/Q137T, A22G/G198Q/I202Y/I248M/I392V, H164E/E221D/P233S/I331V/I440M/R446L,

H164E/P233S/I331V/I440M, and P233S/R446L, wherein the positions are numbered with reference to SEQ ID NO: 858.

[0020] In some further embodiments, the polypeptide sequence of the engineered glycosyltransferase comprises at least one mutation or mutation set at one or more positions selected from 83/202/233, 164, 164/202, 164/202/233/331, 164/202/248/272, 164/202/331, 164/202/331/423, 164/423, 202/233, 202/233/248, 202/233/248/423, 202/248, 202/331, 202/421/423, 202/423, 202/446, 233, 248, and 423, wherein the positions are numbered with reference to SEQ ID NO: 994. In some additional embodiments, the polypeptide sequence of the engineered glycosyltransferase comprises at least one mutation or mutation set selected from 83Y/202I/233S, 164E, 164E/202I, 164E/202I/233S/331V, 164E/202I/248I/272C, 164E/202I/331V, 164E/202I/331V/423L, 164E/423L, 202I/233S, 202I/233S/248I, 202I/233S/248I/423L, 202I/248I, 202I/331V, 202I/421G/423L, 202I/423L, 202I/446L, 233S, 248I, and 423L, wherein the positions are numbered with reference to SEQ ID NO: 994. In some additional embodiments, the polypeptide sequence of the engineered glycosyltransferase comprises at least one mutation or mutation set selected from H83Y/Y202I/P233S, H164E, H164E/Y202I, H164E/Y202I/P233S/I331V, H164E/Y202I/M248I/R272C, H164E/Y202I/I331V, H164E/Y202I/I331V/R423L, H164E/R423L, Y202I/P233S, Y202I/P233S/M248I, Y202I/P233S/M248I/R423L, Y202I/M248I, Y202I/I331V, Y202I/S421G/R423L, Y202I/R423L, Y202I/R446L, P233S, M248I, and R423L, wherein the positions are numbered with reference to SEQ ID NO: 994. In some embodiments, the polypeptide sequence of the engineered glycosyltransferase comprises at least one mutation or mutation set at one or more positions selected from 7, 9, 10, 54, 73, 84, 106, 115, 116, 132, 165, 286, 309, 389, 406, 422, and 438, wherein the positions are numbered with reference to SEQ ID NO: 994. In some additional embodiments, the polypeptide sequence of the engineered glycosyltransferase comprises at least one mutation or mutation set selected from 7T, 9L, 10P, 54L, 54M, 73R, 73S, 84L, 106S, 115A, 116I, 132R, 165P, 286G, 309E, 389E, 406H, 406M, 406Q, 422A, 422T, 422V, 438A, and 438T, wherein the positions are numbered with reference to SEQ ID NO: 994. In some additional embodiments, the polypeptide sequence of the engineered glycosyltransferase comprises at least one mutation or mutation set selected from G7T, S9L, D10P, S54L, S54M, A73R, A73S, Y84L, A106S, N115A, L116I, K132R, E165P, N286G, K309E, D389E, S406H, S406M, S406Q, K422A, K422T, K422V, E438A, and E438T, wherein the positions are numbered with reference to SEQ ID NO: 994. In some further embodiments, the polypeptide sequence of the engineered glycosyltransferase comprises at least one mutation or mutation set at one or more positions selected from 83/202/233, 164, 164/202, 164/423, 202/233/248, and 233, wherein the positions are numbered with reference to SEQ ID NO: 994. In some additional embodiments, the polypeptide sequence of the engineered glycosyltransferase comprises at least one mutation or mutation set selected from 83Y/202I/233S, 164E, 164E/202I, 164E/423L, 202I/233S/248I, and 233S, wherein the positions are numbered with reference to SEQ ID NO: 994. In some additional embodiments, the polypeptide sequence of the engineered glycosyltransferase comprises at least one

mutation or mutation set selected from H83Y/Y202I/P233S, H164E, H164E/Y202I, H164E/R423L, Y202I/P233S/M248I, and P233S, wherein the positions are numbered with reference to SEQ ID NO: 994.

[0021] In yet some further embodiments, the polypeptide sequence of the engineered glycosyltransferase comprises at least one mutation or mutation set at one or more positions selected from 7/9/73/165/286, 7/9/165/286, 7/9/422, 7/116/165/286, 7/165, 9/73/116/165/286/422, 9/286/389, 54, 54/84, 54/406, 73, 73/116/165/286/389, 73/116/286, 73/116/286/422, 73/165/286, 73/286/389, 73/286/422, 73/422, 84, 115, 116, 116/165, 116/165/286/422, 116/389, 165, 165/286, 165/389, 165/389/422, 286, 286/422, 389/422, and 422, wherein the positions are numbered with reference to SEQ ID NO: 1080. In some additional embodiments, the polypeptide sequence of the engineered glycosyltransferase comprises at least one mutation or mutation set selected from 7T/9L/73R/165P/286G, 7T/9L/165P/286G, 7T/9L/422A, 7T/116I/165P/286G, 7T/165P, 9L/73R/116I/165P/286G/422A, 9L/286G/389E, 54L/84L, 54M, 54M/406M, 73R, 73R/116I/165P/286G/389E, 73R/116I/286G, 73R/116I/286G/422A, 73R/165P/286G, 73R/286G/389E, 73R/286G/422A, 73R/286G/422T, 73R/422T, 84L, 115A, 116I, 116I/165P, 116I/165P/286G/422A, 116I/389E, 165P, 165P/286G, 165P/389E, 165P/389E/422T, 286G, 286G/422A, 389E/422A, and 422T, wherein the positions are numbered with reference to SEQ ID NO: 1080. In some additional embodiments, the polypeptide sequence of the engineered glycosyltransferase comprises at least one mutation or mutation set selected from G7T/S9L/A73R/E165P/N286G, G7T/S9L/E165P/N286G, G7T/S9L/K422A, G7T/L116I/E165P/N286G, G7T/E165P, S9L/A73R/L116I/E165P/N286G/K422A, S9L/N286G/D389E, S54L/Y84L, S54M, S54M/S406M, A73R, A73R/L116I/E165P/N286G/D389E, A73R/L116I/N286G, A73R/L116I/N286G/K422A, A73R/E165P/N286G, A73R/N286G/D389E, A73R/N286G/K422A, A73R/N286G/K422T, A73R/K422T, Y84L, N115A, L116I, L116I/E165P, L116I/E165P/N286G/K422A, L116I/D389E, E165P, E165P/N286G, E165P/D389E, E165P/D389E/K422T, N286G, N286G/K422A, D389E/K422A, and K422T, wherein the positions are numbered with reference to SEQ ID NO: 1080. In some further embodiments, the polypeptide sequence of the engineered glycosyltransferase comprises at least one mutation or mutation set at one or more positions selected from 185/190, 219, 220, 255, 257, 302, 385, 395, 395/437, 399, 401, 409, 412, 416, 434, 441, 445, 447, and 449, wherein the positions are numbered with reference to SEQ ID NO: 1080. In some additional embodiments, the polypeptide sequence of the engineered glycosyltransferase comprises at least one mutation or mutation set selected from 185L/190P, 219L, 220A, 255R, 257H, 302A, 302H, 302L, 302R, 302T, 385M, 385P, 385S, 395A, 395D, 395H, 395N, 395S/437L, 399R, 401V, 409L, 412K, 416N, 434I, 441A, 441K, 441L, 441R, 441S, 445K, 445R, 447A, 447R, 447S, 447V, 449A, 449H, and 449T, wherein the positions are numbered with reference to SEQ ID NO: 1080. In some additional embodiments, the polypeptide sequence of the engineered glycosyltransferase comprises at least one mutation or mutation set selected from F185L/T190P,

C219L, T220A, P255R, N257H, N302A, N302H, N302L, N302R, N302T, V385M, V385P, V385S, G395A, G395D, G395H, G395N, G395S/E437L, Q399R, L401V, T409L, I412K, K416N, A434I, Q441A, Q441K, Q441L, Q441R, Q441S, N445K, N445R, N447A, N447R, N447S, N447V, Y449A, Y449H, and Y449T, wherein the positions are numbered with reference to SEQ ID NO: 1080. In some further embodiments, the polypeptide sequence of the engineered glycosyltransferase comprises at least one mutation or mutation set at one or more positions selected from 9/73/116/165/286/422, 54, 54/84, 54/406, 73/116/286/422, 116/165/286/422, and 286/422, wherein the positions are numbered with reference to SEQ ID NO: 1080. In some additional embodiments, the polypeptide sequence of the engineered glycosyltransferase comprises at least one mutation or mutation set selected from 9L/73R/116I/165P/286G/422A, 54L/84L, 54M, 54M/406M, 73R/116I/286G/422A, 116I/165P/286G/422A, and 286G/422A, wherein the positions are numbered with reference to SEQ ID NO: 1080. In some additional embodiments, the polypeptide sequence of the engineered glycosyltransferase comprises at least one mutation or mutation set selected from S9L/A73R/L116I/E165P/N286G/K422A, S54L/Y84L, S54M, S54M/S406M, A73R/L116I/N286G/K422A, L116I/E165P/N286G/K422A, and N286G/K422A, wherein the positions are numbered with reference to SEQ ID NO: 1080.

[0022] In yet some further embodiments, the polypeptide sequence of the engineered glycosyltransferase comprises at least one mutation or mutation set at one or more positions selected from 54, 54/185/190, 54/185/190/219/257/302/385/389/395/445/447/449, 54/185/190/219/385, 54/185/190/219/385/389/395/399/441/445/447/449, 54/185/190/219/385/395/399/441/445/447/449, 54/185/190/219/385/445/447, 54/185/190/219/389/395/399/441/445/447/449, 54/185/190/302, 54/185/190/302/395/399, 54/185/190/385/447/449, 54/185/190/389/395, 54/185/190/389/441/445/447/449, 54/185/190/389/445, 54/185/190/395/399/441/445/447/449, 54/185/219/389, 54/185/257/389/441/445/447/449, 54/185/302/385/399/445/447, 54/185/385/389/395/441/445/449, 54/185/385/395/399/445/447/449, 54/185/389/395/445/447, 54/190/219/257/302, 54/190/219/257/395/445/447/449, 54/190/257, 54/190/257/302/399, 54/190/257/309/385/389/395/399/445, 54/190/257/385/389, 54/190/257/395/445/449, 54/190/302/389, 54/190/302/389/395/399/445/447, 54/190/385/389/395/441/445, 54/190/385/395, 54/190/385/445/447/449, 54/190/395, 54/190/395/445/447, 54/219/302/395/441/445/447, 54/219/385/389/395/441/445/447, 54/257, 54/257/385/449, 54/257/389, 54/257/399, 54/257/441/447, 54/257/441/449, 54/302/385/399/441/445/447, 54/302/385/399/441/445/449, 54/385, 54/385/389/441/445/447/449, 54/389/395/399/447/449, 185/190/219/257/389/395/445/447, 185/190/257/302/395/441/445/447, 185/190/257/385/389/399, 185/190/257/385/399/445/447, 185/190/257/389/395, 185/190/257/389/395/399/441/447/449, 185/190/385/389/441/445/447, 185/190/389/447/449, 185/190/395/399, 185/219/257/399/445/447, 185/257/385/395/399/445/447, 185/302/395/399/441/445/447/449, 185/395/399/441/445/447, 190/219/257/385/389/441/445/447/449, 190/219/302/385/399/445, 190/257/302/385/399,

190/257/385/389/399, 190/257/385/389/445/447, 190/257/385/395, 190/257/385/441/445/447/449, 190/257/389/395/441/445/447, 190/257/399/447/449, 190/302/385/389/395/399/441/445/447, 190/389, 219/257/385/389/395/441/447/449, 219/257/395, 219/385/389/399/445/449, 219/395, 257/385/389/399, 257/389/395/399/445/447, 257/389/395/399/445/449, 257/389/399, 302/389/395/445, 385/389, 385/395, 389/395/445/447, 395/399, and 399, wherein the positions are numbered with reference to SEQ ID NO: 1216.

[0023] In some additional embodiments, the polypeptide sequence of the engineered glycosyltransferase comprises at least one mutation or mutation set selected from 54M, 54M/185L/190P, 54M/185L/190P/219L/257H/302R/385P/389E/395H/445K/447A/449H, 54M/185L/190P/219L/385P/445R/447A, 54M/185L/190P/219L/385S, 54M/185L/190P/219L/385S/389E/395D/399R/441R/445R/447A/449H, 54M/185L/190P/219L/385S/395A/399R/441R/445K/447A/449H, 54M/185L/190P/219L/389E/395H/399R/441S/445K/447A/449H, 54M/185L/190P/302H, 54M/185L/190P/302R/395H/399R, 54M/185L/190P/385P/447A/449H, 54M/185L/190P/389E/395H, 54M/185L/190P/389E/441R/445R/447A/449H, 54M/185L/190P/389E/445K, 54M/185L/190P/395D/399R/441R/445K/447A/449H, 54M/185L/219L/389E, 54M/185L/257H/389E/441S/445R/447A/449H, 54M/185L/302H/385S/399R/445R/447A, 54M/185L/385S/389E/395D/441R/445K/449H, 54M/185L/385S/395D/399R/445R/447A/449H, 54M/185L/389E/395D/445K/447A, 54M/190P/219L/257H/302H, 54M/190P/219L/257H/395H/445R/447A/449H, 54M/190P/257H, 54M/190P/257H/302H/399R, 54M/190P/257H/309N/385S/389E/395D/399R/445K, 54M/190P/257H/385S/389E, 54M/190P/257H/395D/445K/449H, 54M/190P/302H/389E/395D/399R/445K/447S, 54M/190P/302R/389E, 54M/190P/385P/395D, 54M/190P/385P/445R/447A/449H, 54M/190P/385S/389E/395H/441S/445R, 54M/190P/395A, 54M/190P/395H/445K/447A, 54M/219L/302R/395H/441S/445R/447A, 54M/219L/385P/389E/395D/441R/445K/447A, 54M/257H, 54M/257H/385S/449H, 54M/257H/389E, 54M/257H/399R, 54M/257H/441R/447A, 54M/257H/441R/449H, 54M/302H/385P/399R/441S/445K/447S, 54M/302H/385S/399R/441S/445R/449H, 54M/385P, 54M/385P/389E/441R/445K/447A/449H, 54M/389E/395D/399R/447A/449H, 185L/190P/219L/257H/389E/395A/445K/447A, 185L/190P/257H/302R/395D/441S/445R/447S, 185L/190P/257H/385P/389E/399R, 185L/190P/257H/385P/399R/445R/447A, 185L/190P/257H/389E/395A, 185L/190P/257H/389E/395H/399R/441S/447S/449H, 185L/190P/385S/389E/441R/445K/447S, 185L/190P/389E/447S/449H, 185L/190P/395D/399R, 185L/190P/395H/399R, 185L/219L/257H/399R/445R/447A, 185L/257H/385S/395A/399R/445R/447A, 185L/302R/395H/399R/441R/445K/447S/449H, 185L/395D/399R/441R/445R/447A, 190P/219L/257H/385S/389E/441S/445R/447A/449H, 190P/219L/302H/385P/399R/445R, 190P/257H/302H/385P/399R, 190P/257H/385P/389E/399R, 190P/257H/385S/389E/445K/447A,

190P/257H/385S/395D, 190P/257H/385S/441S/445R/447S/449H,
190P/257H/389E/395H/441R/445R/447A, 190P/257H/399R/447A/449H,
190P/302H/385P/389E/395D/399R/441S/445K/447S, 190P/389E,
219L/257H/385S/389E/395D/441R/447A/449H, 219L/257H/395H,
219L/385S/389E/399R/445R/449H, 219L/395H, 257H/385P/389E/399R,
257H/389E/395D/399R/445K/447A, 257H/389E/395D/399R/445K/449H, 257H/389E/399R,
302H/389E/395H/445R, 385S/389E, 385S/395D, 389E/395D/445R/447S, 395D/399R, and 399R,
wherein the positions are numbered with reference to SEQ ID NO: 1216.

[0024] In some additional embodiments, the polypeptide sequence of the engineered glycosyltransferase comprises at least one mutation or mutation set selected from S54M,
S54M/F185L/T190P,
S54M/F185L/T190P/C219L/N257H/N302R/V385P/D389E/G395H/N445K/N447A/Y449H,
S54M/F185L/T190P/C219L/V385P/N445R/N447A, S54M/F185L/T190P/C219L/V385S,
S54M/F185L/T190P/C219L/V385S/D389E/G395D/Q399R/Q441R/N445R/N447A/Y449H,
S54M/F185L/T190P/C219L/V385S/G395A/Q399R/Q441R/N445K/N447A/Y449H,
S54M/F185L/T190P/C219L/D389E/G395H/Q399R/Q441S/N445K/N447A/Y449H,
S54M/F185L/T190P/N302H, S54M/F185L/T190P/N302R/G395H/Q399R,
S54M/F185L/T190P/V385P/N447A/Y449H, S54M/F185L/T190P/D389E/G395H,
S54M/F185L/T190P/D389E/Q441R/N445R/N447A/Y449H, S54M/F185L/T190P/D389E/N445K,
S54M/F185L/T190P/G395D/Q399R/Q441R/N445K/N447A/Y449H, S54M/F185L/C219L/D389E,
S54M/F185L/N257H/D389E/Q441S/N445R/N447A/Y449H,
S54M/F185L/N302H/V385S/Q399R/N445R/N447A,
S54M/F185L/V385S/D389E/G395D/Q441R/N445K/Y449H,
S54M/F185L/V385S/G395D/Q399R/N445R/N447A/Y449H,
S54M/F185L/D389E/G395D/N445K/N447A, S54M/T190P/C219L/N257H/N302H,
S54M/T190P/C219L/N257H/G395H/N445R/N447A/Y449H, S54M/T190P/N257H,
S54M/T190P/N257H/N302H/Q399R,
S54M/T190P/N257H/K309N/V385S/D389E/G395D/Q399R/N445K,
S54M/T190P/N257H/V385S/D389E, S54M/T190P/N257H/G395D/N445K/Y449H,
S54M/T190P/N302H/D389E/G395D/Q399R/N445K/N447S, S54M/T190P/N302R/D389E,
S54M/T190P/V385P/G395D, S54M/T190P/V385P/N445R/N447A/Y449H,
S54M/T190P/V385S/D389E/G395H/Q441S/N445R, S54M/T190P/G395A,
S54M/T190P/G395H/N445K/N447A, S54M/C219L/N302R/G395H/Q441S/N445R/N447A,
S54M/C219L/V385P/D389E/G395D/Q441R/N445K/N447A, S54M/N257H,
S54M/N257H/V385S/Y449H, S54M/N257H/D389E, S54M/N257H/Q399R,
S54M/N257H/Q441R/N447A, S54M/N257H/Q441R/Y449H,
S54M/N302H/V385P/Q399R/Q441S/N445K/N447S,

S54M/N302H/V385S/Q399R/Q441S/N445R/Y449H, S54M/V385P,
S54M/V385P/D389E/Q441R/N445K/N447A/Y449H, S54M/D389E/G395D/Q399R/N447A/Y449H,
F185L/T190P/C219L/N257H/D389E/G395A/N445K/N447A,
F185L/T190P/N257H/N302R/G395D/Q441S/N445R/N447S,
F185L/T190P/N257H/V385P/D389E/Q399R, F185L/T190P/N257H/V385P/Q399R/N445R/N447A,
F185L/T190P/N257H/D389E/G395A,
F185L/T190P/N257H/D389E/G395H/Q399R/Q441S/N447S/Y449H,
F185L/T190P/V385S/D389E/Q441R/N445K/N447S, F185L/T190P/D389E/N447S/Y449H,
F185L/T190P/G395D/Q399R, F185L/T190P/G395H/Q399R,
F185L/C219L/N257H/Q399R/N445R/N447A, F185L/N257H/V385S/G395A/Q399R/N445R/N447A,
F185L/N302R/G395H/Q399R/Q441R/N445K/N447S/Y449H,
F185L/G395D/Q399R/Q441R/N445R/N447A,
T190P/C219L/N257H/V385S/D389E/Q441S/N445R/N447A/Y449H,
T190P/C219L/N302H/V385P/Q399R/N445R, T190P/N257H/N302H/V385P/Q399R,
T190P/N257H/V385P/D389E/Q399R, T190P/N257H/V385S/D389E/N445K/N447A,
T190P/N257H/V385S/G395D, T190P/N257H/V385S/Q441S/N445R/N447S/Y449H,
T190P/N257H/D389E/G395H/Q441R/N445R/N447A, T190P/N257H/Q399R/N447A/Y449H,
T190P/N302H/V385P/D389E/G395D/Q399R/Q441S/N445K/N447S, T190P/D389E,
C219L/N257H/V385S/D389E/G395D/Q441R/N447A/Y449H, C219L/N257H/G395H,
C219L/V385S/D389E/Q399R/N445R/Y449H, C219L/G395H, N257H/V385P/D389E/Q399R,
N257H/D389E/G395D/Q399R/N445K/N447A, N257H/D389E/G395D/Q399R/N445K/Y449H,
N257H/D389E/Q399R, N302H/D389E/G395H/N445R, V385S/D389E, V385S/G395D,
D389E/G395D/N445R/N447S, G395D/Q399R, and Q399R, wherein the positions are numbered with reference to SEQ ID NO: 1216. In some additional embodiments, the polypeptide sequence of the engineered glycosyltransferase comprises at least one mutation or mutation set at one or more positions selected from 5, 14, 96, 108, 157, 181, 188, 278, 293, and 341, wherein the positions are numbered with reference to SEQ ID NO: 1216. In some additional embodiments, the polypeptide sequence of the engineered glycosyltransferase comprises at least one mutation or mutation set selected from 5S, 14T, 96K, 96P, 108E, 157Q, 181L, 188L, 278L, 293V, and 341L

[0025] In some additional embodiments, the polypeptide sequence of the engineered glycosyltransferase comprises at least one mutation or mutation set selected from H5S, R14T, G96K, G96P, N108E, N157Q, M181L, E188L, V278L, A293V, and I341L, wherein the positions are numbered with reference to SEQ ID NO: 1216. In some further embodiments, the polypeptide sequence of the engineered glycosyltransferase comprises at least one mutation or mutation set at one or more positions selected from 54/185/190/219/257/302/385/389/395/445/447/449, 54/185/190/385/447/449, 54/190/257/302/385/395/399, 54/190/302/389/395/399/445/447, 54/257/385/449, 54/257/389, and 54/389/395/399/447/449, wherein the positions are numbered with

reference to SEQ ID NO: 1216. In some additional embodiments, the polypeptide sequence of the engineered glycosyltransferase comprises at least one mutation or mutation set selected from 54M/185L/190P/219L/257H/302R/385P/389E/395H/445K/447A/449H, 54M/185L/190P/385P/447A/449H, 54M/190P/257H/302R/385P/395A/399R, 54M/190P/302H/389E/395D/399R/445K/447S, 54M/257H/385S/449H, 54M/257H/389E, and 54M/389E/395D/399R/447A/449H, wherein the positions are numbered with reference to SEQ ID NO: 1216. In some additional embodiments, the polypeptide sequence of the engineered glycosyltransferase comprises at least one mutation or mutation set selected from S54M/F185L/T190P/C219L/N257H/N302R/V385P/D389E/G395H/N445K/N447A/Y449H, S54M/F185L/T190P/V385P/N447A/Y449H, S54M/T190P/N257H/N302R/V385P/G395A/Q399R, S54M/T190P/N302H/D389E/G395D/Q399R/N445K/N447S, S54M/N257H/V385S/Y449H, S54M/N257H/D389E, and S54M/D389E/G395D/Q399R/N447A/Y449H, wherein the positions are numbered with reference to SEQ ID NO: 1216.

[0026] In some further embodiments, the polypeptide sequence of the engineered glycosyltransferase comprises at least one mutation or mutation set at one or more positions selected from 14/42/51/108/181, 14/42/51/157/341, 14/42/341, 14/51/96/157, 14/51/96/157/341, 14/51/96/341, 14/51/108, 14/51/341, 14/96, 14/96/108/133, 14/96/108/181/341, 14/96/108/188, 14/96/341, 14/108/157, 14/108/157/188, 14/157/181/278, 14/278, 42/96/157/341, 42/188/341, 96/108/181/293, 96/108/188, 96/108/188/341, 96/188, 181, 188, and 293, wherein the positions are numbered with reference to SEQ ID NO: 1488. In some additional embodiments, the polypeptide sequence of the engineered glycosyltransferase comprises at least one mutation or mutation set selected from 14T/42T/51V/108E/181L, 14T/42T/51V/157Q/341L, 14T/42T/341L, 14T/51V/96K/157Q, 14T/51V/96P/157Q/341L, 14T/51V/96P/341L, 14T/51V/108E, 14T/51V/341L, 14T/96K, 14T/96K/108E/133I, 14T/96K/108E/188V, 14T/96K/341L, 14T/96P/108E/181L/341L, 14T/108E/157Q, 14T/108E/157Q/188L, 14T/157Q/181L/278L, 14T/278L, 42T/96P/157Q/341L, 42T/188L/341L, 96K/188L, 96P/108E/181L/293V, 96P/108E/188L, 96P/108E/188L/341L, 181L, 188L, and 293V, wherein the positions are numbered with reference to SEQ ID NO: 1488. In some additional embodiments, the polypeptide sequence of the engineered glycosyltransferase comprises at least one mutation or mutation set selected from R14T/V42T/N51V/N108E/M181L, R14T/V42T/N51V/N157Q/I341L, R14T/V42T/I341L, R14T/N51V/G96K/N157Q, R14T/N51V/G96P/N157Q/I341L, R14T/N51V/G96P/I341L, R14T/N51V/N108E, R14T/N51V/I341L, R14T/G96K, R14T/G96K/N108E/V133I, R14T/G96K/N108E/E188V, R14T/G96K/I341L, R14T/G96P/N108E/M181L/I341L, R14T/N108E/N157Q, R14T/N108E/N157Q/E188L, R14T/N157Q/M181L/V278L, R14T/V278L, V42T/G96P/N157Q/I341L, V42T/E188L/I341L, G96K/E188L, G96P/N108E/M181L/A293V, G96P/N108E/E188L, G96P/N108E/E188L/I341L, M181L, E188L, and A293V, wherein the positions are numbered with reference to SEQ ID NO: 1488. In some further embodiments, the polypeptide

sequence of the engineered glycosyltransferase comprises at least one mutation or mutation set at one or more positions selected from 122, 144, 147, 187, 196, 197, 198, 199, 201, 268, and 324, wherein the positions are numbered with reference to SEQ ID NO: 1488. In some additional embodiments, the polypeptide sequence of the engineered glycosyltransferase comprises at least one mutation or mutation set selected from 122V, 144V, 147F, 187K, 196M, 196P, 196V, 197V, 198A, 198F, 199G, 199Q, 199S, 201P, 268A, 324K, and 324R, wherein the positions are numbered with reference to SEQ ID NO: 1488. In some additional embodiments, the polypeptide sequence of the engineered glycosyltransferase comprises at least one mutation or mutation set selected from L122V, L144V, S147F, T187K, A196M, A196P, A196V, P197V, Q198A, Q198F, N199G, N199Q, N199S, G201P, Y268A, H324K, and H324R, wherein the positions are numbered with reference to SEQ ID NO: 1488. In some further embodiments, the polypeptide sequence of the engineered glycosyltransferase comprises at least one mutation or mutation set at one or more positions selected from 14/42/341, 14/96, 14/96/108/133, 14/96/108/181/341, and 188, wherein the positions are numbered with reference to SEQ ID NO: 1488. In some additional embodiments, the polypeptide sequence of the engineered glycosyltransferase comprises at least one mutation or mutation set selected from 14T/42T/341L, 14T/96K, 14T/96K/108E/133I, 14T/96P/108E/181L/341L, and 188L, wherein the positions are numbered with reference to SEQ ID NO: 1488. In some additional embodiments, the polypeptide sequence of the engineered glycosyltransferase comprises at least one mutation or mutation set selected from R14T/V42T/I341L, R14T/G96K, R14T/G96K/N108E/V133I, R14T/G96P/N108E/M181L/I341L, and E188L, wherein the positions are numbered with reference to SEQ ID NO: 1488.

[0027] In yet some additional embodiments, the polypeptide sequence of the engineered glycosyltransferase comprises at least one mutation or mutation set at one or more positions selected from 147/188/196/201, 152/187/188/324, 152/188, 152/188/196/198/199/324, 152/188/196/199, 152/188/196/201/324, 152/188/324, 188, 188/196/198/199/201, 188/196/198/199/201/324, 188/196/198/201, 188/196/198/324, 188/196/201, 188/198/199/201/324, 188/198/199/268, 188/198/201/324, 188/198/324, 188/199/201, 188/201, 188/201/324, and 188/324, wherein the positions are numbered with reference to SEQ ID NO: 1516. In some additional embodiments, the polypeptide sequence of the engineered glycosyltransferase comprises at least one mutation or mutation set selected from 147F/188E/196V/201P, 152Y/187K/188E/324K, 152Y/188E, 152Y/188E/196V/198A/199Q/324K, 152Y/188E/196V/199Q, 152Y/188E/196V/201P/324K, 152Y/188E/324K, 188E, 188E/196V/198A/199Q/201P, 188E/196V/198A/324K, 188E/196V/198F/199Q/201P/324K, 188E/196V/198F/201P, 188E/196V/201P, 188E/198A/199Q/268A, 188E/198A/201P/324K, 188E/198A/324K, 188E/198F/199Q/201P/324K, 188E/199Q/201P, 188E/201P, 188E/201P/324K, and 188E/324K, wherein the positions are numbered with reference to SEQ ID NO: 1516. In some additional embodiments, the polypeptide sequence of the engineered glycosyltransferase comprises at least one mutation or mutation set selected from

S147F/L188E/A196V/G201P, F152Y/T187K/L188E/H324K, F152Y/L188E, F152Y/L188E/A196V/Q198A/N199Q/H324K, F152Y/L188E/A196V/N199Q, F152Y/L188E/A196V/G201P/H324K, F152Y/L188E/H324K, L188E, L188E/A196V/Q198A/N199Q/G201P, L188E/A196V/Q198A/H324K, L188E/A196V/Q198F/N199Q/G201P/H324K, L188E/A196V/Q198F/G201P, L188E/A196V/G201P, L188E/Q198A/N199Q/Y268A, L188E/Q198A/G201P/H324K, L188E/Q198A/H324K, L188E/Q198F/N199Q/G201P/H324K, L188E/N199Q/G201P, L188E/G201P, L188E/G201P/H324K, and L188E/H324K, wherein the positions are numbered with reference to SEQ ID NO: 1516. In some further embodiments, the polypeptide sequence of the engineered glycosyltransferase comprises at least one mutation or mutation set at one or more positions selected from 152/188/196/198/199/324, 188/196/198/199/201, 188/196/198/201, 188/196/201, 188/198/199/268, 188/201, and 188/324, wherein the positions are numbered with reference to SEQ ID NO: 1516. In some additional embodiments, the polypeptide sequence of the engineered glycosyltransferase comprises at least one mutation or mutation set selected from 152Y/188E/196V/198A/199Q/324K, 188E/196V/198A/199Q/201P, 188E/196V/198F/201P, 188E/196V/201P, 188E/198A/199Q/268A, 188E/201P, and 188E/324K, wherein the positions are numbered with reference to SEQ ID NO: 1516. In some additional embodiments, the polypeptide sequence of the engineered glycosyltransferase comprises at least one mutation or mutation set selected from F152Y/L188E/A196V/Q198A/N199Q/H324K, L188E/A196V/Q198A/N199Q/G201P, L188E/A196V/Q198F/G201P, L188E/A196V/G201P, L188E/Q198A/N199Q/Y268A, L188E/G201P, and L188E/H324K, wherein the positions are numbered with reference to SEQ ID NO: 1516.

[0028] The present invention also provides engineered glycosyltransferase provided herein, wherein the engineered glycosyltransferase is selected from beta-1,2-glycosyltransferases and beta-1,3-glycosyltransferases. In some embodiments, the engineered glycosyltransferase is an NDP-glycosyltransferase selected from ADP-glucose-dependent glycosyltransferases (AGTs), CDP-glucose-dependent glycosyltransferases (CGTs), GDP-glucose-dependent glycosyltransferase (GGTs), TDP-glucose-dependent glycosyltransferases (TGTs), and IDP-glucose-dependent glycosyltransferase (IGTs). In some additional embodiments, the engineered glycosyltransferase is an ADP-glucose-dependent glycosyltransferase. In some further embodiments, the engineered glycosyltransferase preferentially uses a sugar donor other than uracil-diphosphate.

[0029] The present invention also provides engineered polynucleotides encoding at least one engineered glycosyltransferase polypeptides provided herein. The present invention also provides vectors comprising at least one engineered polynucleotide encoding at least one glycosyltransferase provided herein. In some embodiments, the vector further comprises at least one control sequence. In present invention also provides host cells comprising at least one engineered polynucleotide encoding at least one glycosyltransferase provided herein. The present invention further provides host cells

comprising at least one vector provided herein. In some embodiments, the the host cell is selected from eukaryotic and prokaryotic organisms.

[0030] The present invention also provides methods for producing at least one engineered glycosyltransferase provided herein, comprising culturing a host cell provided herein, under conditions such that the engineered glycosyltransferase is produced by the host cell. In some embodiments, the methods further comprise the step of recovering the engineered glycosyltransferase. The present invention also provides compositions comprising at least one engineered glycosyltransferase provided herein.

[0031] The present invention also provides engineered sucrose synthases comprising polypeptide sequences that have at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or more sequence identity to SEQ ID NO: 6. In some embodiments, the engineered sucrose synthase comprising a polypeptide sequence that has at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or more sequence identity to SEQ ID NO: 22. In some embodiments, the polypeptide sequence of the engineered sucrose synthase comprises at least one mutation or mutation set at one or more positions selected from 3/548, 4, 7, 12, 41, 42, 44, 47, 52, 57, 59, 71, 81, 85, 93, 97, 122, 129, 134, 136, 139, 154, 175, 215, 266, 270, 343, 358, 381, 388, 434, 442, 519, 524, 532, 536, 570, 589, 603, 606, 615, 635, 641, 652, 724, 727, and 738, wherein the positions are numbered with reference to SEQ ID NO: 22. In some additional embodiments, the polypeptide sequence of the engineered sucrose synthase comprises at least one mutation or mutation set selected from 3G/548R, 4Q, 4T, 7H, 7L, 12K, 41Q, 41S, 42D, 42E, 42I, 44A, 44I, 44L, 44T, 47A, 47G, 47R, 47T, 52G, 52Q, 52S, 52T, 52V, 57H, 57Y, 59M, 59T, 71Q, 71T, 81H, 81P, 81Q, 85T, 93I, 97S, 97T, 122P, 129Q, 134P, 134Q, 134R, 136G, 139G, 154A, 175S, 215L, 266R, 270L, 343L, 358P, 358Q, 381K, 381R, 381T, 381Y, 388A, 388R, 388S, 434G, 434L, 442Y, 519C, 519V, 524R, 524S, 532E, 532T, 532Y, 536T, 570K, 589Q, 603M, 603T, 606A, 606P, 615T, 635G, 635R, 641I, 641L, 652R, 652Y, 724A, 724S, 727H, 738A, and 738K, wherein the positions are numbered with reference to SEQ ID NO: 22. In some additional embodiments, the polypeptide sequence of the engineered sucrose synthase comprises at least one mutation or mutation set selected from E3G/P548R, E4Q, E4T, Q7H, Q7L, S12K, K41Q, K41S, T42D, T42E, T42I, R44A, R44I, R44L, R44T, P47A, P47G, P47R, P47T, P52G, P52Q, P52S, P52T, P52V, W57H, W57Y, A59M, A59T, R71Q, R71T, L81H, L81P, L81Q, V85T, V93I, A97S, A97T, A122P, E129Q, V134P, V134Q, V134R, Q136G, K139G, H154A, G175S, F215L, N266R, V270L, H343L, E358P, E358Q, S381K, S381R, S381T, S381Y, K388A, K388R, K388S, Y434G, Y434L, H442Y, T519C, T519V, A524R, A524S, S532E, S532T, S532Y, E536T, R570K, S589Q, Q603M, Q603T, M606A, M606P, R615T, S635G, S635R, V641I, V641L, G652R, G652Y, K724A, K724S, E727H, S738A, and S738K, wherein the positions are numbered with reference to SEQ ID NO: 22. In some embodiments, the polypeptide sequence of the engineered sucrose synthase comprises at least one mutation or mutation set at one or more positions selected from 41, 52, 134, 381, 442, 519, 524, and 724, wherein the positions are numbered with reference to SEQ ID NO: 22.

In some additional embodiments, the polypeptide sequence of the engineered sucrose synthase comprises at least one mutation or mutation set selected from 41S, 52V, 134R, 381R, 442Y, 519V, 524R, and 724S, wherein the positions are numbered with reference to SEQ ID NO: 22. In some additional embodiments, the polypeptide sequence of the engineered sucrose synthase comprises at least one mutation or mutation set selected from K41S, P52V, V134R, S381R, H442Y, T519V, A524R, and K724S, wherein the positions are numbered with reference to SEQ ID NO: 22.

[0032] In some futher embodiments, the polypeptide sequence of the engineered sucrose synthase comprises at least one mutation or mutation set at one or more positions selected from 25/44/52/134/434/724, 41/44/52/97/442/719/724, 41/44/52/134/442, 41/44/52/434/442/532/724, 41/44/52/434/724, 41/44/136/329, 41/44/136/442, 41/44/532, 41/52/97/434/442/532/724, 41/52/134/136/434, 41/52/134/442/724, 41/52/136, 41/52/434, 41/52/434/442, 41/52/434/442/724, 41/52/442, 41/97/134/434/442/532/553, 41/134/442/532, 41/329/442, 41/434/442/532, 41/434/442/532/724, 41/434/532, 41/532, 44/52/97/434/442/724, 44/52/97/442/724, 44/52/134/136/329/434/442/532, 44/52/134/434/532, 44/136/329/434/532, 44/136/532, 44/434/442/553, 52, 52/97, 52/97/434/442, 52/97/442, 52/97/532, 52/134, 52/134/136/434, 52/134/136/442, 52/134/329/434, 52/134/329/532, 52/134/434/442/532/553, 52/134/442/724, 52/136, 52/136/434, 52/136/434/442, 52/136/434/442/532, 52/136/434/724, 52/136/442, 52/434, 52/434/442/532, 52/434/442/724, 52/434/532, 52/442, 52/442/532/724, 52/442/553/724, 52/442/724, 52/532, 52/553, 57/97/434/442/724, 97/134/136/442/532, 97/134/136/532, 97/134/442, 97/136/434/442, 97/329/724, 97/442, 134, 134/136/434/442, 134/136/434/442/532/553/724, 134/136/434/442/553/724, 134/136/434/532/724, 134/136/442/532, 134/434/442/724, 136, 136/442, 136/442/724, 136/532/724, 434, 434/442, 442, 442/724, and 532/724, wherein the positions are numbered with reference to SEQ ID NO: 1652. In some additional embodiments, the polypeptide sequence of the engineered sucrose synthase comprises at least one mutation or mutation set selected from 25T/44I/52G/134R/434G/724S, 41S/44I/52G/97T/442Y/719T/724S, 41S/44I/52G/434G/442Y/532Y/724S, 41S/44I/52G/434G/724S, 41S/44I/52V/134R/442Y, 41S/44I/136G/329Q, 41S/44I/136G/442Y, 41S/44I/532Y, 41S/52G/134R/442Y/724S, 41S/52G/136G, 41S/52V/97T/434G/442Y/532Y/724S, 41S/52V/134R/136G/434G, 41S/52V/434G, 41S/52V/434G/442Y, 41S/52V/434G/442Y/724S, 41S/52V/442Y, 41S/97T/134R/434G/442Y/532Y/553A, 41S/134R/442Y/532Y, 41S/329Q/442Y, 41S/434G/442Y/532Y, 41S/434G/442Y/532Y/724S, 41S/434G/532Y, 41S/532Y, 44I/52G/134R/136G/329Q/434G/442Y/532Y, 44I/52V/97T/434G/442Y/724S, 44I/52V/97T/442Y/724S, 44I/52V/134R/434G/532Y, 44I/136G/329Q/434G/532Y, 44I/136G/532Y, 44I/434G/442Y/553A, 52G, 52G/97T, 52G/97T/434G/442Y, 52G/97T/442Y, 52G/97T/532Y, 52G/134R, 52G/134R/136G/434G, 52G/134R/136G/442Y, 52G/134R/329Q/434G, 52G/134R/434G/442Y/532Y/553A, 52G/136G, 52G/434G/442Y/532Y, 52G/442Y, 52G/442Y/553A/724S, 52G/442Y/724S, 52G/532Y, 52G/553A, 52V/134R/329Q/532Y,

52V/134R/442Y/724S, 52V/136G/434G, 52V/136G/434G/442Y, 52V/136G/434G/442Y/532Y, 52V/136G/434G/724S, 52V/136G/442Y, 52V/434G, 52V/434G/442Y/724S, 52V/434G/532Y, 52V/442Y, 52V/442Y/532Y/724S, 52V/442Y/553A/724S, 52V/442Y/724S, 52V/532Y, 57C/97T/434G/442Y/724S, 97T/134R/136G/442Y/532Y, 97T/134R/136G/532Y, 97T/134R/442Y, 97T/136G/434G/442Y, 97T/329Q/724S, 97T/442Y, 134R, 134R/136G/434G/442Y, 134R/136G/434G/442Y/532Y/553A/724S, 134R/136G/434G/442Y/553A/724S, 134R/136G/434G/532Y/724S, 134R/136G/442Y/532Y, 134R/434G/442Y/724S, 136G, 136G/442Y, 136G/442Y/724S, 136G/532Y/724S, 434G, 434G/442Y, 442Y, 442Y/724S, and 532Y/724S, wherein the positions are numbered with reference to SEQ ID NO: 1652. In some additional embodiments, the polypeptide sequence of the engineered sucrose synthase comprises at least one mutation or mutation set selected from A25T/R44I/P52G/V134R/Y434G/K724S, K41S/R44I/P52G/A97T/H442Y/A719T/K724S, K41S/R44I/P52G/Y434G/H442Y/S532Y/K724S, K41S/R44I/P52G/Y434G/K724S, K41S/R44I/P52V/V134R/H442Y, K41S/R44I/Q136G/E329Q, K41S/R44I/Q136G/H442Y, K41S/R44I/S532Y, K41S/P52G/V134R/H442Y/K724S, K41S/P52G/Q136G, K41S/P52V/A97T/Y434G/H442Y/S532Y/K724S, K41S/P52V/V134R/Q136G/Y434G, K41S/P52V/Y434G, K41S/P52V/Y434G/H442Y, K41S/P52V/Y434G/H442Y/K724S, K41S/P52V/H442Y, K41S/A97T/V134R/Y434G/H442Y/S532Y/V553A, K41S/V134R/H442Y/S532Y, K41S/E329Q/H442Y, K41S/Y434G/H442Y/S532Y, K41S/Y434G/H442Y/S532Y/K724S, K41S/Y434G/S532Y, K41S/S532Y, R44I/P52G/V134R/Q136G/E329Q/Y434G/H442Y/S532Y, R44I/P52V/A97T/Y434G/H442Y/K724S, R44I/P52V/A97T/H442Y/K724S, R44I/P52V/V134R/Y434G/S532Y, R44I/Q136G/E329Q/Y434G/S532Y, R44I/Q136G/S532Y, R44I/Y434G/H442Y/V553A, P52G, P52G/A97T, P52G/A97T/Y434G/H442Y, P52G/A97T/H442Y, P52G/A97T/S532Y, P52G/V134R, P52G/V134R/Q136G/Y434G, P52G/V134R/Q136G/H442Y, P52G/V134R/E329Q/Y434G, P52G/V134R/Y434G/H442Y/S532Y/V553A, P52G/Q136G, P52G/Y434G/H442Y/S532Y, P52G/H442Y, P52G/H442Y/V553A/K724S, P52G/H442Y/K724S, P52G/S532Y, P52G/V553A, P52V/V134R/E329Q/S532Y, P52V/V134R/H442Y/K724S, P52V/Q136G/Y434G, P52V/Q136G/Y434G/H442Y, P52V/Q136G/Y434G/H442Y/S532Y, P52V/Q136G/Y434G/K724S, P52V/Q136G/H442Y, P52V/Y434G, P52V/Y434G/H442Y/K724S, P52V/Y434G/S532Y, P52V/H442Y, P52V/H442Y/S532Y/K724S, P52V/H442Y/V553A/K724S, P52V/H442Y/K724S, P52V/S532Y, W57C/A97T/Y434G/H442Y/K724S, A97T/V134R/Q136G/H442Y/S532Y, A97T/V134R/Q136G/S532Y, A97T/V134R/H442Y, A97T/Q136G/Y434G/H442Y, A97T/E329Q/K724S, A97T/H442Y, V134R, V134R/Q136G/Y434G/H442Y, V134R/Q136G/Y434G/H442Y/S532Y/V553A/K724S, V134R/Q136G/Y434G/H442Y/V553A/K724S, V134R/Q136G/Y434G/S532Y/K724S, V134R/Q136G/H442Y/S532Y, V134R/Y434G/H442Y/K724S, Q136G, Q136G/H442Y, Q136G/H442Y/K724S, Q136G/S532Y/K724S, Y434G, Y434G/H442Y, H442Y, H442Y/K724S, and

S532Y/K724S, wherein the positions are numbered with reference to SEQ ID NO: 1652. In some additional embodiments, the polypeptide sequence of the engineered sucrose synthase comprises at least one mutation or mutation set at one or more positions selected from 3, 4, 22, 32, 34, 38, 47/488, 51, 51/433, 62, 75/169, 101, 169, 195/213, 708, and 718, wherein the positions are numbered with reference to SEQ ID NO: 1652. In some additional embodiments, the polypeptide sequence of the engineered sucrose synthase comprises at least one mutation or mutation set selected from 3A, 4V, 22L, 22T, 32S, 34E, 34S, 38L, 38N, 38V, 38W, 47S/488N, 51A, 51H, 51H/433P, 51T, 62I, 75I/169A, 101V, 169E, 195K/213T, 708V, and 718H, wherein the positions are numbered with reference to SEQ ID NO: 1652. In some additional embodiments, the polypeptide sequence of the engineered sucrose synthase comprises at least one mutation or mutation set selected from E3A, E4V, H22L, H22T, L32S, T34E, T34S, R38L, R38N, R38V, R38W, P47S/D488N, Y51A, Y51H, Y51H/L433P, Y51T, V62I, M75I/Q169A, L101V, Q169E, S195K/A213T, T708V, and A718H, wherein the positions are numbered with reference to SEQ ID NO: 1652. In some further embodiments, the polypeptide sequence of the engineered sucrose synthase comprises at least one mutation or mutation set at one or more positions selected from 44/52/97/442/724, 52/97/442, 97/442, and 434/442, wherein the positions are numbered with reference to SEQ ID NO: 1652. In some additional embodiments, the polypeptide sequence of the engineered sucrose synthase comprises at least one mutation or mutation set selected from 44I/52V/97T/442Y/724S, 52G/97T/442Y, 97T/442Y, and 434G/442Y, wherein the positions are numbered with reference to SEQ ID NO: 1652. In some additional embodiments, the polypeptide sequence of the engineered sucrose synthase comprises at least one mutation or mutation set selected from R44I/P52V/A97T/H442Y/K724S, P52G/A97T/H442Y, A97T/H442Y, and Y434G/H442Y, wherein the positions are numbered with reference to SEQ ID NO: 1652.

[0033] In yet some further embodidments, the polypeptide sequence of the engineered sucrose synthase comprises at least one mutation or mutation set at one or more positions selected from 4/22, 4/22/34, 4/22/34/38, 4/22/34/38/169/708, 4/22/34/47/51/169/213, 4/22/51/708, 4/22/101, 4/34/38/101, 4/34/708, 4/62/433/708, 4/169, 4/169/433, 4/169/708, 4/433, 4/708, 22/34, 22/34/38, 22/34/101/169, 22/34/101/169/195, 22/34/169/708, 22/47/169/433, 22/75, 34/38/62, 34/62/169/433, 34/101, 62/708, 75/169, 169/708, and 195/708, wherein the positions are numbered with reference to SEQ ID NO: 1822. In some additional embodiments, the polypeptide sequence of the engineered sucrose synthase comprises at least one mutation or mutation set selected from 4V/22L/34E, 4V/22L/51A/708V, 4V/22L/101V, 4V/22T, 4V/22T/34E/38N, 4V/22T/34E/38V/169A/708V, 4V/22T/34E/47S/51H/169A/213T, 4V/34E/38N/101V, 4V/34E/708V, 4V/62I/433P/708V, 4V/169A, 4V/169A/433P, 4V/169A/708V, 4V/433P, 4V/708V, 22L/34E, 22L/34S/169A/708V, 22L/75I, 22T/34E/38V, 22T/34E/101V/169E, 22T/34E/101V/169E/195K, 22T/47S/169A/433P, 34E/62I/169E/433P, 34E/101V, 34S/38L/62I, 62I/708V, 75I/169A, 169A/708V, and 195K/708V, wherein the positions are numbered with reference to SEQ ID NO: 1822. In some additional embodiments, the polypeptide sequence of the engineered sucrose synthase comprises at least one

mutation or mutation set selected from E4V/H22L/T34E, E4V/H22L/Y51A/T708V, E4V/H22L/L101V, E4V/H22T, E4V/H22T/T34E/R38N, E4V/H22T/T34E/R38V/Q169A/T708V, E4V/H22T/T34E/P47S/Y51H/Q169A/A213T, E4V/T34E/R38N/L101V, E4V/T34E/T708V, E4V/V62I/L433P/T708V, E4V/Q169A, E4V/Q169A/L433P, E4V/Q169A/T708V, E4V/L433P, E4V/T708V, H22L/T34E, H22L/T34S/Q169A/T708V, H22L/M75I, H22T/T34E/R38V, H22T/T34E/L101V/Q169E, H22T/T34E/L101V/Q169E/S195K, H22T/P47S/Q169A/L433P, T34E/V62I/Q169E/L433P, T34E/L101V, T34S/R38L/V62I, V62I/T708V, M75I/Q169A, Q169A/T708V, and S195K/T708V, wherein the positions are numbered with reference to SEQ ID NO: 1822. In some additional embodiments, the polypeptide sequence of the engineered sucrose synthase comprises at least one mutation or mutation set at one or more positions selected from 4, 22, 34, 121, 169, 341, 411, 433, 441, 518, 526, 527, 528, 544, 557, 558, 565, 585, 602, 604, 623, 708, 731, and 770, wherein the positions are numbered with reference to SEQ ID NO: 1822. In some additional embodiments, the polypeptide sequence of the engineered sucrose synthase comprises at least one mutation or mutation set selected from 4V, 22L, 22T, 34E, 121D, 169A, 341N, 411I, 433P, 441A, 441L, 518S, 526H, 526V, 527A, 527Q, 528Q, 544H, 557P, 558G, 565V, 585A, 602P, 604A, 623A, 623K, 623Q, 623R, 708V, 731A, 731M, 731Q, 731T, and 770T, wherein the positions are numbered with reference to SEQ ID NO: 1822. In some additional embodiments, the polypeptide sequence of the engineered sucrose synthase comprises at least one mutation or mutation set selected from E4V, H22L, H22T, T34E, E121D, Q169A, P341N, L411I, L433P, D441A, D441L, R518S, T526H, T526V, E527A, E527Q, E528Q, R544H, R557P, Q558G, M565V, S585A, V602P, N604A, H623A, H623K, H623Q, H623R, T708V, D731A, D731M, D731Q, D731T, and V770T, wherein the positions are numbered with reference to SEQ ID NO: 1822. In some additional embodiments, the polypeptide sequence of the engineered sucrose synthase comprises at least one mutation or mutation set at one or more positions selected from 4, 4/22/34/38, 4/169, 4/169/433, 4/433, 62/708, and 169/708, wherein the positions are numbered with reference to SEQ ID NO: 1822. In some additional embodiments, the polypeptide sequence of the engineered sucrose synthase comprises at least one mutation or mutation set selected from 4V, 4V/22T/34E/38N, 4V/169A, 4V/169A/433P, 4V/433P, 62I/708V, and 169A/708V, wherein the positions are numbered with reference to SEQ ID NO: 1822. In some additional embodiments, the polypeptide sequence of the engineered sucrose synthase comprises at least one mutation or mutation set selected from E4V, E4V/H22T/T34E/R38N, E4V/Q169A, E4V/Q169A/L433P, E4V/L433P, V62I/T708V, and Q169A/T708V, wherein the positions are numbered with reference to SEQ ID NO: 1822.

[0034] In yet some additional embodiments, the polypeptide sequence of the engineered sucrose synthase comprises at least one mutation or mutation set at one or more positions selected from 34/62/411/526/557, 34/62/411/557/602/604, 34/62/441/557/623, 34/62/518/623, 34/62/623/731, 34/121/441/544/604/623, 34/121/526/604/731, 34/411/441, 34/411/441/518/526/557/565/731, 34/411/441/518/544/557/565/708/731, 34/411/441/518/585/604/770, 34/441/518/526/585/604/731,

34/441/526/565/708/770, 34/441/544/557/585/602/623, 34/441/585/623/708/731, 34/526/585/623, 62/121/329/518/557/565/623/708, 62/121/411/441/518/544/557/585/604/623, 62/121/441/518/526/623/770, 62/121/441/565/585, 62/121/518/557/585/604/708/770, 62/411/526/565/604/623, 62/411/585/731, 62/441/518/557/604/623/708/731, 62/441/623/708/770, 62/441/770, 121/441/518/526/602/604, 121/441/518/526/623/708, 121/441/526/557/565/708, 121/604/708/731/770, 411/441/518/557/623, 411/441/518/708, 411/441/604/623/708/731, 411/518/526/604/623/731, 411/565/604/623, 411/585/623, 441/518/526/557/565/602/604/623/708, 441/518/526/557/585/604/623/708, 441/518/526/604/623, 441/518/557/565/585, 441/518/565/623/731/770, 441/518/585, 441/585, 441/585/602/604/623/708/731, 441/708/731, 557/602/604, 557/604, 585/623/708, and 623, wherein the positions are numbered with reference to SEQ ID NO: 2092.

[0035] In some additional embodiments, the polypeptide sequence of the engineered sucrose synthase comprises at least one mutation or mutation set selected from 34E/62I/411I/526H/557P, 34E/62I/411I/557P/602P/604A, 34E/62I/441L/557P/623K, 34E/62I/518S/623K, 34E/62I/623K/731Q, 34E/121D/441L/544H/604A/623K, 34E/121D/526H/604A/731Q, 34E/411I/441L, 34E/411I/441L/518S/526H/557P/565V/731Q, 34E/411I/441L/518S/544H/557P/565V/708V/731Q, 34E/411I/441L/518S/585A/604A/770T, 34E/441L/518S/526H/585A/604A/731Q, 34E/441L/526H/565V/708V/770T, 34E/441L/544H/557P/585A/602P/623K, 34E/441L/585A/623K/708V/731Q, 34E/526H/585A/623K, 62I/121D/329Q/518S/557P/565V/623R/708V, 62I/121D/411I/441L/518S/544H/557P/585A/604A/623K, 62I/121D/441L/518S/526H/623R/770T, 62I/121D/441L/565V/585A, 62I/121D/518S/557P/585A/604A/708V/770T, 62I/411I/526H/565V/604A/623K, 62I/411I/585A/731Q, 62I/441L/518S/557P/604A/623K/708V/731Q, 62I/441L/623R/708V/770T, 62I/441L/770T, 121D/441L/518S/526H/602P/604A, 121D/441L/518S/526H/623K/708V, 121D/441L/526H/557P/565V/708V, 121D/604A/708V/731Q/770T, 411I/441L/518S/557P/623K, 411I/441L/518S/708V, 411I/441L/604A/623K/708V/731Q, 411I/518S/526H/604A/623K/731Q, 411I/565V/604A/623R, 411I/585A/623K, 441L/518S/526H/557P/565V/602P/604A/623K/708V, 441L/518S/526H/557P/585A/604A/623R/708V, 441L/518S/526H/604A/623R, 441L/518S/557P/565V/585A, 441L/518S/565V/623R/731Q/770T, 441L/518S/585A, 441L/585A, 441L/585A/602P/604A/623R/708V/731Q, 441L/708V/731Q, 557P/602P/604A, 557P/604A, 585A/623R/708V, and 623R, wherein the positions are numbered with reference to SEQ ID NO: 2092. In some additional embodiments, the polypeptide sequence of the engineered sucrose synthase comprises at least one mutation or mutation set selected from T34E/V62I/L411I/T526H/R557P, T34E/V62I/L411I/R557P/V602P/N604A, T34E/V62I/D441L/R557P/H623K, T34E/V62I/R518S/H623K, T34E/V62I/H623K/D731Q, T34E/E121D/D441L/R544H/N604A/H623K, T34E/E121D/T526H/N604A/D731Q,

T34E/L411I/D441L, T34E/L411I/D441L/R518S/T526H/R557P/M565V/D731Q, T34E/L411I/D441L/R518S/R544H/R557P/M565V/T708V/D731Q, T34E/L411I/D441L/R518S/S585A/N604A/V770T, T34E/D441L/R518S/T526H/S585A/N604A/D731Q, T34E/D441L/T526H/M565V/T708V/V770T, T34E/D441L/R544H/R557P/S585A/V602P/H623K, T34E/D441L/S585A/H623K/T708V/D731Q, T34E/T526H/S585A/H623K, V62I/E121D/E329Q/R518S/R557P/M565V/H623R/T708V, V62I/E121D/L411I/D441L/R518S/R544H/R557P/S585A/N604A/H623K, V62I/E121D/D441L/R518S/T526H/H623R/V770T, V62I/E121D/D441L/M565V/S585A, V62I/E121D/R518S/R557P/S585A/N604A/T708V/V770T, V62I/L411I/T526H/M565V/N604A/H623K, V62I/L411I/S585A/D731Q, V62I/D441L/R518S/R557P/N604A/H623K/T708V/D731Q, V62I/D441L/H623R/T708V/V770T, V62I/D441L/V770T, E121D/D441L/R518S/T526H/V602P/N604A, E121D/D441L/R518S/T526H/H623K/T708V, E121D/D441L/T526H/R557P/M565V/T708V, E121D/N604A/T708V/D731Q/V770T, L411I/D441L/R518S/R557P/H623K, L411I/D441L/R518S/T708V, L411I/D441L/N604A/H623K/T708V/D731Q, L411I/R518S/T526H/N604A/H623K/D731Q, L411I/M565V/N604A/H623R, L411I/S585A/H623K, D441L/R518S/T526H/R557P/M565V/V602P/N604A/H623K/T708V, D441L/R518S/T526H/R557P/S585A/N604A/H623R/T708V, D441L/R518S/T526H/N604A/H623R, D441L/R518S/R557P/M565V/S585A, D441L/R518S/M565V/H623R/D731Q/V770T, D441L/R518S/S585A, D441L/S585A, D441L/S585A/V602P/N604A/H623R/T708V/D731Q, D441L/T708V/D731Q, R557P/V602P/N604A, R557P/N604A, S585A/H623R/T708V, and H623R, wherein the positions are numbered with reference to SEQ ID NO: 2092. In yet some further embodiments, the polypeptide sequence of the engineered sucrose synthase comprises at least one mutation or mutation set at one or more positions selected from 63, 65, 269, 323, 406, 416, 469, 511, and 640, wherein the positions are numbered with reference to SEQ ID NO: 2092. In some additional embodiments, the polypeptide sequence of the engineered sucrose synthase comprises at least one mutation or mutation set selected from 63G, 63S, 63T, 65C, 65L, 269V, 323G, 323S, 323T, 406G, 416F, 469S, 511L, 640A, 640E, 640H, 640N, 640R, 640V, and 640W, wherein the positions are numbered with reference to SEQ ID NO: 2092. In some additional embodiments, the polypeptide sequence of the engineered sucrose synthase comprises at least one mutation or mutation set selected from A63G, A63S, A63T, T65C, T65L, I269V, A323G, A323S, A323T, N406G, L416F, T469S, V511L, T640A, T640E, T640H, T640N, T640R, T640V, and T640W, wherein the positions are numbered with reference to SEQ ID NO: 2092. In some further embodiments, the polypeptide sequence of the engineered sucrose synthase comprises at least one mutation or mutation set at one or more positions selected from 34/62/441/557/623, 34/441/585/623/708/731, 62/441/623/708/770, 411/441/518/557/623, 411/441/604/623/708/731, 441/518/526/557/585/604/623/708, 441/518/557/565/585, and 441/585/602/604/623/708/731, wherein the positions are numbered with

reference to SEQ ID NO: 2092. In some additional embodiments, the polypeptide sequence of the engineered sucrose synthase comprises at least one mutation or mutation set selected from 34E/62I/441L/557P/623K, 34E/441L/585A/623K/708V/731Q, 62I/441L/623R/708V/770T, 411I/441L/518S/557P/623K, 411I/441L/604A/623K/708V/731Q, 441L/518S/526H/557P/585A/604A/623R/708V, 441L/518S/557P/565V/585A, and 441L/585A/602P/604A/623R/708V/731Q, wherein the positions are numbered with reference to SEQ ID NO: 2092. In some additional embodiments, the polypeptide sequence of the engineered sucrose synthase comprises at least one mutation or mutation set selected from T34E/V62I/D441L/R557P/H623K, T34E/D441L/S585A/H623K/T708V/D731Q, V62I/D441L/H623R/T708V/V770T, L411I/D441L/R518S/R557P/H623K, L411I/D441L/N604A/H623K/T708V/D731Q, D441L/R518S/T526H/R557P/S585A/N604A/H623R/T708V, D441L/R518S/R557P/M565V/S585A, and D441L/S585A/V602P/N604A/H623R/T708V/D731Q, wherein the positions are numbered with reference to SEQ ID NO: 2092.

[0036] In some further embodiments, the polypeptide sequence of the engineered sucrose synthase comprises at least one mutation or mutation set at one or more positions selected from 34, 34/62, 34/62/65/640, 34/62/323, 34/63/65/323/528/640, 34/63/65/406/528/640/713, 34/63/323/526/528/640, 34/63/406/528/640/731, 34/65, 34/65/528/640, 34/323/406/640, 34/323/640, 34/640, 38/640, 62/63, 62/63/65/528/640, 62/63/69/323/406/640, 62/63/526/640/731, 62/323/640, 63/65/323/406/526/528/640/731, 63/65/406/731, 63/65/528/640/731, 63/65/640, 63/323/406/640, 63/406, 63/406/640, 63/526/528/640/731, 63/731, 323/406/640, 323/406/640/731, 323/526/528/640, 323/526/640, 323/640/731, 406/640, 526/528/640/731, and 731, wherein the positions are numbered with reference to SEQ ID NO: 2182. In some additional embodiments, the polypeptide sequence of the engineered sucrose synthase comprises at least one mutation or mutation set selected from 34E, 34E/62I, 34E/62I/65C/640R, 34E/62I/323G, 34E/63S/65C/406G/528D/640R/713V, 34E/63S/323G/526T/528D/640R, 34E/63S/406G/528D/640N/731Q, 34E/63T/65C/323G/528D/640R, 34E/65C, 34E/65C/528D/640R, 34E/323G/406G/640A, 34E/323G/640A, 34E/640A, 34E/640R, 38H/640R, 62I/63S/65C/528D/640A, 62I/63S/526T/640R/731Q, 62I/63T, 62I/63T/69F/323G/406G/640R, 62I/323G/640R, 63S/65C/640R, 63S/323G/406G/640A, 63S/406G, 63S/526T/528D/640R/731Q, 63S/731Q, 63T/65C/323G/406G/526T/528D/640N/731Q, 63T/65C/406G/731Q, 63T/65C/528D/640A/731Q, 63T/406G/640A, 323G/406G/640N, 323G/406G/640R/731Q, 323G/526T/528D/640R, 323G/526T/640R, 323G/640A/731Q, 406G/640R, 526T/528D/640A/731Q, and 731Q, wherein the positions are numbered with reference to SEQ ID NO: 2182. In some additional embodiments, the polypeptide sequence of the engineered sucrose synthase comprises at least one mutation or mutation set selected from T34E, T34E/V62I, T34E/V62I/T65C/T640R, T34E/V62I/A323G, T34E/A63S/T65C/N406G/E528D/T640R/A713V, T34E/A63S/A323G/H526T/E528D/T640R, T34E/A63S/N406G/E528D/T640N/D731Q,

T34E/A63T/T65C/A323G/E528D/T640R, T34E/T65C, T34E/T65C/E528D/T640R, T34E/A323G/N406G/T640A, T34E/A323G/T640A, T34E/T640A, T34E/T640R, R38H/T640R, V62I/A63S/T65C/E528D/T640A, V62I/A63S/H526T/T640R/D731Q, V62I/A63T, V62I/A63T/I69F/A323G/N406G/T640R, V62I/A323G/T640R, A63S/T65C/T640R, A63S/A323G/N406G/T640A, A63S/N406G, A63S/H526T/E528D/T640R/D731Q, A63S/D731Q, A63T/T65C/A323G/N406G/H526T/E528D/T640N/D731Q, A63T/T65C/N406G/D731Q, A63T/T65C/E528D/T640A/D731Q, A63T/N406G/T640A, A323G/N406G/T640N, A323G/N406G/T640R/D731Q, A323G/H526T/E528D/T640R, A323G/H526T/T640R, A323G/T640A/D731Q, N406G/T640R, H526T/E528D/T640A/D731Q, and D731Q, wherein the positions are numbered with reference to SEQ ID NO: 2182. In some further embodiments, the polypeptide sequence of the engineered sucrose synthase comprises at least one mutation or mutation set at one or more positions selected from 28, 30, 30/158, 37, 59, 102, 108, 158, 164, 183, 191, 206, 235, 307, 311, 409, 419, 533, 543, 546, 559, 683, 710, 752, and 793, wherein the positions are numbered with reference to SEQ ID NO: 2182. In some additional embodiments, the polypeptide sequence of the engineered sucrose synthase comprises at least one mutation or mutation set selected from 28H, 30A, 30H/158H, 30M, 30Q, 37L, 37M, 59T, 102N, 102T, 108R, 158A, 158K, 164G, 164Q, 164S, 164T, 183P, 191L, 206I, 235A, 307E, 307H, 311L, 311S, 409S, 419L, 419V, 533K, 533R, 543A, 546N, 546Q, 546T, 559R, 683L, 683V, 710A, 710G, 710S, 710V, 752R, and 793G, wherein the positions are numbered with reference to SEQ ID NO: 2182. In some additional embodiments, the polypeptide sequence of the engineered sucrose synthase comprises at least one mutation or mutation set selected from R28H, S30A, S30H/R158H, S30M, S30Q, Q37L, Q37M, A59T, S102N, S102T, Q108R, R158A, R158K, A164G, A164Q, A164S, A164T, N183P, T191L, L206I, V235A, N307E, N307H, Q311L, Q311S, A409S, T419L, T419V, L533K, L533R, G543A, P546N, P546Q, P546T, K559R, R683L, R683V, E710A, E710G, E710S, E710V, T752R, and E793G, wherein the positions are numbered with reference to SEQ ID NO: 2182. In some further embodiments, the polypeptide sequence of the engineered sucrose synthase comprises at least one mutation or mutation set at one or more positions selected from 34/62, 34/65, 34/323/640, 34/640, 38/640, and 63/65/406/731, wherein the positions are numbered with reference to SEQ ID NO: 2182. In some additional embodiments, the polypeptide sequence of the engineered sucrose synthase comprises at least one mutation or mutation set selected from 34E/62I, 34E/65C, 34E/323G/640A, 34E/640A, 38H/640R, and 63T/65C/406G/731Q, wherein the positions are numbered with reference to SEQ ID NO: 2182. In some additional embodiments, the polypeptide sequence of the engineered sucrose synthase comprises at least one mutation or mutation set selected from T34E/V62I, T34E/T65C, T34E/A323G/T640A, T34E/T640A, R38H/T640R, and A63T/T65C/N406G/D731Q, wherein the positions are numbered with reference to SEQ ID NO: 2182.

[0037] In yet some further embodiments, the polypeptide sequence of the engineered sucrose synthase comprises at least one mutation or mutation set at one or more positions selected from

34/65/323/406/411/565, 34/323/406/411/565, 34/323/406/565/731, 62/65/323/406/565, 62/65/323/406/565/731, 62/65/323/411/565, 62/65/406/411/565/731, 62/323/406/411, 62/323/406/411/565, 62/323/411/565, 63/65/323/406/565/731, 63/65/406/411/565, 65/323/406/565, 323/406/411/565, 323/411/565, and 636, wherein the positions are numbered with reference to SEQ ID NO: 2322. In some additional embodiments, the polypeptide sequence of the engineered sucrose synthase comprises at least one mutation or mutation set selected from 34E/65C/323G/406G/411I/565V, 34E/323G/406G/411I/565V, 34E/323G/406G/565V/731Q, 62I/65C/323G/406G/565V, 62I/65C/323G/406G/565V/731Q, 62I/65C/323G/411I/565V, 62I/65C/406G/411I/565V/731Q, 62I/323G/406G/411I, 62I/323G/406G/411I/565V, 62I/323G/411I/565V, 63T/65C/323G/406G/565V/731Q, 63T/65C/406G/411I/565V, 65C/323G/406G/565V, 323G/406G/411I/565V, 323G/411I/565V, 636A, 636H, and 636N, wherein the positions are numbered with reference to SEQ ID NO: 2322. In some additional embodiments, the polypeptide sequence of the engineered sucrose synthase comprises at least one mutation or mutation set selected from T34E/T65C/A323G/N406G/L411I/M565V, T34E/A323G/N406G/L411I/M565V, T34E/A323G/N406G/M565V/D731Q, V62I/T65C/A323G/N406G/M565V, V62I/T65C/A323G/N406G/M565V/D731Q, V62I/T65C/A323G/L411I/M565V, V62I/T65C/N406G/L411I/M565V/D731Q, V62I/A323G/N406G/L411I, V62I/A323G/N406G/L411I/M565V, V62I/A323G/L411I/M565V, A63T/T65C/A323G/N406G/M565V/D731Q, A63T/T65C/N406G/L411I/M565V, T65C/A323G/N406G/M565V, A323G/N406G/L411I/M565V, A323G/L411I/M565V, Q636A, Q636H, and Q636N, wherein the positions are numbered with reference to SEQ ID NO: 2322. In some further embodiments, the polypeptide sequence of the engineered sucrose synthase comprises at least one mutation or mutation set at one or more positions selected from 34/65/323/406/411/565, 34/323/406/411/565, 62/65/323/406/565, 62/65/406/411/565/636/731, 62/65/406/411/565/731, 62/323/406/411, 62/323/406/411/565, and 62/323/411/565, wherein the positions are numbered with reference to SEQ ID NO: 2322. In some additional embodiments, the polypeptide sequence of the engineered sucrose synthase comprises at least one mutation or mutation set selected from 34E/65C/323G/406G/411I/565V, 34E/323G/406G/411I/565V, 62I/65C/323G/406G/565V, 62I/65C/406G/411I/565V/636H/731Q, 62I/65C/406G/411I/565V/731Q, 62I/323G/406G/411I, 62I/323G/406G/411I/565V, and 62I/323G/411I/565V, wherein the positions are numbered with reference to SEQ ID NO: 2322. In some additional embodiments, the polypeptide sequence of the engineered sucrose synthase comprises at least one mutation or mutation set selected from T34E/T65C/A323G/N406G/L411I/M565V, T34E/A323G/N406G/L411I/M565V, V62I/T65C/A323G/N406G/M565V, V62I/T65C/N406G/L411I/M565V/Q636H/D731Q, V62I/T65C/N406G/L411I/M565V/D731Q, V62I/A323G/N406G/L411I, V62I/A323G/N406G/L411I/M565V, and V62I/A323G/L411I/M565V, wherein the positions are numbered with reference to SEQ ID NO: 2322.

[0038] The present invention also provides engineered polynucleotides encoding at least one engineered sucrose synthase polypeptide provided herein. The present invention further provides vectors comprising at least one engineered polynucleotide encoding at least one engineered sucrose synthase provided herein. In some embodiments, the vectors further comprise at least one control sequence. The present invention also provides host cells comprising at least one engineered polynucleotide encoding at least one sucrose synthase. In some embodiments, the host cell comprises at least one vector comprising at least one polynucleotide encoding at least one sucrose synthase. In some embodimetnts, the host cell is selected from eukaryotic and prokaryotic organisms. The present invention also provides methods for producing at least one engineered sucrose synthase variant provided herein, comprising culturing a host cell provided herein, under conditions such that the engineered sucrose synthase variant is produced by the host cell. In some embodiments, the methods further comprise the step of recovering the engineered sucrose synthase variant. The present invention also provides compositions comprising at least one engineered sucrose synthase variant provided herein.

[0039] The present invention also provides methods for glycosylation of a substrate comprising providing at least one substrate, at least one engineered glycosyl transferase provided herein, and contacting the substrate with the glycosyltransferase under conditions such that the substrate is glycosylated to produce at least one glycosylated product. In some embodiments, the substrate comprises at least one steviol glycoside. In some additional embodiments, the glycosylated product comprises at least one mono-glycosylated and/or polyglycosylated product.

[0040] The present invention also provides methods for producing rebaudioside M, comprising providing a rebaudioside D and/or rebaudioside I substrate, NDP-glucose, and a least one engineered glycosyltransferase provided herein, combining the rebaudioside D and rebaudioside I substrate, NDP-glucose, and the glycosyltransferase under conditions such that rebaudioside M is produced.

[0041] The present invention also provides methods for producing rebaudioside A and/or rebaudioside I, comprising providing a stevioside substrate, NDP-glucose, and at least one engineered glycosyltransferase provided herein, combining the stevioside substrate, NDP-glucose, and glycosyltransferase under conditions such that rebaudioside A and/or rebaudioside I is produced.

[0042] The present invention also provides methods for producing rebaudioside D, comprising providing a stevioside substrate, NDP-glucose, and at least one engineered glycosyltransferase provided herein, combining the stevioside substrate, NDP-glucose, and glycosyltransferase under conditions such that rebaudioside D is produced.

[0043] In some embodiments of the methods provided herein, the NDP-glucose is selected from ADP-glucose, CDP-glucose, TDP-glucose, GDP-glucose, and/or IDT-glucose. In some further embodiments, the NDP-glucose is not UDP-glucose.

[0044] The present invention also provides methods for producing rebaudioside M comprising providing a rebaudioside D and/or rebaudioside I substrate, ADP-glucose, and at least one engineered

glycosyltransferase provided herein, combining the rebaudioside D and/or rebaudioside I substrate, ADP-glucose, and glycosyltransferase under conditions such that rebaudioside M is produced.

[0045] The present invention also provides methods for producing rebaudioside A and/or rebaudioside I, comprising providing a stevioside substrate, ADP-glucose, and at least one engineered ADP-glycosyltransferase provided herein, combining the stevioside substrate, ADP-glucose, and glycosyltransferase under conditions such that rebaudioside A and/or rebaudioside I is produced.

[0046] The present invention also provides methods for producing rebaudioside D, comprising providing a stevioside substrate, ADP-glucose, and at least one engineered glycosyltransferase provided herein, combining the stevioside substrate, ADP-glucose, and glycosyltransferase under conditions such that rebaudioside D is produced.

[0047] The present invention also provides methods for producing rebaudioside M comprising providing a rebaudioside D and/or rebaudioside I substrate, NDP, sucrose, a sucrose synthase, and at least one engineered glycosyltransferase provided herein, combining the rebaudioside D substrate, NDP, sucrose, sucrose synthase, and glycosyltransferase under conditions such that rebaudioside M is produced.

[0048] The present invention also provides methods for producing rebaudioside A and/or rebaudioside I comprising providing a stevioside substrate, NDP, sucrose, a sucrose synthase, and at least one engineered glycosyltransferase provided herein, combining the stevioside substrate, NDP, sucrose, sucrose synthase, and glycosyltransferase under conditions such that rebaudioside A and/or rebaudioside I is produced.

[0049] The present invention also provides methods for producing rebaudioside D, comprising providing a stevioside substrate, NDP, sucrose, a sucrose synthase, and at least one engineered glycosyltransferase provided herein, combining the stevioside substrate, NDP, sucrose, sucrose synthase, and glycosyltransferase under conditions such that rebaudioside D is produced.

[0050] The present invention also provides methods for producing rebaudioside M, comprising providing a stevioside substrate comprising at least one stevioside and/or a mixture of steviosides and rebA, NDP, sucrose, a sucrose synthase, and at least one engineered glycosyltransferase provided herein, combining the stevioside substrate, NDP, sucrose, sucrose synthase, and glycosyltransferase under conditions such that rebaudioside M is produced.

[0051] The present invention also provides methods of producing rebaudioside M, comprising providing a stevioside substrate, NDP, sucrose, at least one sucrose synthase, and at least one engineered glycosyltransferase of provided herein, combining the stevioside substrate, NDP, and glycosyltransferase under conditions such that rebaudioside A is first produced, rebaudioside D and/or rebaudioside I is then produced, and rebaudioside M finally produced.

[0052] In some embodiments of the methods provided herein, the sucrose synthase is an engineered sucrose synthase provided herein. In some further embodiments of the methods provided herein, the method is conducted as a one-pot reaction. In some alternative embodiments, the method is

conducted sequentially. In yet some additional embodiments, the method further comprises repeating the steps of the methods. In some further embodiments, sucrose is recycled during repeated steps. In some additional embodiments, the engineered glycosyltransferase and/or other reaction components are recycled. In yet some further embodiments, the stevioside substrate is extracted from *Stevia rebaudiana*. In some additional embodiments, the stevioside substrate is synthetically produced. In yet some further embodiments, the glycosyltransferase and/or the sucrose synthase is immobilized. In some further embodiments, the method produces a reaction product that includes fructose. In yet some further embodiments, fructose is removed from the reaction product. In some embodiments, the method further comprises a washing step. In some additional embodiments, the method further comprises at least one column chromatography step. In yet some additional embodiments of the methods, at least one engineered glycosyltransferase is a beta-1,2 glycosyltransferase selected from the glycosyltransferase provided herein. In some additional embodiments of the methods, at least one engineered glycosyltransferase is a beta-1,3 glycosyltransferase selected from the glycosyltransferase provided herein. In some embodiments of the methods, at least one engineered glycosyltransferase is a beta-1,2 glycosyltransferase selected from the glycosyltransferases provided herein, and further comprising at least one engineered glycosyltransferase is a beta-1,3 glycosyltransferase selected from the glycosyltransferases provided herein. In some embodiments, the methods further comprise at least one engineered sucrose synthase provided herein.

[0053] The present invention also provides at least one rebaudioside produced according to the methods provided herein. The present invention also provides compositions comprising the rebaudioside produced according to the methods provided herein. In some embodiments, rebaudioside M is produced according to the methods provided herein. The present invention also provides compositions comprising rebaudioside M produced according to the methods provided herein.

[0054] In some embodiments, rebaudioside A is produced according to the methods provided herein. The present invention also provides compositions comprising rebaudioside A produced according to the methods provided herein. In some embodiments, rebaudioside I is produced according to the methods provided herein. The present invention also provides compositions comprising rebaudioside I produced according to the methods provided herein. In some embodiments, rebaudioside D is produced according to the methods provided herein. The present invention also provides compositions comprising rebaudioside D produced according to the methods provided herein. In some embodiments, a mixture of rebaudiosides is produced according to the methods provided herein. The present invention also provides compositions comprising mixtures of rebaudiosides produced according to the methods provided herein. In some embodiments, the mixtures comprise at least two compositions comprising at least one rebaudioside produced according to the methods provided herein. The present invention provides compositions comprising mixtures of at least two rebaudiosides provided herein.

DESCRIPTION OF THE DRAWINGS

[0055] Figure 1 provides an enzymatic reaction scheme in which a glycosyltransferase catalyzes the transfer of a glucosyl group from a nucleoside diphosphoglucose (NDP-glucose), for example ADP-glucose, to an acceptor, for example R-OH, where R is any glycosyl, alkoxy, carboxy, aminocarbonyl, heteroalkyl, heteroalkenyl, heteroalkynyl, carboxyalkyl, aminoalkyl, haloalkyl, alkylthioalkyl, heterocycloalkyl, heteroaryl, or heteroarylalkyl group. In a further embodiment, R-OH is a stevioside or rebaudioside D, and the product is rebaudioside A, rebaudioside I, or rebaudioside M. A nucleoside diphosphate dependent synthase catalyzes the transfer of a glucosyl group from a glucose donor (e.g., sucrose), to a nucleoside diphosphate, regenerating NDP-glucose and releasing a byproduct (e.g., fructose).

[0056] Figure 2 provides the structure of rebaudioside M with the carbons numbered.

[0057] Figure 3 provides the structure of rebaudioside I with the carbons numbered.

DESCRIPTION OF THE INVENTION

[0058] The present invention provides engineered glycosyltransferase (GT) enzymes, polypeptides having GT activity, and polynucleotides encoding these enzymes, as well as vectors and host cells comprising these polynucleotides and polypeptides. The present invention provides engineered sucrose synthase (SuS) enzymes, polypeptides having SuS activity, and polynucleotides encoding these enzymes, as well as vectors and host cells comprising these polynucleotides and polypeptides. The present invention also provides compositions comprising the GT enzymes and methods of using the engineered GT enzymes to make products with β-glucose linkages. The present invention further provides compositions and methods for the production of rebaudiosides (e.g., rebaudioside M, rebaudioside A, rebaudioside I, and rebaudioside D). The present invention also provides compositions comprising the SuS enzymes and methods of using them. Methods for producing GT and SuS enzymes are also provided.

[0059] Unless defined otherwise, all technical and scientific terms used herein generally have the same meaning as commonly understood by one of ordinary skill in the art to which this invention pertains. Generally, the nomenclature used herein and the laboratory procedures of cell culture, molecular genetics, microbiology, organic chemistry, analytical chemistry and nucleic acid chemistry described below are those well-known and commonly employed in the art. Such techniques are well-known and described in numerous texts and reference works well known to those of skill in the art. Standard techniques, or modifications thereof, are used for chemical syntheses and chemical analyses. All patents, patent applications, articles and publications mentioned herein, both *supra* and *infra*, are hereby expressly incorporated herein by reference.

[0060] Although any suitable methods and materials similar or equivalent to those described herein find use in the practice of the present invention, some methods and materials are described herein. It is to be understood that this invention is not limited to the particular methodology, protocols, and

reagents described, as these may vary, depending upon the context they are used by those of skill in the art. Accordingly, the terms defined immediately below are more fully described by reference to the invention as a whole.

[0061] It is to be understood that both the foregoing general description and the following detailed description are exemplary and explanatory only and are not restrictive of the present invention. The section headings used herein are for organizational purposes only and not to be construed as limiting the subject matter described. Numeric ranges are inclusive of the numbers defining the range. Thus, every numerical range disclosed herein is intended to encompass every narrower numerical range that falls within such broader numerical range, as if such narrower numerical ranges were all expressly written herein. It is also intended that every maximum (or minimum) numerical limitation disclosed herein includes every lower (or higher) numerical limitation, as if such lower (or higher) numerical limitations were expressly written herein.

Abbreviations and Definitions

[0062] The abbreviations used for the genetically encoded amino acids are conventional and are as follows: alanine (Ala or A), arginine (Are or R), asparagine (Asn or N), aspartate (Asp or D), cysteine (Cys or C), glutamate (Glu or E), glutamine (Gln or Q), histidine (His or H), isoleucine (Ile or I), leucine (Leu or L), lysine (Lys or K), methionine (Met or M), phenylalanine (Phe or F), proline (Pro or P), serine (Ser or S), threonine (Thr or T), tryptophan (Trp or W), tyrosine (Tyr or Y), and valine (Val or V). When the three-letter abbreviations are used, unless specifically preceded by an "L" or a "D" or clear from the context in which the abbreviation is used, the amino acid may be in either the L- or D-configuration about α-carbon (C_α). For example, whereas "Ala" designates alanine without specifying the configuration about the α-carbon, "D-Ala" and "L-Ala" designate D-alanine and L-alanine, respectively. When the one-letter abbreviations are used, upper case letters designate amino acids in the L-configuration about the α-carbon and lower case letters designate amino acids in the D-configuration about the α-carbon. For example, "A" designates L-alanine and "a" designates D-alanine. When polypeptide sequences are presented as a string of one-letter or three-letter abbreviations (or mixtures thereof), the sequences are presented in the amino (N) to carboxy (C) direction in accordance with common convention.

[0063] The abbreviations used for the genetically encoding nucleosides are conventional and are as follows: adenosine (A); guanosine (G); cytidine (C); thymidine (T); and uridine (U). Unless specifically delineated, the abbreviated nucleosides may be either ribonucleosides or 2'-deoxyribonucleosides. The nucleosides may be specified as being either ribonucleosides or 2'-deoxyribonucleosides on an individual basis or on an aggregate basis. When nucleic acid sequences are presented as a string of one-letter abbreviations, the sequences are presented in the 5' to 3' direction in accordance with common convention, and the phosphates are not indicated.

[0064] In reference to the present invention, the technical and scientific terms used in the descriptions herein will have the meanings commonly understood by one of ordinary skill in the art, unless specifically defined otherwise. Accordingly, the following terms are intended to have the following meanings.

[0065] As used herein, the singular forms "a", "an" and "the" include plural referents unless the context clearly indicates otherwise. Thus, for example, reference to "a polypeptide" includes more than one polypeptide.

[0066] Similarly, "comprise," "comprises," "comprising" "include," "includes," and "including" are interchangeable and not intended to be limiting. Thus, as used herein, the term "comprising" and its cognates are used in their inclusive sense (*i.e.*, equivalent to the term "including" and its corresponding cognates).

[0067] It is to be further understood that where descriptions of various embodiments use the term "comprising," those skilled in the art would understand that in some specific instances, an embodiment can be alternatively described using language "consisting essentially of" or "consisting of."

[0068] As used herein, the term "about" means an acceptable error for a particular value. In some instances "about" means within 0.05%, 0.5%, 1.0%, or 2.0%, of a given value range. In some instances, "about" means within 1, 2, 3, or 4 standard deviations of a given value.

[0069] As used herein, "EC" number refers to the Enzyme Nomenclature of the Nomenclature Committee of the International Union of Biochemistry and Molecular Biology (NC-IUBMB). The IUBMB biochemical classification is a numerical classification system for enzymes based on the chemical reactions they catalyze.

[0070] As used herein, "ATCC" refers to the American Type Culture Collection whose biorepository collection includes genes and strains.

[0071] As used herein, "NCBI" refers to National Center for Biological Information and the sequence databases provided therein.

[0072] "Protein," "polypeptide," and "peptide" are used interchangeably herein to denote a polymer of at least two amino acids covalently linked by an amide bond, regardless of length or post-translational modification (e.g., glycosylation or phosphorylation). Included within this definition are D- and L-amino acids, and mixtures of D- and L-amino acids, as well as polymers comprising D- and L-amino acids, and mixtures of D- and L-amino acids.

[0073] "Amino acids" are referred to herein by either their commonly known three-letter symbols or by the one-letter symbols recommended by IUPAC-IUB Biochemical Nomenclature Commission. Nucleotides, likewise, may be referred to by their commonly accepted single letter codes.

[0074] As used herein, "polynucleotide" and "nucleic acid' refer to two or more nucleotides that are covalently linked together. The polynucleotide may be wholly comprised of ribonucleotides (i.e., RNA), wholly comprised of 2' deoxyribonucleotides (i.e., DNA), or comprised of mixtures of ribo-

and 2' deoxyribonucleotides. While the nucleosides will typically be linked together via standard phosphodiester linkages, the polynucleotides may include one or more non-standard linkages. The polynucleotide may be single-stranded or double-stranded, or may include both single-stranded regions and double-stranded regions. Moreover, while a polynucleotide will typically be composed of the naturally occurring encoding nucleobases (i.e., adenine, guanine, uracil, thymine and cytosine), it may include one or more modified and/or synthetic nucleobases, such as, for example, inosine, xanthine, hypoxanthine, etc. In some embodiments, such modified or synthetic nucleobases are nucleobases encoding amino acid sequences.

[0075] As used herein, "nucleoside" refers to glycosylamines comprising a nucleobase (i.e., a nitrogenous base), and a 5-carbon sugar (e.g., ribose or deoxyribose). Non-limiting examples of nucleosides include cytidine, uridine, adenosine, guanosine, thymidine, and inosine. In contrast, the term "nucleotide" refers to the glycosylamines comprising a nucleobase, a 5-carbon sugar, and one or more phosphate groups. In some embodiments, nucleosides can be phosphorylated by kinases to produce nucleotides.

[0076] As used herein, "nucleoside diphosphate" refers to glycosylamines comprising a nucleobase (i.e., a nitrogenous base), a 5-carbon sugar (e.g., ribose or deoxyribose), and a diphosphate (i.e., pyrophosphate) moiety. In some embodiments herein, "nucleoside diphosphate" is abbreviated as "NDP." Non-limiting examples of nucleoside diphosphates include cytidine diphosphate (CDP), uridine diphosphate (UDP), adenosine diphosphate (ADP), guanosine diphosphate (GDP), thymidine diphosphate (TDP), and inosine diphosphate. The terms "nucleoside" and "nucleotide" may be used interchangeably in some contexts.

[0077] As used herein, "coding sequence" refers to that portion of a nucleic acid (e.g., a gene) that encodes an amino acid sequence of a protein.

[0078] As used herein, the terms "biocatalysis," "biocatalytic," "biotransformation," and "biosynthesis" refer to the use of enzymes to perform chemical reactions on organic compounds.

[0079] As used herein, "glycosyltransferase" (GT) refers to a polypeptide having an enzymatic capability of transferring glycosyl residues from an activated sugar donor to monomeric and polymeric acceptor molecules. In some embodiments, the glycosyltransferases are referred to as "glycosyltransferase variants" or "glycosyltransferase combinatorial variants." In some embodiments, "glycosyltransferase" refers to an UDP-glucuronosyltransferase enzyme of the classification EC 2.4.1.17, which catalyzes the transfer of glucose from UDP-α-D-glucuronate (also known as UDP-glucose) to an acceptor, releasing UDP and forming acceptor β-D-glucuronoside. The Carbohydrate-Active Enzymes database (CAZy) provides a continuously updated list of the glycosyltransferase families. In some embodiments, the glycosyltransferases include, but are not limited to, enzymes classified in the GT1 family. In some preferred embodiments, the glycosyltransferase variants of the present invention preferentially utilize ADP-glucose. In some additional embodiments, the glycosyltransferase enzymes of the present invention do not utilize UDP-glucose. In some further

embodiments, the glycosyltransferase variants of the present invention utilize ADP-glucose, CDP-glucose, TDP-glucose, GDP-glucose, and/or IDT-glucose, but not UDP-glucose. Thus, in some preferred embodiments, the present invention provides ADP-glucose-dependent glycosyltransferases (ADP-glycosyltransferases; AGTs), CDP-glucose-dependent glycosyltransferases (CDP-glycosyltransferases; CGTs), GDP-glucose-dependent glycosyltransferases (GDP-glycosyltransferases; GGTs), TDP-glucose-dependent glycosyltransferases (TDP-glycosyltransferases; TGTs), and IDP-glucose-dependent glycosyltransferase (IDP-glycosyltransferases; IGTs).

[0080] As used herein, "NDP-glycosyltransferase" (NDP-GT) refers to a polypeptide having an enzymatic capability of transferring glycosyl residues from an activated sugar donor that is an NDP to monomeric and polymeric acceptor molecules. In some embodiments, NDP-glycosyltransferases are generally referred to as "glycosyltransferases." Indeed, the term "glycosyltransferase" as used herein encompasses NDP-glycosyltransferases, including, but not limited to ADP-glucose-dependent glycosyltransferases (ADP-glycosyltransferases; AGTs), CDP-glucose-dependent glycosyltransferases (CDP-glycosyltransferases; CGTs), GDP-glucose-dependent glycosyltransferase (GDP-glycosyltransferases; GGTs), TDP-glucose-dependent glycosyltransferases (TDP-glycosyltransferases; TGTs), and IDP-glucose-dependent glycosyltransferase (IDP-glycosyltransferases; IGTs). In some embodiments, the glycosyltransferase enzymes of the present invention utilize ADP-glucose, CDP-glucose, TDP-glucose, GDP-glucose, and/or IDT-glucose, but not UDP-glucose. In some additional embodiments the enzymes are referred to as "variants" or "combinatorial variants" (e.g., ADP-glycosyltransferase variants).

[0081] As used herein, "transglycosylation" refers to a reaction in which a glycosyl residue is transferred from a disaccharide, trisaccharide, or oligosaccharide donor to an aglycosylated or glycosylated acceptor molecule.

[0082] As used herein, "transglucosylation" refers to a transglycosylation reaction in which the glycosyl residue that is transferred is a glucose and the disaccharide, trisaccharide, or oligosaccharide donor contains glucose.

[0083] As used herein, "glycosylation" refers to the formation of a glycosidic linkage between a glycosyl residue and an acceptor molecule.

[0084] As used herein, "glucosylation" refers to the formation of a glycosidic linkage between a glucose residue and an acceptor molecule.

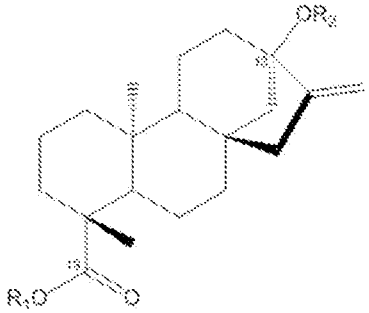
[0085] As used herein, "glycosyl" refers to an organic group that is a univalent free radical or substituent structure obtained by removing the hemiacetal hydroxyl group from the cyclic form of a monosaccharide, lower oligosaccharide or oligosaccharide derivative. Glycosyl groups react with inorganic acids (e.g., phosphoric acid) to form esters (e.g., glucose 1-phosphate).

[0086] As used herein, "glycoside" refers to a molecule in which a carbohydrate (e.g., sugar) is bound to another functional group by a glycosidic bond. Glycosides can be hydrolyzed to produce a sugar and a non-sugar (i.e., aglycone) component.

[0087] As used herein, the term "steviol glycoside" refers to a glycoside of steviol, including but not limited to, naturally occurring steviol glycosides (e.g., stevioside, steviolmonoside, steviolbioside, rubusoside, dulcoside B, dulcoside A, rebaudioside B, rebaudioside G, rebaudioside C, rebaudioside F, rebaudioside A, rebaudioside I, rebaudioside E, rebaudioside H, rebaudioside L, rebaudioside K, rebaudioside J, rebaudioside M (also referred to as rebaudioside X), rebaudioside D, rebaudioside N, rebaudioside O), and synthetic steviol glycosides (e.g., enzymatically glucosylated steviol glycosides), and combinations thereof. The chemical structures of steviol and its glycosides are below (See, WO 2013/176738).

[0088] As used herein, "stevioside substrate" refers to any suitable material comprising at least one steviol glycoside.

Chemical Structure of Steviol and Its Glycosides



Glycoside	R_1	R_2
Steviol	H	H
Steviolmonoside	H	Glcβ1-
Steviol monoglucosyl ester	Glcβ1-	H
Rubusoside	Glcβ1-	Glcβ1-
Steviolbioside	H	Glcβ (1-2) Glcβ1-
Dulcoside A	Glcβ1-	Rhaα(1-2) Glcβ1-
Stevioside	Glcβ1-	Glcβ (1-2) Glcβ1-
Rebaudioside B	H	Glcβ (1-2)[Glcβ (1-3)] Glcβ1-
Rebaudioside C	Glcβ1-	Rhaα(1-2)[Glcβ (1-3)] Glcβ1-

Chemical Structure of Steviol and Its Glycosides		
Rebaudioside A	Glcβ1-	Glcβ (1-2)[Glcβ (1-3)] Glcβ1-
Rebaudioside D	Glcβ (1-2) Glcβ1-	Glcβ (1-2)[Glcβ (1-3)] Glcβ1-
Rebaudioside I	Glcβ (1-3) Glcβ1-	Glcβ (1-2)[Glcβ (1-3)] Glcβ1-
Rebaudioside M	Glcβ (1-2)[Glcβ (1-3)] Glcβ1-	Glcβ (1-2)[Glcβ (1-3)] Glcβ1-
(Glc=glucose, Rha=rhamnose)		

[0089] As used herein, "sucrose synthase" refers to a glycosyltransferase enzyme (EC 2.4.1.1.13) that reversibly catalyzes the chemical reaction NDP-glucose + D-fructose to NDP and sucrose. In some embodiments, the present invention provides variants of *Acidithiobacillus caldus* sucrose synthase ("AcSuS"). In some embodiments, these enzymes are referred to as "sucrose synthase variants," "SuS," "SUS," "SuS variants," "SUS variants," "sucrose synthase combinatorial variants," or "SuS combinatorial variants," or "SUS combinatorial variants." In some embodiments, these variants preferentially utilize NDPs other than uridine (i.e., ADP-glucose, CDP-glucose, TDP-glucose, GDP-glucose, and/or IDP-glucose are utilized, rather than UDP-glucose). In some embodiments, these variants do not utilize UDP-glucose.

[0090] As used herein, the term "one-pot reaction" refers to the production of rebaudioside of interest in one reaction vessel. In some embodiments, the term is used in reference to the production of rebM from a starting material, including but not limited to as rebA and/or steviosides with the intermediate production of other rebaudiosides (e.g., rebD and/or rebI). In some embodiments, the conversion of stevioside to RebA, RebA to RebD and/or RebI and RebD and/or RebI to Reb M, are conducted as a multiple enzyme cascade in one reaction vessel.

[0091] As used herein, "wild-type" and "naturally-occurring" refer to the form found in nature. For example a wild-type polypeptide or polynucleotide sequence is a sequence present in an organism that can be isolated from a source in nature and which has not been intentionally modified by human manipulation.

[0092] As used herein, "recombinant," "engineered," and "non-naturally occurring" when used with reference to a cell, nucleic acid, or polypeptide, refers to a material, or a material corresponding to the natural or native form of the material, that has been modified in a manner that would not otherwise exist in nature. In some embodiments, the cell, nucleic acid or polypeptide is identical a naturally occurring cell, nucleic acid or polypeptide, but is produced or derived from synthetic materials and/or by manipulation using recombinant techniques. Non-limiting examples include, among others, recombinant cells expressing genes that are not found within the native (non-recombinant) form of the cell or express native genes that are otherwise expressed at a different level.

[0093] The term "percent (%) sequence identity" is used herein to refer to comparisons among polynucleotides or polypeptides, and are determined by comparing two optimally aligned sequences over a comparison window, wherein the portion of the polynucleotide or polypeptide sequence in the comparison window may comprise additions or deletions (i.e., gaps) as compared to the reference sequence for optimal alignment of the two sequences. The percentage may be calculated by determining the number of positions at which the identical nucleic acid base or amino acid residue occurs in both sequences to yield the number of matched positions, dividing the number of matched positions by the total number of positions in the window of comparison and multiplying the result by 100 to yield the percentage of sequence identity. Alternatively, the percentage may be calculated by determining the number of positions at which either the identical nucleic acid base or amino acid residue occurs in both sequences or a nucleic acid base or amino acid residue is aligned with a gap to yield the number of matched positions, dividing the number of matched positions by the total number of positions in the window of comparison and multiplying the result by 100 to yield the percentage of sequence identity. Those of skill in the art appreciate that there are many established algorithms available to align two sequences. Optimal alignment of sequences for comparison can be conducted by any suitable method, including, but not limited to the local homology algorithm of Smith and Waterman (Smith and Waterman, Adv. Appl. Math., 2:482 [1981]), by the homology alignment algorithm of Needleman and Wunsch (Needleman and Wunsch, J. Mol. Biol., 48:443 [1970]), by the search for similarity method of Pearson and Lipman (Pearson and Lipman, Proc. Natl. Acad. Sci. USA 85:2444 [1988]), by computerized implementations of these algorithms (e.g., GAP, BESTFIT, FASTA, and TFASTA in the GCG Wisconsin Software Package), or by visual inspection, as known in the art. Examples of algorithms that are suitable for determining percent sequence identity and sequence similarity include, but are not limited to the BLAST and BLAST 2.0 algorithms, which are described by Altschul et al. (See Altschul et al., J. Mol. Biol., 215: 403-410 [1990]; and Altschul et al., Nucl. Acids Res., 3389-3402 [1977], respectively). Software for performing BLAST analyses is publicly available through the National Center for Biotechnology Information website. This algorithm involves first identifying high scoring sequence pairs (HSPs) by identifying short words of length W in the query sequence, which either match or satisfy some positive-valued threshold score T when aligned with a word of the same length in a database sequence. T is referred to as, the neighborhood word score threshold (See, Altschul et al., *supra*). These initial neighborhood word hits act as seeds for initiating searches to find longer HSPs containing them. The word hits are then extended in both directions along each sequence for as far as the cumulative alignment score can be increased. Cumulative scores are calculated using, for nucleotide sequences, the parameters M (reward score for a pair of matching residues; always >0) and N (penalty score for mismatching residues; always <0). For amino acid sequences, a scoring matrix is used to calculate the cumulative score. Extension of the word hits in each direction are halted when: the cumulative alignment score falls off by the quantity X from its maximum achieved value; the cumulative score goes to zero or below, due to the

accumulation of one or more negative-scoring residue alignments; or the end of either sequence is reached. The BLAST algorithm parameters W, T, and X determine the sensitivity and speed of the alignment. The BLASTN program (for nucleotide sequences) uses as defaults a wordlength (W) of 11, an expectation (E) of 10, M=5, N=-4, and a comparison of both strands. For amino acid sequences, the BLASTP program uses as defaults a wordlength (W) of 3, an expectation (E) of 10, and the BLOSUM62 scoring matrix (See, Henikoff and Henikoff, Proc. Natl. Acad. Sci. USA 89:10915 [1989]). Exemplary determination of sequence alignment and % sequence identity can employ the BESTFIT or GAP programs in the GCG Wisconsin Software package (Accelrys, Madison WI), using default parameters provided.

[0094] As used herein, "reference sequence" refers to a defined sequence used as a basis for a sequence and/or activity comparison. A reference sequence may be a subset of a larger sequence, for example, a segment of a full-length gene or polypeptide sequence. Generally, a reference sequence is at least 20 nucleotide or amino acid residues in length, at least 25 residues in length, at least 50 residues in length, at least 100 residues in length or the full length of the nucleic acid or polypeptide. Since two polynucleotides or polypeptides may each (1) comprise a sequence (i.e., a portion of the complete sequence) that is similar between the two sequences, and (2) may further comprise a sequence that is divergent between the two sequences, sequence comparisons between two (or more) polynucleotides or polypeptides are typically performed by comparing sequences of the two polynucleotides or polypeptides over a "comparison window" to identify and compare local regions of sequence similarity. In some embodiments, a "reference sequence" can be based on a primary amino acid sequence, where the reference sequence is a sequence that can have one or more changes in the primary sequence.

[0095] As used herein, "comparison window" refers to a conceptual segment of at least about 20 contiguous nucleotide positions or amino acid residues wherein a sequence may be compared to a reference sequence of at least 20 contiguous nucleotides or amino acids and wherein the portion of the sequence in the comparison window may comprise additions or deletions (i.e., gaps) of 20 percent or less as compared to the reference sequence (which does not comprise additions or deletions) for optimal alignment of the two sequences. The comparison window can be longer than 20 contiguous residues, and includes, optionally 30, 40, 50, 100, or longer windows.

[0096] As used herein, "corresponding to," "reference to," and "relative to" when used in the context of the numbering of a given amino acid or polynucleotide sequence refer to the numbering of the residues of a specified reference sequence when the given amino acid or polynucleotide sequence is compared to the reference sequence. In other words, the residue number or residue position of a given polymer is designated with respect to the reference sequence rather than by the actual numerical position of the residue within the given amino acid or polynucleotide sequence. For example, a given amino acid sequence, such as that of an engineered glycosyltransferase, can be aligned to a reference sequence by introducing gaps to optimize residue matches between the two sequences. In these cases,

although the gaps are present, the numbering of the residue in the given amino acid or polynucleotide sequence is made with respect to the reference sequence to which it has been aligned.

[0097] As used herein, "substantial identity" refers to a polynucleotide or polypeptide sequence that has at least 80 percent sequence identity, at least 85 percent identity, at least between 89 to 95 percent sequence identity, or more usually, at least 99 percent sequence identity as compared to a reference sequence over a comparison window of at least 20 residue positions, frequently over a window of at least 30-50 residues, wherein the percentage of sequence identity is calculated by comparing the reference sequence to a sequence that includes deletions or additions which total 20 percent or less of the reference sequence over the window of comparison. In some specific embodiments applied to polypeptides, the term "substantial identity" means that two polypeptide sequences, when optimally aligned, such as by the programs GAP or BESTFIT using default gap weights, share at least 80 percent sequence identity, preferably at least 89 percent sequence identity, at least 95 percent sequence identity or more (e.g., 99 percent sequence identity). In some embodiments, residue positions that are not identical in sequences being compared differ by conservative amino acid substitutions.

[0098] As used herein, "amino acid difference" and "residue difference" refer to a difference in the amino acid residue at a position of a polypeptide sequence relative to the amino acid residue at a corresponding position in a reference sequence. In some cases, the reference sequence has a histidine tag, but the numbering is maintained relative to the equivalent reference sequence without the histidine tag. The positions of amino acid differences generally are referred to herein as "Xn," where n refers to the corresponding position in the reference sequence upon which the residue difference is based. For example, a "residue difference at position X93 as compared to SEQ ID NO:4" refers to a difference of the amino acid residue at the polypeptide position corresponding to position 93 of SEQ ID NO:4. Thus, if the reference polypeptide of SEQ ID NO:4 has a serine at position 93, then a "residue difference at position X93 as compared to SEQ ID NO:4" an amino acid substitution of any residue other than serine at the position of the polypeptide corresponding to position 93 of SEQ ID NO:4. In most instances herein, the specific amino acid residue difference at a position is indicated as "XnY" where "Xn" specified the corresponding position as described above, and "Y" is the single letter identifier of the amino acid found in the engineered polypeptide (i.e., the different residue than in the reference polypeptide). In some instances (e.g., in the Tables presented in the Examples), the present invention also provides specific amino acid differences denoted by the conventional notation "AnB", where A is the single letter identifier of the residue in the reference sequence, "n" is the number of the residue position in the reference sequence, and B is the single letter identifier of the residue substitution in the sequence of the engineered polypeptide. In some instances, a polypeptide of the present invention can include one or more amino acid residue differences relative to a reference sequence, which is indicated by a list of the specified positions where residue differences are present relative to the reference sequence. In some embodiments, where more than one amino acid can be

used in a specific residue position of a polypeptide, the various amino acid residues that can be used are separated by a "/" (e.g., X307H/X307P or X307H/P). The slash may also be used to indicate multiple substitutions within a given variant (i.e., there is more than one substitution present in a given sequence, such as in a combinatorial variant). In some embodiments, the present invention includes engineered polypeptide sequences comprising one or more amino acid differences comprising conservative or non-conservative amino acid substitutions. In some additional embodiments, the present invention provides engineered polypeptide sequences comprising both conservative and non-conservative amino acid substitutions.

[0099] As used herein, "conservative amino acid substitution" refers to a substitution of a residue with a different residue having a similar side chain, and thus typically involves substitution of the amino acid in the polypeptide with amino acids within the same or similar defined class of amino acids. By way of example and not limitation, in some embodiments, an amino acid with an aliphatic side chain is substituted with another aliphatic amino acid (e.g., alanine, valine, leucine, and isoleucine); an amino acid with an hydroxyl side chain is substituted with another amino acid with an hydroxyl side chain (e.g., serine and threonine); an amino acids having aromatic side chains is substituted with another amino acid having an aromatic side chain (e.g., phenylalanine, tyrosine, tryptophan, and histidine); an amino acid with a basic side chain is substituted with another amino acid with a basis side chain (e.g., lysine and arginine); an amino acid with an acidic side chain is substituted with another amino acid with an acidic side chain (e.g., aspartic acid or glutamic acid); and/or a hydrophobic or hydrophilic amino acid is replaced with another hydrophobic or hydrophilic amino acid, respectively.

[0100] As used herein, "non-conservative substitution" refers to substitution of an amino acid in the polypeptide with an amino acid with significantly differing side chain properties. Non-conservative substitutions may use amino acids between, rather than within, the defined groups and affects (a) the structure of the peptide backbone in the area of the substitution (e.g., proline for glycine) (b) the charge or hydrophobicity, or (c) the bulk of the side chain. By way of example and not limitation, an exemplary non-conservative substitution can be an acidic amino acid substituted with a basic or aliphatic amino acid; an aromatic amino acid substituted with a small amino acid; and a hydrophilic amino acid substituted with a hydrophobic amino acid.

[0101] As used herein, "deletion" refers to modification to the polypeptide by removal of one or more amino acids from the reference polypeptide. Deletions can comprise removal of 1 or more amino acids, 2 or more amino acids, 5 or more amino acids, 10 or more amino acids, 15 or more amino acids, or 20 or more amino acids, up to 10% of the total number of amino acids, or up to 20% of the total number of amino acids making up the reference enzyme while retaining enzymatic activity and/or retaining the improved properties of an engineered glycosyltransferase enzyme. Deletions can be directed to the internal portions and/or terminal portions of the polypeptide. In various

embodiments, the deletion can comprise a continuous segment or can be discontinuous. Deletions are typically indicated by "-" in amino acid sequences.

[0102] As used herein, "insertion" refers to modification to the polypeptide by addition of one or more amino acids from the reference polypeptide. Insertions can be in the internal portions of the polypeptide, or to the carboxy or amino terminus. Insertions as used herein include fusion proteins as is known in the art. The insertion can be a contiguous segment of amino acids or separated by one or more of the amino acids in the naturally occurring polypeptide.

[0103] A "functional fragment" and "biologically active fragment" are used interchangeably herein to refer to a polypeptide that has an amino-terminal and/or carboxy-terminal deletion(s) and/or internal deletions, but where the remaining amino acid sequence is identical to the corresponding positions in the sequence to which it is being compared (e.g., a full-length engineered glycosyltransferase of the present invention) and that retains substantially all of the activity of the full-length polypeptide.

[0104] As used herein, "isolated polypeptide" refers to a polypeptide which is substantially separated from other contaminants that naturally accompany it (e.g., protein, lipids, and polynucleotides). The term embraces polypeptides which have been removed or purified from their naturally-occurring environment or expression system (e.g., within a host cell or via in vitro synthesis). The recombinant glycosyltransferase polypeptides may be present within a cell, present in the cellular medium, or prepared in various forms, such as lysates or isolated preparations. As such, in some embodiments, the recombinant glycosyltransferase polypeptides can be an isolated polypeptide.

[0105] As used herein, "substantially pure polypeptide" or "purified protein" refers to a composition in which the polypeptide species is the predominant species present (i.e., on a molar or weight basis it is more abundant than any other individual macromolecular species in the composition), and is generally a substantially purified composition when the object species comprises at least about 50 percent of the macromolecular species present by mole or % weight. However, in some embodiments, the composition comprising glycosyltransferase comprises glycosyltransferase that is less than 50% pure (e.g., about 10%, about 20%, about 30%, about 40%, or about 50%) Generally, a substantially pure glycosyltransferase composition comprises about 60% or more, about 70% or more, about 80% or more, about 90% or more, about 95% or more, and about 98% or more of all macromolecular species by mole or % weight present in the composition. In some embodiments, the object species is purified to essential homogeneity (i.e., contaminant species cannot be detected in the composition by conventional detection methods) wherein the composition consists essentially of a single macromolecular species. Solvent species, small molecules (<500 Daltons), and elemental ion species are not considered macromolecular species. In some embodiments, the isolated recombinant glycosyltransferase polypeptides are substantially pure polypeptide compositions.

[0106] As used herein, "improved enzyme property" refers to at least one improved property of an enzyme. In some embodiments, the present invention provides engineered glycosyltransferase

polypeptides that exhibit an improvement in any enzyme property as compared to a reference glycosyltransferase polypeptide and/or a wild-type glycosyltransferase polypeptide, and/or another engineered glycosyltransferase polypeptide. Thus, the level of "improvement" can be determined and compared between various glycosyltransferase polypeptides, including wild-type, as well as engineered glycosyltransferases. Improved properties include, but are not limited, to such properties as increased protein expression, increased thermoactivity, increased thermostability, increased pH activity, increased stability, increased enzymatic activity, increased substrate specificity or affinity, increased specific activity, increased resistance to substrate or end-product inhibition, increased chemical stability, improved chemoselectivity, improved solvent stability, increased tolerance to acidic pH, increased tolerance to proteolytic activity (i.e., reduced sensitivity to proteolysis), reduced aggregation, increased solubility, and altered temperature profile. In additional embodiments, the term is used in reference to the at least one improved property of sucrose synthase enzymes. In some embodiments, the present invention provides engineered sucrose synthase polypeptides that exhibit an improvement in any enzyme property as compared to a reference sucrose synthase polypeptide and/or a wild-type sucrose synthase polypeptide, and/or another engineered sucrose synthase polypeptide. Thus, the level of "improvement" can be determined and compared between various sucrose synthase polypeptides, including wild-type, as well as engineered sucrose synthases.

[0107] As used herein, "increased enzymatic activity" and "enhanced catalytic activity" refer to an improved property of the engineered polypeptides, which can be represented by an increase in specific activity (e.g., product produced/time/weight protein) or an increase in percent conversion of the substrate to the product (e.g., percent conversion of starting amount of substrate to product in a specified time period using a specified amount of enzyme) as compared to the reference enzyme. In some embodiments, the terms refer to an improved property of engineered glycosyltransferase polypeptides provided herein, which can be represented by an increase in specific activity (e.g., product produced/time/weight protein) or an increase in percent conversion of the substrate to the product (e.g., percent conversion of starting amount of substrate to product in a specified time period using a specified amount of glycosyltransferase) as compared to the reference glycosyltransferase enzyme. In some embodiments, the terms are used in reference to improved sucrose synthase enzymes provided herein. Exemplary methods to determine enzyme activity of the engineered glycosyltransferases and sucrose synthases of the present invention are provided in the Examples. Any property relating to enzyme activity may be affected, including the classical enzyme properties of K_m, V_{max} or k_{cat}, changes of which can lead to increased enzymatic activity. For example, improvements in enzyme activity can be from about 1.1 fold the enzymatic activity of the corresponding wild-type enzyme, to as much as 2-fold, 5-fold, 10-fold, 20-fold, 25-fold, 50-fold, 75-fold, 100-fold, 150-fold, 200-fold or more enzymatic activity than the naturally occurring glycosyltransferase or another engineered glycosyltransferase from which the glycosyltransferase polypeptides were derived.

[0108] As used herein, "conversion" refers to the enzymatic conversion (or biotransformation) of a substrate(s) to the corresponding product(s). "Percent conversion" refers to the percent of the substrate that is converted to the product within a period of time under specified conditions. Thus, the "enzymatic activity" or "activity" of a glycosyltransferase polypeptide can be expressed as "percent conversion" of the substrate to the product in a specific period of time.

[0109] Enzymes with "generalist properties" (or "generalist enzymes") refer to enzymes that exhibit improved activity for a wide range of substrates, as compared to a parental sequence. Generalist enzymes do not necessarily demonstrate improved activity for every possible substrate. In some embodiments, the present invention provides glycosyltransferase variants with generalist properties, in that they demonstrate similar or improved activity relative to the parental gene for a wide range of sterically and electronically diverse substrates. In addition, the generalist enzymes provided herein were engineered to be improved across a wide range of diverse molecules to increase the production of metabolites/products.

[0110] The term "stringent hybridization conditions" is used herein to refer to conditions under which nucleic acid hybrids are stable. As known to those of skill in the art, the stability of hybrids is reflected in the melting temperature (T_m) of the hybrids. In general, the stability of a hybrid is a function of ion strength, temperature, G/C content, and the presence of chaotropic agents. The T_m values for polynucleotides can be calculated using known methods for predicting melting temperatures (See e.g., Baldino et al., Meth. Enzymol., 168:761-777 [1989]; Bolton et al., Proc. Natl. Acad. Sci. USA 48:1390 [1962]; Bresslauer et al., Proc. Natl. Acad. Sci. USA 83:8893-8897 [1986]; Freier et al., Proc. Natl. Acad. Sci. USA 83:9373-9377 [1986]; Kierzek et al., Biochem., 25:7840-7846 [1986]; Rychlik et al., Nucl. Acids Res., 18:6409-6412 [1990] (erratum, Nucl. Acids Res., 19:698 [1991]); Sambrook et al., *supra*); Suggs et al., 1981, *in* Developmental Biology Using Purified Genes, Brown et al. [eds.], pp. 683-693, Academic Press, Cambridge, MA [1981]; and Wetmur, Crit. Rev. Biochem. Mol. Biol. 26:227-259 [1991]). In some embodiments, the polynucleotide encodes the polypeptide disclosed herein and hybridizes under defined conditions, such as moderately stringent or highly stringent conditions, to the complement of a sequence encoding an engineered glycosyltransferase enzyme of the present invention.

[0111] As used herein, "hybridization stringency" relates to hybridization conditions, such as washing conditions, in the hybridization of nucleic acids. Generally, hybridization reactions are performed under conditions of lower stringency, followed by washes of varying but higher stringency. The term "moderately stringent hybridization" refers to conditions that permit target-DNA to bind a complementary nucleic acid that has about 60% identity, preferably about 75% identity, about 85% identity to the target DNA, with greater than about 90% identity to target-polynucleotide. Exemplary moderately stringent conditions are conditions equivalent to hybridization in 50% formamide, 5× Denhart's solution, 5×SSPE, 0.2% SDS at 42°C, followed by washing in 0.2×SSPE, 0.2% SDS, at 42°C. "High stringency hybridization" refers generally to conditions that are about 10°C or less from

the thermal melting temperature T_m as determined under the solution condition for a defined polynucleotide sequence. In some embodiments, a high stringency condition refers to conditions that permit hybridization of only those nucleic acid sequences that form stable hybrids in 0.018M NaCl at 65°C (i.e., if a hybrid is not stable in 0.018M NaCl at 65°C, it will not be stable under high stringency conditions, as contemplated herein). High stringency conditions can be provided, for example, by hybridization in conditions equivalent to 50% formamide, 5× Denhart's solution, 5×SSPE, 0.2% SDS at 42°C, followed by washing in 0.1×SSPE, and 0.1% SDS at 65°C. Another high stringency condition is hybridizing in conditions equivalent to hybridizing in 5X SSC containing 0.1% (w/v) SDS at 65°C and washing in 0.1x SSC containing 0.1% SDS at 65°C. Other high stringency hybridization conditions, as well as moderately stringent conditions, are described in the references cited above.

[0112] As used herein, "codon optimized" refers to changes in the codons of the polynucleotide encoding a protein to those preferentially used in a particular organism such that the encoded protein is efficiently expressed in the organism of interest. Although the genetic code is degenerate in that most amino acids are represented by several codons, called "synonyms" or "synonymous" codons, it is well known that codon usage by particular organisms is nonrandom and biased towards particular codon triplets. This codon usage bias may be higher in reference to a given gene, genes of common function or ancestral origin, highly expressed proteins versus low copy number proteins, and the aggregate protein coding regions of an organism's genome. In some embodiments, the polynucleotides encoding the glycosyltransferase enzymes may be codon optimized for optimal production in the host organism selected for expression.

[0113] As used herein, "preferred," "optimal," and "high codon usage bias" codons when used alone or in combination refer(s) interchangeably to codons that are used at higher frequency in the protein coding regions than other codons that code for the same amino acid. The preferred codons may be determined in relation to codon usage in a single gene, a set of genes of common function or origin, highly expressed genes, the codon frequency in the aggregate protein coding regions of the whole organism, codon frequency in the aggregate protein coding regions of related organisms, or combinations thereof. Codons whose frequency increases with the level of gene expression are typically optimal codons for expression. A variety of methods are known for determining the codon frequency (e.g., codon usage, relative synonymous codon usage) and codon preference in specific organisms, including multivariate analysis, for example, using cluster analysis or correspondence analysis, and the effective number of codons used in a gene (See e.g., GCG CodonPreference, Genetics Computer Group Wisconsin Package; CodonW, Peden, University of Nottingham; McInerney, Bioinform., 14:372-73 [1998]; Stenico et al., Nucl. Acids Res., 222437-46 [1994]; and Wright, Gene 87:23-29 [1990]). Codon usage tables are available for many different organisms (See e.g., Wada et al., Nucl. Acids Res., 20:2111-2118 [1992]; Nakamura et al., Nucl. Acids Res., 28:292 [2000]; Duret, et al., supra; Henaut and Danchin, *in* *Escherichia coli* and *Salmonella*, Neidhardt, et al.

(eds.), ASM Press, Washington D.C., p. 2047-2066 [1996]). The data source for obtaining codon usage may rely on any available nucleotide sequence capable of coding for a protein. These data sets include nucleic acid sequences actually known to encode expressed proteins (e.g., complete protein coding sequences-CDS), expressed sequence tags (ESTS), or predicted coding regions of genomic sequences (See e.g., Mount, Bioinformatics: Sequence and Genome Analysis, Chapter 8, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y. [2001]; Uberbacher, Meth. Enzymol., 266:259-281 [1996]; and Tiwari et al., Comput. Appl. Biosci., 13:263-270 [1997]).

[0114] As used herein, "control sequence" includes all components, which are necessary or advantageous for the expression of a polynucleotide and/or polypeptide of the present invention. Each control sequence may be native or foreign to the nucleic acid sequence encoding the polypeptide. Such control sequences include, but are not limited to, a leader, polyadenylation sequence, propeptide sequence, promoter sequence, signal peptide sequence, initiation sequence and transcription terminator. At a minimum, the control sequences include a promoter, and transcriptional and translational stop signals. The control sequences may be provided with linkers for the purpose of introducing specific restriction sites facilitating ligation of the control sequences with the coding region of the nucleic acid sequence encoding a polypeptide.

[0115] "Operably linked" is defined herein as a configuration in which a control sequence is appropriately placed (i.e., in a functional relationship) at a position relative to a polynucleotide of interest such that the control sequence directs or regulates the expression of the polynucleotide and/or polypeptide of interest.

[0116] "Promoter sequence" refers to a nucleic acid sequence that is recognized by a host cell for expression of a polynucleotide of interest, such as a coding sequence. The promoter sequence contains transcriptional control sequences, which mediate the expression of a polynucleotide of interest. The promoter may be any nucleic acid sequence which shows transcriptional activity in the host cell of choice including mutant, truncated, and hybrid promoters, and may be obtained from genes encoding extracellular or intracellular polypeptides either homologous or heterologous to the host cell.

[0117] The phrase "suitable reaction conditions" refers to those conditions in the enzymatic conversion reaction solution (e.g., ranges of enzyme loading, substrate loading, temperature, pH, buffers, co-solvents, etc.) under which a glycosyltransferase polypeptide of the present invention is capable of converting a substrate to the desired product compound. Some exemplary "suitable reaction conditions" are provided herein.

[0118] As used herein, "loading," such as in "compound loading" or "enzyme loading" refers to the concentration or amount of a component in a reaction mixture at the start of the reaction.

[0119] As used herein, "substrate" in the context of an enzymatic conversion reaction process refers to the compound or molecule acted on by the engineered enzymes provided herein (e.g., engineered glycosyltransferase polypeptides).

[0120] As used herein, the terms "biomass," "biomass substrate," "cellulosic biomass," "cellulosic feedstock," and "cellulosic substrate" refer to any materials that contain cellulose. Biomass can be derived from plants, animals, or microorganisms, and may include, but is not limited to agricultural, industrial, and forestry residues, industrial and municipal wastes, and terrestrial and aquatic crops grown for energy purposes. Examples of cellulosic substrates include, but are not limited to, wood, wood pulp, paper pulp, corn fiber, corn grain, corn cobs, crop residues such as corn husks, corn stover, grasses, wheat, wheat straw, barley, barley straw, hay, rice, rice straw, switchgrass, waste paper, paper and pulp processing waste, woody or herbaceous plants, fruit or vegetable pulp, corn cobs, distillers grain, grasses, rice hulls, cotton, hemp, flax, sisal, sugar cane bagasse, sorghum, soy, switchgrass, components obtained from milling of grains, trees, branches, roots, leaves, wood chips, sawdust, shrubs and bushes, vegetables, fruits, and flowers and any suitable mixtures thereof In some embodiments, the cellulosic biomass comprises, but is not limited to cultivated crops (e.g., grasses, including C4 grasses, such as switch grass, cord grass, rye grass, miscanthus, reed canary grass, or any combination thereof), sugar processing residues, for example, but not limited to, bagasse (e.g., sugar cane bagasse, beet pulp [e.g., sugar beet], or a combination thereof), agricultural residues (e.g., soybean stover, corn stover, corn fiber, rice straw, sugar cane straw, rice, rice hulls, barley straw, corn cobs, wheat straw, canola straw, oat straw, oat hulls, corn fiber, hemp, flax, sisal, cotton, or any combination thereof), fruit pulp, vegetable pulp, distillers' grains, forestry biomass (e.g., wood, wood pulp, paper pulp, recycled wood pulp fiber, sawdust, hardwood, such as aspen wood, softwood, or a combination thereof). Furthermore, in some embodiments, the cellulosic biomass comprises cellulosic waste material and/or forestry waste materials, including but not limited to, paper and pulp processing waste, newsprint, cardboard and the like. In some embodiments, the cellulosic biomass comprises one species of fiber, while in some alternative embodiments, the cellulosic biomass comprises a mixture of fibers that originate from different cellulosic biomasses. In some embodiments, the biomass may also comprise transgenic plants that express ligninase and/or cellulase enzymes (See e.g., US 2008/0104724, incorporated by reference herein).

[0121] As used herein, the term "slurry" refers to an aqueous solution in which are dispersed one or more solid components, such as a cellulosic substrate.

[0122] As used herein, "increasing" yield of a product (e.g., a steviol glycoside) from a reaction occurs when a particular component present during the reaction (e.g., a GH enzyme) causes more product to be produced, compared with a reaction conducted under the same conditions with the same substrate and other substituents, but in the absence of the component of interest.

[0123] As used herein, "hydrolyzing" cellulose or other polysaccharide occurs when at least some of the glycosidic bonds between two monosaccharides present in the substrate are hydrolyzed, thereby detaching from each other the two monomers that were previously bonded.

[0124] A reaction is the to be "substantially free" of a particular enzyme if the amount of that enzyme compared with other enzymes that participate in catalyzing the reaction is less than about 2%, about 1%, or about 0.1% (wt/wt).

[0125] As used herein, "fractionating" a liquid (e.g., a culture broth) means applying a separation process (e.g., salt precipitation, column chromatography, size exclusion, and filtration) or a combination of such processes to provide a solution in which a desired protein (e.g., rebaudiosides) comprises a greater percentage of total protein in the solution than in the initial liquid product.

[0126] As used herein, "starting composition" refers to any composition that comprises at least one substrate. In some embodiments, the starting composition comprises any cellulosic substrate.

[0127] In some alternative embodiments, the term "starting composition" refers to any composition comprising at least one steviol glycoside, wherein one or more of the steviol glycosides act as substrate(s) for a biotransformation. In some embodiments, the starting composition is provided as an aqueous solution. In some embodiments, the starting composition comprises at least one steviol glycoside selected from stevioside, steviolmonoside, steviolbioside, rubusoside, dulcoside B, dulcoside A, rebaudioside B, rebaudioside G, rebaudioside C, rebaudioside F, rebaudioside A, rebaudioside I, rebaudioside E, rebaudioside H, rebaudioside L, rebaudioside K, rebaudioside J, rebaudioside M (also referred to as rebaudioside X), rebaudioside D, rebaudioside N, rebaudioside O, and synthetic steviol glycosides (e.g., enzymatically glucosylated steviol glycosides), In some embodiments, the starting composition comprises two or more steviol glycosides. In some embodiments, the starting composition comprises an extract obtained from purification of *Stevia rebaudiana* plant material (e.g., leaves). In some alternative embodiments, the starting composition comprises commercially available stevia extract(s). Additional starting compositions comprise by-products of processes used to isolate and purify steviol glycosides. In some embodiments, the starting composition comprises purified or partially purified steviol glycoside substrate(s). In some embodiments, the starting composition comprises greater than about 99% of a particular steviol glycoside by weight.

[0128] In some embodiments, the starting composition comprises at least one glycoside and a cellulosic component as the substrate to produce at least one steviol glycoside (e.g., rebaudioside A, D, etc.).

[0129] As used herein, "product" in the context of an enzymatic conversion process refers to the compound or molecule resulting from the action of an enzymatic polypeptide on a substrate. As used herein, in some embodiments, the term refers to the compound or molecule resulting from the action of the glycosyltransferase polypeptide on a substrate. In some embodiments, the product provided by the present invention is a steviol glycoside. In some embodiments, the product comprises at least one steviol glycoside selected from stevioside, steviolmonoside, steviolbioside, rubusoside, dulcoside B, dulcoside A, rebaudioside B, rebaudioside G, rebaudioside C, rebaudioside F, rebaudioside A, rebaudioside I, rebaudioside E, rebaudioside H, rebaudioside L, rebaudioside K, rebaudioside J,

rebaudioside M (also referred to as rebaudioside X), rebaudioside D, rebaudioside N, rebaudioside O, and synthetic steviol glycosides (e.g., enzymatically glucosylated steviol glycosides),

[0130] As used herein the term "culturing" refers to the growing of a population of microbial cells under any suitable conditions (e.g., using a liquid, gel or solid medium).

[0131] Recombinant polypeptides can be produced using any suitable methods known in the art. Genes encoding the wild-type polypeptide of interest can be cloned in vectors, such as plasmids, and expressed in desired hosts, such as *E. coli,* etc. Variants of recombinant polypeptides can be generated by various methods known in the art. Indeed, there is a wide variety of different mutagenesis techniques well known to those skilled in the art. In addition, mutagenesis kits are also available from many commercial molecular biology suppliers. Methods are available to make specific substitutions at defined amino acids (site-directed), specific or random mutations in a localized region of the gene (regio-specific), or random mutagenesis over the entire gene (e.g., saturation mutagenesis). Numerous suitable methods are known to those in the art to generate enzyme variants, including but not limited to site-directed mutagenesis of single-stranded DNA or double-stranded DNA using PCR, cassette mutagenesis, gene synthesis, error-prone PCR, shuffling, and chemical saturation mutagenesis, or any other suitable method known in the art. Mutagenesis and directed evolution methods can be readily applied to enzyme-encoding polynucleotides to generate variant libraries that can be expressed, screened, and assayed. Any suitable mutagenesis and directed evolution methods find use in the present invention and are well known in the art (See e.g., US Patent Nos. 5,605,793, 5,811,238, 5,830,721, 5,834,252, 5,837,458, 5,928,905, 6,096,548, 6,117,679, 6,132,970, 6,165,793, 6,180,406, 6,251,674, 6,265,201, 6,277,638, 6,287,861, 6,287,862, 6,291,242, 6,297,053, 6,303,344, 6,309,883, 6,319,713, 6,319,714, 6,323,030, 6,326,204, 6,335,160, 6,335,198, 6,344,356, 6,352,859, 6,355,484, 6,358,740, 6,358,742, 6,365,377, 6,365,408, 6,368,861, 6,372,497, 6,337,186, 6,376,246, 6,379,964, 6,387,702, 6,391,552, 6,391,640, 6,395,547, 6,406,855, 6,406,910, 6,413,745, 6,413,774, 6,420,175, 6,423,542, 6,426,224, 6,436,675, 6,444,468, 6,455,253, 6,479,652, 6,482,647, 6,483,011, 6,484,105, 6,489,146, 6,500,617, 6,500,639, 6,506,602, 6,506,603, 6,518,065, 6,519,065, 6,521,453, 6,528,311, 6,537,746, 6,573,098, 6,576,467, 6,579,678, 6,586,182, 6,602,986, 6,605,430, 6,613,514, 6,653,072, 6,686,515, 6,703,240, 6,716,631, 6,825,001, 6,902,922, 6,917,882, 6,946,296, 6,961,664, 6,995,017, 7,024,312, 7,058,515, 7,105,297, 7,148,054, 7,220,566, 7,288,375, 7,384,387, 7,421,347, 7,430,477, 7,462,469, 7,534,564, 7,620,500, 7,620,502, 7,629,170, 7,702,464, 7,747,391, 7,747,393, 7,751,986, 7,776,598, 7,783,428, 7,795,030, 7,853,410, 7,868,138, 7,783,428, 7,873,477, 7,873,499, 7,904,249, 7,957,912, 7,981,614, 8,014,961, 8,029,988, 8,048,674, 8,058,001, 8,076,138, 8,108,150, 8,170,806, 8,224,580, 8,377,681, 8,383,346, 8,457,903, 8,504,498, 8,589,085, 8,762,066, 8,768,871, 9,593,326, 9,665,694, 9,684,771, and all related US, as well as PCT and non-US counterparts; Ling et al., Anal. Biochem., 254(2):157-78 [1997]; Dale et al., Meth. Mol. Biol., 57:369-74 [1996]; Smith, Ann. Rev. Genet., 19:423-462 [1985]; Botstein et al., Science, 229:1193-1201 [1985]; Carter, Biochem. J., 237:1-7 [1986]; Kramer et al., Cell, 38:879-887 [1984]; Wells et al., Gene, 34:315-323

[1985]; Minshull et al., Curr. Op. Chem. Biol., 3:284-290 [1999]; Christians et al., Nat. Biotechnol., 17:259-264 [1999]; Crameri et al., Nature, 391:288-291 [1998]; Crameri, et al., Nat. Biotechnol., 15:436-438 [1997]; Zhang et al., Proc. Nat. Acad. Sci. U.S.A., 94:4504-4509 [1997]; Crameri et al., Nat. Biotechnol., 14:315-319 [1996]; Stemmer, Nature, 370:389-391 [1994]; Stemmer, Proc. Nat. Acad. Sci. USA, 91:10747-10751 [1994]; WO 95/22625; WO 97/0078; WO 97/35966; WO 98/27230; WO 00/42651; WO 01/75767; and WO 2009/152336, all of which are incorporated herein by reference).

[0132] In some embodiments, the enzyme clones obtained following mutagenesis treatment are screened by subjecting the enzyme preparations to a defined temperature (or other assay conditions) and measuring the amount of enzyme activity remaining after heat treatments or other suitable assay conditions. Clones containing a polynucleotide encoding a polypeptide are then isolated from the gene, sequenced to identify the nucleotide sequence changes (if any), and used to express the enzyme in a host cell. Measuring enzyme activity from the expression libraries can be performed using any suitable method known in the art (e.g., standard biochemistry techniques, such as HPLC analysis).

[0133] After the variants are produced, they can be screened for any desired property (e.g., high or increased activity, or low or reduced activity, increased thermal activity, increased thermal stability, and/or acidic pH stability, etc.). In some embodiments, "recombinant glycosyltransferase polypeptides" (also referred to herein as "engineered glycosyltransferase polypeptides," "variant glycosyltransferase enzymes," "glycosyltransferase variants," and "glycosyltransferase combinatorial variants") find use. In some embodiments, "recombinant sucrose synthase polypeptides" (also referred to as "engineered sucrose synthase polypeptides," "variant sucrose synthase enzymes," "sucrose synthase variants," and "sucrose synthase combinatorial variants") find use.

[0134] As used herein, a "vector" is a DNA construct for introducing a DNA sequence into a cell. In some embodiments, the vector is an expression vector that is operably linked to a suitable control sequence capable of effecting the expression in a suitable host of the polypeptide encoded in the DNA sequence. In some embodiments, an "expression vector" has a promoter sequence operably linked to the DNA sequence (e.g., transgene) to drive expression in a host cell, and in some embodiments, also comprises a transcription terminator sequence.

[0135] As used herein, the term "expression" includes any step involved in the production of the polypeptide including, but not limited to, transcription, post-transcriptional modification, translation, and post-translational modification. In some embodiments, the term also encompasses secretion of the polypeptide from a cell.

[0136] As used herein, the term "produces" refers to the production of proteins and/or other compounds by cells. It is intended that the term encompass any step involved in the production of polypeptides including, but not limited to, transcription, post-transcriptional modification, translation, and post-translational modification. In some embodiments, the term also encompasses secretion of the polypeptide from a cell.

[0137] As used herein, an amino acid or nucleotide sequence (e.g., a promoter sequence, signal peptide, terminator sequence, etc.) is "heterologous" to another sequence with which it is operably linked if the two sequences are not associated in nature. For example a "heterologous polynucleotide" is any polynucleotide that is introduced into a host cell by laboratory techniques, and includes polynucleotides that are removed from a host cell, subjected to laboratory manipulation, and then reintroduced into a host cell.

[0138] As used herein, the terms "host cell" and "host strain" refer to suitable hosts for expression vectors comprising DNA provided herein (e.g., the polynucleotides encoding the glycosyltransferase variants). In some embodiments, the host cells are prokaryotic or eukaryotic cells that have been transformed or transfected with vectors constructed using recombinant DNA techniques as known in the art.

[0139] The term "analogue" means a polypeptide having more than 70% sequence identity but less than 100% sequence identity (e.g., more than 75%, 78%, 80%, 83%, 85%, 88%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% sequence identity) with a reference polypeptide. In some embodiments, analogues means polypeptides that contain one or more non-naturally occurring amino acid residues including, but not limited, to homoarginine, ornithine and norvaline, as well as naturally occurring amino acids. In some embodiments, analogues also include one or more D-amino acid residues and non-peptide linkages between two or more amino acid residues.

[0140] The term "effective amount" means an amount sufficient to produce the desired result. One of general skill in the art may determine what the effective amount by using routine experimentation.

[0141] The terms "isolated" and "purified" are used to refer to a molecule (e.g., an isolated nucleic acid, polypeptide, etc.) or other component that is removed from at least one other component with which it is naturally associated. The term "purified" does not require absolute purity, rather it is intended as a relative definition.

[0142] As used herein, "stereoselectivity" refers to the preferential formation in a chemical or enzymatic reaction of one stereoisomer over another. Stereoselectivity can be partial, where the formation of one stereoisomer is favored over the other, or it may be complete where only one stereoisomer is formed. When the stereoisomers are enantiomers, the stereoselectivity is referred to as enantioselectivity, the fraction (typically reported as a percentage) of one enantiomer in the sum of both. It is commonly alternatively reported in the art (typically as a percentage) as the enantiomeric excess ("e.e.") calculated therefrom according to the formula [major enantiomer − minor enantiomer]/[major enantiomer + minor enantiomer]. Where the stereoisomers are diastereoisomers, the stereoselectivity is referred to as diastereoselectivity, the fraction (typically reported as a percentage) of one diastereomer in a mixture of two diastereomers, commonly alternatively reported as the diastereomeric excess ("d.e."). Enantiomeric excess and diastereomeric excess are types of stereomeric excess.

[0143] As used herein, "regioselectivity" and "regioselective reaction" refer to a reaction in which one direction of bond making or breaking occurs preferentially over all other possible directions. Reactions can completely (100%) regioselective if the discrimination is complete, substantially regioselective (at least 75%), or partially regioselective (x%, wherein the percentage is set dependent upon the reaction of interest), if the product of reaction at one site predominates over the product of reaction at other sites.

[0144] As used herein, "thermostable" refers to a glycosyltransferase polypeptide that maintains similar activity (more than 60% to 80% for example) after exposure to elevated temperatures (e.g., 40-80°C) for a period of time (e.g., 0.5-24 h) compared to the wild-type enzyme exposed to the same elevated temperature.

[0145] As used herein, "solvent stable" refers to a glycosyltransferase polypeptide that maintains similar activity (more than e.g., 60% to 80%) after exposure to varying concentrations (e.g., 5-99%) of solvent (ethanol, isopropyl alcohol, dimethylsulfoxide [DMSO], tetrahydrofuran, 2-methyltetrahydrofuran, acetone, toluene, butyl acetate, methyl tert-butyl ether, etc.) for a period of time (e.g., 0.5-24 h) compared to the wild-type enzyme exposed to the same concentration of the same solvent.

[0146] As used herein, "thermo- and solvent stable" refers to a glycosyltransferase polypeptide that is both thermostable and solvent stable.

[0147] As used herein, "reductant" refers to a compound or agent capable of converting Fe^{+3} to Fe^{+2}. An exemplary reductant is ascorbic acid, which is generally in the form of L-ascorbic acid.

[0148] As used herein, "optional" and "optionally" mean that the subsequently described event or circumstance may or may not occur, and that the description includes instances where the event or circumstance occurs and instances in which it does not. One of ordinary skill in the art would understand that with respect to any molecule described as containing one or more optional substituents, only sterically practical and/or synthetically feasible compounds are meant to be included. "Optionally substituted" refers to all subsequent modifiers in a term or series of chemical groups.

Glycosylation

[0149] Glycosylation can alter many properties of natural and synthetic products including stability, pharmacodynamics, solubility, and membrane transport. The present invention provides compositions, methods and enzymes suitable for generating new glycosylated compounds from various aglycone and glycosylated substrates. In some embodiments, the present invention provides means to efficiently generate known glycosylated compounds from easily obtained precursors. In some cases, glycosylation is achieved through chemical synthesis methods. However, these methods typically require undesirable chemicals and processes and can result in mixed products (e.g., with linkages in

incorrect positions and/or with undesired anomeric configurations). Furthermore, carbohydrate chemistry requires multiple protection and deprotection steps.

[0150] In contrast, glycosylating enzymes can be active under mild conditions and can confer high positional selectivity and stereospecificity in a single step. Many naturally-occurring glycosylated metabolites are generated *in vivo* using glycosyltransferases that transfer sugar moieties from various sugar nucleosides. Many molecules, including many secondary metabolites with antimicrobial, antitumor, natural sweetness properties, etc., comprise non-ribosomal peptide, polyketide, or terpenoid backbones modified with β-glycosidic linkages. Many of the diterpene glycosides extracted from the plant, *Stevia rebaudiana* Bertoni, contain β-linked glucose molecules. Naturally, these molecules are glycosylated *in vivo* using UDP-glucose dependent glycosyl transferase enzymes. The present invention provides a method (See, Figure 1), in which a new engineered glycosyltransferase is used to transfer the glucose moiety from a nucleoside diphosphoglucose to a substrate (e.g., rebaudioside D or stevioside), to produce one or more β-glucose linked products (e.g., rebaudioside M, rebaudioside A, or rebaudioside I). However, when used *in vitro*, the UDP-glucose can be prohibitively expensive and/or unavailable. In the some additional embodiments, a synthase (e.g., sucrose synthase or trehalose synthase) acts in the reverse direction to form a nucleoside diphosphoglucose compound from a nucleoside diphosphate and a glucose donor (e.g., sucrose, trehalose, or starch).

[0151] Thus, glycosylation finds use in the production of natural sweeteners, such as those derived from the sweet herb, *Stevia rebaudiana* Bertoni. As indicated above, this plant produces a number of diterpene glycosides which feature high intensity sweetness and sensory properties superior to those of many other high potency sweeteners. The above-mentioned sweet glycosides, have a common aglycone (i.e., steviol), and differ by the number and type of carbohydrate residues at the C13 and C19 positions. Steviol glycosides differ from each other not only in their molecular structure, but also by their taste properties. Usually, stevioside is reported to be 89-143 times sweeter than sucrose, while rebaudioside A is reported to be between 85 and 242 times sweeter than sucrose (See e.g., Kasai et al., Nippon Kagaku Kaishi, 1981:726-735 [1981]). Of these common compounds, rebaudioside A has the least astringent, the least bitter, and the least persistent aftertaste. Thus, it has the most favorable sensory attributes of the major steviol glycosides and has been commercialized. However, rebaudioside A only constitutes a smaller fraction (about 20%) of total glycosides isolated from *Stevia rebaudiana* Bertoni, with stevioside (about 70%) and minor steviol glycosides making up the rest (See e.g., FAO, Chemical and Technical Assessment, 63rd JECFA, Steviol Glycosides [2004]). The naturally occurring but even less abundant compound rebaudioside M, also known as rebaudioside X, is 200-350 times sweeter than sucrose and has a reduced aftertaste relative to rebaudioside A (See e.g., Prakash et al., Food, 3:162-175 [2014]).. Thus, there is interest in the commercialization of rebaudioside M, for example as a natural sweetener, but currently no viable commercial route to synthesize this compound.

Engineered Glycosyltransferase Polypeptides

[0152] The present invention provides glycosyltransferase polypeptides, polynucleotides encoding the polypeptides, methods of preparing the polypeptides, and methods for using the polypeptides. Where the description relates to polypeptides, it is to be understood that it also describes the polynucleotides encoding the polypeptides. In some embodiments, the present invention provides engineered, non-naturally occurring GT enzymes with improved properties as compared to wild-type GT enzymes. Any suitable reaction conditions find use in the present invention. In some embodiments, methods are used to analyze the improved properties of the engineered polypeptides to carry out the transferase reaction. In some embodiments, the reaction conditions are modified with regard to concentrations or amounts of polypeptide, substrate, co-substrate, buffer, co-solvent, pH, conditions including temperature and reaction time, and/or conditions with the polypeptide immobilized on a solid support, as further described below and in the Examples.

[0153] In some embodiments, the engineered GT polypeptides described herein have improved properties as compared to wild-type GT enzymes such as in the conversion of steviol glycosides to further glycosylated steviol glycosides (e.g., stevioside to rebaudioside A or rebaudioside D to rebaudioside M) and in the use of adenine diphosphoglucose or other nucleoside diphosphates.

[0154] In some embodiments, additional reaction components or additional techniques are utilized to supplement the reaction conditions. In some embodiments, these include taking measures to stabilize or prevent inactivation of the enzyme, reduce product inhibition, shift reaction equilibrium to glucosylated product formation.

[0155] In some further embodiments, any of the above described process for the conversion of substrate compound to product compound can further comprise one or more steps selected from: extraction; isolation; purification; and crystallization, filtration, or lyophilization of product compound. Methods, techniques, and protocols for extracting, isolating, purifying, and/or crystallizing the glucosylated product from biocatalytic reaction mixtures produced by the above disclosed processes are known to the ordinary artisan and/or accessed through routine experimentation. Additionally, illustrative methods are provided in the Examples below.

Engineered Sucrose Synthase Polypeptides

[0156] The present invention provides engineered sucrose synthase (SuS) polypeptides, polynucleotides encoding the polypeptides, methods of preparing the polypeptides, and methods for using the polypeptides. Where the description relates to polypeptides, it is to be understood that it also describes the polynucleotides encoding the polypeptides. In some embodiments, the present invention provides engineered, non-naturally occurring SuS enzymes with improved properties as compared to wild-type SuS enzymes. Any suitable reaction conditions find use in the present invention. In some embodiments, methods are used to analyze the improved properties of the engineered polypeptides to

carry out the synthase reaction. In some embodiments, the reaction conditions are modified with regard to concentrations or amounts of engineered SuS, substrate(s), buffer(s), solvent(s), pH, conditions including temperature and reaction time, and/or conditions with the engineered SuS polypeptide immobilized on a solid support, as further described below and in the Examples.

[0157] In some embodiments, the engineered SuS polypeptides described herein have improved properties as compared to wild-type SuS enzymes such as in the reactions described herein.

[0158] In some embodiments, additional reaction components or additional techniques are utilized to supplement the reaction conditions. In some embodiments, these include taking measures to stabilize or prevent inactivation of the enzyme, reduce product inhibition, shift reaction equilibrium to glucosylated product formation.

[0159] In some further embodiments, any of the above described process for the conversion of substrate compound to product compound can further comprise one or more steps selected from: extraction, isolation, purification, crystallization, filtration, and/or lyophilization of product compound(s). Methods, techniques, and protocols for extracting, isolating, purifying, and/or crystallizing the product(s) (e.g., rebaudiosides) from biocatalytic reaction mixtures produced by the processes provided herein are known to the ordinary artisan and/or accessed through routine experimentation. Additionally, illustrative methods are provided in the Examples below.

Polynucleotides Encoding Engineered Polypeptides, Expression Vectors and Host Cells

[0160] The present invention provides polynucleotides encoding the engineered enzyme polypeptides described herein. In some embodiments, the polynucleotides are operatively linked to one or more heterologous regulatory sequences that control gene expression to create a recombinant polynucleotide capable of expressing the polypeptide. In some embodiments, expression constructs containing at least one heterologous polynucleotide encoding the engineered enzyme polypeptide(s) is introduced into appropriate host cells to express the corresponding enzyme polypeptide(s).

[0161] As will be apparent to the skilled artisan, availability of a protein sequence and the knowledge of the codons corresponding to the various amino acids provide a description of all the polynucleotides capable of encoding the subject polypeptides. The degeneracy of the genetic code, where the same amino acids are encoded by alternative or synonymous codons, allows an extremely large number of nucleic acids to be made, all of which encode an engineered enzyme (e.g., GT or SuS) polypeptide. Thus, the present invention provides methods and compositions for the production of each and every possible variation of enzyme polynucleotides that could be made that encode the enzyme polypeptides described herein by selecting combinations based on the possible codon choices, and all such variations are to be considered specifically disclosed for any polypeptide described herein, including the amino acid sequences presented in the Examples (e.g., in the various Tables).

[0162] In some embodiments, the codons are preferably optimized for utilization by the chosen host cell for protein production. For example, preferred codons used in bacteria are typically used for

expression in bacteria. Consequently, codon optimized polynucleotides encoding the engineered enzyme polypeptides contain preferred codons at about 40%, 50%, 60%, 70%, 80%, 90%, or greater than 90% of the codon positions in the full length coding region.

[0163] In some embodiments, the enzyme polynucleotide encodes an engineered polypeptide having enzyme activity with the properties disclosed herein, wherein the polypeptide comprises an amino acid sequence having at least 60%, 65%, 70%, 75%, 80%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or more identity to a reference sequence selected from the SEQ ID NOS provided herein, or the amino acid sequence of any variant (e.g., those provided in the Examples), and one or more residue differences as compared to the reference polynucleotide(s), or the amino acid sequence of any variant as disclosed in the Examples (for example 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more amino acid residue positions).

[0164] In some embodiments, the polynucleotides are capable of hybridizing under highly stringent conditions to a reference polynucleotide sequence selected from any polynucleotide sequence provided herein, or a complement thereof, or a polynucleotide sequence encoding any of the variant enzyme polypeptides provided herein. In some embodiments, the polynucleotide capable of hybridizing under highly stringent conditions encodes a enzyme polypeptide comprising an amino acid sequence that has one or more residue differences as compared to a reference sequence.

[0165] In some embodiments, an isolated polynucleotide encoding any of the engineered enzyme polypeptides herein is manipulated in a variety of ways to facilitate expression of the enzyme polypeptide. In some embodiments, the polynucleotides encoding the enzyme polypeptides comprise expression vectors where one or more control sequences is present to regulate the expression of the enzyme polynucleotides and/or polypeptides. Manipulation of the isolated polynucleotide prior to its insertion into a vector may be desirable or necessary depending on the expression vector utilized. Techniques for modifying polynucleotides and nucleic acid sequences utilizing recombinant DNA methods are well known in the art. In some embodiments, the control sequences include among others, promoters, leader sequences, polyadenylation sequences, propeptide sequences, signal peptide sequences, and transcription terminators. In some embodiments, suitable promoters are selected based on the host cells selection. For bacterial host cells, suitable promoters for directing transcription of the nucleic acid constructs of the present disclosure, include, but are not limited to promoters obtained from the *E. coli* lac operon, *Streptomyces coelicolor* agarase gene (dagA), *Bacillus subtilis* levansucrase gene (sacB), *Bacillus licheniformis* alpha-amylase gene (amyL), *Bacillus stearothermophilus* maltogenic amylase gene (amyM), *Bacillus amyloliquefaciens* alpha-amylase gene (amyQ), *Bacillus licheniformis* penicillinase gene (penP), *Bacillus subtilis* xylA and xylB genes, and prokaryotic beta-lactamase gene (See e.g., Villa-Kamaroff et al., Proc. Natl Acad. Sci. USA 75: 3727-3731 [1978]), as well as the *tac* promoter (See e.g., DeBoer et al., Proc. Natl Acad. Sci. USA 80: 21-25 [1983]). Exemplary promoters for filamentous fungal host cells, include, but are not limited to promoters obtained from the genes for *Aspergillus oryzae* TAKA amylase, *Rhizomucor miehei*

aspartic proteinase, *Aspergillus niger* neutral alpha-amylase, *Aspergillus niger* acid stable alpha-amylase, *Aspergillus niger* or *Aspergillus awamori* glucoamylase (glaA), *Rhizomucor miehei* lipase, *Aspergillus oryzae* alkaline protease, *Aspergillus oryzae* triose phosphate isomerase, *Aspergillus nidulans* acetamidase, and *Fusarium oxysporum* trypsin-like protease (See e.g., WO 96/00787), as well as the NA2-tpi promoter (a hybrid of the promoters from the genes for *Aspergillus niger* neutral alpha-amylase and *Aspergillus oryzae* triose phosphate isomerase), and mutant, truncated, and hybrid promoters thereof. Exemplary yeast cell promoters can be from the genes can be from the genes for *Saccharomyces cerevisiae* enolase (ENO-1), *Saccharomyces cerevisiae* galactokinase (GAL1), *Saccharomyces cerevisiae* alcohol dehydrogenase/glyceraldehyde-3-phosphate dehydrogenase (ADH2/GAP), and *Saccharomyces cerevisiae* 3-phosphoglycerate kinase. Other useful promoters for yeast host cells are known in the art (See e.g., Romanos et al., Yeast 8:423-488 [1992]).

[0166] In some embodiments, the control sequence is also a suitable transcription terminator sequence (i.e., a sequence recognized by a host cell to terminate transcription). In some embodiments, the terminator sequence is operably linked to the 3' terminus of the nucleic acid sequence encoding the enzyme polypeptide. Any suitable terminator which is functional in the host cell of choice finds use in the present invention. Exemplary transcription terminators for filamentous fungal host cells can be obtained from the genes for *Aspergillus oryzae* TAKA amylase, *Aspergillus niger* glucoamylase, *Aspergillus nidulans* anthranilate synthase, *Aspergillus niger* alpha-glucosidase, and *Fusarium oxysporum* trypsin-like protease. Exemplary terminators for yeast host cells can be obtained from the genes for *Saccharomyces cerevisiae* enolase, *Saccharomyces cerevisiae* cytochrome C (CYC1), and *Saccharomyces cerevisiae* glyceraldehyde-3-phosphate dehydrogenase. Other useful terminators for yeast host cells are known in the art (See e.g., Romanos et al., *supra*).

[0167] In some embodiments, the control sequence is also a suitable leader sequence (i.e., a non-translated region of an mRNA that is important for translation by the host cell). In some embodiments, the leader sequence is operably linked to the 5' terminus of the nucleic acid sequence encoding the enzyme polypeptide. Any suitable leader sequence that is functional in the host cell of choice find use in the present invention. Exemplary leaders for filamentous fungal host cells are obtained from the genes for *Aspergillus oryzae* TAKA amylase, and *Aspergillus nidulans* triose phosphate isomerase. Suitable leaders for yeast host cells are obtained from the genes for *Saccharomyces cerevisiae* enolase (ENO-1), *Saccharomyces cerevisiae* 3-phosphoglycerate kinase, *Saccharomyces cerevisiae* alpha-factor, and *Saccharomyces cerevisiae* alcohol dehydrogenase/glyceraldehyde-3-phosphate dehydrogenase (ADH2/GAP).

[0168] In some embodiments, the control sequence is also a polyadenylation sequence (i.e., a sequence operably linked to the 3' terminus of the nucleic acid sequence and which, when transcribed, is recognized by the host cell as a signal to add polyadenosine residues to transcribed mRNA). Any suitable polyadenylation sequence which is functional in the host cell of choice finds use in the present invention. Exemplary polyadenylation sequences for filamentous fungal host cells include, but

are not limited to the genes for *Aspergillus oryzae* TAKA amylase, *Aspergillus niger* glucoamylase, *Aspergillus nidulans* anthranilate synthase, *Fusarium oxysporum* trypsin-like protease, and *Aspergillus niger* alpha-glucosidase. Useful polyadenylation sequences for yeast host cells are known (See e.g., Guo and Sherman, Mol. Cell. Bio., 15:5983-5990 [1995]).

[0169] In some embodiments, the control sequence is also a signal peptide (i.e., a coding region that codes for an amino acid sequence linked to the amino terminus of a polypeptide and directs the encoded polypeptide into the cell's secretory pathway). In some embodiments, the 5' end of the coding sequence of the nucleic acid sequence inherently contains a signal peptide coding region naturally linked in translation reading frame with the segment of the coding region that encodes the secreted polypeptide. Alternatively, in some embodiments, the 5' end of the coding sequence contains a signal peptide coding region that is foreign to the coding sequence. Any suitable signal peptide coding region which directs the expressed polypeptide into the secretory pathway of a host cell of choice finds use for expression of the engineered polypeptide(s). Effective signal peptide coding regions for bacterial host cells are the signal peptide coding regions include, but are not limited to those obtained from the genes for *Bacillus* NClB 11837 maltogenic amylase, *Bacillus stearothermophilus* alpha-amylase, *Bacillus licheniformis* subtilisin, *Bacillus licheniformis* beta-lactamase, *Bacillus stearothermophilus* neutral proteases (nprT, nprS, nprM), and *Bacillus subtilis* prsA. Further signal peptides are known in the art (See e.g., Simonen and Palva, Microbiol. Rev., 57:109-137 [1993]). In some embodiments, effective signal peptide coding regions for filamentous fungal host cells include, but are not limited to the signal peptide coding regions obtained from the genes for *Aspergillus oryzae* TAKA amylase, *Aspergillus niger* neutral amylase, *Aspergillus niger* glucoamylase, *Rhizomucor miehei* aspartic proteinase, *Humicola insolens* cellulase, and *Humicola lanuginosa* lipase. Useful signal peptides for yeast host cells include, but are not limited to those from the genes for *Saccharomyces cerevisiae* alpha-factor and *Saccharomyces cerevisiae* invertase.

[0170] In some embodiments, the control sequence is also a propeptide coding region that codes for an amino acid sequence positioned at the amino terminus of a polypeptide. The resultant polypeptide is referred to as a “proenzyme,” “propolypeptide,” or “zymogen.” A propolypeptide can be converted to a mature active polypeptide by catalytic or autocatalytic cleavage of the propeptide from the propolypeptide. The propeptide coding region may be obtained from any suitable source, including, but not limited to the genes for *Bacillus subtilis* alkaline protease (aprE), *Bacillus subtilis* neutral protease (nprT), *Saccharomyces cerevisiae* alpha-factor, *Rhizomucor miehei* aspartic proteinase, and *Myceliophthora thermophila* lactase (See e.g., WO 95/33836). Where both signal peptide and propeptide regions are present at the amino terminus of a polypeptide, the propeptide region is positioned next to the amino terminus of a polypeptide and the signal peptide region is positioned next to the amino terminus of the propeptide region.

[0171] In some embodiments, regulatory sequences are also utilized. These sequences facilitate the regulation of the expression of the polypeptide relative to the growth of the host cell. Examples of

regulatory systems are those that cause the expression of the gene to be turned on or off in response to a chemical or physical stimulus, including the presence of a regulatory compound. In prokaryotic host cells, suitable regulatory sequences include, but are not limited to the *lac*, *tac*, and *trp* operator systems. In yeast host cells, suitable regulatory systems include, but are not limited to the ADH2 system or GAL1 system. In filamentous fungi, suitable regulatory sequences include, but are not limited to the TAKA alpha-amylase promoter, *Aspergillus niger* glucoamylase promoter, and *Aspergillus oryzae* glucoamylase promoter.

[0172] In another aspect, the present invention is directed to a recombinant expression vector comprising a polynucleotide encoding an engineered enzyme polypeptide, and one or more expression regulating regions such as a promoter and a terminator, a replication origin, etc., depending on the type of hosts into which they are to be introduced. In some embodiments, the various nucleic acid and control sequences described herein are joined together to produce recombinant expression vectors which include one or more convenient restriction sites to allow for insertion or substitution of the nucleic acid sequence encoding the enzyme polypeptide at such sites. Alternatively, in some embodiments, the nucleic acid sequence of the present invention is expressed by inserting the nucleic acid sequence or a nucleic acid construct comprising the sequence into an appropriate vector for expression. In some embodiments involving the creation of the expression vector, the coding sequence is located in the vector so that the coding sequence is operably linked with the appropriate control sequences for expression.

[0173] The recombinant expression vector may be any suitable vector (e.g., a plasmid or virus), that can be conveniently subjected to recombinant DNA procedures and bring about the expression of the enzyme polynucleotide sequence. The choice of the vector typically depends on the compatibility of the vector with the host cell into which the vector is to be introduced. The vectors may be linear or closed circular plasmids.

[0174] In some embodiments, the expression vector is an autonomously replicating vector (i.e., a vector that exists as an extra-chromosomal entity, the replication of which is independent of chromosomal replication, such as a plasmid, an extra-chromosomal element, a minichromosome, or an artificial chromosome). The vector may contain any means for assuring self-replication. In some alternative embodiments, the vector is one in which, when introduced into the host cell, it is integrated into the genome and replicated together with the chromosome(s) into which it has been integrated. Furthermore, in some embodiments, a single vector or plasmid, or two or more vectors or plasmids which together contain the total DNA to be introduced into the genome of the host cell, and/or a transposon is utilized.

[0175] In some embodiments, the expression vector contains one or more selectable markers, which permit easy selection of transformed cells. A "selectable marker" is a gene, the product of which provides for biocide or viral resistance, resistance to heavy metals, prototrophy to auxotrophs, and the like. Examples of bacterial selectable markers include, but are not limited to the *dal* genes from

Bacillus subtilis or *Bacillus licheniformis*, or markers, which confer antibiotic resistance such as ampicillin, kanamycin, chloramphenicol or tetracycline resistance. Suitable markers for yeast host cells include, but are not limited to ADE2, HIS3, LEU2, LYS2, MET3, TRP1, and URA3. Selectable markers for use in filamentous fungal host cells include, but are not limited to, amdS (acetamidase; e.g., from *A. nidulans* or *A. orzyae*), argB (ornithine carbamoyltransferases), bar (phosphinothricin acetyltransferase; e.g., from S. *hygroscopicus*), hph (hygromycin phosphotransferase), niaD (nitrate reductase), pyrG (orotidine-5'-phosphate decarboxylase; e.g., from *A. nidulans* or *A. orzyae*), sC (sulfate adenyltransferase), and trpC (anthranilate synthase), as well as equivalents thereof. In another aspect, the present invention provides a host cell comprising at least one polynucleotide encoding at least one engineered enzyme polypeptide of the present invention, the polynucleotide(s) being operatively linked to one or more control sequences for expression of the engineered enzyme enzyme(s) in the host cell. Host cells suitable for use in expressing the polypeptides encoded by the expression vectors of the present invention are well known in the art and include but are not limited to, bacterial cells, such as *E. coli*, *Vibrio fluvialis*, *Streptomyces* and *Salmonella typhimurium* cells; fungal cells, such as yeast cells (*e.g.*, *Saccharomyces cerevisiae* or *Pichia pastoris* (ATCC Accession No. 201178)); insect cells such as *Drosophila* S2 and *Spodoptera* Sf9 cells; animal cells such as CHO, COS, BHK, 293, and Bowes melanoma cells; and plant cells. Exemplary host cells also include various *Escherichia coli* strains (e.g., W3110 (ΔfhuA) and BL21).

[0176] Accordingly, in another aspect, the present invention provides methods of producing the engineered enzyme polypeptides, where the methods comprise culturing a host cell capable of expressing a polynucleotide encoding the engineered enzyme polypeptide under conditions suitable for expression of the polypeptide. In some embodiments, the methods further comprise the steps of isolating and/or purifying the enzyme polypeptides, as described herein.

[0177] Appropriate culture media and growth conditions for host cells are well known in the art. It is contemplated that any suitable method for introducing polynucleotides for expression of the enzyme polypeptides into cells will find use in the present invention. Suitable techniques include, but are not limited to electroporation, biolistic particle bombardment, liposome mediated transfection, calcium chloride transfection, and protoplast fusion.

[0178] Various features and embodiments of the present invention are illustrated in the following representative examples, which are intended to be illustrative, and not limiting.

EXPERIMENTAL

[0179] The following Examples, including experiments and results achieved, are provided for illustrative purposes only and are not to be construed as limiting the present invention. Indeed, there are various suitable sources for many of the reagents and equipment described below. It is not intended that the present invention be limited to any particular source for any reagent or equipment item.

[0180] In the experimental disclosure below, the following abbreviations apply: M (molar); mM (millimolar), uM and μM (micromolar); nM (nanomolar); mol (moles); gm and g (gram); mg (milligrams); ug and μg (micrograms); L and l (liter); ml and mL (milliliter); cm (centimeters); mm (millimeters); um and μη (micrometers); sec. (seconds); min(s) (minute(s)); h(s) and hr(s) (hour(s)); U (units); MW (molecular weight); rpm (rotations per minute); psi and PSI (pounds per square inch); °C (degrees Centigrade); RT and rt (room temperature); CV (coefficient of variability); CAM and cam (chloramphenicol); PMBS (polymyxin B sulfate); IPTG (isopropyl β-D-l - thiogalactopyranoside); LB (Luria broth); TB (terrific broth); SFP (shake flask powder); CDS (coding sequence); DNA (deoxyribonucleic acid); RNA (ribonucleic acid); nt (nucleotide; polynucleotide); aa (amino acid; polypeptide); *E. coli* W3110 (commonly used laboratory *E. coli* strain, available from the Coli Genetic Stock Center [CGSC], New Haven, CT); AcSus (*Acidithiobacillus caldus* sucrose synthase); SUS, SuS, and SuSy (sucrose synthase, also known as sucrose synthetase); NDP (nucleoside diphosphate); adenosine diphosphate (ADP); cytidine diphosphate (CDP); guanosine diphosphate (GDP); thymidine diphosphate (TDP); uridine diphosphate (UDP); inosine diphosphate (IDP); GT (glycosyltransferase); UGT (UDP-glucose-dependent glycosyltransferase); NGT (NDP-nucleoside diphosphate-dependent glycosyltransferase); AGT (ADP-glucose-dependent glycosyltransferase); CGT (CDP-glucose-dependent glycosyltransferase); GGT (GDP-glucose-dependent glycosyltransferase); TGT (TDP-glucose-dependent glycosyltransferase); IGT (IDP-glucose-dependent glycosyltransferase); UGT (UDP-glucose-dependent glycosyltransferase); reb (rebaudioside); rebA (rebaudioside A); rebD (rebaudioside D); rebI (rebaudioside I); rebM (rebaudioside M); "Reb A 60" is a ~1:2 mixture of stevioside and rebaudioside A respectively; HTP (high throughput); HPLC (high pressure liquid chromatography); HPLC-UV (HPLC-Ultraviolet Visible Detector); 1H NMR (proton nuclear magnetic resonance spectroscopy); HSQC NMR (heteronuclear single quantum coherence spectroscopy NMR); COSY NMR (homonuclear correlation spectroscopy NMR); Acorn (Acorn NMR, Livermore, CA); FIOPC (fold improvements over positive control); Sigma and Sigma-Aldrich (Sigma-Aldrich, St. Louis, MO; Difco (Difco Laboratories, BD Diagnostic Systems, Detroit, MI); Microfluidics (Microfluidics, Westwood, MA); ChromaDex (ChromaDex, Inc., Irvine, CA); Life Technologies (Life Technologies, a part of Fisher Scientific, Waltham, MA); Amresco (Amresco, LLC, Solon, OH); Carbosynth (Carbosynth, Ltd., Berkshire, UK); Varian (Varian Medical Systems, Palo Alto, CA); Agilent (Agilent Technologies, Inc., Santa Clara, CA); and Thermotron (Thermotron, Inc., Holland, MI).

EXAMPLE 1

Synthesis, Optimization, and Assays

[0181] In this Example, methods used in the synthesis, optimization and assaying of UGT enzymes with glucosylation activity are described.

Gene Synthesis and Optimization:

[0182] The polynucleotide sequence (SEQ ID NO: 1) encoding the wild-type *Stevia rebaudiana* polypeptide (SEQ ID NO: 2) reported to glucosylate steviolbioside to rebaudioside B and glucosylate stevioside to rebaudioside A (See e.g., Richman et al., Plant J., 41:56-67 [2005]), was codon-optimized and synthesized as the gene of SEQ ID NO: 3. This synthetic gene (SEQ ID NO: 3) was cloned into a pCK110900 vector system (See e.g., US Pat. Appln. Publn. No. 2006/0195947, which is hereby incorporated by reference herein) and subsequently expressed in *E. coli* W3110 (ΔfhuA). The *E. coli* strain W3110 expressed the UGT enzymes under the control of the lac promoter. Rounds of evolution were conducted as described in the following Examples.

Production of Shake Flask Powders (SFP):

[0183] A shake-flask procedure was used to generate the glycosyltransferase polypeptide shake flask powders (SFP) for characterization assays used in the biocatalytic processes described herein. Shake flask powder (SFP) preparation of enzymes provides a more purified preparation (e.g., up to >30% of total protein) of the enzyme as compared to the cell lysate used in HTP assays and also allows for the use of more concentrated enzyme solutions. A single colony of *E. coli* containing a plasmid encoding an engineered polypeptide of interest was inoculated into 5 mL Luria Bertani broth containing 30 μg/ml chloramphenicol and 1% glucose. Cells were grown overnight (at least 16 hours) in an incubator at 30°C with shaking at 250 rpm. The culture was diluted into 250 mL Terrific Broth (12 g/L bacto-tryptone, 24 g/L yeast extract, 4 mL/L glycerol, 65 mM potassium phosphate, pH 7.0, 1 mM MgS04) containing 30 μg/ml CAM, in a 1 L flask to an optical density of 600 nm (OD600) of 0.2 and allowed to grow at 30°C.

[0184] Expression of the glycosyltransferase gene was induced by addition of IPTG to a final concentration of 1 mM when the OD600 of the culture was 0.6 to 0.8. Incubation was then continued overnight (at least 16 hours). Cells were harvested by centrifugation (5000 rpm, 15 min, 4 °C) and the supernatant discarded. The cell pellet was resuspended in two volumes of 25 mM triethanolamine buffer, pH 7.5, and passed through a MICROFLUIDIZER® high pressure homogenizer (Microfluidics), with standard *E. coli* lysis settings and maintained at 4°C. Cell debris was removed by centrifugation (10,000 rpm, 45 minutes, 4°C). The cleared lysate supernatant was collected and frozen at -80°C and then either His-affinity purified and dialyzed to produce purified protein or lyophilized to produce a dry shake-flask powder of crude protein.

Assay of SFP for Stevioside Glucosylation:

[0185] SFP was reconstituted to provide 20 g/L powder. Then, 50 μL of these stocks were diluted in 200μL total reaction volume of 50 mM Tris-HCl buffer, pH 7.5, with 3 mM MgSO4 and 1 mM stevioside (ChromaDex, >94% purity), with 2 mM uridine diphosphoglucose. The reaction was performed at 30 °C in a THERMOTRON® titre-plate shaker with 300 RPM shaking for 16-18h.

HPLC-MS/MS Analysis:

[0186] The reaction described above was quenched with 0.5 volume/volume acetonitrile with 0.2% formic acid and precipitated by centrifugation. Glycosylated stevioside products were detected in the supernatant by LC-MS/MS with the following instrument and parameters:

Table 1.1 HPLC-MS/MS Analysis of Steviol Glycosides	
Instrument	Agilent HPLC 1200 series, Sciex 4000 QTrap
Column	Poroshell 120 EC C18 50 x 3.0 mm, 2.7 μm with Poroshell 120 EC C18 5 x 3.0, 2.7 μm guard column (Agilent Technologies)
Mobile phase	Gradient (A: 0.1% formic acid in water, B: 0.1% formic acid in methanol) Time (m) %B 0 60 0.50 60 1.00 70 4.33 70 5.00 95 5.33 9 5.34 60 6.00 60
Flow rate	0.8 mL/m
Run time	6 m
Peak retention times	Rebaudioside A: 2.35 m
Column temperature	40 °C
Injection volume	10 μL
MS detection	MRM 990/828 (for steviol tetraglycosides, e.g., rebaudioside A), 1152/828 (for steviol pentaglycosides, e.g., rebaudioside D), 1314/828 (steviol hexaglycosides, e.g., rebaudioside M), 828/666 (for steviol triglycosides, e.g., stevioside), 666/504 (steviol diglycosides, e.g., rubusoside)
MS conditions	MODE: MRM; CUR: 30; IS: 4750; CAD: high; TEM: 550 °C; GS1: 50; GS2: 50; DP: 150; EP: 10; CXP: 14; DT: 50

Table 1.1 HPLC-MS/MS Analysis of Steviol Glycosides	
	ms for each transition. For the first three transitions CE: 85; for the last two transitions CE: 60.

[0187] Activity was detected for SEQ ID NO:4. High conversion (i.e., >95%), of stevioside to rebaudioside A was observed in the LC-MS/MS analysis of the assay samples described above.

EXAMPLE 2

GT Variants of SEQ ID NO: 4

[0188] In this Example, experiments for evolution and screening of GT polypeptides derived from SEQ ID NO: 4 for improved glucosylation of steviol glycosides using ADP-glucose are described. Directed evolution of the GT encoded by SEQ ID NO: 3 (i.e., SEQ ID NO:4) was carried out by constructing libraries of variant genes in which positions associated with certain structural features of the enzyme were subjected to mutagenesis. These libraries were then plated, grown, and screened using the high-throughput (HTP) assay described below to provide a first round ("Round 1") of engineered GT variant polypeptides with glucosyltransferase activity toward ADP-glucose and steviol glycosides. Many additional rounds of evolution were conducted by constructing libraries of variant genes in which mutations associated with improved activity in Round 1 and then subsequent rounds of evolution were recombined. These libraries were then plated, grown, and screened using the high-throughput (HTP) growth, expression, lysate preparation, and assay described below.

HTP Growth, Expression, and Lysate Preparation

[0189] Cells were picked into 96-well plates and grown overnight in LB media containing 1% glucose and 30 μg/mL CAM, 30 °C, 200 rpm, 85% humidity. Then, 20 μL of overnight growth were transferred to a deep-well plate containing 380 μL TB growth media containing 30 μg/mL CAM, induced with 1 mM IPTG, and incubated for 18 h at 30 °C, 200 rpm, 85% humidity. Cell cultures were centrifuged at 4000 rpm, 4 °C for 10 m, and the media discarded. Cell pellets thus obtained were frozen at -80°C, and lysed in 250 μL lysis buffer (0.5 g/L lysozyme and 0.5 g/L PMBS in 20 mM Tris-HCl buffer, pH 7.5) with low-speed shaking for 2 h on titre-plate shaker at room temperature. The plates were then centrifuged at 4000 rpm and 4°C for 20 min and the cleared lysate supernatants were used in the HTP assay reactions described below.

HTP Assay for Glucose Transfer from ADP-Glucose to Stevioside:

[0190] Assays were performed on 96-well plates of cleared *E. coli* culture lysates expressing SEQ ID NO: 3 variants with lysate loading of 50 μL lysate in 200 μL reactions and with substrate loading of 1 mM stevioside (ChromaDex, >94% purity), from a 20 mM stock solution in 50% ethanol and co-substrate loading of 0.5 mM ADP-glucose (Sigma, >93% purity). The following reaction conditions

were used: 50 mM Tris-HCl buffer, pH 7.5, 3 mM $MgCl_2$, 30 °C in a THERMOTRON® titre-plate shaker with 300 RPM shaking for 18 h. The reactions were quenched with 100 μL/well acetonitrile with 0.2% formic acid, centrifuged 10 m at 4 °C, and the supernatants were analyzed by HPLC-MS/MS as described in Example 1, Table 1.1.

[0191] Formation of rebaudioside A from stevioside in the presence of wild-type UGT76G1 (SEQ ID NO:4) with ADP-glucose was indistinguishable from a no enzyme control. In contrast to the wild-type enzyme of SEQ ID NO:4, glycosyltransferase variant polypeptides were identified that produced rebaudioside A from stevioside with ADP-glucose. The engineered polypeptides are listed in Table 2.1. Although the parent and variant constructs contain an N-terminal histidine tag for affinity purification, the mutations were numbered relative to the untagged reference sequence for clarity. Shake-flask scale cultures of the variants produced during multiple rounds of evolution were grown for protein purification as described in Example 1.

EXAMPLE 3

Synthesis, Optimization, and Assaying of Glycosyltransferase Enzymes with Glucosylation Activity

[0192] In this Example, methods used in the synthesis, optimization and assaying of UGT enzymes with glucosylation activity are described.

Gene Synthesis and Optimization

[0193] The polynucleotide sequence encoding a beta-1,2 glycosyltransferase *Solanum tuberosum* was codon-optimized and synthesized as the gene of SEQ ID NO: 11. These synthetic genes were cloned into a pCK110900 vector system (See e.g., US Pat. No. 9,714,437, which is hereby incorporated by reference) and subsequently expressed in *E. coli* W3110 (ΔfhuA). The *E. coli* strain W3110 expressed the UGT enzymes under the control of the lac promoter.

Production of Shake Flask Powders (SFP)

[0194] A shake-flask procedure was used to generate the glycosyltransferase polypeptide shake flask powders (SFP) for characterization assays used in the biocatalytic processes described herein. Shake flask powder (SFP) preparation of enzymes provides a more purified preparation (e.g., up to >30% of total protein) of the enzyme as compared to the cell lysate used in HTP assays and also allows for the use of more concentrated enzyme solutions. A single colony of *E. coli* containing a plasmid encoding an engineered polypeptide of interest was inoculated into 5 mL Luria Bertani broth containing 30 μg/ml chloramphenicol and 1% glucose. Cells were grown overnight (at least 16 hours) in an incubator at 30°C with shaking at 250 rpm. The culture was diluted into 250 mL Terrific Broth (12 g/L bacto-tryptone, 24 g/L yeast extract, 4 mL/L glycerol, 65 mM potassium phosphate, pH 7.0, 1

mM $MgSO_4$) containing 30 μg/ml CAM, in a 1 L flask to an optical density of 600 nm (OD600) of 0.2 and allowed to grow at 30 °C.

[0195] Expression of the glycosyltransferase gene was induced by addition of IPTG to a final concentration of 1 mM when the OD600 of the culture was 0.6 to 0.8. Incubation was then continued overnight (at least 16 hours). Cells were harvested by centrifugation (5000 rpm, 15 min, 4°C) and the supernatant discarded. The cell pellet was resuspended in two volumes of 25 mM triethanolamine buffer, pH 7.5, and passed through a MICROFLUIDIZER® high pressure homogenizer (Microfluidics), with standard *E. coli* lysis settings and maintained at 4°C. Cell debris was removed by centrifugation (10,000 rpm, 45 minutes, 4°C). The cleared lysate supernatant was collected and frozen at -80 °C and then either His-affinity purified and dialyzed to produce purified protein or lyophilized to produce a dry shake-flask powder of crude protein.

Assay for Rebaudioside A Glucosylation with Purified Proteins

[0196] First, 50 μL purified protein was diluted in 200 μL total reaction volume consisting of 50 mM Tris-HCl buffer pH 7.5, 3 mM magnesium chloride, 1 mM rebaudioside A, and 0.5 mM uridine diphosphoglucose. The reaction was performed at 30°C in a THERMOTRON® titre-plate shaker with 300 RPM shaking for 18 h. Boiled enzyme reaction was used as the negative control. Ten μL of the reaction was quenched with 90 μL acetonitrile with 0.2% formic acid and precipitated by centrifugation. Glycosylated rebaudioside A products were detected in the supernatant by LC-MS/MS as described in Example 1, Table 1.1. Despite poor soluble expression, SEQ ID NO: 12 demonstrated high specific activity and good selectivity toward producing β-1,2-glucose linkages in the steviol glycoside substrates.

Assay for Rebaudioside A Glucosylation with Shake Flask Powder

[0197] Lyophilized shake flask powder was reconstituted to 20 mg/mL. Then, 10 μL of these stocks were diluted in 100 μL total reaction volume of 50 mM potassium phosphate (KPhos) buffer, pH 7, with 3 mM $MgCl_2$,1 mM rebaudioside A (>97% purity), and 2 mM uridine diphosphoglucose (UDP-glucose). The reaction was performed at 40 °C in a THERMOTRON® titre-plate shaker with 300 RPM shaking for 16-18 h. Activity was detected for SEQ ID NO: 12 over negative control. Low conversion (i.e., <10%), of rebaudioside A to rebaudioside D was observed in the LC-MS/MS analysis.

EXAMPLE 4

GT Variants of SEQ ID NO: 12

[0198] In this Example, experiments for evolution and screening of GT polypeptides derived from SEQ ID NO: 12 for improved glucosylation of steviol glycosides are described. Directed evolution of the GT encoded by SEQ ID NO: 11 (i.e., SEQ ID NO: 12) was carried out by constructing

combinatorial libraries of variant genes in which positions associated with surface residues of the enzyme were subjected to mutagenesis. These libraries were then plated, grown, and screened using the high-throughput (HTP) assay described below to provide a first round ("Round 1") of engineered GT variant polypeptides with β-1,2-glucosyltransferase activity toward steviol glycosides. Many additional rounds of evolution were conducted by constructing libraries of variant genes in which mutations associated with improved activity in Round 1 and then subsequent rounds of evolution were recombined. These libraries were then plated, grown, and screened using the high-throughput (HTP) growth, expression, lysate preparation, and assay described below.

HTP Growth, Expression, and Lysate Preparation

[0199] Cells were picked into 96-well plates and grown overnight in LB media containing 1% glucose and 30 μg/mL CAM, 30 °C, 200 rpm, 85% humidity. Then, 20 μL of overnight growth were transferred to a deep-well plate containing 380 μL TB growth media containing 30 μg/mL CAM, induced with 1 mM IPTG, and incubated for 18 h at 30 °C, 200 rpm, 85% humidity. Cell cultures were centrifuged at 4000 rpm, 4 °C for 10 m, and the media discarded. Cell pellets thus obtained were frozen at -80 °C, and lysed in 250 μL lysis buffer (0.5 g/L lysozyme and 0.5 g/L PMBS in 20 mM Tris-HCl buffer, pH 7.5) with low-speed shaking for 2 h on titre-plate shaker at room temperature. The plates were then centrifuged at 4000 rpm and 4 °C for 20 min and the cleared lysate supernatants were used in the HTP assay reactions described below.

HTP Assay for Rebaudioside A Glucosylation

[0200] Assays were performed on 96-well plates of cleared *E. coli* culture lysates expressing enzyme variants with lysate loading of 25 μL lysate in 100 μL reactions and with substrate loading of 1 mM rebaudioside A (Sigma, >96% purity), from a 20 mM stock solution in 50% ethanol, and co-substrate loading of 0.5 mM UDP-glucose (Sigma, >98% purity). The following reaction conditions were used: 50 mM Tris-HCl buffer, pH 7.5, 3 mM $MgCl_2$, 30 °C in a THERMOTRON® titre-plate shaker with 300 RPM shaking for 4 h. The reactions were quenched with 0.5 volume/volume acetonitrile with 0.2% formic acid and precipitated by centrifugation for 10 m at 4 °C. Glycosylated products were detected in the supernatant by LC-MS/MS following 1:20 dilution in water with the instrument and parameters described in Example 1, Table 1.1. Glycosyltransferase variant polypeptides that produced rebaudioside D from rebaudioside A at greater quantities than the reference backbone sequence were identified and used in subsequent rounds of evolution. Shake-flask scale cultures were grown for lyophilized powder production as described in Example 1 for analysis of variants.

Shake Flask Lysate Characterization Assay and Analysis for Rebaudioside A Glucosylation

[0201] First, 250 mL shake flask cultures were grown, induced, and lysed. Cell debris was removed by centrifugation as described in Example 1, and the cleared lysate supernatant was collected. Then,

10 µL of the lysate were diluted in 100 µL total reaction volume of 50 mM Tris-HCl buffer, pH 7.5, 3 mM $MgCl_2$, 1 mM rebaudioside A (Sigma, >96% purity), and 2 mM UDP-glucose (Sigma,>98% purity). The reaction was performed at 30 °C in a THERMOTRON® titre-plate shaker with 300 RPM shaking for 0-18 h. The reaction described above was quenched with 0.5 volume/volume acetonitrile with 0.2% formic acid and precipitated by centrifugation. Glycosylated products were detected in the supernatant by LC-MS/MS following 1:20 dilution in water with the instrument and parameters described in Example 1, Table 1.1.

EXAMPLE 5

Sucrose Synthase Variants of SEQ ID NO: 6

[0202] In this Example, experiments for the evolution and screening of sucrose synthase (SuS) polypeptides derived from a codon-optimized *Acidothiobacillus caldus* sucrose synthase polynucleotide (SEQ ID NO: 5) for improved production of ADP-glucose from sucrose and ADP are described. Directed evolution of the SuS encoded by SEQ ID NO: 5 (i.e., SEQ ID NO: 6) was carried out by constructing libraries of variant genes in which positions associated with certain structural features of the enzyme were subjected to saturation mutagenesis and diversity from homologs in publically available databases was recombined. These libraries were then plated, grown, and screened using the high-throughput (HTP) assay described below to provide a first round ("Round 1") of engineered SuS variant polypeptides with improved activity toward synthesizing ADP-glucose. Many additional rounds of evolution were conducted by constructing libraries of variant genes in which mutations associated with improved activity in Round 1 and then subsequent rounds of evolution were recombined. These libraries were then plated, grown, and screened using the high-throughput (HTP) growth, expression, lysate preparation, and assay described below.

HTP Assay for Glucose Transfer from Sucrose to ADP

[0203] Assays were performed on 96-well plates of cleared *E. coli* culture lysates expressing SEQ ID NO: 5 variants (i.e., variants of SEQ ID NO: 6) with lysate loading of 25 µL lysate in 100 µL reactions and with substrate loading of 30% w/v sucrose (Sigma) from a 60% stock solution in water and co-substrate loading of 2 mM ADP (Sigma, >95%). The following reaction conditions were used: 50 mM Tris-HCl buffer, pH 7.5, 3 mM $MgCl_2$, 30 °C in a Thermocycler for 2 h. The reactions were heat quenched at 95 °C for 10 minutes, and then analyzed by a colorimetric D-fructose dehydrogenase assay adapted from the literature (See e.g., Ameyama et al., J. Bacteriol., 145:814-823 [1981]; and Ameyama, Meth. Enzymol., 89:20-29 [1982]). Briefly, an overnight enzyme-coupled assay was conducted in 96-well plates with 20 µL sample, diluted such that fructose concentration is <1 g/L, 20 µL 100 mM potassium ferricyanide (Sigma P-8131), and 160 µL 0.8 units/mL fructose dehydrogenase (Sigma F4892) dissolved in pH 4.6 McIlvaine buffer with 0.1% Triton X-100. This reaction quantitatively converts fructose to $K_4Fe(CN)_6$, which is then quantified colorimetrically by

adding 67 µL of the overnight reaction to 33 µL of stop solution (0.3% w/v sodium dodecyl sulfate, Sigma L-4509, 8.1% v/v phosphoric acid, Sigma P-6560, and 0.5% w/v ferric sulfate, Sigma F-1135) and shaking for 20 minutes to allow for complete conversion of $K_4Fe(CN)_6$ to Prussian blue, the absorbance of which is read on a plate reader at a wavelength of 690 nm. Following the primary assay, engineered sucrose synthase (SuS) variant polypeptides with higher fructose, and therefore higher stoichiometric ADP-glucose, formation activity than SEQ ID NO: 6 were screened in triplicate at a lower substrate load of 2% w/v sucrose (Sigma) and co-substrate load of 1 mM ADP (Sigma, >95%).

HTP Coupled Assay for Glucose Transfer from Sucrose to ADP to Rebaudioside D

[0204] Libraries were screened using the following HTP enzyme-coupled assay. Pelleted *E. coli* cultures were lysed with 250 µL of Tris-HCl, pH 7.5, with 1 mM magnesium sulfate and 0.5 mg/mL lysozyme and polymyxin B sulfate (PMBS) and cleared by centrifugation. Lysate was diluted 20x into Tris-HCl, pH 7.5. Then, 10 µL diluted SuS lysate and 2 g/L GT SEQ ID NO: 8 were combined in 100 µL reaction volume with substrate loading of ~1 mM rebaudioside D and co-substrate loadings of 1 Mm ADP (Sigma, >95%) and 10 mM sucrose (Sigma). The following reaction conditions were used: 50 mM potassium phosphate buffer, pH 7, 3 mM $MgCl_2$, 50 °C, in a THERMOTRON® titre-plate shaker with 300 RPM shaking for 2 h. The reaction described above was quenched by adding 10 µL assay mixture to 90 µL acetonitrile with 0.2% formic acid, and precipitated by centrifugation. The supernatant was diluted 10x in water and steviol glycoside products were detected by RapidFire SPE-MS/MS, with the instrument and parameters described in Table 5.1.

Table 5.1 RapidFire SPE-MS/MS Conditions for Steviol Glycoside Detection.

Agilent RapidFire Conditions	
Buffer A	0.1% formic acid in LC/MS grade water; 1.5 mL/min flow rate
Buffer B	0.1% formic acid in LC/MS grade methanol; 0.8 mL/min flow rate
Aqueous wash	Water
Organic wash	Acetonitrile
SPE cartridge	Agilent RapidFire cartridge A (C4)
RF state 1	600 ms
RF state 2	2500 ms
RF state 3	0
RF state 4	5000 ms
RF state 5	1000 ms
Agilent Jet Stream Source Parameters	
Drying gas temperature	300 °C

Table 5.1 RapidFire SPE-MS/MS Conditions for Steviol Glycoside Detection. Agilent RapidFire Conditions						
Drying gas flow	10 L/min					
Nebulizer pressure	45 psi					
Sheath gas temperature	350 °C					
Sheath gas flow	11 L/min					
Capillary voltage	+3500 V					
Nozzle voltage	+2000 V					
Agilent 6470 Triple Quadrupole MRM Parameters						
Compound	Q1	Q3	Dwell	Fragmentor	CE	CAV
Stevioside	827.4	665.3	50	150	50	5
RebA	989.5	827.5	50	350	60	5
RebD or RebI	1151.7	827.5	50	350	55	5
RebM	1313.7	827.5	50	350	70	5

Production of Shake Flask Powders (SFP)

[0205] A shake-flask procedure was used to generate the glycosyltransferase polypeptide shake flask powders (SFP) for characterization assays used in the biocatalytic processes described herein. Shake flask powder (SFP) preparation of enzymes provides a more purified preparation (e.g., up to >30% of total protein) of the enzyme, as compared to the cell lysate used in HTP assays, and also allows for the use of more concentrated enzyme solutions. A single colony of *E. coli* containing a plasmid encoding an engineered polypeptide of interest was inoculated into 5 mL Luria Bertani broth containing 30 µg/ml chloramphenicol and 1% glucose. Cells were grown overnight (at least 16 hours) in an incubator at 30°C with shaking at 250 rpm. The culture was diluted into 250 mL Terrific Broth (12 g/L bacto-tryptone, 24 g/L yeast extract, 4 mL/L glycerol, 65 mM potassium phosphate, pH 7.0, 1 mM $MgS0_4$) containing 30 µg/ml CAM, in a 1 L flask to an optical density of 600 nm (OD600) of 0.2, and allowed to grow at 30°C.

Shake Flask Powder Characterization Assay and Analysis for Glucosyl Transfer from Sucrose to Rebaudioside D

[0206] An experiment was performed to characterize the activity of the engineered SUS variants on sucrose and ADP to facilitate the formation of rebaudioside M from rebaudioside D. Shake flask powder (SFP) was added to a 100 µL total reaction volume at 0.125 g/L concentration containing 50 mM potassium phosphate buffer, pH 7, 3 mM magnesium chloride, 1 g/L rebaudioside D, 10 mM sucrose, 1 mM ADP, and 2 g/L GT SEQ ID NO: 10. The reaction was performed at 50°C in a THERMOTRON® titre-plate shaker with 300 RPM shaking for 1 h. The reaction was quenched by

adding 10 µL of the reaction mixture to 90 µL acetonitrile with 0.2% formic acid, and precipitated by centrifugation. The supernatant was diluted 10x in water and analyzed for steviol glycosides by RapidFire SPE-MS/MS, with the instrument and parameters described in Table 5.1. Based on these results, additional rounds of evolution were conducted to optimize the enzymes.

EXAMPLE 6

Sucrose Synthase Variants

[0207] Directed evolution of the sucrose synthase enzymes was continued by constructing libraries of variant genes in which mutations associated with improved activity in earlier rounds of evolution were recombined. These libraries were then plated, grown, and screened using the high-throughput (HTP) assay described below, to provide additional iterative rounds engineered SuS variant polypeptides with activity toward the generation of ADP-glucose.

HTP Coupled Assay for Glucose Transfer from Sucrose to ADP to Rebaudioside A

[0208] Combinatorial libraries were screened using the following HTP enzyme-coupled assay. Pelleted *E. coli* cultures were lysed with 400 µL of Tris-HCl, pH 7.5 with 1 mM magnesium sulfate and 0.5 mg/mL lysozyme and polymyxin B sulfate (PMBS), and cleared by centrifugation. Lysate was diluted ~90x into Tris-HCl, pH 7.5. Then, 10 µL diluted SuS lysate and 1 g/L GT SEQ ID NO: 14 were combined in 100 µL reaction volume with substrate loading of 4.5-7.5 mM rebaudioside A 97 and co-substrate loadings of 0.2-0.25 mM ADP (Sigma, >95%) and 30 mM sucrose (Sigma). The following reaction conditions were used: 50 mM potassium phosphate buffer, pH 6, 55°C in a THERMOTRON® titre-plate shaker with 300 RPM shaking for 2 h. The reaction described above was solubilized by adding 10 µL assay to 90-190 µL water, quenched by adding 10 µL solubilized assay to 90 µL acetonitrile with 0.2% formic acid, and precipitated by centrifugation. The supernatant was diluted 4.4-6.7x in water and steviol glycoside products were detected by RapidFire SPE-MS/MS, with the instrument and parameters described in Table 5.1. After analysis, engineered SuS variant polypeptides that showed improved activity coupled with a GT on rebaudioside A were identified.

Shake Flask Powder Characterization Assay and Analysis for Glucosyl Transfer from Sucrose to Rebaudioside A

[0209] Experiments were performed to characterize the activity of the engineered SUS variants on sucrose and ADP to facilitate the formation of rebaudioside D from rebaudioside A. Shake flask powder (SFP) was added to a 100 µL total reaction volume at 0.02 g/L concentration containing 50 mM potassium phosphate buffer, pH 6, 7.5 mM rebaudioside A 97, 30 mM sucrose, 0.2 mM ADP, and 1 g/L GT SEQ ID NO: 14. The reaction was performed at 50°C in a THERMOTRON® titre-plate shaker with 300 RPM shaking for 1 h. The reaction was solubilized by diluting 20x into water, quenched by adding 10 µL of the diluted reaction to 90 µL acetonitrile with 0.2% formic acid, and

precipitated by centrifugation. The supernatant was diluted 4.4x in water and analyzed for steviol glycosides by RapidFire SPE-MS/MS, with the instrument and parameters described in Table 5.1. Variants with higher activities than the reference sequences and higher levels of rebaudioside D produced from rebaudioside A were selected for further directed evolution for the catalysis of the recycling reaction transferring a glucose from sucrose to ADP.

EXAMPLE 7

Beta-1,3-ADP-Glycosyltransferase Variants of SEQ ID NO: 20

[0210] In this Example, experiments for evolution and screening of β1,3-glycosyltransferase (β1,3GT) polypeptides derived from SEQ ID NO: 8598 for improved glucosylation of steviol glycosides using in situ synthesized ADP-glucose are described. Directed evolution of the GT encoded by SEQ ID NO: 19 (i.e., SEQ ID NO: 20) was carried out by constructing libraries of variant genes in which mutations associated with improved production identified during the development of the present invention were recombined and in which certain structural features were subjected to saturation mutagenesis. These libraries were then plated, grown, and screened using the high-throughput (HTP) assay described below to provide another round of 72 engineered GT variant polypeptides with glucosyltransferase activity toward ADP-glucose and steviol glycosides.

HTP Assay for Glucose Transfer from Sucrose to ADP and then from ADP-Glucose to Rebaudioside A 60

[0211] Assays were performed on 96-well plates of cleared *E. coli* culture lysates expressing SEQ ID NO: 19 variants (i.e., variants of SEQ ID NO: 20). Pellets were lysed, and lysate was cleared as described in Example 6, and then diluted 10x into 50 mM potassium phosphate buffer, pH 6. In order to thermally challenge the lysates, they were pre-incubated at 75°C in a Thermotron® titre-plate shaker with 300 RPM shaking for 1 h. Assays were conducted with 10 µL pre-incubated lysate in 100 µL reactions and with 20 g/L rebaudioside A 60% substrate, 0.025 g/L ADP (Amresco, ultra pure grade) co-substrate, 0.05 g/L SUS SFP SEQ ID NO: 18, 0.12 g/L β-1,2-glycosyltransferase (β12GT) SEQ ID NO: 16, and 40 g/L sucrose (cane sugar). The following reaction conditions were used: 50 mM potassium phosphate buffer, pH 6, 60°C in a THERMOTRON® titre-plate shaker with 300 RPM shaking for 16 h. Reactions were solubilized by 20x dilution with water, quenched by 5x dilution with acetonitrile with 0.2% formic acid, and diluted 20x with water for analysis. Samples were analyzed by RapidFire-MS/MS as described in Example 5, Table 5.1. Glycosyltransferase variant polypeptides that produced rebaudioside M from rebaudioside A 60 with *in situ* synthesized ADP-glucose at greater quantities than SEQ ID NO: 20 were identified. The engineered polypeptides are listed in Table 7.1 and Table 7.2. Shake-flask scale cultures were grown, lysed, cleared, and lyophilized to powder as described in Example 1 for analysis of variants shown in Table 7.3 relative to SEQ ID NO: 20.

Table 7.1 β1,3GT Combinatorial Variants and RebM Levels		
SEQ ID NO: (nt/aa)	**Amino Acid Differences (Relative to SEQ ID NO: 20)**	**Increased RebM[a]**
25/26	H81T/K139N/M144Q/H195Q/K200R/D204T/I207V/W233Q	+++
27/28	H81T/Q197K/K200R/I207V/W233Q/C338A	+++
29/30	A41E/T72P/C338A	+++
31/32	M144Q/W233T/C338V	+++
33/34	T72P/M144Q/C338V	+++
35/36	A41E/T72P/W233Q/C338A	+++
37/38	R76S/M144Q/Q197K/K200R	+++
39/40	K200R/D204T/I207V/W233T	+++
41/42	H81T/M144Q/W233Q	+++
43/44	T72P/K200R/D204T/I207V	++
45/46	T72P/H81T/H195Q/W233Q	++
47/48	Q61D/E259T/K428I	++
49/50	A41E/W233T/C338V	++
51/52	A41E/W233Q	++
53/54	M144Q/W233T	++
55/56	W233Q/C338V	++
57/58	R76S/Q197K/I207V/W233Q	++
59/60	H81T/C338V	++
61/62	A41E/W233Q/C338V	++
63/64	K139N/M144Q/W233Q	++
65/66	A41E/M144Q/W233T	++
67/68	R76S/H195Q/Q197K/D204T/I207V/W233T	++
69/70	T72P/R76S/L163A/Q197K	++
71/72	T72P/I207V	++
73/74	T72P/R76T/Q197K/D204T	+
75/76	L163A/W233T/C338A	+
77/78	T72P/R76S/I207V/C338V	+
79/80	T72P/R76S	+
81/82	T72P	+
83/84	T72P/R76T	+
85/86	A41E/W233T	+
87/88	T72P/R76T/I207V/W233Q	+

Table 7.1 β1,3GT Combinatorial Variants and RebM Levels		
SEQ ID NO: (nt/aa)	**Amino Acid Differences (Relative to SEQ ID NO: 20)**	**Increased RebM[a]**
89/90	T72P/R76T/H195Q/W233T	+
91/92	T72P/H81T	+
93/94	T72P/K139N/H195Q/D204T	+
95/96	R76S/W233T	+
97/98	H81T/W233Q/C338V	+
99/100	Q61D	+
101/102	Q61E/R411T	+
103/104	Q61D/A87K/Q91L/A107L	+
105/106	Q61E/Q91L/D431M	+
107/108	Q61D/A87K/Q91L/E259T	+
109/110	A107V/E259T	+
111/112	A107V	+
113/114	Q61D/I407T/K428I	+
115/116	Q61D/A107V	+
117/118	C156S/I407T	+
119/120	E259T	+
121/122	Q61E/A87K/Q91L/A107V	+
[a]Levels of increased production were determined relative to the reference polypeptide of SEQ ID NO: 20, and defined as follows: "+" = at least 1.24-fold that of the reference but less than 1.36-fold increased production; "++" = at least 1.36-fold but less than 1.43-fold increased production relative to reference polypeptide; and "+++" = at least 1.43-fold increased production.		

Table 7.2 β1,3GT Saturation Mutagenesis Variants and RebM Levels			
SEQ ID NO: (nt/aa)	**Amino Acid Differences (Relative to SEQ ID NO: 20)**	**Increased RebM at 60 °C[a]**	**Increased RebM at 65 °C[a]**
123/124	A85V	++	++
125/126	H81L	++	+
127/128	A124P	++	-
129/130	T80P	++	++
131/132	H81A	++	++
133/134	H81M	++	+
135/136	H81V/Q270K	++	++

Table 7.2 β1,3GT Saturation Mutagenesis Variants and RebM Levels			
SEQ ID NO: (nt/aa)	**Amino Acid Differences (Relative to SEQ ID NO: 20)**	**Increased RebM at 60 °C[a]**	**Increased RebM at 65 °C[a]**
137/138	H81T	++	++
139/140	H81V	++	+
141/142	V286L	++	-
143/144	H81S	++	+
145/146	T80D	++	++
147/148	A85T	++	+
149/150	P83D	+	-
151/152	Q71G	+	+
153/154	A97V	+	-
155/156	S456K	+	-
157/158	E420R	+	+
159/160	T263C	+	+
161/162	I334V	+	-
163/164	Q71T	+	-
165/166	T80Q	-	+
167/168	E402I	-	++

[a]Levels of increased production were determined relative to the reference polypeptide of SEQ ID NO: 20, and defined as follows: "-" = less than the activity of the reference; "+" = at least the activity of the reference but less than 1.1-fold increased production; and "++" = at least 1.1-fold increased production.

SFP Characterization Assay and Analysis for Glucosyl Transfer from Sucrose to ADP and then from ADP-Glucose to Stevioside and Rebaudioside D

[0212] Shake flask powders (SFP) were reconstituted to a concentration of 20 g/L and diluted to 0.005-0.15 g/L SFP in 100 µL total reaction volume of 50 mM potassium phosphate buffer, pH 6, 15 mM stevioside (>95% purity) or rebaudioside D, 0.02 g/L ADP (Amresco, ultra pure grade) co-substrate, 0.05 g/L SUS SFP SEQ ID NO: 18, and 37.5 mM sucrose (cane sugar). The reactions were incubated in a Thermotron® titre-plate shaker with 300 RPM shaking for 4 hours at 60°C. The reactions were solubilized by 20x dilution with water, quenched by 5x dilution with acetonitrile with 0.2% formic acid, cleared by centrifugation, and diluted 15x with water for analysis. Glycosylated products were detected by SPE-QQQ as described in Example 5, Table 5.1. A one-pot reaction was conducted with 0.01-0.3 g/L SFP in 100 µL total reaction volume of 50 mM potassium phosphate buffer, pH 6, 20 g/L RebA60, 0.02 g/L ADP (Amresco, ultra pure grade) co-substrate, 0.05 g/L SUS SFP SEQ ID NO: 18, 0.12 g/L β1,2 GT SFP SEQ ID NO: 16, and 30 g/L sucrose (cane sugar). The

reactions were incubated in a THERMOTRON® titre-plate shaker with 300 RPM shaking for 16 hours at 60°C. The reactions were solubilized by 20x dilution with water, quenched by 5x dilution with acetonitrile with 0.2% formic acid, cleared by centrifugation, and diluted 20x with water for analysis. Similar one-pot reactions were also performed with 10 µL of crude shake flask lysate after diluting 20-fold in 50 mM potassium phosphate buffer, pH 6, and incubating for 24 h at 75.1 °C.

Table 7.3 β1,3GT Shake Flask Powder Variants and RebA, RebM from RebD, and RebM from RebA60, and Thermostability Levels

SEQ ID NO: (nt/aa)	Amino Acid Differences (Relative to SEQ ID NO: 20)	Increased RebA, 60 °C[a]	Increased RebM, 60 °C[a]	Increased RebM (from A60), 60 °C[a]	% Activity Remaining[b]
25/26	H81T/K139N/M144Q/H195Q/K200R/D204T/I207V/W233Q	++	+++	+++	-
27/28	H81T/Q197K/K200R/I207V/W233Q/C338A	-	+	+++	-
29/30	A41E/T72P/C338A	++	++	++	-
35/36	A41E/T72P/W233Q/C338A	+	+	+++	+
47/48	Q61D/E259T/K428I	+	+	+	-
99/100	Q61D	-	-	-	-
105/106	Q61E/Q91L/D431M	+	+	-	+
113/114	Q61D/I407T/K428I	-	+	+	-

[a]Levels of increased production were determined relative to the reference polypeptide of SEQ ID NO: 20, at 0.019 g/L shake flask powder for single substrate and 0.04 g/L for one-pot and defined as follows: "-" = production less than that of the reference polypeptide; "+" = production at least that of the reference but less than 1.1-fold reference polypeptide; and "++" = at least 1.1-fold increased production relative to reference polypeptide.

[b]The percent of activity remaining for each variant was determined following 24 h pre-incubation at 75.1°C, relative to the production from each variant following 24 h pre-incubation at 60°C and is defined as follows: and "-" = less than 20% of activity remained following 24 h pre-incubation at 75.1 °C; and "+" = at least 20% activity remained.

[0213] In these experiments, at least 4 variants in Table 7.3 produced more rebaudioside M from rebaudioside A 60 than SEQ ID NO: 20, and three variants also produced more rebaudioside A from stevioside and rebaudioside M from rebaudioside D. All variants were less thermostable than SEQ ID NO: 20. SEQ ID NO: 36 was selected as the starting point for further enzyme engineering, because of its superior performance in the one-pot assay and limited decrease in thermostability.

EXAMPLE 8

Beta-1,3-ADP-Glycosyltransferase Variants of SEQ ID NO: 36

[0214] In this Example, experiments for evolution and screening of β1,3-glycosyltransferase (β1,3GT) polypeptides derived from SEQ ID NO: 36 for improved glucosylation of steviol glycosides using in situ synthesized ADP-glucose are described. Directed evolution of the GT encoded by SEQ ID NO: 35 (i.e., SEQ ID NO: 36) was carried out by constructing libraries of variant genes in which mutations associated with improved production identified during the development of the present invention were recombined and in which the N-terminal coding region was targeted. These libraries were then plated, grown, and screened using the high-throughput (HTP) assay described below to provide another round of 58 engineered GT variant polypeptides with glucosyltransferase activity toward ADP-glucose and steviol glycosides.

HTP Assay for Glucose Transfer from Sucrose to ADP and then from ADP-Glucose to Rebaudioside A 60

[0215] Assays were performed on 96-well plates of cleared *E. coli* culture lysates expressing SEQ ID NO: 35 (i.e., variants of SEQ ID NO: 36). Pellets were lysed, and lysate was cleared as described in Example 6, and then diluted 20x into 50 mM potassium phosphate buffer, pH 6. In order to thermally challenge the lysates, they were pre-incubated at 75°C in a THERMOTRON® titre-plate shaker with 300 RPM shaking for 2 h. Assays were conducted with 10 µL pre-incubated lysate in 100 µL reactions and with 20 g/L rebaudioside A 60% substrate, 0.02 g/L ADP (Amresco, ultra pure grade) co-substrate, 0.03 g/L SUS SFP SEQ ID NO: 22, 0.08 g/L β-1,2-glycosyltransferase (β12GT) SEQ ID NO: 858, and 30 g/L sucrose (cane sugar). The following reaction conditions were used: 50 mM potassium phosphate buffer, pH 6, 60°C in a THERMOTRON® titre-plate shaker with 300 RPM shaking for 16 h. Reactions were solubilized by 20x dilution with water, quenched by 5x dilution with acetonitrile with 0.2% formic acid, and diluted 20x with water for analysis. Samples were analyzed by RapidFire-MS/MS as described in Example 5, Table 5.1. Glycosyltransferase variant polypeptides that produced rebaudioside M from rebaudioside A 60 with *in situ* synthesized ADP-glucose at greater quantities than SEQ ID NO: 36 were identified. The engineered polypeptides are listed in Table 8.1. Four additional variants with modified N-terminal DNA coding sequences but no amino acid mutations were also identified in SEQ ID NO: 277/278, 279/280, 281/282, and 283/284. Shake-flask scale cultures were grown, lysed, cleared, and lyophilized to powder as described in Example 1 for analysis of variants shown in Table 8.2 relative to SEQ ID NO: 36.

Table 8.1 β1,3GT Combinatorial Variants and RebM Levels		
SEQ ID NO: (nt/aa)	**Amino Acid Differences (Relative to SEQ ID NO: 36)**	**Increased RebM[a]**
169/170	M144Q/I207V/E259T/E285S/V451Q	+++
171/172	M144Q/S252P/E259T/A426I	+++
173/174	H81T/S111T/M144Q/I207V/E285S/V451Q	+++
175/176	M144Q/E285S/V451Q	+++
177/178	M144Q/I207V/S252P/E259T	+++
179/180	E73S/M144Q/I207V/E259T/E285S/V451Q	+++
181/182	M144Q/I207V/V451Q	+++
183/184	E73S/H81T/M144Q/I207V/E285S/V451Q	+++
185/186	M144Q/S252P/E259T/A426I/V451Q	+++
187/188	E73S/E259T/V451Q	+++
189/190	E73S/M144Q/I207V/E259T/E285S/A426I/V451Q	+++
191/192	H81T/M144Q/I207V/S252P/E285S/A426I	+++
193/194	H81T/M144Q/E259T/E285S	++
195/196	I207V/E259T/A426I/V451Q	++
197/198	M144Q/E259T/E285S	++
199/200	M144Q/I207V/E259T	++
201/202	E73S/M144Q/E259T/E285S	++
203/204	H81T/M144Q/I207V/E259T/V451Q	++
205/206	M144Q/E285S	++
207/208	E73S/H81T/M144Q/I207V/E259T	++
209/210	E73S/S111T/E285S/V451Q	++
211/212	E73S/M144Q/I207V/S252P/A426I/V451Q	++
213/214	M144Q/I207V/E285S/A426I	++
215/216	S111T/M144Q/E259T/E285S/A426I/V451Q	++
217/218	H81T/M144Q/E259T	++
219/220	H81T/M144Q/E259T/V451Q	++
221/222	H81T/I207V/S252P/E259T/V451Q	++
223/224	M144Q/I207V/E259T/A426I/V451Q	++
225/226	H81T/S111T/E259T/V451Q	++
227/228	E73S/M144Q/I207V/E259T/A426I/V451Q	+
229/230	E259T/E285S/V451Q	+
231/232	S111T/M144Q/S252P/E259T/E285S/V451Q	+
233/234	M144Q/S252P/E259T/E285S/V451Q	+

Table 8.1 β1,3GT Combinatorial Variants and RebM Levels		
SEQ ID NO: (nt/aa)	**Amino Acid Differences (Relative to SEQ ID NO: 36)**	**Increased RebM[a]**
235/236	E259T	+
237/238	E285S	+
239/240	M144Q/V451Q	+
241/242	S111T/M144Q/I207V/S252P/E285S/A426I/V451Q	+
243/244	E73S/E259T/A426I/V451Q	+
245/246	M144Q/S252P/E259T/E285S	+
247/248	I207V/E285S/A426I/V451Q	+
249/250	I207V/S252P/E259T/V451Q	+
251/252	H81T/E285S	+
253/254	H81T/M144Q/S252P/E259T/E285S/V451Q	+
255/256	I207V	+
257/258	H81T/I207V/E285S/A426I/V451Q	+
259/260	I207V/S252P/V451Q	+
261/262	E285S/V451Q	+
263/264	I207V/V451Q	+
265/266	I207V/E259T	+
267/268	E73S/H81T/E259T/E285S/A426I/V451Q	+
269/270	S252P/E259T	+
271/272	H81T/E285S/V451Q	+
273/274	S111T/I207V/E285S	+
275/276	E73S/H81T/E259T/V451Q	+

[a]Levels of increased production were determined relative to the reference polypeptide of SEQ ID NO: 36, and defined as follows: "+" = at least that of the reference but less than 1.28-fold increased production; "++" = at least 1.28-fold but less than 1.38-fold increased production relative to reference polypeptide; and "+++" = at least 1.38-fold increased production.

SFP Characterization Assay and Analysis for Glucosyl Transfer from Sucrose to ADP and then from ADP-Glucose to Stevioside and Rebaudioside D

[0216] Shake flask powders (SFP) were reconstituted to a concentration of 20 g/L and diluted to 0.005-0.15 g/L SFP in 100 µL total reaction volume of 50 mM potassium phosphate buffer, pH 6, 15 mM stevioside (>95% purity) or rebaudioside D, 0.02 g/L ADP (Amresco, ultra pure grade) co-substrate, 0.05 g/L SUS SFP SEQ ID NO: 22, and 37.5 mM sucrose (cane sugar). The reactions were incubated in a Thermotron® titre-plate shaker with 300 RPM shaking for 4 hours at 60°C. The

reactions were solubilized by 20x dilution with water, quenched by 5x dilution with acetonitrile with 0.2% formic acid, cleared by centrifugation, and diluted 15x with water for analysis. Glycosylated products were detected by SPE-QQQ as described in Example 5, Table 5.1. A one-pot reaction was conducted with 0.01-0.3 g/L SFP in 100 µL total reaction volume of 50 mM potassium phosphate buffer, pH 6, 20 g/L RebA60, 0.02 g/L ADP (Amresco, ultra pure grade) co-substrate, 0.03 g/L SUS SFP SEQ ID NO: 22, 0.08 g/L β1,2 GT SFP SEQ ID NO: 858, and 30 g/L sucrose (cane sugar). The reactions were incubated in a THERMOTRON® titre-plate shaker with 300 RPM shaking for 16 hours at 60°C. The reactions were solubilized by 20x dilution with water, quenched by 5x dilution with acetonitrile with 0.2% formic acid, cleared by centrifugation, and diluted 20x with water for analysis. Similar one-pot reactions were also performed with 10 µL of crude shake flask lysate after diluting 20-fold in 50 mM potassium phosphate buffer, pH 6, and incubating for 24 h at 75.2 °C.

Table 8.2 β1,3GT Shake Flask Powder Variants and RebA, RebM from RebD, and RebM from RebA60, and Thermostability Levels

SEQ ID NO: (nt/aa)	Amino Acid Differences (Relative to SEQ ID NO: 36)	Increased RebA[a]	Increased RebM[a]	Increased RebM (from A60)[a]	% Activity Remaining[b]
279/280	N/A	+	+	+	+
277/279	N/A	+	+	+	+
185/186	M144Q/S252P/E259T/A426I/V451Q	++	++	++	-
173/174	H81T/S111T/M144Q/I207V/E285S/V451Q	+++	+++	+++	-
169/107	M144Q/I207V/E259T/E285S/V451Q	+	+++	+++	-
171/172	M144Q/S252P/E259T/A426I	++	++	+++	+
175/176	M144Q/E285S/V451Q	++	+++	+++	-
177/178	M144Q/I207V/S252P/E259T	+	+++	+++	+

[a]Levels of increased production were determined relative to the reference polypeptide of SEQ ID NO: 36, at 0.019 g/L shake flask powder for single substrate and 0.04 g/L for one-pot and defined as follows: "-" = production less than that of the reference polypeptide; "+" = production at least 0.92-fold that of the reference but less than 1.2-fold reference polypeptide; "++" = at least 1.2-fold increased production but less than 1.4-fold; and "+++"= at least 1.4-fold increased production relative to reference polypeptide.

[b]The percent of activity remaining for each variant was determined following 24 h pre-incubation at 75.2°C, relative to the production from each variant following 24 h pre-incubation at 65°C and is

Table 8.2 β1,3GT Shake Flask Powder Variants and RebA, RebM from RebD, and RebM from RebA60, and Thermostability Levels					
SEQ ID NO: (nt/aa)	**Amino Acid Differences (Relative to SEQ ID NO: 36)**	**Increased RebA[a]**	**Increased RebM[a]**	**Increased RebM (from A60)[a]**	**% Activity Remaining[b]**
defined as follows: "-" = less than 40% of activity remained following 24 h pre-incubation at 75.2 °C; and "+" = at least 40% activity remained.					

[0217] In these experiments, the 2 N-terminally modified sequences in Table 8.2 performed the same as SEQ ID NO: 36, and the 6 combinatorial variants performed better on all three substrates. SEQ ID NO: 174 was selected as the starting point for further enzyme engineering, because of its superior performance on all three substrates.

EXAMPLE 9

Beta-1,3-ADP-Glycosyltransferase Variants of SEQ ID NO: 174

[0218] In this Example, experiments for evolution and screening of β1,3-glycosyltransferase (β1,3GT) polypeptides derived from SEQ ID NO: 174 for improved glucosylation of steviol glycosides using in situ synthesized ADP-glucose are described. Directed evolution of the GT encoded by SEQ ID NO: 173 (i.e., SEQ ID NO: 174) was carried out by constructing libraries of variant genes in which mutations associated with improved production identified during the development of the present invention were recombined and in which certain structural features were subjected to saturation mutagenesis. These libraries were then plated, grown, and screened using the high-throughput (HTP) assay described below to provide another round of 60 engineered GT variant polypeptides with glucosyltransferase activity toward ADP-glucose and steviol glycosides.

HTP Assay for Glucose Transfer from Sucrose to ADP and then from ADP-Glucose to Rebaudioside A 60

[0219] Assays were performed on 96-well plates of cleared *E. coli* culture lysates expressing SEQ ID NO: 173 variants (i.e., variants of SEQ ID NO: 174). Pellets were lysed, and lysate was cleared as described in Example 6, and then diluted 25-33.3x into 50 mM potassium phosphate buffer, pH 6. In order to thermally challenge the lysates, they were pre-incubated at 75°C in a THERMOTRON® titre-plate shaker with 300 RPM shaking for 2 h. Assays were conducted with 10 µL pre-incubated lysate in 100 µL reactions and with 20 g/L rebaudioside A 60% substrate, 0.02 g/L ADP (Amresco, ultra pure grade) co-substrate, 0.03 g/L SUS SFP SEQ ID NO: 1652, 0.08 g/L β-1,2-glycosyltransferase (β12GT) SEQ ID NO: 858, and 30 g/L sucrose (cane sugar). The following reaction conditions were used: 50 mM potassium phosphate buffer, pH 6, 60°C in a THERMOTRON® titre-plate shaker with 300 RPM shaking for 16 h. Reactions were solubilized by 20x dilution with water, quenched by 5x dilution with acetonitrile with 0.2% formic acid, and diluted 20x with water for analysis. Samples

were analyzed by RapidFire-MS/MS as described in Example 5, Table 5.1. Glycosyltransferase variant polypeptides that produced rebaudioside M from rebaudioside A 60 with *in situ* synthesized ADP-glucose at greater quantities than SEQ ID NO: 174 were identified. The engineered polypeptides are listed in Table 9.1 and Table 9.2. The saturation mutagenesis variants listed in Table 9.2 were additionally assayed under 4 more conditions. One was the same as the assay described above, except incubated for 16 h at 70 °C. Another was the same as above, except 40 g/L RebA60 was included in the pre-incubation potassium phosphate dilution buffer. The other two were single substrate reaction conditions in which clarified lysate was diluted 25 x into 50 mM potassium phosphate buffer, pH 6, pre-incubated at 75 °C for 2 h, and then diluted 10x into 100 µL reactions containing 15 mM steviol glycoside 95% or rebaudioside D, 30 g/L sucrose, 0.02 g/L ADP, 50 mM potassium phosphate pH 6, and 0.03 g/L SUS SFP SEQ ID NO: 1652. These reactions were incubated for 16 h at 60 °C, solubilized by diluting 20x in water, quenched by diluting 5x into acetonitrile with 0.2% formic acid, and precipitated by cold centrifugation. The supernatants were then diluted 15x in water and alyzed by RapidFire-MS/MS. Shake-flask scale cultures were grown, lysed, cleared, and lyophilized to powder as described in Example 1 for analysis of variants shown in Table 9.3 relative to SEQ ID NO: 174.

Table 9.1 β1,3GT Combinatorial Variants and RebM Levels

SEQ ID NO: (nt/aa)	Amino Acid Differences (Relative to SEQ ID NO: 174)	Increased RebM[a]
285/286	T80D/A85V/A251I/E259T	+++
287/288	A85V	+++
289/290	T80P/A85V	+++
291/292	A85V/A251M/S252P/L256G	+++
293/294	T80D/T81A/A85V	+++
295/296	T80D/T81A/A85V/A238G	+++
297/298	T80P/T81A/A85V/E259T	++
299/300	T80D/A85V/A251I/S252P/L256G/E259T	++
301/302	T80D/A85V/E259T	++
303/304	A85V/A238G	++
305/306	T81A/A85V/E259T	++
307/308	T80D/A85V	++
309/310	T80D/A85V/A251M	++
311/312	T80P/T81A/A251I/S252P/E259T	++
313/314	T80D/A85V/A238G	++
315/316	T80D/A85V/A251M/S252P	++

Table 9.1 β1,3GT Combinatorial Variants and RebM Levels		
SEQ ID NO: (nt/aa)	**Amino Acid Differences (Relative to SEQ ID NO: 174)**	**Increased RebM[a]**
317/318	T81A/A85V	+
319/320	T81A/A85V/A251M/S252P	+
321/322	T80D/A85V/A251M/S252P/L256G/E259T	+
323/324	T80P/A85V/A251M/S252P/E259T	+
325/326	T80D/E259T	+
327/328	T80D/T81A/A85V/A251M/E259T	+
329/330	E259T	+
331/332	Q270K/E402I	+
333/334	A85V/A251F	+
335/336	A251I/S252P/E259T	+
337/338	T80D/T81A	+
339/340	E420R	+
341/342	T80D/A85V/A238G/A251M/E259T	+
343/344	A85V/L256G/E259T	+
345/346	T80D/T81A/A85V/S252P/L256G/E259T	+
347/348	A85V/A251F/E259T	+
349/350	E175D/E402I	+
[a]Levels of increased production were determined relative to the reference polypeptide of SEQ ID NO: 174, and defined as follows: "+" = at least 1.12-fold that of the reference but less than 1.21-fold increased production; "++" = at least 1.21-fold but less than 1.24-fold increased production relative to reference polypeptide; and "+++" = at least 1.24-fold increased production.		

Table 9.2 β1,3GT Saturation Mutagenesis Variants and RebM Levels						
SEQ ID NO: (nt/aa)	**Amino Acid Differences (Relative to SEQ ID NO: 174)**	**Increased RebA[a]**	**Increased RebM[a]**	**Increased RebM (from A60, 60 °C)[a]**	**Increased RebM (from A60, 70 °C)[a]**	**Increased RebM (from A60, A60 preincubated)[a]**
351/352	G326E	++	++	+++	++	+++
353/354	G404V	++	++	+++	+++	+++
355/356	S443A	++	++	+++	++	+
357/358	S5L	+++	++	+++	+++	++
359/360	G326A	++	++	+++	++	++

Table 9.2 β1,3GT Saturation Mutagenesis Variants and RebM Levels						
SEQ ID NO: (nt/aa)	**Amino Acid Differences (Relative to SEQ ID NO: 174)**	**Increased RebA[a]**	**Increased RebM[a]**	**Increased RebM (from A60, 60 °C)[a]**	**Increased RebM (from A60, 70 °C)[a]**	**Increased RebM (from A60, A60 preincubated)[a]**
361/362	N3S	++	+	++	++	+++
363/364	S5T	++	+	++	+	++
365/366	M422I	+	++	++	++	++
367/368	S455M	++	+	++	++	++
369/370	T7A	++	+	++	+	++
371/372	V299M	+	+	++	-	-
373/374	G404M	+	++	++	++	++
375/376	G326S	++	+	++	++	+
377/378	V232I/Y317L	-	+	++	-	++
379/380	G326N	++	+	++	+	+
381/382	V393I	+	-	++	+	+
383/384	S5A	++	+	++	++	++
385/386	A153C	+	+	+	++	++
387/388	S5H	++	+	+	+	+
389/390	T7Q	+	+	+	+	+
391/392	A409S	++	+	+	+	-
393/394	Q451V	+	-	+	+	+
395/396	S455R	+	+	+	+++	+
397/398	Q451E	+	+	+	+	-
399/400	E99V	-	-	+	+	+
401/402	S252A	-	+	+	-	+
403/404	S273K	+	+	+	+	+

[a]Levels of increased production were determined relative to the reference polypeptide of SEQ ID NO: 174, and defined as follows: "-" = production less than that of the reference polypeptide, "+" = at least that of the reference but less than 1.1-fold increased production; "++" = at least 1.1-fold that of the reference but less than 1.2-fold; and "+++" at least 1.2-fold increased production relative to the reference.

SFP Characterization Assay and Analysis for Glucosyl Transfer from Sucrose to ADP and then from ADP-Glucose to Stevioside and Rebaudioside D

[0220] Shake flask powders (SFP) were reconstituted to a concentration of 20 g/L and diluted to 0.005-0.15 g/L SFP in 100 µL total reaction volume of 50 mM potassium phosphate buffer, pH 6, 15 mM stevioside (>95% purity) or rebaudioside D, 0.02 g/L ADP (Amresco, ultra pure grade) co-

substrate, 0.03 g/L SUS SFP SEQ ID NO: 1652, and 37.5 mM sucrose (cane sugar). The reactions were incubated in a THERMOTRON® titre-plate shaker with 300 RPM shaking for 4 hours at 60°C. The reactions were solubilized by 20x dilution with water, quenched by 5x dilution with acetonitrile with 0.2% formic acid, cleared by centrifugation, and diluted 15x with water for analysis. Glycosylated producs were detected by SPE-QQQ as described in Example 5, Table 5.1. A one-pot reaction was conducted with 0.01-0.3 g/L SFP in 100 µL total reaction volume of 50 mM potassium phosphate buffer, pH 6, 20 g/L RebA60, 0.02 g/L ADP (Amresco, ultra pure grade) co-substrate, 0.03 g/L SUS SFP SEQ ID NO: 1652, 0.08 g/L β1,2 GT SFP SEQ ID NO: 858, and 30 g/L sucrose (cane sugar). The reactions were incubated in a THERMOTRON® titre-plate shaker with 300 RPM shaking for 16 hours at 60°C. The reactions were solubilized by 20x dilution with water, quenched by 5x dilution with acetonitrile with 0.2% formic acid, cleared by centrifugation, and diluted 20x with water for analysis. Similar one-pot reactions were also performed with 10 µL of crude shake flask lysate after diluting 20-fold in 50 mM potassium phosphate buffer, pH 6, and incubating for 24 h at 75.2 °C.

Table 9.3 β1,3GT Shake Flask Powder Variants and RebA, RebM from RebD, and RebM from RebA60, and Thermostability Levels

SEQ ID NO: (nt/aa)	Amino Acid Differences (Relative to SEQ ID NO: 174)	Increased RebA, 60 °C[a]	Increased RebM, 60 °C[a]	Increased RebM (from A60), 60 °C[a]	% Activity Remaining[b]
287/288	A85V	++	++	+++	++
285/286	T80D/A85V/A251I/E259T	++	++	+++	+++
291/292	A85V/A251M/S252P/L256G	-	++	+++	-
293/294	T80D/T81A/A85V	+	+	+++	+
289/290	T80P/A85V	++	-	++	++
349/350	E175D/E402I	+	-	-	+++
331/332	Q270K/E402I	-	-	-	-

[a]Levels of increased production were determined relative to the reference polypeptide of SEQ ID NO: 174, at 0.019 g/L shake flask powder for single substrate and 0.04 g/L for one-pot and defined as follows: "-" = production less than that of the reference polypeptide; "+" = production at least that of the reference but less than 1.1-fold reference polypeptide; "++" = at least 1.1-fold increased production but less than 1.2-fold; and "+++"= at least 1.2-fold increased production relative to reference polypeptide.

[b]The percent of activity remaining for each variant was determined following 24 h pre-incubation at 75.2°C, relative to the production from each variant following 24 h pre-incubation at 65 °C and is defined as follows: "-" = less than 20% of activity remained following 24 h pre-incubation at 75.2 °C; "+" = at least 20% activity remained, but less than 30% activity remaining; "++" = at least 30%

Table 9.3 β1,3GT Shake Flask Powder Variants and RebA, RebM from RebD, and RebM from RebA60, and Thermostability Levels					
SEQ ID NO: (nt/aa)	**Amino Acid Differences (Relative to SEQ ID NO: 174)**	**Increased RebA, 60 °C[a]**	**Increased RebM, 60 °C[a]**	**Increased RebM (from A60), 60 °C[a]**	**% Activity Remaining[b]**
activity remained, but less than 40% activity remaining; and "+++" = at least 40% activity remained.					

[0221] In these experiments, five variants in Table 9.3 produced more rebaudioside M from rebaudioside A 60 than SEQ ID NO: 174, and four variants also produced more rebaudioside A from stevioside and of those three also produced more rebaudioside M from rebaudioside D. Several variants were significantly more thermostable than SEQ ID NO: 190. The most thermostable variant, SEQ ID NO: 350, was re-cloned with the best N-terminal DNA sequence from Example 8 (SEQ ID NO: 279/280) and this enzyme, SEQ ID NO: 406, was used as the starting point for further enzyme engineering.

EXAMPLE 10

Beta-1,3-ADP-Glycosyltransferase Variants of SEQ ID NO: 406

[0222] In this Example, experiments for evolution and screening of β1,3-glycosyltransferase (β1,3GT) polypeptides derived from SEQ ID NO: 406 for improved glucosylation of steviol glycosides using in situ synthesized ADP-glucose are described. Directed evolution of the GT encoded by SEQ ID NO: 405 (i.e., SEQ ID NO: 406) was carried out by constructing libraries of variant genes in which mutations associated with improved production identified during the development of the present invention were recombined. These libraries were then plated, grown, and screened using the high-throughput (HTP) assay described below to provide another round of 16 engineered GT variant polypeptides with glucosyltransferase activity toward ADP-glucose and steviol glycosides.

HTP Assay for Glucose Transfer from Sucrose to ADP and then from ADP-Glucose to Rebaudioside A 60

[0223] Assays were performed on 96-well plates of cleared *E. coli* culture lysates expressing SEQ ID NO: 405 variants (i.e., variants of SEQ ID NO: 406). Pellets were lysed, and lysate was cleared as described in Example 6, and then diluted 15x into 50 mM potassium phosphate buffer, pH 6 and separately the same buffer with 40 g/L rebaudioside A 60% substrate. In order to thermally challenge the lysates, they were pre-incubated at 75°C in a THERMOTRON® titre-plate shaker with 300 RPM shaking for 2 h. Assays were conducted with 10 µL pre-incubated lysate in 100 µL reactions and with 20 g/L rebaudioside A 60% substrate, 0.02 g/L ADP (Amresco, ultra pure grade) co-substrate, 0.03 g/L SUS SFP SEQ ID NO: 1822, 0.06 g/L β-1,2-glycosyltransferase (β12GT) SEQ ID NO: 994, and

30 g/L sucrose (cane sugar). The following reaction conditions were used: 50 mM potassium phosphate buffer, pH 6, 60°C in a THERMOTRON® titre-plate shaker with 300 RPM shaking for 16 h. Reactions were solubilized by 20x dilution with water, quenched by 5x dilution with acetonitrile with 0.2% formic acid, and diluted 20x with water for analysis. Samples were analyzed by RapidFire-MS/MS as described in Example 5, Table 5.1. Glycosyltransferase variant polypeptides that produced rebaudioside M from rebaudioside A 60 with *in situ* synthesized ADP-glucose at greater quantities than SEQ ID NO: 406 were identified. The engineered polypeptides are listed in Table 10.1.

Table 10.1 β1,3 GT Combinatorial Variants and RebM Levels

SEQ ID NO: (nt/aa)	Amino Acid Differences (Relative to SEQ ID NO: 406)	Increased RebM[a]	Increased RebM (A60 preincubated)[a]
407/408	A85V/E259T	++	++
409/410	A85V/D175E/E259T	++	+++
411/412	A85V/D175E/A251I/E259T/I402E	++	++
413/414	A85V	++	++
415/416	A85V/D175E/E259T/I402E	++	+++
417/418	A85V/D175E/I402E	++	+
419/420	T81A/A85V/A251I/E259T	++	++
421/422	T81A/A85V/A251I	++	++
423/424	T81A/A85V/E259T/I402E	++	++
425/426	T81A/A85V/D175E/E259T/I402E	++	+
427/428	A85V/I402E	++	++
429/430	A85V/E259T/I402E	++	++
431/432	T81A/A85V	++	++
433/434	A85V/D175E	++	++
435/436	A85V/D175E/A251I	+	++
437/438	T81A/A85V/E259T	+	++

[a]Levels of increased production were determined relative to the reference polypeptide of SEQ ID NO: 406, and defined as follows: "+" = at least 1.1-fold that of the reference but less than 1.25-fold increased production; "++" = at least 1.25-fold but less than 1.5-fold increased production relative to reference polypeptide; and "+++" = at least 1.5-fold increased production.

[0224] From the variants listed in Table 10.1, SEQ ID NO: 408 was selected on the basis of the improvement in stability to pre-incubation in potassium phosphate buffer with and without rebaudioside A 60% and the benefit associated with the two mutations A85V and E259T as the starting point for further enzyme engineering.

EXAMPLE 11

Beta-1,3-ADP-Glycosyltransferase Variants of SEQ ID NO: 408

[0225] In this Example, experiments for evolution and screening of β1,3-glycosyltransferase (β1,3GT) polypeptides derived from SEQ ID NO: 408 for improved glucosylation of steviol glycosides using in situ synthesized ADP-glucose are described. Directed evolution of the GT encoded by SEQ ID NO: 407 (i.e., SEQ ID NO: 408) was carried out by constructing libraries of variant genes in which mutations associated with improved production identified during the development of the present invention were recombined and in which certain structural features were subjected to saturation mutagenesis. These libraries were then plated, grown, and screened using the high-throughput (HTP) assay described below to provide another round of 39 engineered GT variant polypeptides with glucosyltransferase activity toward ADP-glucose and steviol glycosides.

HTP Assay for Glucose Transfer from Sucrose to ADP and then from ADP-Glucose to Rebaudioside A 60

[0226] Assays were performed on 96-well plates of cleared *E. coli* culture lysates expressing SEQ ID NO: 407 variants (i.e., variants of SEQ ID NO: 408). Pellets were lysed, and lysate was cleared as described in Example 6, and then diluted 20x into 50 mM potassium phosphate buffer, pH 6 with 60 g/L of rebaudioside A 60%. In order to thermally challenge the lysates, they were pre-incubated at 75°C in a THERMOTRON® titre-plate shaker with 300 RPM shaking for 2 h. Assays were conducted with 10 µL pre-incubated lysate in 100 µL reactions and with 20 g/L rebaudioside A 60% substrate, 0.02 g/L ADP (Amresco, ultra pure grade) co-substrate, 0.03 g/L SUS SFP SEQ ID NO: 1822, 0.06 g/L β-1,2-glycosyltransferase (β12GT) SEQ ID NO: 1080, and 30 g/L sucrose (cane sugar). The following reaction conditions were used: 50 mM potassium phosphate buffer, pH 6, 60°C in a THERMOTRON® titre-plate shaker with 300 RPM shaking for 16 h. Reactions were solubilized by 20x dilution with water, quenched by 5x dilution with acetonitrile with 0.2% formic acid, and diluted 20x with water for analysis. Samples were analyzed by RapidFire-MS/MS as described in Example 5, Table 5.1. Glycosyltransferase variant polypeptides that produced rebaudioside M from rebaudioside A 60 with *in situ* synthesized ADP-glucose at greater quantities than SEQ ID NO: 408 were identified. The engineered polypeptides are listed in Table 11.1 and Table 11.2. Shake-flask scale cultures were grown, lysed, cleared, and lyophilized to powder as described in Example 1 for analysis of variants shown in Table 11.3 relative to SEQ ID NO: 408.

Table 11.1 β1,3GT Combinatorial Variants and RebM Levels		
SEQ ID NO: (nt/aa)	**Amino Acid Differences (Relative to SEQ ID NO: 408)**	**Increased RebM[a]**
439/440	A153C	+++
441/442	A153C/G326S/S443A	++
443/444	A153C/G326S	++
445/446	V232I/V393I/Q451E	++
447/448	G326E	++
449/450	V232I	+
451/452	G404V	+
453/454	A153C/G326S/S443A/S455M	+
455/456	G326S	+
457/458	Q451E	+
459/460	V232I/S273K/V299M	+
461/462	V299M/Q451E	+
[a]Levels of increased production were determined relative to the reference polypeptide of SEQ ID NO: 408, and defined as follows: "+" = at least 1.1-fold that of the reference but less than 1.15-fold increased production; "++" = at least 1.15-fold but less than 1.3-fold increased production relative to reference polypeptide; and "+++" = at least 1.3-fold increased production.		

Table 11.2 β1,3GT Saturation Mutagenesis Variants and RebM Levels		
SEQ ID NO: (nt/aa)	**Amino Acid Differences (Relative to SEQ ID NO: 408)**	**Increased RebM[a]**
463/464	D170P	+++
465/466	D170G	+++
467/468	A300S	+++
469/470	S146G	+++
471/472	R412A	+++
473/474	D170C	++
475/476	E116R	++
477/478	D170T	++
479/480	D170S	++
481/482	R412K	++
483/484	S227T	++
485/486	A438Q	++

Table 11.2 β1,3GT Saturation Mutagenesis Variants and RebM Levels		
SEQ ID NO: (nt/aa)	**Amino Acid Differences (Relative to SEQ ID NO: 408)**	**Increased RebM[a]**
487/488	E330T	++
489/490	K315T	+
491/492	E448H	+
493/494	N408C	+
495/496	T361C	+
497/498	E330G	+
499/500	R296V	+
501/502	E116D	+
503/504	F327Y	+
505/506	R449S	+
507/508	R173S	+
509/510	L25A	+
511/512	R412S	+
513/514	E116S	+
515/516	N408T	+
[a]Levels of increased production were determined relative to the reference polypeptide of SEQ ID NO: 408, and defined as follows: "+" = at least 1.10-fold that of the reference but less than 1.14-fold increased production; "++" = at least 1.14-fold but less than 1.19-fold increased production; and "+++" = greater than 1.19-fold increased production.		

SFP Characterization Assay and Analysis for Glucosyl Transfer from Sucrose to ADP and then from ADP-Glucose to Stevioside and Rebaudioside D

[0227] Shake flask powders (SFP) were reconstituted to a concentration of 20 g/L and diluted to 0.005-0.15 g/L SFP in 100 µL total reaction volume of 50 mM potassium phosphate buffer, pH 6, 15 mM stevioside (>95% purity) or rebaudioside D, 0.02 g/L ADP (Amresco, ultra pure grade) co-substrate, 0.03 g/L SUS SFP SEQ ID NO: 1822, and 37.5 mM sucrose (cane sugar). The reactions were incubated in a THERMOTRON® titre-plate shaker with 300 RPM shaking for 4 hours at 60°C. The reactions were solubilized by 20x dilution with water, quenched by 5x dilution with acetonitrile with 0.2% formic acid, cleared by centrifugation, and diluted 15x with water for analysis. Glycosylated products were detected by SPE-QQQ as described in Example 5, Table 5.1. A one-pot reaction was conducted with 0.01-0.3 g/L SFP in 100 µL total reaction volume of 50 mM potassium phosphate buffer, pH 6, 20 g/L RebA60, 0.02 g/L ADP (Amresco, ultra pure grade) co-substrate, 0.03 g/L SUS SFP SEQ ID NO: 1822, 0.06 g/L β1,2 GT SFP SEQ ID NO: 1080, and 30 g/L sucrose (cane sugar). One set of SFP dilutions was pre-incubated at 75 °C for 2 h in 50 mM potassium phosphate

buffer, pH 6, while another set was not preincubated. The reactions were incubated in a THERMOTRON® titre-plate shaker with 300 RPM shaking for 16 hours at 60 °C. The reactions were solubilized by 20x dilution with water, quenched by 5x dilution with acetonitrile with 0.2% formic acid, cleared by centrifugation, and diluted 20x with water for analysis. Similar one-pot reactions were also performed with 10 μL of crude shake flask lysate after diluting 20-fold in 50 mM potassium phosphate buffer, pH 6, and incubating for 24 h at 75.2 °C.

Table 11.3 β1,3GT Shake Flask Powder Variants and RebA, RebM from RebD, and RebM from RebA60, and Thermostability Levels

SEQ ID NO: (nt/aa)	Amino Acid Differences (Relative to SEQ ID NO: 408)	Increased RebA[a]	Increased RebM[a]	Increased RebM (from A60)[a]	Increased RebM (from A60, A60 preincubated)[a]	% Activity Remaining[b]
439/440	A153C	++	++	+	+	++
449/450	V232I	++	+	+	++	+
445/446	V232I/V393I/ Q451E	+++	+	++	++	-
457/458	Q451E	+++	+	+	+	-

[a]Levels of increased production were determined relative to the reference polypeptide of SEQ ID NO: 408, at 0.009 g/L shake flask powder for single substrate and 0.02 g/L for one-pot and defined as follows: "+" = production less than 1.1-fold that of the reference; "++" = production at least 1.1-fold that of the reference but less than 1.25-fold reference polypeptide; and "+++" = at least 1.25-fold increased production relative to reference polypeptide.

[b]The percent of activity remaining for each variant was determined following 24 h pre-incubation at 75.2°C, relative to the production from each variant following 24 h pre-incubation at 65 °C and is defined as follows: "-" = less than 30% of activity remained following 24 h pre-incubation at 75.2 °C; "+" = at least 30% activity remained, but there was less than 50% activity remaining; and "++" = at least 50% activity remained.

[0228] In these experiments, all four variants in Table 11.3 produced more rebaudioside M from rebaudioside A 60 than SEQ ID NO: 408, more rebaudioside A from stevioside, and more rebaudioside M from rebaudioside D. The most thermostable variant, SEQ ID NO: 440, was used as the starting point for further enzyme engineering.

EXAMPLE 12

Beta-1,3-ADP-Glycosyltransferase Variants of SEQ ID NO: 440

[0229] In this Example, experiments for evolution and screening of β1,3-glycosyltransferase (β1,3GT) polypeptides derived from SEQ ID NO: 440 for improved glucosylation of steviol

glycosides using in situ synthesized ADP-glucose are described. Directed evolution of the GT encoded by SEQ ID NO: 439 (i.e., SEQ ID NO: 440) was carried out by constructing libraries of variant genes in which mutations associated with improved production identified during the development of the present invention were recombined and in which certain structural features were subjected to saturation mutagenesis. These libraries were then plated, grown, and screened using the high-throughput (HTP) assay described below to provide another round of 36 engineered GT variant polypeptides with glucosyltransferase activity toward ADP-glucose and steviol glycosides.

HTP Assay for Glucose Transfer from Sucrose to ADP and then from ADP-Glucose to Rebaudioside A 60

[0230] Assays were performed on 96-well plates of cleared *E. coli* culture lysates expressing SEQ ID NO: 440 variants. Pellets were lysed, and lysate was cleared as described in Example 6, and then diluted 20x into 50 mM potassium phosphate buffer, pH 6 with 80 g/L of rebaudioside A 60%. In order to thermally challenge the lysates, they were pre-incubated at 75°C in a THERMOTRON® titre-plate shaker with 300 RPM shaking for 2 h. Assays were conducted with 10 µL pre-incubated lysate in 100 µL reactions and with 20 g/L rebaudioside A 60% substrate, 0.02 g/L ADP (Amresco, ultra pure grade) co-substrate, 0.03 g/L SUS SFP SEQ ID NO: 1822, 0.06 g/L β-1,2-glycosyltransferase (β12GT) SEQ ID NO: 1080, and 30 g/L sucrose (cane sugar). The following reaction conditions were used: 50 mM potassium phosphate buffer, pH 6, 60°C in a THERMOTRON® titre-plate shaker with 300 RPM shaking for 16 h. Reactions were solubilized by 20x dilution with water, quenched by 5x dilution with acetonitrile with 0.2% formic acid, and diluted 20x with water for analysis. Samples were analyzed by RapidFire-MS/MS as described in Example 5, Table 5.1. Glycosyltransferase variant polypeptides that produced rebaudioside M from rebaudioside A 60 with *in situ* synthesized ADP-glucose at greater quantities than SEQ ID NO: 440 were identified. The engineered polypeptides are listed in Table 12.1 and Table 12.2. Shake-flask scale cultures were grown, lysed, cleared, and lyophilized to powder as described in Example 1 for analysis of variants shown in Table 12.3 relative to SEQ ID NO: 440.

Table 12.1 β1,3 GT Combinatorial Variants and RebM Levels

SEQ ID NO: (nt/aa)	Amino Acid Differences (Relative to SEQ ID NO: 440)	Increased RebM[a]
517/518	S146G/D170S/V232I	+++
519/520	S146G/D170T/W196I/V232I	+++
521/522	S146G/D170S/V232I/R423K/E448H/Q451E/S455M	+++
523/524	S146G/W196I/V232I	+++
525/526	S146G/W196I/V232I/T259E/E448H/Q451E	++
527/528	S146G/D170T/W196I/V232I/Q451E/S455R	++

Table 12.1 β1,3 GT Combinatorial Variants and RebM Levels		
SEQ ID NO: (nt/aa)	**Amino Acid Differences (Relative to SEQ ID NO: 440)**	**Increased RebM[a]**
529/530	S146G/D170T/V232I/R423K	++
531/532	S146G/V232I/K315S/R423K/Q451E/S455M	++
533/534	S146G/V232I/G326E	++
535/536	S146G/D170T/W196I/V232I/R423K	++
537/538	S146G/D170S/W196I	+
539/540	S146G/V232I/Q451E	+
541/542	S146G/V232I/E448H	+
543/544	S146G/W196I	+
545/546	S146G/V413M/Q451E/S455M	+
547/548	S146G/V232I/K315S	+
549/550	S146G/D170T/T259E	+
551/552	S146G/V232I/T259E/S455M	+
553/554	S146G/W196I/S455R	+
555/556	S146G/D170T/W196I/N408T/Q451E	+
[a]Levels of increased production were determined relative to the reference polypeptide of SEQ ID NO: 440, and defined as follows: "+" = at least 1.36-fold that of the reference but less than 1.52-fold increased production; "++" = at least 1.52-fold but less than 1.65-fold increased production relative to reference polypeptide; and "+++" = at least 1.65-fold increased production.		

Table 12.2 β1,3GT Saturation Mutagenesis Variants and RebM Levels		
SEQ ID NO: (nt/aa)	**Amino Acid Differences (Relative to SEQ ID NO: 440)**	**Increased RebM[a]**
557/558	E209Y	+++
559/560	D204M	+++
561/562	A107C	+++
563/564	K289A	++
565/566	L161E	++
567/568	V9L	++
569/570	R12Q	++
571/572	P417V	++
573/574	E169T	+
575/576	S337W	+
577/578	C156S	+

Table 12.2 β1,3GT Saturation Mutagenesis Variants and RebM Levels		
SEQ ID NO: (nt/aa)	**Amino Acid Differences (Relative to SEQ ID NO: 440)**	**Increased RebM[a]**
579/580	P131A	+
581/582	S337N	+
583/584	A199S	+
585/586	Q233K	+
587/588	S262A	+
[a]Levels of increased production were determined relative to the reference polypeptide of SEQ ID NO: 440, and defined as follows: "+" = at least 1.07-fold that of the reference but less than 1.12-fold increased production; "++" = at least 1.12-fold that of the reference but less than 1.16-fold increased production; and "+++" = at least 1.16-fold increased production.		

SFP Characterization Assay and Analysis for Glucosyl Transfer from Sucrose to ADP and then from ADP-Glucose to Stevioside and Rebaudioside D

[0231] Shake flask powders (SFP) were reconstituted to a concentration of 20 g/L and diluted to 0.005-0.15 g/L SFP in 100 µL total reaction volume of 50 mM potassium phosphate buffer, pH 6, 15 mM stevioside (>95% purity) or rebaudioside D, 0.02 g/L ADP (Amresco, ultra pure grade) co-substrate, 0.03 g/L SUS SFP SEQ ID NO: 1822, and 37.5 mM sucrose (cane sugar). The reactions were incubated in a Thermotron® titre-plate shaker with 300 RPM shaking for 4 hours at 60°C. The reactions were solubilized by 20x dilution with water, quenched by 5x dilution with acetonitrile with 0.2% formic acid, cleared by centrifugation, and diluted 15x with water for analysis. Glycosylated products were detected by SPE-QQQ as described in Example 5, Table 5.1. A one-pot reaction was conducted with 0.01-0.3 g/L SFP in 100 µL total reaction volume of 50 mM potassium phosphate buffer, pH 6, 20 g/L RebA60, 0.02 g/L ADP (Amresco, ultra pure grade) co-substrate, 0.03 g/L SUS SFP SEQ ID NO: 1822, 0.06 g/L β1,2GT SFP SEQ ID NO: 1080, and 30 g/L sucrose (cane sugar). One set of SFP dilutions was pre-incubated at 75 °C for 2 h in 50 mM potassium phosphate buffer, pH 6 with 80 g/L rebaudioside A 60%, while another set was not preincubated. The reactions were incubated in a THERMOTRON® titre-plate shaker with 300 RPM shaking for 16 hours at 60 °C. The reactions were solubilized by 20x dilution with water, quenched by 5x dilution with acetonitrile with 0.2% formic acid, cleared by centrifugation, and diluted 20x with water for analysis. Similar one-pot reactions were also performed with 10 µL of 1.5 mg/mL shake flask powder in 50 mM potassium phosphate buffer, pH 6, that had been incubated 24 h at 78.5 °C.

Table 12.3 β1,3 GT Shake Flask Powder Variants and RebA, RebM from RebD, and RebM from RebA60, and Thermostability Levels						
SEQ ID NO: (nt/aa)	**Amino Acid Differences (Relative to SEQ ID NO: 440)**	**Increased RebA[a]**	**Increased RebM[a]**	**Increased RebM (from A60)[a]**	**Increased RebM (from A60, A60 preincubated)[a]**	**% Activity Remaining[b]**
541/542	S146G/V232I/E448H	-	+	+	+	-
537/538	S146G/D170S/W196I	-	+	+++	++	-
523/524	S146G/W196I/V232I	-	+	+++	++	-
525/526	S146G/W196I/V232I/T259E/E448H/Q451E	-	+	++	+	-
527/528	S146G/D170T/W196I/V232I/Q451E/S455R	-	++	+++	+++	++
519/520	S146G/D170T/W196I/V232I	-	++	+++	+++	-
517/518	S146G/D170S/V232I	-	++	++	++	+
521/522	S146G/D170S/V232I/R423K/E448H/Q451E/S455M	-	++	++	++	-

[a]Levels of increased production were determined relative to the reference polypeptide of SEQ ID NO: 440, at 0.009 g/L shake flask powder for single substrate and 0.02 g/L for one-pot and defined as follows: "-" = production less than 0.9-fold that of the reference polypeptide; "+" = production at least 0.9-fold that of the reference but less than 1.25-fold reference polypeptide; "++" = at least 1.25-fold increased production but less than 1.5-fold; and "+++"= at least 1.5-fold increased production relative to reference polypeptide.

[b]The percent of activity remaining for each variant was determined following 24 h pre-incubation at 78.5°C, relative to the production from each variant following 24 h pre-incubation at 65 °C and is defined as follows: "-" = less than 50% of activity remained following 24 h pre-incubation at 78.5

Table 12.3 β1,3 GT Shake Flask Powder Variants and RebA, RebM from RebD, and RebM from RebA60, and Thermostability Levels						
SEQ ID NO: (nt/aa)	**Amino Acid Differences (Relative to SEQ ID NO: 440)**	**Increased RebA[a]**	**Increased RebM[a]**	**Increased RebM (from A60)[a]**	**Increased RebM (from A60, A60 preincubated)[a]**	**% Activity Remaining[b]**
°C; "+" = at least 50% activity remained, but less than 60% activity remaining; and "++" = at least 60% activity remained.						

[0232] In these experiments, all eight variants in Table 12.3 produced more rebaudioside M from rebaudioside A 60 and from rebaudioside D than SEQ ID NO: 440. All of the variants were also less active in catalyzing glucosylation of stevioside to form rebaudioside A. The variant with the most activity on all three substrates, SEQ ID NO: 520, was used as the starting point for further enzyme engineering.

EXAMPLE 13

Beta-1,3-ADP-Glycosyltransferase Variants of SEQ ID NO: 520

[0233] In this Example, experiments for evolution and screening of β1,3-glycosyltransferase (β1,3GT) polypeptides derived from SEQ ID NO: 520 for improved glucosylation of steviol glycosides using in situ synthesized ADP-glucose are described. Directed evolution of the GT encoded by SEQ ID NO: 519 (i.e., SEQ ID NO: 520) was carried out by constructing libraries of variant genes in which mutations associated with improved production identified during the development of the present invention were recombined and in which certain structural features were subjected to saturation mutagenesis. These libraries were then plated, grown, and screened using the high-throughput (HTP) assay described below to provide another round of 44 engineered GT variant polypeptides with glucosyltransferase activity toward ADP-glucose and steviol glycosides.

HTP Assay for Glucose Transfer from Sucrose to ADP and then from ADP-Glucose to Rebaudioside A 60

[0234] Assays were performed on 96-well plates of cleared *E. coli* culture lysates expressing SEQ ID NO: 519 variants (i.e., variants of SEQ ID NO 520). Pellets were lysed, and lysate was cleared as described in Example 6, and then diluted 49x into 50 mM potassium phosphate buffer, pH 6 with 100 g/L of rebaudioside A 60%. In order to thermally challenge the lysates, they were pre-incubated at 75°C in a THERMOTRON® titre-plate shaker with 300 RPM shaking for 2 h. Assays were conducted with 10 µL pre-incubated lysate in 100 µL reactions and with 40 g/L rebaudioside A 60% substrate, 0.02 g/L ADP (Amresco, ultra pure grade) co-substrate, 0.06 g/L SUS SFP SEQ ID NO: 2182, 0.12 g/L β-1,2-glycosyltransferase (β12GT) SEQ ID NO: 1216, and 60 g/L sucrose (cane sugar). The following reaction conditions were used: 50 mM potassium phosphate buffer, pH 6, 60°C in a

THERMOTRON® titre-plate shaker with 300 RPM shaking for 16 h. Reactions were solubilized by 40x dilution with water, quenched by 5x dilution with acetonitrile with 0.2% formic acid, and diluted 20x with water for analysis. Samples were analyzed by RapidFire-MS/MS as described in Example 5, Table 5.1. Glycosyltransferase variant polypeptides that produced rebaudioside M from rebaudioside A 60 with *in situ* synthesized ADP-glucose at greater quantities than SEQ ID NO: 520 were identified. The engineered polypeptides are listed in Table 13.1 and Table 13.2. Shake-flask scale cultures were grown, lysed, cleared, and lyophilized to powder as described in Example 1 for analysis of variants shown in Table 13.3 relative to SEQ ID NO: 520.

Table 13.1 β1,3GT Combinatorial Variants and RebM Levels

SEQ ID NO: (nt/aa)	Amino Acid Differences (Relative to SEQ ID NO: 520)	Increased RebM[a]
589/590	D204M/Q451E/S455R	+++
591/592	V9L/R12Q/A199S/D204M/E209Y/S337N/S455R	+++
593/594	V9L/D204M	+++
595/596	L161E/D204M/P417V	+++
597/598	V9L/D204M/T259E/K289A	+++
599/600	V9L/R12Q/L161E/E169T/A199S/E209Y/S337W	++
601/602	V9L/A107C/C156S/L161E/A199S/D204M/P417V/S455R	++
603/604	A107C/A199S/E209Y/T259E/Q451E/S455R	++
605/606	Q451E/S455R	++
607/608	V9L/E169T/D204M/K289A/S337W/Q451E/S455R	++
609/610	V9L/P131A/C156S/E209Y/K289A/S337N	++
611/612	V9L/R12Q/A107C/L161E/A199S/E209Y/S337N	++
613/614	C156S/E169T/A199S/D204M/E209Y/T259E/K289A	++
615/616	K289A	+
617/618	R12Q/V14I/A107C/D204M/K289A/S455R	+
619/620	V9L/A107C/L161E/E209Y/T259E/K289A/Q451E/S455R	+
621/622	V9L/P131A/D204M/S337N/Q451E	+
623/624	V9L/S337W	+
625/626	A107C/A199S/D204M/K289A	+
627/628	R12Q/A107C/C156S/E209Y/K289A/S455R	+
629/630	V9L/R12Q/A199S/E209Y/S337W/Q451E/S455R	+
631/632	V9L/C156S/E169T/D204M/S337N	+
633/634	R12Q/A107C/P131A/D204M/K289A/S337W/P417V/Q451E/S455R	+
635/636	V9L/A107C/P131A/D204M/T259E/P417V/Q451E	+

Table 13.1 β1,3GT Combinatorial Variants and RebM Levels		
SEQ ID NO: (nt/aa)	**Amino Acid Differences (Relative to SEQ ID NO: 520)**	**Increased RebM[a]**
637/638	A107C/D204M	+
639/640	A107C/L161E/E169T/A199S/D204M/T259E/Q451E	+
[a]Levels of increased production were determined relative to the reference polypeptide of SEQ ID NO: 520, and defined as follows: "+" = at least 1.15-fold that of the reference but less than 1.25-fold increased production; "++" = at least 1.25-fold but less than 1.36-fold increased production relative to reference polypeptide; and "+++" = at least 1.36-fold increased production.		

Table 13.2 β1,3GT Saturation Mutagenesis Variants and RebM Levels		
SEQ ID NO: (nt/aa)	**Amino Acid Differences (Relative to SEQ ID NO: 520)**	**Increased RebM[a]**
641/642	L257A	++
643/644	K430Q	++
645/646	V413L	++
647/648	K336R	++
649/650	T111C	++
651/652	I410V	++
653/654	T80A	+
655/656	K53Q	+
657/658	F221A	+
659/660	T111S	+
661/662	L16M	+
663/664	H95W	+
665/666	L78V	+
667/668	A426S	+
669/670	L391R	+
671/672	I349V	+
673/674	T54P	+
675/676	T111G	+
[a]Levels of increased production were determined relative to the reference polypeptide of SEQ ID NO: 520, and defined as follows: "+" = at least 1.02-fold that of the reference but less than 1.1-fold increased production; and "++" = at least 1.1-fold increased production.		

SFP Characterization Assay and Analysis for Glucosyl Transfer from Sucrose to ADP and then from ADP-Glucose to Stevioside and Rebaudioside D

[0235] Shake flask powders (SFP) were reconstituted to a concentration of 20 g/L and diluted to 0.005-0.15 g/L SFP in 100 µL total reaction volume of 50 mM potassium phosphate buffer, pH 6, 15 mM stevioside (>95% purity) or rebaudioside D, 0.02 g/L ADP (Amresco, ultra pure grade) co-substrate, 0.03 g/L SUS SFP SEQ ID NO: 2182, and 37.5 mM sucrose (cane sugar). The reactions were incubated in a THERMOTRON® titre-plate shaker with 300 RPM shaking for 4 hours at 60°C. The reactions were solubilized by 20x dilution with water, quenched by 5x dilution with acetonitrile with 0.2% formic acid, cleared by centrifugation, and diluted 15x with water for analysis. Glycosylated products were detected by SPE-QQQ as described in Example 5, Table 5.1. A one-pot reaction was conducted with 0.01-0.3 g/L SFP in 100 µL total reaction volume of 50 mM potassium phosphate buffer, pH 6, 40 g/L RebA60, 0.02 g/L ADP (Amresco, ultra pure grade) co-substrate, 0.03 g/L SUS SFP SEQ ID NO: 2182, 0.12 g/L β1,2 GT SFP SEQ ID NO: 1216, and 60 g/L sucrose (cane sugar). One set of SFP dilutions was pre-incubated at 75 °C for 2 h in 50 mM potassium phosphate buffer, pH 6 with 100 g/L rebaudioside A 60%, while another set was not preincubated. The reactions were incubated in a THERMOTRON® titre-plate shaker with 300 RPM shaking for 16 hours at 60 °C. The reactions were solubilized by 40x dilution with water, quenched by 5x dilution with acetonitrile with 0.2% formic acid, cleared by centrifugation, and diluted 20x with water for analysis. Similar one-pot reactions were also performed with 10 µL of crude clarified lysate that had been diluted 25x in 50 mM potassium phosphate buffer, pH 6, with 100 g/L rebaudioside A 60% and incubated 24 h at 75.2 °C.

Table 13.3 β1,3 GT Shake Flask Powder Variants and RebA, RebM from RebD, and RebM from RebA60, and Thermostability Levels

SEQ ID NO: (nt/aa)	Amino Acid Differences (Relative to SEQ ID NO: 520)	Increased RebA[a]	Increased RebM[a]	Increased RebM (from A60)[a]	Increased RebM (from A60, A60 preincubated)[a]	% Activity Remaining[b]
625/626	A107C/A199S /D204M/K289 A	+++	+	++	++	+
617/618	R12Q/V14I/A 107C/D204M/ K289A/S455R	+++	+	++	++	+
589/590	D204M/Q451 E/S455R	++	+	++	+++	+

Table 13.3 β1,3 GT Shake Flask Powder Variants and RebA, RebM from RebD, and RebM from RebA60, and Thermostability Levels

SEQ ID NO: (nt/aa)	Amino Acid Differences (Relative to SEQ ID NO: 520)	Increased RebA[a]	Increased RebM[a]	Increased RebM (from A60)[a]	Increased RebM (from A60, A60 preincubated)[a]	% Activity Remaining[b]
591/592	V9L/R12Q/A199S/D204M/E209Y/S337N/S455R	+++	+	+	++	-
639/640	A107C/L161E/E169T/A199S/D204M/T259E/Q451E	+++	+	+	++	-
593/594	V9L/D204M	+	+	+	++	-
609/610	V9L/P131A/C156S/E209Y/K289A/S337N	+	+	+	++	+

[a]Levels of increased production were determined relative to the reference polypeptide of SEQ ID NO: 520, at 0.02 g/L shake flask powder for single substrate and 0.04 g/L for one-pot and defined as follows: "+" = production at least that of the reference but less than 1.1-fold reference polypeptide; "++" = at least 1.1-fold increased production but less than 1.25-fold; and "+++"= at least 1.25-fold increased production relative to reference polypeptide.

[b]The percent of activity remaining for each variant was determined following 24 h pre-incubation at 75.2°C, relative to the production from each variant following 24 h pre-incubation at 65 °C and is defined as follows: "-" = less than 30% of activity remained following 24 h pre-incubation at 65 °C; and "+" = at least 30% activity remained.

[0236] In these experiments, all seven variants in Table 13.3 produced more rebaudioside M from rebaudioside A 60 and from rebaudioside D and more rebaudioside A from stevioside than SEQ ID NO: 520. Four variants had similar thermostability to SEQ ID NO: 520. The variant with the most activity on stevioside and RebA60 without pre-incubation, SEQ ID NO: 626, was used as the starting point for further enzyme engineering.

EXAMPLE 14

Beta-1,3-ADP-Glycosyltransferase Variants of SEQ ID NO: 626

[0237] In this Example, experiments for evolution and screening of β1,3-glycosyltransferase (β1,3GT) polypeptides derived from SEQ ID NO: 626 for improved glucosylation of steviol glycosides using in situ synthesized ADP-glucose are described. Directed evolution of the GT encoded by SEQ ID NO: 625 (i.e., SEQ ID NO: 626) was carried out by constructing libraries of variant genes in which mutations associated with improved production identified during the development of the present invention were recombined and in which certain structural features were subjected to saturation mutagenesis. These libraries were then plated, grown, and screened using the high-throughput (HTP) assay described below to provide another round of 43 engineered GT variant polypeptides with glucosyltransferase activity toward ADP-glucose and steviol glycosides.

HTP Assay for Glucose Transfer from Sucrose to ADP and then from ADP-Glucose to Rebaudioside A 60

[0238] Assays were performed on 96-well plates of cleared *E. coli* culture lysates expressing SEQ ID NO: 625 variants (i.e., variants of SEQ ID NO: 626). Pellets were lysed, and lysate was cleared as described in Example 6, and then diluted 100x into 50 mM potassium phosphate buffer, pH 6 with 100 g/L of rebaudioside A 60%. In order to thermally challenge the lysates, they were pre-incubated at 75°C in a THERMOTRON® titre-plate shaker with 300 RPM shaking for 2 h. Assays were conducted with 10 µL pre-incubated lysate in 100 µL reactions and with 40 g/L rebaudioside A 60% substrate, 0.02 g/L ADP (Amresco, ultra pure grade) co-substrate, 0.06 g/L SUS SFP SEQ ID NO: 2182, 0.09 g/L β-1,2-glycosyltransferase (β12GT) SEQ ID NO: 1488, and 60 g/L sucrose (cane sugar). The following reaction conditions were used: 50 mM potassium phosphate buffer, pH 6, 60°C in a THERMOTRON® titre-plate shaker with 300 RPM shaking for 16 h. Reactions were solubilized by 40x dilution with water, quenched by 5x dilution with acetonitrile with 0.2% formic acid, and diluted 20x with water for analysis. Samples were analyzed by RapidFire-MS/MS as described in Example 5, Table 5.1. Glycosyltransferase variant polypeptides that produced rebaudioside M from rebaudioside A 60 with *in situ* synthesized ADP-glucose at greater quantities than SEQ ID NO: 626 were identified. The engineered polypeptides are listed in Table 14.1 and Table 14.2. Shake-flask scale cultures were grown, lysed, cleared, and lyophilized to powder as described in Example 1 for analysis of variants shown in Table 14.3 relative to SEQ ID NO: 626.

Table 14.1 β1,3GT Combinatorial Variants and RebM Levels

SEQ ID NO: (nt/aa)	Amino Acid Differences (Relative to SEQ ID NO: 626)	Increased RebM[a]
693/694	L16M/F221A/I410V	+++
695/696	L16M/T80A/F221A/L257A/K336R/I410V	+++
681/682	L16M/F221A/L257A	+++

Table 14.1 β1,3GT Combinatorial Variants and RebM Levels		
SEQ ID NO: (nt/aa)	**Amino Acid Differences (Relative to SEQ ID NO: 626)**	**Increased RebM[a]**
683/684	L16M/T111S/F221A	+++
685/686	T80A/T111S/F221A/L257A/I410V	++
687/688	F221A	++
689/690	L16M/F221A/L257A/K336R/L391R	++
691/692	L16M/L257A	++
693/694	L16M/F221A	++
695/696	T111S/F221A/L257A/L391R	++
697/698	L16M	+
699/700	L16M/T80A/T111C	+
701/702	L16M/T80A/F221A/K336R/I410V	+
703/704	L16M/T80A	+
705/706	F221A/L257A	+
707/708	L16M/L257A/K336R/V413L/E420G	+
709/710	T111S/F221A/L257A/K336R/L391R	+
711/712	L16M/T80A/L257A	+
713/714	L16M/T111C/F221A/L257A/L391R	+
715/716	L16M/T80A/T111S/L257A	+
717/718	L16M/H59Q/T80A/V413L	+

[a]Levels of increased production were determined relative to the reference polypeptide of SEQ ID NO: 626, and defined as follows: "+" = at least 1.15-fold that of the reference but less than 1.22-fold increased production; "++" = at least 1.22-fold but less than 1.28-fold increased production relative to reference polypeptide; and "+++" = at least 1.28-fold increased production.

Table 14.2 β1,3GT Saturation Mutagenesis Variants and RebM Levels				
SEQ ID NO: (nt/aa)	**Amino Acid Differences (Relative to SEQ ID NO: 626)**	**Increased RebA[a]**	**Increased RebM[a]**	**Increased RebM (from A60)[a]**
719/720	S194P	+	+++	+++
721/722	S5N	+	+++	+++
723/724	S5M	+	+++	+++
725/726	S5L	+	+++	+++
727/728	A118V	+	++	++
729/730	V286L	+	++	++

Table 14.2 β1,3GT Saturation Mutagenesis Variants and RebM Levels				
SEQ ID NO: (nt/aa)	**Amino Acid Differences (Relative to SEQ ID NO: 626)**	**Increased RebA[a]**	**Increased RebM[a]**	**Increased RebM (from A60)[a]**
731/732	S254T	+	+++	++
733/734	E418D	+	+++	++
735/736	K102R	+	++	++
737/738	A118S	+	++	++
739/740	Q91V	+	+	++
741/742	V14L	+	+	+
743/744	E99S	+	+	+
745/746	E99L	+	+	+
747/748	D416E	+	+	+
749/750	S254G	+	+	+
751/752	T7L	+	++	+
753/754	L138I	+	+	+
755/756	S254V	+	+	+
757/758	S5Q	+	+	+
759/760	E420A	+	+	+
761/762	I65V	+	+	+
[a]Levels of increased production were determined relative to the reference polypeptide of SEQ ID NO: 626, and defined as follows: "+" = at least 0.95-fold that of the reference but less than 1.15-fold increased production; "++" = at least 1.15-fold that of the reference but less than 1.3-fold increased production; and "+++" = at least 1.3-fold increased production.				

SFP Characterization Assay and Analysis for Glucosyl Transfer from Sucrose to ADP and then from ADP-Glucose to Stevioside and Rebaudioside D

[0239] Shake flask powders (SFP) were reconstituted to a concentration of 20 g/L and diluted to 0.005-0.15 g/L SFP in 100 µL total reaction volume of 50 mM potassium phosphate buffer, pH 6, 15 mM stevioside (>95% purity) or rebaudioside D, 0.02 g/L ADP (Amresco, ultra pure grade) co-substrate, 0.03 g/L SUS SFP SEQ ID NO: 2182, and 37.5 mM sucrose (cane sugar). The reactions were incubated in a THERMOTRON® titre-plate shaker with 300 RPM shaking for 4 hours at 60°C. The reactions were solubilized by 20x dilution with water, quenched by 5x dilution with acetonitrile with 0.2% formic acid, cleared by centrifugation, and diluted 15x with water for analysis. Glycosylated products were detected by SPE-QQQ as described in Example 5, Table 5.1. A one-pot reaction was conducted with 0.01-0.3 g/L SFP in 100 µL total reaction volume of 50 mM potassium phosphate buffer, pH 6, 50 g/L RebA60, 0.025 g/L ADP (Amresco, ultra pure grade) co-substrate, 0.075 g/L SUS SFP SEQ ID NO: 2182, 0.09 g/L β1,2 GT SFP SEQ ID NO: 1488, and 75 g/L sucrose

(cane sugar). One set of SFP dilutions was pre-incubated at 75 °C for 2 h in 50 mM potassium phosphate buffer, pH 6 with 100 g/L rebaudioside A 60%, while another set was not preincubated. The reactions were incubated in a THERMOTRON® titre-plate shaker with 300 RPM shaking for 16 hours at 60 °C. The reactions were solubilized by 50x dilution with water, quenched by 5x dilution with acetonitrile with 0.2% formic acid, cleared by centrifugation, and diluted 20x with water for analysis. Similar one-pot reactions were also performed with 10 µL of crude clarified lysate that had been diluted 25x in 50 mM potassium phosphate buffer, pH 6, with 100 g/L rebaudioside A 60% and incubated 24 h at 75.2 °C.

Table 14.3 β1,3GT Shake Flask Powder Variants and RebA, RebM from RebD, and RebM from RebA60, and Thermostability Levels

SEQ ID NO: (nt/aa)	Amino Acid Differences (Relative to SEQ ID NO: 626)	Increased RebA[a]	Increased RebM[a]	Increased RebM (from A60)[a]	Increased RebM (from A60, A60 preincubated)[a]	% Activity Remaining[b]
687/688	F221A	+	-	-	+	+
681/682	L16M/F221A/ L257A	++	+	+	+	++
691/692	L16M/L257A	+	-	+	+	+
683/684	L16M/T111S/ F221A	-	-	-	-	+
679/680	L16M/T80A/F 221A/L257A/ K336R/I410V	+++	-	+	+	+++
685/686	T80A/T111S/ F221A/L257A /I410V	++	-	+	+	+
677/678	L16M/F221A/ I410V	++	+	+	++	++

Table 14.3 β1,3GT Shake Flask Powder Variants and RebA, RebM from RebD, and RebM from RebA60, and Thermostability Levels

SEQ ID NO: (nt/aa)	Amino Acid Differences (Relative to SEQ ID NO: 626)	Increased RebA[a]	Increased RebM[a]	Increased RebM (from A60)[a]	Increased RebM (from A60, A60 preincubated)[a]	% Activity Remaining[b]

[a]Levels of increased production were determined relative to the reference polypeptide of SEQ ID NO: 626, at 0.02 g/L shake flask powder for single substrate and 0.04 g/L for one-pot and defined as follows: "-" = production less than 0.9-fold that of the reference polypeptide; "+" = production at least 0.9-fold that of the reference but less than 1.15-fold reference polypeptide; "++" = at least 1.15-fold increased production but less than 1.3-fold; and "+++"= at least 1.3-fold increased production relative to reference polypeptide.

[b]The percent of activity remaining for each variant was determined following 24 h pre-incubation at 75.2 °C, relative to the production from each variant following 24 h pre-incubation at 65 °C and is defined as follows: "+" = at least 27% of activity remained following 24 h pre-incubation at 75.2 °C but less than 57%; "+" = at least 57% of activity remained but less than 70%; and "+++" = at least 70% activity remained.

[0240] In these experiments, five variants in Table 14.3 produced more rebaudside M from rebaudioside A 60 than SEQ ID NO: 626. Of these, one variant, SEQ ID NO: 678, was also improved for catalyzing the glucosylation of stevioside to rebaudioside A and rebaudioside D to rebaudioside M, as well as more thermostable than SEQ ID NO: 626. This active and thermostable variant, SEQ ID NO: 678, was used as the starting point for further enzyme engineering.

EXAMPLE 15

Beta-1,3-ADP-Glycosyltransferase Variants of SEQ ID NO: 678

[0241] In this Example, experiments for evolution and screening of β1,3-glycosyltransferase (β1,3GT) polypeptides derived from SEQ ID NO: 678 for improved glucosylation of steviol glycosides using in situ synthesized ADP-glucose are described. Directed evolution of the GT encoded by SEQ ID NO: 677 (i.e., SEQ ID NO: 678) was carried out by constructing libraries of variant genes in which mutations associated with improved production identified during the development of the present invention were recombined. These libraries were then plated, grown, and screened using the high-throughput (HTP) assay described below to provide another round of 47 engineered GT variant polypeptides with glucosyltransferase activity toward ADP-glucose and steviol glycosides.

HTP Assay for Glucose Transfer from Sucrose to ADP and then from ADP-Glucose to Rebaudioside A 60

[0242] Assays were performed on 96-well plates of cleared *E. coli* culture lysates expressing SEQ ID NO: 677 variants (i.e., variants of SEQ ID NO: 678). Pellets were lysed, and lysate was cleared as described in Example 6, and then diluted 70x into 50 mM potassium phosphate buffer, pH 6 with 100 g/L of rebaudioside A 60%. In order to thermally challenge the lysates, they were pre-incubated at 75°C in a THERMOTRON® titre-plate shaker with 300 RPM shaking for 2 h. Assays were conducted with 10 µL pre-incubated lysate in 100 µL reactions and with 50 g/L rebaudioside A 60% substrate, 0.025 g/L ADP (Amresco, ultra pure grade) co-substrate, 0.075 g/L SUS SFP SEQ ID NO: 2322, 0.09 g/L β-1,2-glycosyltransferase (β12GT) SEQ ID NO: 1488, and 75 g/L sucrose (cane sugar). The following reaction conditions were used: 50 mM potassium phosphate buffer, pH 6, 60°C in a THERMOTRON® titre-plate shaker with 300 RPM shaking for 16 h. Reactions were solubilized by 50x dilution with water, quenched by 5x dilution with acetonitrile with 0.2% formic acid, and diluted 20x with water for analysis. Samples were analyzed by RapidFire-MS/MS as described in Example 5, Table 5.1. Glycosyltransferase variant polypeptides that produced rebaudioside M from rebaudioside A 60 with *in situ* synthesized ADP-glucose at greater quantities than SEQ ID NO: 678 were identified. The engineered polypeptides are listed in Table 15.1. Shake-flask scale cultures were grown, lysed, cleared, and lyophilized to powder as described in Example 1 for analysis of variants shown in Table 15.2 relative to SEQ ID NO: 678.

Table 15.1 β1,3 GT Combinatorial Variants and RebM Levels

SEQ ID NO: (nt/aa)	Amino Acid Differences (Relative to SEQ ID NO: 678)	Increased RebM[a]
763/764	S5N/Q91V/S194P/E418D	+++
765/766	S5N/Q91V/A118S/V286L	+++
767/768	S5N/Q91V/S106I/S254T/V286L	+++
769/770	S5N/A118S/S194P/V286L/E418D	+++
771/772	S5N/S194P	+++
773/774	Q91V/A118V/S194P	+++
775/776	S5N/Q91V	+++
777/778	S5N/Q91V/S254T/V286L	+++
779/780	V14L/E99L	+++
781/782	S5N/Q91V/A118V/S194P/S254T/V286L	++
783/784	S5N/A118V/S254T	++
785/786	T7L/V14L/I65V/E99L	++
787/788	Q91V/A118V/S194P/V286L	++
789/790	V14L/E99L/S254G	++
791/792	S5N/Q91V/S194P/S254T	++

Table 15.1 β1,3 GT Combinatorial Variants and RebM Levels		
SEQ ID NO: (nt/aa)	**Amino Acid Differences (Relative to SEQ ID NO: 678)**	**Increased RebM[a]**
793/794	A118V/V286L/E418D	++
795/796	S5N/K102R/E418D	++
797/798	V14L/S455R	++
799/800	V14L/E99S/D416E	++
801/802	S5N/A118S/V286L	++
803/804	V14L/D167E	++
805/806	S5L/V14L/E99L	++
807/808	V14L/E99L/S254G/D416E/S455R	++
809/810	Q91V/A118V/V286L	+
811/812	S5N/Q91V/A118V/S194P/E418D	+
813/814	S5N/Q91V/K102R/S254T/E418D	+
815/816	V14L/E99L/D416E	+
817/818	V14L/S254G	+
819/820	V14L	+
821/822	V14L/I65V/E99L/D416E	+
823/824	S5N/Q91V/A118S/S254T/V286L/E418D	+
825/826	T7L/V14L/E99S/D416E	+
827/828	S5N/Q91V/A118S/S194P/S254T/E418D	+
829/830	S5N/Q91V/K102R/A118V	+
831/832	Q91V/A118V	+
833/834	Q91V/K102R/S194P	+
835/836	V14L/E99L/S254G/D416E	+
837/839	Q91V	+
841/840	S5N	+
841/842	T7L/V14L	+
843/844	Q91V/S194P/S254T/V286L	+
845/846	S5N/Q91V/S254T	+
847/848	S5N/E418D	+
849/850	Q91V/A118V/S194P/E418D	+
851/852	S5L/V14L/D416E	+
853/854	Q91V/V286L	+
855/856	S5N/S254T/V286L	+

Table 15.1 β1,3 GT Combinatorial Variants and RebM Levels		
SEQ ID NO: (nt/aa)	**Amino Acid Differences (Relative to SEQ ID NO: 678)**	**Increased RebM[a]**
[a]Levels of increased production were determined relative to the reference polypeptide of SEQ ID NO: 678, and defined as follows: "+" = at least 1.32-fold that of the reference but less than 1.46-fold increased production; "++" = at least 1.46-fold but less than 1.62-fold increased production relative to reference polypeptide; and "+++" = at least 1.62-fold increased production.		

SFP Characterization Assay and Analysis for Glucosyl Transfer from Sucrose to ADP and then from ADP-Glucose to Stevioside and Rebaudioside D

[0243] Shake flask powders (SFP) were reconstituted to a concentration of 20 g/L and diluted to 0.0125-0.2 g/L SFP in 100 µL total reaction volume of 50 mM potassium phosphate buffer, pH 6, 15 mM stevioside (>95% purity) or rebaudioside D or rebaudioside E (prepared in house from stevioside treated with β1,2GT), 0.02 g/L ADP (Amresco, ultra pure grade) co-substrate, 0.025 g/L SUS SFP SEQ ID NO: 2322, and 37.5 mM sucrose (cane sugar). The reactions were incubated in a THERMOTRON® titre-plate shaker with 300 RPM shaking for 4 hours at 60°C. The reactions were solubilized by 20x dilution with water, quenched by 5x dilution with acetonitrile with 0.2% formic acid, cleared by centrifugation, and diluted 15x with water for analysis. Glycosylated products were detected by SPE-QQQ as described in Example 5, Table 5.1. A one-pot reaction was conducted with 0.0125-0.2 g/L SFP in 100 µL total reaction volume of 50 mM potassium phosphate buffer, pH 6, 100 g/L RebA60, 0.05 g/L ADP (Amresco, ultra pure grade) co-substrate, 0.2 g/L SUS SFP SEQ ID NO: 2322, 0.2 g/L β1,2GT SFP SEQ ID NO: 1516, and 150 g/L sucrose (cane sugar). One set of SFP dilutions was pre-incubated at 75 °C for 2 h in 50 mM potassium phosphate buffer, pH 6 with 100 g/L rebaudioside A 60%, while another set was not preincubated. The reactions were incubated in a THERMOTRON® titre-plate shaker with 300 RPM shaking for 16 hours at 60 °C. The reactions were solubilized by 100x dilution with water, quenched by 5x dilution with acetonitrile with 0.2% formic acid, cleared by centrifugation, and diluted 20x with water for analysis. Similar one-pot reactions were also performed with 10 µL of crude clarified lysate that had been diluted 25x in 50 mM potassium phosphate buffer, pH 6, with 100 g/L rebaudioside A 60% and incubated 24 h at 75.2 °C.

Table 15.2 β1,3 GT Shake Flask Powder Variants and RebA; RebM from RebD, RebE, and RebA60; and Thermostability Levels

SEQ ID NO: (nt/ aa)	Amino Acid Differences (Relative to SEQ ID NO: 678)	Increased RebA[a]	Increased RebM (from RebE)[a]	Increased RebM (from RebD)[a]	Increased RebM (from A60)[a]	Increased RebM (from A60, A60 pre-incubated)[a]	% Activity Remain-ing[b]
767/768	S5N/Q91V/S106I/S254T/V286L	+	+++	+	++	+++	++
765/766	S5N/Q91V/A118S/V286L	-	+++	+	++	+++	-
777/778	S5N/Q91V/S254T/V286L	-	++	+	+	++	-
819/820	V14L	+	-	+	-	+	-
779/780	V14L/E99L	+	+	+	+	+	+
821/822	V14L/I65V/E99L/D416E	+	-	-	-	+	+
789/790	V14L/E99L/S254G	+	+	+	+	+	+

[a]Levels of increased production were determined relative to the reference polypeptide of SEQ ID NO: 678, at 0.025 g/L shake flask powder and defined as follows: "-" = production less than 0.9-fold that of the reference polypeptide; "+" = production at least 0.9-fold that of the reference but less than 1.1-fold reference polypeptide; "++" = at least 1.1-fold increased production but less than 1.3-fold; and "+++"= at least 1.3-fold increased production relative to reference polypeptide.

[b]The percent of activity remaining for each variant was determined following 24 h pre-incubation at 75.2 °C, relative to the production from each variant following 24 h pre-incubation at 65 °C and is defined as follows: "-" = less than 30% of activity remained following 24 h pre-incubation at 75.2 °C; "+" = at least 30% activity remained, but there was less than 39% activity remaining; and "++" = at least 39% activity remained.

[0244] In these experiments, three variants in Table 15.2 produced more rebaudioside M from rebaudioside A 60 than SEQ ID NO: 678. Of these, one variant, SEQ ID NO: 768, was also at least as thermostable as SEQ ID NO: 678. Therefore, SEQ ID NO: 768 was selected as the best enzyme for the catalysis of glycosyltransfer from ADP-glucose to stevioside and rebaudioside D for the formation of rebaudioside A and rebaudioside M, respectively.

EXAMPLE 16

Beta-1,2-ADP-Glycosyltransferase Variants of SEQ ID NO: 24

[0245] In this Example, experiments for evolution and screening of GT polypeptides derived from SEQ ID NO: 24, for improved glucosylation of steviol glycosides using ADP-glucose are described. Directed evolution of the GT encoded by SEQ ID NO: 23 was carried out by constructing libraries of variant genes. Libraries recombined mutations associated with improved production identified during the development of the present invention and subjected certain structural features of the enzyme with saturation mutagenesis. These libraries were then plated, grown, and screened using the HTP assay described below to provide another round of engineered GT variant polypeptides with glucosyltransferase activity toward ADP-glucose and steviol glycosides. Sixteen engineered variants were identified from the combinatorial libraries (Table 16.1), and fifty-one were identified from the saturation mutagenesis libraries (Table 16.2).

HTP Coupled Assay for Glucose Transfer from Sucrose to ADP to Rebaudioside A 60

[0246] Assays were performed on 96-well plates of cleared *E. coli* culture lysates expressing SEQ ID NO: 23 variants (i.e., variants of SEQ ID NO: 24). Lysis buffer volume was 400 µL, and the lysate was diluted 150-fold into 50 mM potassium phosphate, pH 6.0, and pre-incubated for 1 h at 75°C. Assays were then conducted with 10 µL diluted lysate, 0.03 g/L SUS SFP SEQ ID NO: 22, and 0.1 g/L β-1,3-glycosyltransferase (β1,3GT) SFP SEQ ID NO: 20, in 100 µL reaction volume with 20 g/L rebaudioside A 60% (RebA60) substrate, 0.02 g/L ADP (Amresco, ultra pure grade) co-substrate, and 30 g/L sucrose. The following reaction conditions were used: 50 mM potassium phosphate buffer, pH 6, 60°C in a THERMOTRON® titre-plate shaker with 300 RPM shaking for 16-18 h. The reactions were solubilized by 20x dilution into water, quenched by 5x dilution into acetonitrile with 0.2% formic acid, precipitated by centrifugation, and diluted 20x into water for analysis as described above. The resulting engineered variants with glucosyltransferase activity on RebA60 are listed in Table 16.1 and 16.2. Shake-flask scale cultures were grown, lysed, and lyophilized to powder for variants listed in Table 16.3.

Table 16.1 β1,2 GT Combinatorial Variants and RebM Levels

SEQ ID NO: (nt/aa)	Amino Acid Differences (Relative to SEQ ID NO: 24)	Increased RebM[a]
857/858	L16T/Q127V/P169E	+++
859/860	L16T/R423L/R427S	+++
861/862	L16T/P169E	+++
863/864	L16T/P169E/A398M/Q399K	++
865/866	R423L/R427S	++
867/868	L16T	++
869/870	L16T/P169E/R423L	++
871/872	L16T/R423L	++

Table 16.1 β1,2 GT Combinatorial Variants and RebM Levels		
SEQ ID NO: (nt/aa)	**Amino Acid Differences (Relative to SEQ ID NO: 24)**	**Increased RebM[a]**
873/874	A134S	+
875/876	Q399K/R423L	+
877/878	L16T/A398M/R427S	+
879/880	P143G	+
881/882	L16T/P143G/R423L	+
883/884	L16T/A134S	+
885/886	L16T/A398M	+
887/888	L16T/A398M/Q399K	+
[a]Levels of increased production were determined relative to the reference polypeptide of SEQ ID NO: 24, and defined as follows: "+" = production at least 1.15-fold that of the reference but less than 1.18-fold reference polypeptide; "++" = at least 1.18-fold increased production but less than 1.21-fold; and "+++" = at least 1.21-fold increased production.		

Table 16.2 β1,2GT Saturation Mutagenesis Variants and RebM Levels		
SEQ ID NO: (nt/aa)	**Amino Acid Differences (Relative to SEQ ID NO: 24)**	**Increased RebM[a]**
889/890	Y44E	+++
891/892	C443G	+++
893/894	H164A	+++
895/896	P233S	+++
897/898	E221D	+++
899/900	H164E	+++
901/902	I248M	+++
903/904	R446L	+++
905/906	Q137T	+++
907/908	S421G	+++
909/910	H3I	++
911/912	P60T	++
913/914	K56A	++
915/916	I392V	++
917/918	I440M	++
919/920	L322R	++
921/922	G281S	++
923/924	V400C	++

Table 16.2 β1,2GT Saturation Mutagenesis Variants and RebM Levels		
SEQ ID NO: (nt/aa)	**Amino Acid Differences (Relative to SEQ ID NO: 24)**	**Increased RebM[a]**
925/926	N433G	++
927/928	A426S	++
929/930	H164Q	++
931/932	R427W	++
933/934	H3V	++
935/936	F158V	++
937/938	F41M	++
939/940	F158H	+
941/942	I139T	+
943/944	L122C	+
945/946	R427A	+
947/948	V8R	+
949/950	S285A	+
951/952	L135V	+
953/954	Q301G	+
955/956	P232A	+
957/958	P233T	+
959/960	H164S	+
961/962	C443H	+
963/964	Q235M	+
965/966	Q11L	+
967/968	G138K	+
969/970	L284V	+
971/972	D249R	+
973/974	N433T	+
975/976	A372S	+
977/978	H164N	+
979/980	G138R	+
981/982	I440L	+
983/984	R176S	+
985/986	S421A	+
987/988	Q235L	+
989/990	Q11P	+

Table 16.2 β1,2GT Saturation Mutagenesis Variants and RebM Levels		
SEQ ID NO: (nt/aa)	**Amino Acid Differences (Relative to SEQ ID NO: 24)**	**Increased RebM[a]**
[a]Levels of increased production were determined relative to the reference polypeptide of SEQ ID NO: 24, and defined as follows: "+" = production at least that of the reference but less than 1.06-fold reference polypeptide; "++" = at least 1.06-fold increased production but less than 1.18-fold; and "+++" = at least 1.18-fold increased production.		

Shake Flask Powder Characterization Assay and Analysis for Glucosyl Transfer from Sucrose to ADP to Rebaudioside A 60

[0247] Shake flask powders (SFP) were reconstituted to a concentration of 4 g/L and diluted to 0.0013-0.04 g/L in 100 µL total reaction volume containing 50 mM potassium phosphate buffer, pH 6, 20 g/L RebA97 (single substrate) or RebA60 (one-pot), 0.02 g/L ADP, 20 (single substrate) or 30 g/L (one-pot) sucrose, 0.04 g/L SUS SFP SEQ ID NO: 22, and, for the one-pot reaction only, 0.15 g/L β-1,3-glycosyltransferase (β1,3GT) SFP SEQ ID NO: 36. The reactions were performed at 60 °C in a THERMOTRON® titre-plate shaker at 300 RPM for 4 h (single substrate) or 16-18 h (one-pot). One set of SFP dilutions was pre-incubated for 1 h at 75 °C in 50 mM potassium phosphate buffer, pH 6, while another set was not pre-incubated. The reactions were solubilized by 20x dilution with water, quenched by 5x dilution with acetonitrile with 0.2% formic acid, cleared by centrifugation, and diluted 20x with water for analysis. Glycosylated products were detected by SPE-QQQ as described in Example 5, Table 5.1. To assess thermostability, similar single substrate rebaudioside A reactions were also performed with 10 µL of crude clarified lysate that had been diluted 200x in 50 mM potassium phosphate buffer, pH 6, and incubated 24 h at 66.2 °C. The thermostability results and production levels of rebaudioside D in the single substrate and rebaudioside M in the one-pot reactions by these variants at 0.01 g/L SFP loading are shown in Table 16.3.

Table 16.3 β1,2GT Shake Flask Variants and RebD and RebM Levels					
SEQ ID NO: (nt/aa)	**Amino Acid Differences (Relative to SEQ ID NO: 24)**	**Increased RebD[a]**	**Increased RebM[a]**	**Increased RebM (pre-incubated)[a]**	**% Activity Remaining[b]**
867/868	L16T	++	++	+	++
857/858	L16T/Q127V/P169E	++	++	++	++
861/862	L16T/P169E	+	+	+	+

Table 16.3 β1,2GT Shake Flask Variants and RebD and RebM Levels					
SEQ ID NO: (nt/aa)	**Amino Acid Differences (Relative to SEQ ID NO: 24)**	**Increased RebD[a]**	**Increased RebM[a]**	**Increased RebM (pre-incubated)[a]**	**% Activity Remaining[b]**
[a]Levels of increased production were determined relative to the reference polypeptide of SEQ ID NO: 24, and defined as follows: "+" = production at least that of the reference but less than 1.1-fold reference polypeptide; and "++" = at least 1.1-fold increased production. [b]The percent of activity remaining for each variant was determined following 23 h pre-incubation at 66.2 °C, relative to the production from each variant following 24 h pre-incubation at 60 °C and is defined as follows: "+" = at least 50% but less than 60% of activity remained following 23 h pre-incubation at 66.2 °C and "++" = at least 60% activity remained.					

[0248] In these experiments, the three variants in Table 16.3 were improved relative to SEQ ID NO: 24, for catalyzing the glucosylation of rebaudioside A to rebaudioside D and for catalyzing the β1,2-glucosylations involved in converting rebaudioside A 60% to rebaudioside M. Two of these variants were also more thermostable than SEQ ID NO: 24. Of these, the variant that was most improved with pre-incubation, SEQ ID NO: 858, was selected as the starting point for further enzyme engineering.

EXAMPLE 17

Beta-1,2-ADP-Glycosyltransferase Variants of SEQ ID NO: 858

[0249] In this Example, experiments for evolution and screening of GT polypeptides derived from SEQ ID NO: 858, for improved glucosylation of steviol glycosides using ADP-glucose are described. Directed evolution of the GT encoded by SEQ ID NO: 857 was carried out by constructing libraries of variant genes. Libraries recombined mutations associated with improved production identified during the development of the present invention. These libraries were then plated, grown, and screened using the HTP assay described below to provide another round of engineered GT variant polypeptides with glucosyltransferase activity toward ADP-glucose and steviol glycosides. Forty-four engineered variants were identified from the combinatorial libraries (Table 17.1).

HTP Coupled Assay for Glucose Transfer from Sucrose to ADP to Rebaudioside A 60

[0250] Assays were performed on 96-well plates of cleared *E. coli* culture lysates expressing SEQ ID NO: 857 variants (i.e., variants of SEQ ID NO: 858). Lysis buffer volume was 400 µL, and the lysate was diluted 200-fold into 50 mM potassium phosphate, pH 6.0, and pre-incubated for 2 h at 75°C. Assays were then conducted with 10 µL diluted lysate, 0.03 g/L SUS SFP SEQ ID NO: 1652, and 0.1 g/L β-1,3-glycosyltransferase (β1,3GT) SFP SEQ ID NO: 174, in 100 µL reaction volume with 20 g/L rebaudioside A 60% (RebA60) substrate, 0.02 g/L ADP (Amresco, ultra pure grade) co-substrate, and 30 g/L sucrose. The following reaction conditions were used: 50 mM potassium phosphate buffer, pH

6, 60°C in a THERMOTRON® titre-plate shaker with 300 RPM shaking for 16-18 h. The reactions were solubilized by 20x dilution into water, quenched by 5x dilution into acetonitrile with 0.2% formic acid, precipitated by centrifugation, and diluted 20x into water for analysis as described above. The resulting engineered variants with glucosyltransferase activity on RebA60 are listed in Table 17.1. Shake-flask scale cultures were grown, lysed, and lyophilized to powder for variants listed in Table 17.2.

Table 17.1 β1,2GT Combinatorial Variants and RebM Levels

SEQ ID NO: (nt/aa)	Amino Acid Differences (Relative to SEQ ID NO: 858)	Increased RebM[a]
991/992	H3I/A22G/G198Q/S421G	+++
993/994	A22G/G198Q/I202Y/I248M/I392V	+++
995/996	A22G/K56A/S421G/R423L	+++
997/998	H164E/P233S/I331V/I440M	+++
999/1000	F125L/P233S/C443G/R446L	+++
1001/1002	A22G/K56A	+++
1003/1004	P233S	+++
1005/1006	A22G/S421G/R423L	+++
1007/1008	A22G/I392V	+++
1009/1010	A22G/K56A/Q137T/G198Q/G201A/I248M/L322R/I392V	++
1011/1012	L13Q/P233S/I331V/I440M	++
1013/1014	H3I/A22G/S421G	++
1015/1016	A22G/R423L	++
1017/1018	H164A/I331V	++
1019/1020	A22G/K56A/G198Q/I248M/S421G/R423L	++
1021/1022	H164E/E221D/P233S/I331V/I440M/R446L	++
1023/1024	A22G/K56A/G198Q/I248M/L322R/I392V/S421G	++
1025/1026	H164A/P233S/R446L	++
1027/1028	A22G/Q137T	++
1029/1030	A22G/K56A/G198Q/I202Y/I248M	++
1031/1032	C443G/R446L	++
1033/1034	A22G/I248M/I392V	++
1035/1036	G198Q/I202Y/I392V	+
1037/1038	A329L	+
1039/1040	A22G/K56A/Q137T/G198Q/I248M/I392V/S421G	+
1041/1042	H3I/G201A/I248M/L322R/I392V	+

Table 17.1 β1,2GT Combinatorial Variants and RebM Levels		
SEQ ID NO: (nt/aa)	**Amino Acid Differences (Relative to SEQ ID NO: 858)**	**Increased RebM[a]**
1043/1044	H3I/G201A/I248M/L322R	+
1045/1046	A22G/S421G	+
1047/1048	P233S/R446L	+
1049/1050	Y44E/H164E/P233S	+
1051/1052	H164A/I440M	+
1053/1054	A22G/K56A/Q137T/L322R	+
1055/1056	H164A/C443G	+
1057/1058	F125L	+
1059/1060	Y44E/H164A/P233S	+
1061/1062	S421G/R423L	+
1063/1064	A22G/K56A/Q137T/L322R/I392V/R423L	+
1065/1066	A22G/Q137T/G198Q/I202Y/I248M	+
1067/1068	A22G/I32L/K56A/P60T/G198Q/I248M/L322R/I392V/S421G	+
1069/1070	E221D/A329L/I331V	+
1071/1072	L13Q/I440M/C443G	+
1073/1074	A22G/P60T/I392V/S421G	+
1075/1076	Y44E/I331V	+
1077/1078	Y44E/H164E/P233S/A329L/I331V	+

[a]Levels of increased production were determined relative to the reference polypeptide of SEQ ID NO: 858, and defined as follows: "+" = production at least 1.22-fold that of the reference but less than 1.27-fold reference polypeptide; "++" = at least 1.27-fold increased production but less than 1.38-fold; and "+++" = at least 1.38-fold increased production.

Shake Flask Powder Characterization Assay and Analysis for Glucosyl Transfer from Sucrose to ADP to Rebaudioside A 60

[0251] Shake flask powders (SFP) were reconstituted to a concentration of 4 g/L and diluted to 0.0013-0.04 g/L in 100 µL total reaction volume containing 50 mM potassium phosphate buffer, pH 6, 20 g/L RebA97 (single substrate) or RebA60 (one-pot), 0.02 g/L ADP, 20 (single substrate) or 30 g/L (one-pot) sucrose, 0.03 g/L SUS SFP SEQ ID NO: 1652, and, for the one-pot reaction only, 0.1 g/L β-1,3-glycosyltransferase (β1,3GT) SFP SEQ ID NO: 174. The reactions were performed at 60 °C in a THERMOTRON® titre-plate shaker at 300 RPM for 4 h (single substrate) or 16-18 h (one-pot). One set of SFP dilutions was pre-incubated for 2 h at 75 °C in 50 mM potassium phosphate buffer, pH 6, while another set was not pre-incubated. The reactions were solubilized by 20x dilution with water, quenched by 5x dilution with acetonitrile with 0.2% formic acid, cleared by

centrifugation, and diluted 20x with water for analysis. Glycosylated products were detected by SPE-QQQ as described in Example 5, Table 5.1. To assess thermostability, similar single substrate rebaudioside A reactions were also performed with 10 µL of crude clarified lysate that had been diluted 70x in 50 mM potassium phosphate buffer, pH 6, and incubated 24 h at 71.8 °C. The thermostability results and production levels of rebaudioside D in the single substrate and rebaudioside M in the one-pot reactions by these variants at 0.005 g/L SFP loading are shown in Table 17.2.

Table 17.2 β1,2GT Shake Flask Variants and RebD and RebM Levels

SEQ ID NO: (nt/aa)	Amino Acid Differences (Relative to SEQ ID NO: 858)	Increased RebD[a]	Increased RebM[a]	Increased RebM (pre-incubated)[a]	% Activity Remaining[b]
997/998	H164E/P233S/I331V/I440M	+	++	+	++
1071/1072	L13Q/I440M/C443G	++	++	+	++
1021/1022	H164E/E221D/P233S/I331V /I440M/R446L	++	++	+	+
1047/1048	P233S/R446L	+	+	+	+
1027/1028	A22G/Q137T	++	++	+	-
993/994	A22G/G198Q/I202Y/I248M /I392V	+++	+++	+	+
1029/1030	A22G/K56A/G198Q/I202Y/ I248M	+++	+++	+	+
991/992	H3I/A22G/G198Q/S421G	+++	++	++	+++

[a]Levels of increased production were determined relative to the reference polypeptide of SEQ ID NO: 858 and defined as follows: "+" = production at least that of the reference but less than 1.3-fold reference polypeptide; "++" = at least 1.3-fold but less than 1.6-fold increased production; and "+++" = at least 1.6-fold increased production.

[b]The percent of activity remaining for each variant was determined following 23 h pre-incubation at 71.8 °C, relative to the production from each variant following 24 h pre-incubation at 65 °C and is defined as follows: "-" = less than 27% of activity remained following 23 h pre-incubation at 71.8 °C; "+" = at least 27% but less than 37% activity remained; "++" = at least 37% but less than 42% activity remained; and "+++" = at least 42% activity remained.

[0252] In these experiments, all eight variants in Table 17.2 were improved relative to SEQ ID NO: 858, for catalyzing the glucosylation of rebaudioside A to rebaudioside D and for catalyzing the β1,2-glucosylations involved in converting rebaudioside A 60% to rebaudioside M. The most active variant

on both substrates, SEQ ID NO: 994, was selected as the starting point for further enzyme engineering.

EXAMPLE 18

Beta-1,2-ADP-Glycosyltransferase Variants of SEQ ID NO: 994

[0253] In this Example, experiments for evolution and screening of GT polypeptides derived from SEQ ID NO: 994 for improved glucosylation of steviol glycosides using ADP-glucose are described. Directed evolution of the GT encoded by SEQ ID NO: 994 was carried out by constructing libraries of variant genes. Libraries recombined mutations associated with improved production identified during the development of the present invention and subjected certain structural features of the enzyme with saturation mutagenesis. These libraries were then plated, grown, and screened using the HTP assay described below to provide another round of engineered GT variant polypeptides with glucosyltransferase activity toward ADP-glucose and steviol glycosides. Nineteen engineered variants were identified from the combinatorial libraries (Table 18.1), and twenty-five were identified from the saturation mutagenesis libraries (Table 18.2).

HTP Coupled Assay for Glucose Transfer from Sucrose to ADP to Rebaudioside A 60

[0254] Assays were performed on 96-well plates of cleared *E. coli* culture lysates expressing SEQ ID NO: 994 variants (i.e., variants of SEQ ID NO: 994). Lysis buffer volume was 400 µL, and the lysate was diluted 100-fold into 50 mM potassium phosphate, pH 6.0 with and without 20 g/L (combinatorial libraries) or 30 g/L (saturation mutagenesis libraries) rebaudioside A 60%, and pre-incubated for 2 h at 75°C. Assays were then conducted with 10 µL diluted lysate, 0.03 g/L SUS SFP SEQ ID NO: 1822, and 0.1 g/L β-1,3-glycosyltransferase (β1,3GT) SFP SEQ ID NO: 350, in 100 µL reaction volume with 20 g/L rebaudioside A 60% (RebA60) substrate, 0.02 g/L ADP (Amresco, ultra pure grade) co-substrate, and 30 g/L sucrose. The following reaction conditions were used: 50 mM potassium phosphate buffer, pH 6, 60°C in a THERMOTRON® titre-plate shaker with 300 RPM shaking for 16-18 h. The reactions were solubilized by 20x dilution into water, quenched by 5x dilution into acetonitrile with 0.2% formic acid, precipitated by centrifugation, and diluted 20x into water for analysis as described above. The resulting engineered variants with glucosyltransferase activity on RebA60 are listed in Table 18.1 and 90.2. Shake-flask scale cultures were grown, lysed, and lyophilized to powder for variants listed in Table 18.3.

Table 18.1 β1,2GT Combinatorial Variants and RebM Levels

SEQ ID NO: (nt/aa)	Amino Acid Differences (Relative to SEQ ID NO: 994)	Increased RebM[a]	Increased RebM (A60 preincubated)[a]
1079/1080	H164E/Y202I	++	++
1081/1082	H83Y/Y202I/P233S	++	+

Table 18.1 β1,2GT Combinatorial Variants and RebM Levels			
SEQ ID NO: (nt/aa)	**Amino Acid Differences (Relative to SEQ ID NO: 994)**	**Increased RebM[a]**	**Increased RebM (A60 preincubated)[a]**
1083/1084	Y202I/P233S/M248I	++	+
1085/1086	R423L	++	+
1087/1088	Y202I/P233S/M248I/R423L	+	+
1089/1090	P233S	+	-
1091/1092	H164E/Y202I/P233S/I331V	+	+
1093/1094	M248I	+	+
1095/1096	H164E/R423L	+	+
1097/1098	Y202I/I331V	+	+
1099/1100	Y202I/P233S	+	+++
1101/1102	H164E	+	+
1103/1104	Y202I/S421G/R423L	+	+++
1105/1106	Y202I/M248I	+	+++
1107/1108	H164E/Y202I/I331V/R423L	+	+++
1109/1110	Y202I/R423L	+	+++
1111/1112	H164E/Y202I/I331V	+	+++
1113/1114	Y202I/R446L	+	++
1115/1116	H164E/Y202I/M248I/R272C	+	++

[a]Levels of increased production were determined relative to the reference polypeptide of SEQ ID NO: 994, and defined as follows: "-" = production less than 0.9-fold that of the reference polypeptide; "+" = production at least 0.9-fold that of the reference but less than 1.5-fold reference polypeptide; "++" = at least 1.5-fold increased production but less than 3-fold; and "+++" = at least 3-fold increased production.

Table 18.2 β1,2GT Saturation Mutagenesis Variants and RebM Levels			
SEQ ID NO: (nt/aa)	**Amino Acid Differences (Relative to SEQ ID NO: 994)**	**Increased RebM[a]**	**Increased RebM (A60 preincubated)[a]**
1117/1118	K422T	++	++
1119/1120	S54L	++	++
1121/1122	K422A	++	+++
1123/1124	K422V	++	+
1125/1126	L116I	++	++
1127/1128	K132R	++	+

Table 18.2 β1,2GT Saturation Mutagenesis Variants and RebM Levels			
SEQ ID NO: (nt/aa)	**Amino Acid Differences (Relative to SEQ ID NO: 994)**	**Increased RebM[a]**	**Increased RebM (A60 preincubated)[a]**
1129/1130	G7T	++	+
1131/1132	Y84L	++	++
1133/1134	S9L	++	+
1135/1136	S54M	+	+
1137/1138	D10P	+	+
1139/1140	K309E	+	+
1141/1142	A106S	+	+
1143/1144	E165P	+	++
1145/1146	S406Q	+	+
1147/1148	A73S	+	+
1149/1150	D389E	+	++
1151/1152	N286G	+	+++
1153/1154	N115A	+	+
1155/1156	S406H	+	++
1157/1158	E438A	+	+++
1159/1160	A73R	+	+
1161/1162	E438T	+	+++
1163/1164	None – N-terminal DNA expression variant	+	++
1165/1166	S406M	+	+
[a]Levels of increased production were determined relative to the reference polypeptide of SEQ ID NO: 994, and defined as follows: "+" = production at least 1.1-fold that of the reference but less than 1.18-fold reference polypeptide; "++" = at least 1.18-fold increased production but less than 1.27-fold; and "+++" = at least 1.27-fold increased production.			

Shake Flask Powder Characterization Assay and Analysis for Glucosyl Transfer from Sucrose to ADP to Rebaudioside A 60

[0255] Shake flask powders (SFP) were reconstituted to a concentration of 4 g/L and diluted to 0.0013-0.04 g/L in 100 µL total reaction volume containing 50 mM potassium phosphate buffer, pH 6, 20 g/L RebA97 (single substrate) or RebA60 (one-pot), 0.02 g/L ADP, 20 (single substrate) or 30 g/L (one-pot) sucrose, 0.03 g/L SUS SFP SEQ ID NO: 1822, and, for the one-pot reaction only, 0.1 g/L β-1,3-glycosyltransferase (β1,3GT) SFP SEQ ID NO: 350. The reactions were performed at 60 °C in a THERMOTRON® titre-plate shaker at 300 RPM for 4 h (single substrate) or 16-18 h (one-pot). One set of SFP dilutions was pre-incubated for 2 h at 75 °C in 50 mM potassium phosphate buffer, pH 6 with 10 g/L rebaudioside A 60 %, while another set was not pre-incubated. The reactions

were solubilized by 20x dilution with water, quenched by 5x dilution with acetonitrile with 0.2% formic acid, cleared by centrifugation, and diluted 20x with water for analysis. Glycosylated products were detected by SPE-QQQ as described in Example 5, Table 5.1. To assess thermostability, similar one-pot RebA60 reactions were also performed with 10 µL of crude clarified lysate that had been diluted 70x in 50 mM potassium phosphate buffer, pH 6 with 10 g/L RebA60, and incubated 24 h at 71.8 °C. The thermostability results and production levels of rebaudioside D in the single substrate and rebaudioside M in the one-pot reactions by these variants at 0.005 g/L SFP loading are shown in Table 18.3.

Table 18.3 β1,2GT Shake Flask Variants and RebD and RebM Levels

SEQ ID NO: (nt/aa)	Amino Acid Differences (Relative to SEQ ID NO: 994)	Increased RebD[a]	Increased RebM[a]	Increased RebM (preincubated)[a]	% Activity Remaining[b]
1079/1080	H164E/Y202I	-	+	++	+
1081/1082	H83Y/Y202I/P233S	+	-	++	+
1083/1084	Y202I/P233S/M248I	+	+	++	+
1101/1102	H164E	+	+	+	-
1095/1096	H164E/R423L	+	+	-	-
1089/1090	P233S	++	+	-	-

[a]Levels of increased production were determined relative to the reference polypeptide of SEQ ID NO: 994, and defined as follows: "-" = production less than 0.9-fold that of the reference polypeptide; "+" = production at least 0.9-fold that of the reference but less than 1.1-fold reference polypeptide; and "++" = at least 1.1-fold increased production.

[b]The percent of activity remaining for each variant was determined following 24 h pre-incubation at 71.8 °C, relative to the production from each variant following 24 h pre-incubation at 65 °C and is defined as follows: "-" = less than 16% of activity remained following 24 h pre-incubation at 71.8 °C; and "+" = at least 16% activity remained.

[0256] In these experiments, three variants in Table 18.3 were significantly improved following preincubation relative to SEQ ID NO: 994 for catalyzing the glucosylation of rebaudioside A to rebaudioside D. The most thermostable variant, SEQ ID NO: 1080, was selected as the starting point for further enzyme engineering.

EXAMPLE 19

Beta-1,2-ADP-Glycosyltransferase Variants of SEQ ID NO: 1080

[0257] In this Example, experiments for evolution and screening of GT polypeptides derived from SEQ ID NO: 1080 for improved glucosylation of steviol glycosides using ADP-glucose are described.

Directed evolution of the GT encoded by SEQ ID NO: 1079 was carried out by constructing libraries of variant genes. Libraries recombined mutations associated with improved production identified during the development of the present invention and subjected certain structural features of the enzyme with saturation mutagenesis. These libraries were then plated, grown, and screened using the HTP assay described below to provide another round of engineered GT variant polypeptides with glucosyltransferase activity toward ADP-glucose and steviol glycosides. Thirty-three engineered variants were identified from the combinatorial libraries (Table 19.1), and thirty-eight were identified from the saturation mutagenesis libraries (Table 19.2).

HTP Coupled Assay for Glucose Transfer from Sucrose to ADP to Rebaudioside A 60

[0258] Assays were performed on 96-well plates of cleared *E. coli* culture lysates expressing SEQ ID NO: 1079 variants (i.e., variants of SEQ ID NO: 1080). Lysis buffer volume was 400 µL, and the lysate was diluted 100-fold into 50 mM potassium phosphate, pH 6.0 with 30 g/L (combinatorial libraries) or 10 g/L (saturation mutagenesis retest) rebaudioside A 60%, and pre-incubated for 2 h at 75°C. Assays were then conducted with 10 µL diluted lysate, 0.03 g/L SUS SFP SEQ ID NO: 1822, and 0.1 g/L β-1,3-glycosyltransferase (β1,3GT) SFP SEQ ID NO: 350, in 100 µL reaction volume with 20 g/L rebaudioside A 60% (RebA60) substrate, 0.02 g/L ADP (Amresco, ultra pure grade) co-substrate, and 30 g/L sucrose. The following reaction conditions were used: 50 mM potassium phosphate buffer, pH 6, 60°C in a THERMOTRON® titre-plate shaker with 300 RPM shaking for 16-18 h. The reactions were solubilized by 20x dilution into water, quenched by 5x dilution into acetonitrile with 0.2% formic acid, precipitated by centrifugation, and diluted 20x into water for analysis as described above. The resulting engineered variants with glucosyltransferase activity on RebA60 are listed in Table 19.1 and 19.2. Shake-flask scale cultures were grown, lysed, and lyophilized to powder for variants listed in Table 19.3.

Table 19.1 β1,2 GT Combinatorial Variants and RebM Levels

SEQ ID NO: (nt/aa)	Amino Acid Differences (Relative to SEQ ID NO: 1080)	Increased RebM[a]
1167/1168	S9L/A73R/L116I/E165P/N286G/K422A	+++
1169/1170	D389E/K422A	+++
1171/1172	E165P	+++
1173/1174	A73R/L116I/N286G	+++
1175/1176	N286G/K422A	+++
1177/1178	A73R/N286G/D389E	+++
1179/1180	A73R/N286G/K422A	++
1181/1182	L116I/E165P/N286G/K422A	++
1183/1184	A73R/K422T	++

Table 19.1 β1,2 GT Combinatorial Variants and RebM Levels		
SEQ ID NO: (nt/aa)	**Amino Acid Differences (Relative to SEQ ID NO: 1080)**	**Increased RebM[a]**
1185/1186	A73R/N286G/K422T	++
1187/1188	G7T/E165P	++
1189/1190	N286G	++
1191/1192	A73R/L116I/E165P/N286G/D389E	++
1193/1194	A73R	++
1195/1196	G7T/S9L/A73R/E165P/N286G	++
1197/1198	G7T/L116I/E165P/N286G	++
1199/1200	G7T/S9L/E165P/N286G	+
1201/1202	S54M/S406M	+
1203/1204	L116I/E165P	+
1205/1206	E165P/D389E	+
1207/1208	K422T	+
1209/1210	L116I	+
1211/1212	S9L/N286G/D389E	+
1213/1214	E165P/N286G	+
1215/1216	A73R/L116I/N286G/K422A	+
1217/1218	G7T/S9L/K422A	+
1219/1220	L116I/D389E	+
1221/1222	N115A	+
1223/1224	E165P/D389E/K422T	+
1225/1226	A73R/E165P/N286G	+
1227/1228	Y84L	+
1229/1230	S54M	+
1231/1232	S54L/Y84L	+

[a]Levels of increased production were determined relative to the reference polypeptide of SEQ ID NO: 1080, and defined as follows: "+" = production at least 1.37-fold that of the reference but less than 1.65-fold reference polypeptide; "++" = at least 1.65-fold increased production but less than 2.02-fold; and "+++" = at least 2.02-fold increased production.

Table 19.2 β1,2GT Saturation Mutagenesis Variants and RebM Levels		
SEQ ID NO: (nt/aa)	**Amino Acid Differences (Relative to SEQ ID NO: 1080)**	**Increased RebM[a]**
1233/1234	Q441R	+++

Table 19.2 β1,2GT Saturation Mutagenesis Variants and RebM Levels		
SEQ ID NO: (nt/aa)	**Amino Acid Differences (Relative to SEQ ID NO: 1080)**	**Increased RebM[a]**
1235/1236	V385P	+++
1237/1238	G395D	+++
1239/1240	F185L/T190P	+++
1241/1242	Q441S	+++
1243/1244	Q399R	+++
1245/1246	Q441A	+++
1247/1248	G395H	+++
1249/1250	N445R	++
1251/1252	N447A	++
1253/1254	N445K	++
1255/1256	Y449H	++
1257/1258	V385S	++
1259/1260	G395A	++
1261/1262	N302H	++
1263/1264	G395N	++
1265/1266	N257H	++
1267/1268	Q441K	++
1269/1270	N447S	++
1271/1272	N447R	+
1273/1274	N302R	+
1275/1276	V385M	+
1277/1278	C219L	+
1279/1280	T220A	+
1281/1282	N302T	+
1283/1284	N302L	+
1285/1286	P255R	+
1287/1288	Q441L	+
1289/1290	N447V	+
1291/1292	N302A	+
1293/1294	Y449A	+
1295/1296	T409L	+
1297/1298	A434I	+
1299/1300	G395S/E437L	+

Table 19.2 β1,2GT Saturation Mutagenesis Variants and RebM Levels		
SEQ ID NO: (nt/aa)	**Amino Acid Differences (Relative to SEQ ID NO: 1080)**	**Increased RebM[a]**
1301/1302	I412K	+
1303/1304	K416N	+
1305/1306	L401V	+
1307/1308	Y449T	+
[a]Levels of increased production were determined relative to the reference polypeptide of SEQ ID NO: 1080, and defined as follows: "+" = production at least 1.11-fold that of the reference but less than 1.22-fold reference polypeptide; "++" = at least 1.22-fold increased production but less than 1.40-fold; and "+++" = at least 1.40-fold increased production.		

Shake Flask Powder Characterization Assay and Analysis for Glucosyl Transfer from Sucrose to ADP to Rebaudioside A 60

[0259] Shake flask powders (SFP) were reconstituted to a concentration of 4 g/L and diluted to 0.0013-0.04 g/L in 100 µL total reaction volume containing 50 mM potassium phosphate buffer, pH 6, 20 g/L RebA97 or steviol glycoside 95% (single substrate) or RebA60 (one-pot), 0.02 g/L ADP, 20 (single substrate) or 30 g/L (one-pot) sucrose, 0.03 g/L SUS SFP SEQ ID NO: 1822, and, for the one-pot reaction only, 0.1 g/L β-1,3-glycosyltransferase (β1,3GT) SFP SEQ ID NO: 440. The reactions were performed at 60 °C in a THERMOTRON® titre-plate shaker at 300 RPM for 4 h (single substrate) or 16-18 h (one-pot). One set of SFP dilutions was pre-incubated for 2 h at 75 °C in 50 mM potassium phosphate buffer, pH 6 with 30 g/L rebaudioside A 60 %, while another set was not pre-incubated. The reactions were solubilized by 20x dilution with water, quenched by 5x dilution with acetonitrile with 0.2% formic acid, cleared by centrifugation, and diluted 20x with water for analysis. Glycosylated products were detected by SPE-QQQ as described in Example 5, Table 5.1. To assess thermostability, similar one-pot RebA60 reactions were also performed with 10 µL of crude clarified lysate that had been diluted 70x in 50 mM potassium phosphate buffer, pH 6 with 30 g/L RebA60, and incubated 24 h at 71.8 °C. The thermostability results and production levels of rebaudioside D and rebaudioside E in the single substrate and rebaudioside M in the one-pot reactions by these variants at 0.01 g/L SFP loading are shown in Table 19.3.

Table 19.3 β1,2GT Shake Flask Variants and RebD and RebM Levels						
SEQ ID NO: (nt/aa)	**Amino Acid Differences (Relative to SEQ ID NO: 1080)**	**Increased RebD[a]**	**Increased RebE[a]**	**Increased RebM[a]**	**Increased RebM (pre-incubated)[a]**	**% Activity Remaining[b]**
1167/1168	S9L/A73R/L116I/E165P/N286G/K422A	-	-	-	++	++
1215/1216	A73R/L116I/N286G/K422A	+	+	+	++	++
1175/1176	N286G/K422A	-	+	+	++	+
1181/1182	L116I/E165P/N286G/K422A	+	+	-	+++	+
1229/1230	S54M	+	++	-	+++	+
1231/1232	S54L/Y84L	-	+	-	++	+
1201/1202	S54M/S406M	-	+	-	++	+

[a]Levels of increased production were determined relative to the reference polypeptide of SEQ ID NO: 1080, and defined as follows: "-" = production less than 0.9-fold that of the reference polypeptide; "+" = production at least 0.9-fold that of the reference but less than 1.1-fold reference polypeptide; "++" = at least 1.1-fold but less than 1.5-fold increased production; and "+++" = at least 1.5-fold increased production.

[b]The percent of activity remaining for each variant was determined following 24 h pre-incubation at 71.8 °C, relative to the production from each variant following 24 h pre-incubation at 65 °C and is defined as follows: "+" = less than 35% of activity remained following 24 h pre-incubation at 71.8 °C; and "++" = at least 35% activity remained.

[0260] In these experiments, two variants in Table 19.3 had similar activity relative to SEQ ID NO: 1080 without pre-incubation and greater activity with pre-incubation. The more thermostable variant, SEQ ID NO: 1216, was selected as the starting point for further enzyme engineering.

EXAMPLE 20

Beta-1,2-ADP-Glycosyltransferase Variants of SEQ ID NO: 1216

[0261] In this Example, experiments for evolution and screening of GT polypeptides derived from SEQ ID NO: 1216 for improved glucosylation of steviol glycosides using ADP-glucose are described. Directed evolution of the GT encoded by SEQ ID NO: 1215 was carried out by constructing libraries of variant genes. Libraries recombined mutations associated with improved production identified during the development of the present invention and subjected certain structural features of the enzyme with saturation mutagenesis. These libraries were then plated, grown, and screened using the HTP assay described below to provide another round of engineered GT variant polypeptides with

glucosyltransferase activity toward ADP-glucose and steviol glycosides. Ninety-one engineered variants were identified from the combinatorial libraries (Table 20.1), and eleven were identified from the saturation mutagenesis libraries (Table 20.2).

HTP Coupled Assay for Glucose Transfer from Sucrose to ADP to Rebaudioside A 60

[0262] Assays were performed on 96-well plates of cleared *E. coli* culture lysates expressing SEQ ID NO: 1215 variants (i.e., variants of SEQ ID NO: 1216). Lysis buffer volume was 400 µL, and the lysate was diluted 60-fold into 50 mM potassium phosphate, pH 6.0 with 60 g/L (combinatorial libraries) or 80 g/L (saturation mutagenesis retest) rebaudioside A 60%, and pre-incubated for 2 h at 75°C. Assays were then conducted with 10 µL diluted lysate, 0.03 g/L SUS SFP SEQ ID NO: 2182, and 0.1 g/L β-1,3-glycosyltransferase (β1,3GT) SFP SEQ ID NO: 520, in 100 µL reaction volume with 20 g/L rebaudioside A 60% (RebA60) substrate, 0.01 g/L ADP (Amresco, ultra pure grade) co-substrate, and 30 g/L sucrose. The following reaction conditions were used: 50 mM potassium phosphate buffer, pH 6, 60 °C in a THERMOTRON® titre-plate shaker with 300 RPM shaking for 16-18 h. The reactions were solubilized by 20x dilution into water, quenched by 5x dilution into acetonitrile with 0.2% formic acid, precipitated by centrifugation, and diluted 20x into water for analysis as described above. The resulting engineered variants with glucosyltransferase activity on RebA60 are listed in Table 20.1 and 20.2. Shake-flask scale cultures were grown, lysed, and lyophilized to powder for variants listed in Table 20.3.

Table 20.1 β1,2 GT Combinatorial Variants and RebM Levels

SEQ ID NO: (nt/aa)	Amino Acid Differences (Relative to SEQ ID NO: 1216)	Increased RebM[a]
1309/1310	F185L/T190P/V385S/D389E/Q441R/N445K/N447S	+++
1311/1312	S54M/D389E/G395D/Q399R/N447A/Y449H	+++
1313/1314	S54M/F185L/T190P/C219L/N257H/N302R/V385P/D389E/G395H/N445K/N447A/Y449H	+++
1315/1316	S54M/F185L/T190P/V385P/N447A/Y449H	+++
1317/1318	V385S/D389E	+++
1319/1320	T190P/N257H/V385S/D389E/N445K/N447A	+++
1321/1322	N257H/D389E/G395D/Q399R/N445K/Y449H	+++
1323/1324	S54M/N257H/Q441R/Y449H	+++
1325/1326	F185L/C219L/N257H/Q399R/N445R/N447A	+++
1327/1328	S54M/F185L/V385S/G395D/Q399R/N445R/N447A/Y449H	+++
1329/1330	S54M/T190P/N257H/V385S/D389E	+++
1331/1332	S54M/T190P/N302H/D389E/G395D/Q399R/N445K/N447S	+++
1333/1334	S54M/C219L/N302R/G395H/Q441S/N445R/N447A	+++

Table 20.1 β1,2 GT Combinatorial Variants and RebM Levels		
SEQ ID NO: (nt/aa)	**Amino Acid Differences (Relative to SEQ ID NO: 1216)**	**Increased RebM[a]**
1335/1336	S54M/N257H/Q441R/N447A	+++
1337/1338	S54M/F185L/T190P/D389E/Q441R/N445R/N447A/Y449H	+++
1339/1340	F185L/T190P/N257H/N302R/G395D/Q441S/N445R/N447S	+++
1341/1342	T190P/N257H/D389E/G395H/Q441R/N445R/N447A	+++
1343/1344	F185L/T190P/G395D/Q399R	+++
1345/1346	S54M/F185L/V385S/D389E/G395D/Q441R/N445K/Y449H	++
1347/1348	F185L/T190P/C219L/N257H/D389E/G395A/N445K/N447A	++
1349/1350	S54M/V385P	++
1351/1352	N257H/D389E/Q399R	++
1353/1354	S54M/T190P/G395A	++
1355/1356	S54M/F185L/T190P	++
1357/1358	S54M/V385P/D389E/Q441R/N445K/N447A/Y449H	++
1359/1360	S54M/F185L/T190P/C219L/D389E/G395H/Q399R/Q441S/N445K/N447A/Y449H	++
1361/1362	S54M/F185L/T190P/C219L/V385S	++
1363/1364	S54M/N257H	++
1365/1366	S54M/F185L/T190P/D389E/G395H	++
1367/1368	F185L/N302R/G395H/Q399R/Q441R/N445K/N447S/Y449H	++
1369/1370	F185L/T190P/G395H/Q399R	++
1371/1372	S54M/F185L/N302H/V385S/Q399R/N445R/N447A	++
1373/1374	F185L/G395D/Q399R/Q441R/N445R/N447A	++
1375/1376	S54M/F185L/T190P/N302R/G395H/Q399R	++
1377/1378	S54M/F185L/T190P/G395D/Q399R/Q441R/N445K/N447A/Y449H	++
1379/1380	S54M/N257H/D389E	++
1381/1382	F185L/T190P/D389E/N447S/Y449H	++
1383/1384	C219L/N257H/G395H	++
1385/1386	S54M/T190P/G395H/N445K/N447A	++
1387/1388	T190P/N257H/V385S/G395D	++
1389/1390	S54M/T190P/V385S/D389E/G395H/Q441S/N445R	++
1391/1392	S54M/N257H/V385S/Y449H	++
1392/1394	T190P/C219L/N302H/V385P/Q399R/N445R	++
1395/1396	S54M	++
1397/1398	S54M/T190P/N257H/N302H/Q399R	++

Table 20.1 β1,2 GT Combinatorial Variants and RebM Levels		
SEQ ID NO: (nt/aa)	**Amino Acid Differences (Relative to SEQ ID NO: 1216)**	**Increased RebM[a]**
1399/1400	S54M/N257H/Q399R	+
1401/1402	S54M/F185L/C219L/D389E	+
1403/1404	S54M/T190P/C219L/N257H/N302H	+
1405/1406	N257H/V385P/D389E/Q399R	+
1407/1408	S54M/T190P/V385P/N445R/N447A/Y449H	+
1409/1410	S54M/T190P/N257H	+
1411/1412	S54M/T190P/N302R/D389E	+
1413/1414	S54M/C219L/V385P/D389E/G395D/Q441R/N445K/N447A	+
1415/1416	C219L/G395H	+
1417/1418	S54M/F185L/T190P/N302H	+
1719/1420	G395D/Q399R	+
1421/1422	S54M/T190P/N257H/K309N/V385S/D389E/G395D/Q399R/N445K	+
1423/1424	S54M/F185L/T190P/C219L/V385S/D389E/G395D/Q399R/Q441R/N445R/N447A/Y449H	+
1425/1426	V385S/G395D	+
1427/1428	S54M/F185L/T190P/C219L/V385S/G395A/Q399R/Q441R/N445K/N447A/Y449H	+
1429/1430	S54M/T190P/C219L/N257H/G395H/N445R/N447A/Y449H	+
1431/1432	S54M/F185L/T190P/D389E/N445K	+
1433/1434	N257H/D389E/G395D/Q399R/N445K/N447A	+
1435/1436	T190P/N257H/Q399R/N447A/Y449H	+
1467/1438	F185L/T190P/N257H/D389E/G395H/Q399R/Q441S/N447S/Y449H	+
1439/1440	F185L/N257H/V385S/G395A/Q399R/N445R/N447A	+
1441/1442	F185L/T190P/N257H/D389E/G395A	+
1443/1444	T190P/N257H/N302H/V385P/Q399R	+
1445/1446	T190P/D389E	+
1447/1448	D389E/G395D/N445R/N447S	+
1449/1450	Q399R	+
1451/1452	S54M/T190P/V385P/G395D	+
1453/1454	S54M/F185L/D389E/G395D/N445K/N447A	+
1455/1456	N302H/D389E/G395H/N445R	+
1457/1458	S54M/N302H/V385S/Q399R/Q441S/N445R/Y449H	+
1459/1460	S54M/F185L/N257H/D389E/Q441S/N445R/N447A/Y449H	+

Table 20.1 β1,2 GT Combinatorial Variants and RebM Levels		
SEQ ID NO: (nt/aa)	**Amino Acid Differences (Relative to SEQ ID NO: 1216)**	**Increased RebM[a]**
1461/1462	F185L/T190P/N257H/V385P/D389E/Q399R	+
1463/1464	S54M/F185L/T190P/C219L/V385P/N445R/N447A	+
1465/1466	T190P/C219L/N257H/V385S/D389E/Q441S/N445R/N447A/Y449H	+
1467/1468	T190P/N302H/V385P/D389E/G395D/Q399R/Q441S/N445K/N447S	+
1469/1470	C219L/N257H/V385S/D389E/G395D/Q441R/N447A/Y449H	+
1471/1472	S54M/T190P/N257H/G395D/N445K/Y449H	+
1473/1474	T190P/N257H/V385P/D389E/Q399R	+
1475/1476	T190P/N257H/V385S/Q441S/N445R/N447S/Y449H	+
1477/1478	F185L/T190P/N257H/V385P/Q399R/N445R/N447A	+
1479/1480	S54M/N302H/V385P/Q399R/Q441S/N445K/N447S	+
1481/1482	C219L/V385S/D389E/Q399R/N445R/Y449H	+
[a]Levels of increased production were determined relative to the reference polypeptide of SEQ ID NO: 1216, and defined as follows: "+" = production at least 1.87-fold that of the reference but less than 2.17-fold reference polypeptide; "++" = at least 2.17-fold increased production but less than 2.42-fold; and"+++" = at least 2.42-fold increased production.		

Table 20.2 β1,2GT Saturation Mutagenesis Variants and RebM Levels		
SEQ ID NO: (nt/aa)	**Amino Acid Differences (Relative to SEQ ID NO: 1216)**	**Increased RebM[a]**
1491/1492	I341L	+++
1493/1494	N157Q	+++
1495/1496	V278L	++
1497/1498	N108E	++
1499/1500	M181L	++
1501/1502	E188L	+
1503/1504	A293V	+
1505/1506	G96K	+
1507/1508	R14T	+
1509/1510	H5S	+
1511/1512	G96P	+

Table 20.2 β1,2GT Saturation Mutagenesis Variants and RebM Levels		
SEQ ID NO: (nt/aa)	**Amino Acid Differences (Relative to SEQ ID NO: 1216)**	**Increased RebM[a]**
[a]Levels of increased production were determined relative to the reference polypeptide of SEQ ID NO: 1216, and defined as follows: "+" = production at least 1.17-fold that of the reference but less than 1.40-fold reference polypeptide; "++" = at least 1.40-fold increased production but less than 1.83-fold; and "+++" = at least 1.83-fold increased production.		

Shake Flask Powder Characterization Assay and Analysis for Glucosyl Transfer from Sucrose to ADP to Rebaudioside A 60

[0263] Shake flask powders (SFP) were reconstituted to a concentration of 4 g/L and diluted to 0.0013-0.04 g/L in 100 µL total reaction volume containing 50 mM potassium phosphate buffer, pH 6, 20 g/L RebA97 or steviol glycoside 95% (single substrate) or RebA60 (one-pot), 0.02 g/L ADP, 20 (single substrate) or 30 g/L (one-pot) sucrose, 0.03 g/L SUS SFP SEQ ID NO: 2182, and, for the one-pot reaction only, 0.1 g/L β-1,3-glycosyltransferase (β1,3GT) SFP SEQ ID NO: 520. The reactions were performed at 60 °C in a THERMOTRON® titre-plate shaker at 300 RPM for 4 h (single substrate) or 16-18 h (one-pot). One set of SFP dilutions was pre-incubated for 2 h at 75 °C in 50 mM potassium phosphate buffer, pH 6 with 80 g/L rebaudioside A 60 %, while another set was not pre-incubated. The reactions were solubilized by 20x dilution with water, quenched by 5x dilution with acetonitrile with 0.2% formic acid, cleared by centrifugation, and diluted 20x with water for analysis. Glycosylated products were detected by SPE-QQQ as described in Example 5, Table 5.1. To assess thermostability, similar one-pot RebA60 reactions were also performed with 10 µL of crude clarified lysate that had been diluted 70x in 50 mM potassium phosphate buffer, pH 6 with 100 g/L RebA60, and incubated 24 h at 71.8 °C. The thermostability results and production levels of rebaudioside D and rebaudioside E in the single substrate and rebaudioside M in the one-pot reactions by these variants at 0.005 g/L SFP loading are shown in Table 20.3.

Table 20.3 β1,2GT Shake Flask Variants and RebD and RebM Levels						
SEQ ID NO: (nt/aa)	**Amino Acid Differences (Relative to SEQ ID NO: 1216)**	**Increased RebD[a]**	**Increased RebE[a]**	**Increased RebM[a]**	**Increased RebM (pre-incubated)[a]**	**% Activity Remaining[b]**
1315/1316	S54M/F185L/T190P/V385P/N447A/Y449H	+	+	++	+++	++
1487/1488	S54M/T190P/N257H/N302R/V385P/G395A/Q399R	+	+	+	+++	+++
1379/1380	S54M/N257H/D389E	+	+	+	++	+

Table 20.3 β1,2GT Shake Flask Variants and RebD and RebM Levels						
SEQ ID NO: (nt/aa)	**Amino Acid Differences (Relative to SEQ ID NO: 1216)**	**Increased RebD[a]**	**Increased RebE[a]**	**Increased RebM[a]**	**Increased RebM (pre-incubated)[a]**	**% Activity Remaining[b]**
1331/1332	S54M/T190P/N302H/D389E/G395D/Q399R/N445K/N447S	-	+	+	+++	+
1313/1314	S54M/F185L/T190P/C219L/N257H/N302R/V385P/D389E/G395H/N445K/N447A/Y449H	-	+	+	+++	++
1391/1392	S54M/N257H/V385S/Y449H	+	+	++	++	+
1311/1312	S54M/D389E/G395D/Q399R/N447A/Y449H	+	+	+	++	+

[a]Levels of increased production were determined relative to the reference polypeptide of SEQ ID NO: 1216, and defined as follows: "-" = production less than 0.9-fold that of the reference polypeptide; "+" = production at least 0.9-fold that of the reference but less than 1.1-fold reference polypeptide; "++" = at least 1.1-fold but less than 1.8-fold increased production; and "+++" = at least 1.8-fold increased production relative to reference polypeptide.

[b]The percent of activity remaining for each variant was determined following 24 h pre-incubation at 71.8 °C, relative to the production from each variant following 24 h pre-incubation at 65 °C and is defined as follows: "+" = at least 17% but less than 33% activity remained following 24 h pre-incubation at 71.8 °C; "++" = at least 33% but less than 35% activity remained; and "+++" = at least 35% activity remained.

[0264] In these experiments, all seven variants in Table 20.3 were improved relative to SEQ ID NO: 1216 with pre-incubation. The more thermostable variant, SEQ ID NO: 1488, was selected as the starting point for further enzyme engineering.

EXAMPLE 21

Beta-1,2-ADP-Glycosyltransferase Variants of SEQ ID NO: 1488

[0265] In this Example, experiments for evolution and screening of GT polypeptides derived from SEQ ID NO: 1488 for improved glucosylation of steviol glycosides using ADP-glucose are described. Directed evolution of the GT encoded by SEQ ID NO: 1487 was carried out by constructing libraries of variant genes. Libraries recombined mutations associated with improved production identified during the development of the present invention and subjected certain structural features of the enzyme with saturation mutagenesis. These libraries were then plated, grown, and screened using the

HTP assay described below to provide another round of engineered GT variant polypeptides with glucosyltransferase activity toward ADP-glucose and steviol glycosides. Twenty-six engineered variants were identified from the combinatorial libraries (Table 21.1), and seventeen were identified from the saturation mutagenesis libraries (Table 21.2).

HTP Coupled Assay for Glucose Transfer from Sucrose to ADP to Rebaudioside A 60

[0266] Assays were performed on 96-well plates of cleared *E. coli* culture lysates expressing SEQ ID NO: 1487 variants (i.e., variants of SEQ ID NO:1488). Lysis buffer volume was 400 µL, and the lysate was diluted 60 or 100-fold into 50 mM potassium phosphate, pH 6.0 with 100 g/L rebaudioside A 60%, and pre-incubated for 2 h at 75°C. Assays were then conducted with 10 µL diluted lysate, 0.075 g/L SUS SFP SEQ ID NO: 2182, and 0.25 g/L β-1,3-glycosyltransferase (β1,3GT) SFP SEQ ID NO: 626, in 100 µL reaction volume with 50 g/L rebaudioside A 60% (RebA60) substrate, 0.025 g/L ADP (Amresco, ultra pure grade) co-substrate, and 75 g/L sucrose. The following reaction conditions were used: 50 mM potassium phosphate buffer, pH 6, 60 °C in a THERMOTRON® titre-plate shaker with 300 RPM shaking for 16-18 h. The reactions were solubilized by 50x dilution into water, quenched by 5x dilution into acetonitrile with 0.2% formic acid, precipitated by centrifugation, and diluted 20x into water for analysis as described above. The resulting engineered variants with glucosyltransferase activity on RebA60 are listed in Table 21.1 and 21.2. Shake-flask scale cultures were grown, lysed, and lyophilized to powder for variants listed in Table 21.3.

Table 21.1 β1,2 GT Combinatorial Variants and RebM Levels

SEQ ID NO: (nt/aa)	Amino Acid Differences (Relative to SEQ ID NO: 1488)	Increased RebM[a]
1513/1514	R14T/G96P/N108E/M181L/I341L	+++
1515/1516	E188L	+++
1517/1518	V42T/E188L/I341L	+++
1519/1520	M181L	+++
1521/1522	G96P/N108E/E188L/I341L	+++
1523/1524	R14T/G96K	++
1525/1526	R14T/G96K/N108E/V133I	++
1527/1528	R14T/N108E/N157Q/E188L	++
1529/1530	R14T/N51V/G96P/N157Q/I341L	++
1531/1532	R14T/V42T/I341L	++
1533/1534	G96K/E188L	++
1535/1536	R14T/V42T/N51V/N157Q/I341L	++
1537/1538	R14T/N51V/N108E	++
1539/1540	G96P/N108E/E188L	+

Table 21.1 β1,2 GT Combinatorial Variants and RebM Levels		
SEQ ID NO: (nt/aa)	**Amino Acid Differences (Relative to SEQ ID NO: 1488)**	**Increased RebM[a]**
1541/1542	R14T/V42T/N51V/N108E/M181L	+
1543/1544	R14T/N51V/G96K/N157Q	+
1545/1546	R14T/V278L	+
1547/1548	R14T/N51V/I341L	+
1549/1550	R14T/N108E/N157Q	+
1551/1552	G96P/N108E/M181L/A293V	+
1553/1554	R14T/G96K/N108E/E188V	+
1555/1556	R14T/G96K/I341L	+
1557/1558	R14T/N157Q/M181L/V278L	+
1559/1560	V42T/G96P/N157Q/I341L	+
1561/1562	A293V	+
1563/1564	R14T/N51V/G96P/I341L	+
[a]Levels of increased production were determined relative to the reference polypeptide of SEQ ID NO: 1488, and defined as follows: "+" = production at least 1.11-fold that of the reference but less than 1.21-fold reference polypeptide; "++" = at least 1.21-fold increased production but less than 1.35-fold; and "+++" = at least 1.35-fold increased production.		

Table 21.2 β1,2 GT Saturation Mutagenesis Variants and RebM Levels			
SEQ ID NO: (nt/aa)	**Amino Acid Differences (Relative to SEQ ID NO: 1488)**	**Increased RebM[a]**	**Increased RebM (A60 preincubated)[a]**
1565/1566	H324K	+++	+
1567/1568	A196V	+++	++
1569/1570	P197V	++	+
1571/1572	Y268A	++	+
1573/1574	A196P	++	+
1575/1576	G201P	++	+++
1577/1578	N199Q	+	+
1579/1580	T187K	+	+
1581/1582	H324R	+	+
1583/1584	A196M	+	++
1585/1586	S147F	+	+++
1587/1588	Q198F	+	+++
1589/1590	N199G	+	+

Table 21.2 β1,2 GT Saturation Mutagenesis Variants and RebM Levels			
SEQ ID NO: (nt/aa)	**Amino Acid Differences (Relative to SEQ ID NO: 1488)**	**Increased RebM[a]**	**Increased RebM (A60 preincubated)[a]**
1591/1592	Q198A	+	++
1593/1594	L144V	+	++
1595/1596	L122V	+	++
1597/1598	N199S	+	+
[a]Levels of increased production were determined relative to the reference polypeptide of SEQ ID NO: 1488, and defined as follows: "+" = production at least that of the reference but less than 1.22-fold reference polypeptide; "++" = at least 1.22-fold increased production but less than 1.45-fold; and "+++" = at least 1.45-fold increased production.			

Shake Flask Powder Characterization Assay and Analysis for Glucosyl Transfer from Sucrose to ADP to Rebaudioside A 60

[0267] Shake flask powders (SFP) were reconstituted to a concentration of 4 g/L and diluted to 0.0013-0.04 g/L in 100 µL total reaction volume containing 50 mM potassium phosphate buffer, pH 6. Single substrate reactions consisted of 20 g/L RebA97, 0.02 g/L ADP, 20 g/L sucrose, and 0.03 g/L SUS SFP SEQ ID NO: 2182. One pot reactions consisted of 50 g/L RebA60, 0.025 g/L ADP, 75 g/L sucrose, 0.075 g/L SUS SFP SEQ ID NO: 2182, and 0.25 g/L β-1,3-glycosyltransferase (β1,3GT) SFP SEQ ID NO: 626. The reactions were performed at 60 °C in a THERMOTRON® titre-plate shaker at 300 RPM for 4 h (single substrate) or 16-18 h (one-pot). One set of SFP dilutions was pre-incubated for 2 h at 75 °C in 50 mM potassium phosphate buffer, pH 6 with 100 g/L rebaudioside A 60 %, while another set was not pre-incubated. The reactions were solubilized by diluting to 1 g/L substrate in water, quenched by 5x dilution with acetonitrile with 0.2% formic acid, cleared by centrifugation, and diluted 20x with water for analysis. Glycosylated products were detected by SPE-QQQ as described in Example 5, Table 5.1. To assess thermostability, similar one-pot RebA60 reactions were also performed with 10 µL of crude clarified lysate that had been diluted 70x in 50 mM potassium phosphate buffer, pH 6 with 100 g/L RebA60, and incubated 24 h at 71.8 °C. The thermostability results and production levels of rebaudioside D and rebaudioside E in the single substrate and rebaudioside M in the one-pot reactions by these variants at 0.005 g/L SFP loading are shown in Table 21.3.

Table 21.3 β1,2 GT Shake Flask Variants and RebD and RebM Levels					
SEQ ID NO: (nt/aa)	**Amino Acid Differences (Relative to SEQ ID NO: 1488)**	**Increased RebD[a]**	**Increased RebM[a]**	**Increased RebM (pre-incubated)[a]**	**% Activity Remaining[b]**
1523/1524	R14T/G96K	-	-	+	+
1525/1526	R14T/G96K/N108E/V133I	-	-	+	+

Table 21.3 β1,2 GT Shake Flask Variants and RebD and RebM Levels					
SEQ ID NO: (nt/aa)	**Amino Acid Differences (Relative to SEQ ID NO: 1488)**	**Increased RebD[a]**	**Increased RebM[a]**	**Increased RebM (pre-incubated)[a]**	**% Activity Remaining[b]**
1515/1516	E188L	++	++	+	+
1513/1514	R14T/G96P/N108E/M181L/ I341L	-	-	++	+
1531/1532	R14T/V42T/I341L	-	-	++	+
1523/1524	R14T/G96K	-	-	+	+
1525/1526	R14T/G96K/N108E/V133I	-	-	+	+

[a]Levels of increased production were determined relative to the reference polypeptide of SEQ ID NO: 1488, and defined as follows: "-" = production less than 0.95-fold that of the reference polypeptide; "+" = production at least 0.95-fold that of the reference but less than 1.05-fold reference polypeptide; and "++" = at least 1.05-fold increased production.

[b]The percent of activity remaining for each variant was determined following 24 h pre-incubation at 71.8 °C, relative to the production from each variant following 24 h pre-incubation at 65 °C and is defined as follows: "+" = at least 17% and less than 23% of activity remained following 24 h pre-incubation at 71.8 °C.

[0268] In these experiments, one variant in Table 21.3 performed similarly or better than SEQ ID NO: 1488 under the single substrate, one-pot with and without pre-incubation, and 24 h thermostability conditions. This variant, SEQ ID NO: 1516, was selected as the starting point for further enzyme engineering.

EXAMPLE 22

Beta-1,2-ADP-Glycosyltransferase Variants of SEQ ID NO: 1516

[0269] In this Example, experiments for evolution and screening of GT polypeptides derived from SEQ ID NO: 1516 for improved glucosylation of steviol glycosides using ADP-glucose are described. Directed evolution of the GT encoded by SEQ ID NO: 1516 was carried out by constructing libraries of variant genes. Libraries recombined mutations associated with improved production identified during the development of the present invention. These libraries were then plated, grown, and screened using the HTP assay described below to provide another round of engineered GT variant polypeptides with glucosyltransferase activity toward ADP-glucose and steviol glycosides. Twenty-one engineered variants were identified from the combinatorial libraries (Table 22.1).

HTP Coupled Assay for Glucose Transfer from Sucrose to ADP to Rebaudioside A 60

[0270] Assays were performed on 96-well plates of cleared *E. coli* culture lysates expressing SEQ ID NO: 1515 variants (i.e., variants of SEQ ID NO: 1516). Lysis buffer volume was 400 µL, and the lysate was diluted 33-fold into 50 mM potassium phosphate, pH 6.0 with and without 100 g/L rebaudioside A 60%, and the plates with 100g/L RebA60 were pre-incubated for 2 h at 75°C. Assays were then conducted with 10 µL diluted lysate, 0.075 g/L SUS SFP SEQ ID NO: 2322, and 0.25 g/L β-1,3-glycosyltransferase (β1,3GT) SFP SEQ ID NO: 678, in 100 µL reaction volume with 50 g/L rebaudioside A 60% (RebA60) substrate, 0.025 g/L ADP (Amresco, ultra pure grade) co-substrate, and 75 g/L sucrose. The following reaction conditions were used: 50 mM potassium phosphate buffer, pH 6, 60 °C in a THERMOTRON® titre-plate shaker with 300 RPM shaking for 16-18 h. The reactions were solubilized by 50x dilution into water, quenched by 5x dilution into acetonitrile with 0.2% formic acid, precipitated by centrifugation, and diluted 20x into water for analysis as described above. The resulting engineered variants with glucosyltransferase activity on RebA60 are listed in Table 22.1. Shake-flask scale cultures were grown, lysed, and lyophilized to powder for variants listed in Table 22.2.

Table 22.1 β1,2 GT Combinatorial Variants and RebM Levels

SEQ ID NO: (nt/aa)	Amino Acid Differences (Relative to SEQ ID NO: 1516)	Increased RebM[a]	Increased RebM (A60 preincubated)[a]
1599/1600	L188E/Q198A/N199Q/Y268A	+	+++
1601/1602	L188E/H324K	++	++
1603/1604	L188E/A196V/G201P	+	+++
1605/1606	F152Y/L188E/A196V/Q198A/N199Q/H324K	+	+++
1607/1608	L188E/A196V/Q198A/N199Q/G201P	+	+++
1609/1610	L188E/A196V/Q198F/G201P	+	+++
1611/1612	L188E/G201P	++	+++
1613/1614	F152Y/L188E/A196V/G201P/H324K	-	+++
1615/1616	L188E/Q198F/N199Q/G201P/H324K	-	++
1617/1618	S147F/L188E/A196V/G201P	-	++
1619/1620	L188E/Q198A/H324K	+	++
1621/1622	L188E/N199Q/G201P	+	++
1623/1624	F152Y/L188E/A196V/N199Q	+	++
1625/1626	L188E	+	++
1627/1628	L188E/A196V/Q198A/H324K	+	++
1629/1630	L188E/A196V/Q198F/N199Q/G201P/H324K	+	++
1631/1632	F152Y/L188E	+	++
1633/1634	L188E/G201P/H324K	+	++

Table 22.1 β1,2 GT Combinatorial Variants and RebM Levels			
SEQ ID NO: (nt/aa)	**Amino Acid Differences (Relative to SEQ ID NO: 1516)**	**Increased RebM[a]**	**Increased RebM (A60 preincubated)[a]**
1635/1636	F152Y/T187K/L188E/H324K	+	++
1637/1638	L188E/Q198A/G201P/H324K	-	++
1639/1640	F152Y/L188E/H324K	+	++

[a]Levels of increased production were determined relative to the reference polypeptide of SEQ ID NO: 1516, and defined as follows: "-" = production less than 0.9-fold that of the reference; "+" = production at least 0.9-fold that of the reference but less than 1.2-fold reference; "++" = at least 1.2-fold but less than 1.4-fold increased production; and "+++" = at least 1.4-fold increased production.

Shake Flask Powder Characterization Assay and Analysis for Glucosyl Transfer from Sucrose to ADP to Rebaudioside A 60

[0271] Shake flask powders (SFP) were reconstituted to a concentration of 4 g/L and diluted to 0.013-0.2 g/L in 100 µL total reaction volume containing 50 mM potassium phosphate buffer, pH 6. Single substrate reactions consisted of 20 g/L rebaudioside A 97% or steviol glycoside 95%, 0.02 g/L ADP, 20 g/L sucrose, and 0.03 g/L SUS SFP SEQ ID NO: 2322. One pot reactions consisted of 100 g/L RebA60, 0.05 g/L ADP, 150 g/L sucrose, 0.2 g/L SUS SFP SEQ ID NO: 2322, and 0.3 g/L β-1,3-glycosyltransferase (β1,3GT) SFP SEQ ID NO: 678. The reactions were performed at 60 °C in a THERMOTRON® titre-plate shaker at 300 RPM for 4 h (single substrate) or 16-18 h (one-pot). One set of SFP dilutions was pre-incubated for 2 h at 75 °C in 50 mM potassium phosphate buffer, pH 6 with 100 g/L rebaudioside A 60 %, while another set was not pre-incubated. The reactions were solubilized by diluting to 1 g/L substrate in water, quenched by 5x dilution with acetonitrile with 0.2% formic acid, cleared by centrifugation, and diluted 20x with water for analysis. Glycosylated products were detected by SPE-QQQ as described in Example 5, Table 5.1. To assess thermostability, similar one-pot RebA60 reactions were also performed with 10 µL of crude clarified lysate that had been diluted 70x in 50 mM potassium phosphate buffer, pH 6 with 100 g/L RebA60, and incubated 24 h at 71.8 °C. The thermostability results and production levels of rebaudioside D and rebaudioside E in the single substrate and rebaudioside M in the one-pot reactions by these variants at 0.0125 g/L SFP loading are shown in Table 22.2.

Table 22.2 β1,2 GT Shake Flask Variants and RebD and RebM Levels						
SEQ ID NO: (nt/aa)	**Amino Acid Differences (Relative to SEQ ID NO: 1516)**	**Increased RebD[a]**	**Increased RebE[a]**	**Increased RebM[a]**	**Increased RebM (pre-incubated)[a]**	**% Activity Remaining[b]**
1599/1600	L188E/Q198A/N199Q/Y268A	++	++	++	+++	++
1601/1602	L188E/H324K	++	++	++	+	+
1603/1604	L188E/A196V/G201P	++	+	++	+++	+
1605/1606	F152Y/L188E/A196V/Q198A/N199Q/H324K	+	+	++	+++	+
1607/1608	L188E/A196V/Q198A/N199Q/G201P	+	+	+	+++	+
1609/1610	L188E/A196V/Q198F/G201P	+	++	++	+++	+
1611/1612	L188E/G201P	++	+++	++	++	+

[a]Levels of increased production were determined relative to the reference polypeptide of SEQ ID NO: 1516, and defined as follows: "+" = production at least that of the reference but less than 1.3-fold reference polypeptide; "++" = production at least 1.3-fold that of the reference but less than 1.6-fold; and "+++" = at least 1.6-fold increased production.

[b]The percent of activity remaining for each variant was determined following 24 h pre-incubation at 71.8 °C, relative to the production from each variant following 24 h pre-incubation at 65 °C and is defined as follows: "+" = at least 20% but less than 25% of activity remained following 24 h pre-incubation at 71.8 °C; and "++" = at least 25% activity remained.

[0272] In these experiments, all eight variants in Table 22.2 were improved relative to SEQ ID NO: 1516 under both single substrate and both one-pot assays. One variant was also more stable in the 24 h thermostability conditions. This variant, SEQ ID NO: 1516, was further engineered by introducing the beneficial amino acid mutation G96K that was present in several of these shake flask powders (Table 22.3) to produce SEQ ID NO: 1641/1642. This enzyme, SEQ ID NO: 1642 was selected as the best enzyme for the catalysis of glycosyltransfer from ADP-glucose to stevioside and rebaudioside A for the formation of rebaudioside E and rebaudioside D, respectively, and for formation of rebaudioside M in a one-pot reaction with a sucrose synthase and a β-1,3-glycosyltransferase.

EXAMPLE 23

Sucrose Synthase Variants of SEQ ID NO: 22

[0273] Directed evolution of the sucrose synthase encoded by SEQ ID NO:21 was continued by constructing a library of variant genes in which certain structural features of the enzyme were subjected to saturation mutagenesis. This library was then plated, grown, and screened using the high-throughput (HTP) assay described below, to provide another round of 88 engineered SuS variant polypeptides with increased activity toward the generation of ADP-glucose.

HTP Coupled Assay for Glucose Transfer from Sucrose to ADP to Rebaudioside A 60 to Form Rebaudioside M

[0274] Libraries were screened using the following HTP enzyme-coupled assay. Pelleted *E. coli* cultures were lysed with 400 µL of Tris-HCl, pH 7.5 with 1 mM magnesium sulfate and 0.5 mg/mL lysozyme and polymyxin B sulfate (PMBS) and cleared by centrifugation. Lysate was diluted 60-120x into potassium phosphate buffer, pH 6.0, with 14.5 g/L RebA60 and pre-incubated for 1-1.5 hour at 75-77 °C. Then, 10 µL diluted, pre-incubated SuS lysate, 0.08 g/L β1,2GT SFP SEQ ID NO: 24, and 0.2 g/L β1,3GT SFP SEQ ID NO: 20, were used in 100 µL reaction volume with 20 g/L RebA60, 0.02 g/L ADP (Amresco, ultra pure grade), 30 g/L sucrose (cane sugar), and 7.2 g/L fructose. The following reaction conditions were used: 50 mM potassium phosphate buffer, pH 6, 60°C in a THERMOTRON® titre-plate shaker with 300 RPM shaking for 16-18 h. The reaction described above was solubilized by diluting 20x in water, quenched by diluting 5x in acetonitrile with 0.2% formic acid, and precipitated by centrifugation. The supernatant was diluted 20x in water and steviol glycoside products were detected by RapidFire SPE-MS/MS with the instrument and parameters described in Table 5.1. After analysis, the engineered SuS variant polypeptides that showed improved activity in this one-pot reaction were identified and are listed in Table 23.1. Shake-flask scale cultures were grown for protein characterization as described in Example 1 for variants with the amino acid mutations shown in Table 23.2.

Table 23.1 SUS Saturation Mutagenesis Variants and RebM Levels

SEQ ID NO: (nt/aa)	Amino Acid Differences (Relative to SEQ ID NO: 22)	Increased RebM, 1 h 77 °C preincubated[a]	Increased RebM, 1.5 h 75 °C preincubated[a]
1643/1644	H442Y	+	+++
1645/1646	V134R	+	+++
1647/1648	P52V	+	+++
1649/1650	A524R	+	+++
1651/1652	S381R	+	+++
1653/1654	K41S	+	+++
1655/1656	T519V	+	+++

Table 23.1 SUS Saturation Mutagenesis Variants and RebM Levels			
SEQ ID NO: (nt/aa)	**Amino Acid Differences (Relative to SEQ ID NO: 22)**	**Increased RebM, 1 h 77 °C preincubated[a]**	**Increased RebM, 1.5 h 75 °C preincubated[a]**
1657/1658	P52G	+	+++
1659/1660	K724S	+	+++
1661/1662	S532Y	+	+++
1663/1664	Y434G	+	+++
1665/1666	Q136G	+	+++
1667/1668	S381K	+	+++
1669/1670	R44I	++	+++
1671/1672	S381Y	+	+++
1672/1674	S738K	++	+++
1675/1676	H154A	+	++
1677/1678	V134P	++	++
1679/1680	K41Q	+	++
1681/1682	A97T	++	++
1683/1684	S635R	+	++
1685/1686	K724A	+	++
1687/1688	P52Q	++	++
1689/1690	S381T	+	++
1691/1692	A59M	+	++
1693/1694	R44L	++	++
1695/1696	T42D	++	++
1697/1698	E358Q	+	++
1699/1700	P52S	++	++
1701/1702	R44T	+	++
1703/1704	E4T	+	++
1705/1706	P47A	+	++
1707/1708	V85T	+	++
1709/1710	A97S	+	++
1711/1712	V641I	+	++
1713/1714	P52T	+	++
1715/1716	P47R	+	++
1717/1718	H343L	+	++
1719/1720	Y434L	+	++

Table 23.1 SUS Saturation Mutagenesis Variants and RebM Levels			
SEQ ID NO: (nt/aa)	**Amino Acid Differences (Relative to SEQ ID NO: 22)**	**Increased RebM, 1 h 77 °C preincubated[a]**	**Increased RebM, 1.5 h 75 °C preincubated[a]**
1721/1722	V270L	+	++
1723/1724	E4Q	+	++
1725/1726	V641L	+	++
1727/1728	A59T	++	++
1729/1730	S12K	+	++
1731/1732	E3G/P548R	+	++
1733/1734	R44A	+	+
1735/1736	R71T	+	+
1737/1738	E536T	+	+
1739/1740	E129Q	+	+
1741/1742	S589Q	+	+
1743/1744	Q603T	+	+
1745/1746	R570K	+	+
1747/1748	N266R	+	+
1749/1750	W57H	+	+
1751/1752	V134Q	+	+
1753/1754	W57Y	+	+
1755/1756	S532T	+	+
1757/1758	T519C	+	+
1759/1760	K139G	+	+
1761/1762	T42E	++	+
1762/1764	M606A	+	+
1765/1766	P47G	+	+
1767/1768	G652Y	+	+
1769/1770	L81H	+	+
1771/1772	A122P	+	+
1773/1774	E358P	++	+
1775/1776	G652R	+	+
1777/1778	E727H	+	+
1779/1780	Q7L	+	+
1781/1782	S635G	+	+
1783/1784	K388A	+	+

Table 23.1 SUS Saturation Mutagenesis Variants and RebM Levels			
SEQ ID NO: (nt/aa)	**Amino Acid Differences (Relative to SEQ ID NO: 22)**	**Increased RebM, 1 h 77 °C preincubated[a]**	**Increased RebM, 1.5 h 75 °C preincubated[a]**
1785/1786	T42I	+	+
1781/1788	S738A	+	+
1789/1790	F215L	++	+
1791/1792	R615T	+	+
1793/1794	V93I	+	+
1795/1796	Q7H	+	+
1797/1798	R71Q	+	+
1799/1800	S532E	+	+
1801/1802	G175S	+	+
1803/1804	L81P	+	+
1805/1806	A524S	+	+
1807/1808	K388R	+	+
1809/1810	P47T	+	+
1811/1812	K388S	+	+
1812/1814	L81Q	+	+
1815/1816	Q603M	+	+
1817/1818	M606P	+	+

[a]Levels of increased production were determined relative to the reference polypeptide of SEQ ID NO: 22, and defined as follows: "+" = production at least that of the reference but less than 1.3-fold reference polypeptide; "++" = at least 1.3-fold increased production but less than 1.5-fold increased production; and "+++" = at least 1.5-fold increased production relative to the reference polypeptide.

Shake Flask Powder Characterization Assay and Analysis for Glucosyl Transfer from Sucrose to Rebaudioside A 60 to form Rebaudioside M

[0275] An experiment was performed to characterize the activity of these engineered SUS variants on sucrose and ADP to facilitate the formation of rebaudioside M from rebaudioside A 60%. Shake flask powder (SFP) was made up to 0.016-0.5 g/L in 14.5 g/L RebA60 in potassium phosphate buffer, pH 6, and an aliquot was pre-incubated at 75 °C for 1.5 hour. 10 µL diluted, either pre-incubated or not pre-incubated SuS lysate, 0.08 g/L β1,2 GT SFP SEQ ID NO: 24, and 0.2 g/L β1,3GT SFP SEQ ID NO: 20, were used in 100 µL reaction volume with 20 g/L RebA60, 0.02 g/L ADP (Amresco, ultra pure grade), 30 g/L sucrose (cane sugar), and 7.2 g/L fructose. The following reaction conditions were used: 50 mM potassium phosphate buffer, pH 6, 60°C in a THERMOTRON® titre-plate shaker with 300 RPM shaking for 16-18 h. The reaction described above was solubilized by diluting 20x in water,

quenched by diluting 5x in acetonitrile with 0.2% formic acid, and precipitated by centrifugation. The supernatant was diluted 20x in water and steviol glycoside products were detected by RapidFire SPE-MS/MS with the instrument and parameters described in Table 5.1. To assess thermostability, reactions were also performed with 10 µL of crude clarified lysate that had been diluted 20x in 50 mM potassium phosphate buffer, pH 6, and incubated 20 h at 73.5 °C in 100 µL reaction volume with 15 mM rebaudioside A substrate, 0.02 mM ADP (Amresco, ultra pure grade) cofactor, 37.5 mM sucrose co-substrate, 9 mM fructose, and 0.5 g/L β1,2 GT SFP SEQ ID NO: 24. These single substrate reactions were incubated 4 h at 60 °C and then solubilized by diluting 40x in water, quenched by diluting 5x in acetonitrile with 0.2% formic acid and precipitating by centrifugation, and then the supernatant was diluted 7.5x in water and analyzed as described above. The thermostability results and production levels of rebaudioside M in the one-pot reactions by these variants at 0.006 g/L SFP loading are shown in Table 23.2.

Table 23.2 SUS SFP Variants and RebM Levels

SEQ ID NO: (nt/aa)	Amino Acid Differences (Relative to SEQ ID NO: 22)	Increased RebM[a]	Increased RebM (pre-incubated)[a]	% Activity Remaining[b]
1651/1562	S381R	++	++	+
1643/1644	H442Y	+	++	+++
1649/1670	A524R	+	+	+
1645/1646	V134R	-	+	++
1655/1656	T519V	+	+	+
1659/1660	K724S	+	++	+
1647/1648	P52V	++	+	+
1653/1654	K41S	+	+	+

[a]Levels of increased production were determined relative to the reference polypeptide of SEQ ID NO: 22, and defined as follows: "-" = production less than 0.9-fold that of the reference polypeptide; "+" = production at least 0.9-fold that of the reference but less than 1.1-fold reference polypeptide; and "++" = production at least 1.1-fold increased relative to that of the reference polypeptide.

[b]The percent of activity remaining for each variant was determined following 20 h pre-incubation at 73.5 °C, relative to the production from each variant following 20 h pre-incubation at 60 °C and is defined as follows: "+" = at least 13% but less than 20% of activity remained following 24 h pre-incubation at 73.5 °C; "++" = at least 20% but less than 30% activity remained; and "++" = at least 30% activity remained.

[0276] All 8 of the variants listed in Table 23.2 performed similarly or better than SEQ ID NO: 22, in all three assays. SEQ ID NO: 1652 was selected as the starting point for further enzyme engineering.

EXAMPLE 24

Sucrose Synthase Variants of SEQ ID NO: 1652

[0277] Directed evolution of the sucrose synthase encoded by SEQ ID NO: 1651 was continued by constructing libraries of variant genes in which certain structural features of the enzyme were subjected to saturation mutagenesis and libraries in which mutations associated with improved production identified during the development of the present invention were recombined. These libraries were then plated, grown, and screened using the high-throughput (HTP) assay described below, to provide another round of 111 engineered SuS variant polypeptides with increased activity toward the generation of ADP-glucose.

HTP Coupled Assay for Glucose Transfer from Sucrose to ADP to Rebaudioside A 60 to Form Rebaudioside M

[0278] Libraries were screened using the following HTP enzyme-coupled assay. Pelleted *E. coli* cultures were lysed with 400 µL of Tris-HCl, pH 7.5 with 1 mM magnesium sulfate and 0.5 mg/mL lysozyme and polymyxin B sulfate (PMBS) and cleared by centrifugation. Lysate was diluted 120x into potassium phosphate buffer, pH 6.0 with 14.5 g/L RebA60, or 20x (for the combinatorial libraries) or 60x (for the saturation mutagenesis library) into the same buffer with 40 g/L RebA60 and pre-incubated for 2 hours at 75 °C. Then, 10 µL diluted, pre-incubated SuS lysate, 0.08 g/L β1,2GT SFP SEQ ID NO: 858, and 0.14 g/L β1,3GT SFP SEQ ID NO: 174, were used in 100 µL reaction volume with 20 g/L RebA60, 0.02 g/L ADP (Amresco, ultra pure grade), 30 g/L sucrose (cane sugar), and 7.2 g/L fructose. The following reaction conditions were used: 50 mM potassium phosphate buffer, pH 6, 60°C in a THERMOTRON® titre-plate shaker with 300 RPM shaking for 16-18 h. The saturation mutagenesis library was also assayed on the single substrate rebaudioside A 97% at 20 g/L with 0.02 g/L ADP, 30 g/L sucrose, 7.2 g/L fructose, and 0.12 g/L β1,2 GT SFP SEQ ID NO: 994, using lysate that had been diluted 200x in potassium phosphate buffer, pH 6.0 with 40 g/L RebA60 and pre-incubated 2 h at 75 °C. The reaction described above was solubilized by diluting 20x in water, quenched by diluting 5x in acetonitrile with 0.2% formic acid, and precipitated by centrifugation. The supernatant was diluted 20x in water and steviol glycoside products were detected by RapidFire SPE-MS/MS with the instrument and parameters described in Table 5.1. The resulting engineered sucrose synthase variants that showed improved activity in the one-pot reaction are listed in Table 24.1 and 24.2. Shake-flask scale cultures were grown, lysed, and lyophilized to powder as described in Example 1 for variants listed in Table 24.3.

Table 24.1 SUS Combinatorial Variants and RebM Levels			
SEQ ID NO: (nt/aa)	**Amino Acid Differences (Relative to SEQ ID NO: 1652)**	**Increased RebM, 14.5 g/L RebA60 preincubated[a]**	**Increased RebM, 40 g/L RebA60 preincubated[a]**
1819/1820	R44I/P52V/A97T/Y434G/H442Y/K724S	+++	++
1821/1822	Y434G/H442Y	+++	++
1823/1824	A25T/R44I/P52G/V134R/Y434G/K724S	+++	+
1825/1826	A97T/H442Y	+++	++
1827/1828	K41S/P52V/H442Y	+++	++
1829/1830	Q136G/H442Y/K724S	+++	+++
1831/1832	R44I/Q136G/E329Q/Y434G/S532Y	+++	+
1833/1834	V134R/Q136G/Y434G/S532Y/K724S	+++	+
1835/1836	P52G/A97T/H442Y	+++	+++
1837/1838	K41S/P52V/Y434G/H442Y/K724S	+++	+++
1839/1840	R44I/P52V/V134R/Y434G/S532Y	+++	+++
1841/1842	P52V/Q136G/Y434G/K724S	+++	++
1843/1844	P52V/H442Y/V553A/K724S	+++	+
1845/1846	P52V/H442Y	+++	+
1847/1848	K41S/P52V/Y434G	++	+
1849/1850	P52V/Q136G/Y434G/H442Y	++	+
1851/1852	P52V/Q136G/Y434G	++	+
1853/1854	K41S/Y434G/H442Y/S532Y	++	+++
1855/1856	P52V/Y434G	++	+
1857/1858	H442Y	++	+
1859/1860	P52V/Q136G/H442Y	++	+
1861/1862	R44I/Y434G/H442Y/V553A	++	+
1863/1864	K41S/R44I/P52G/A97T/H442Y/A719T/K724S	++	+
1865/1866	K41S/P52V/V134R/Q136G/Y434G	++	++
1867/1868	R44I/P52V/A97T/H442Y/K724S	++	+
1869/1870	P52G/A97T/Y434G/H442Y	++	++
1817/1872	P52G/Y434G/H442Y/S532Y	++	+++
1873/1874	K41S/R44I/P52G/Y434G/K724S	++	+
1875/1876	K41S/Y434G/S532Y	++	+
1877/1878	K41S/Y434G/H442Y/S532Y/K724S	++	++
1879/1880	P52G/V134R	++	+

Table 24.1 SUS Combinatorial Variants and RebM Levels			
SEQ ID NO: (nt/aa)	**Amino Acid Differences (Relative to SEQ ID NO: 1652)**	**Increased RebM, 14.5 g/L RebA60 preincubated[a]**	**Increased RebM, 40 g/L RebA60 preincubated[a]**
1881/1882	V134R/Y434G/H442Y/K724S	++	+
1883/1884	P52G/A97T	++	+
1885/1886	P52G/V134R/E329Q/Y434G	++	+
1887/1888	P52G/H442Y/K724S	++	++
1889/1890	K41S/R44I/Q136G/H442Y	++	+
1891/1892	P52G/V134R/Q136G/H442Y	++	+++
1893/1894	A97T/V134R/H442Y	++	++
1895/1896	K41S/S532Y	+	+
1897/1898	K41S/P52V/A97T/Y434G/H442Y/S532Y/K724S	+	++
1899/1900	Q136G/S532Y/K724S	+	+
1901/1902	P52V/Q136G/Y434G/H442Y/S532Y	+	+
1903/1904	K41S/E329Q/H442Y	+	+
1905/1906	V134R/Q136G/Y434G/H442Y/V553A/K724S	+	++
1907/1908	A97T/Q136G/Y434G/H442Y	+	+++
1909/1910	R44I/Q136G/S532Y	+	+
1911/1912	P52G/H442Y/V553A/K724S	+	+
1913/1914	V134R/Q136G/Y434G/H442Y/S532Y/V553A/K724S	+	+
1915/1916	K41S/R44I/P52V/V134R/H442Y	+	++
1917/1918	K41S/P52V/Y434G/H442Y	+	+
1919/1920	P52V/S532Y	+	+
1921/1922	V134R	+	+
1923/1924	K41S/R44I/Q136G/E329Q	+	+
1925/1926	K41S/R44I/S532Y	+	++
1927/1928	Y434G	+	+
1929/1930	K41S/P52G/Q136G	+	++
1931/1932	K41S/A97T/V134R/Y434G/H442Y/S532Y/V553A	+	+++
1933/1934	P52G/V134R/Y434G/H442Y/S532Y/V553A	+	++
1935/1936	P52V/H442Y/K724S	+	+
1937/1938	Q136G/H442Y	+	++

Table 24.1 SUS Combinatorial Variants and RebM Levels			
SEQ ID NO: (nt/aa)	**Amino Acid Differences (Relative to SEQ ID NO: 1652)**	**Increased RebM, 14.5 g/L RebA60 preincubated[a]**	**Increased RebM, 40 g/L RebA60 preincubated[a]**
1939/1940	H442Y/K724S	+	+
1941/1942	P52G/Q136G	+	++
1943/1944	P52G/V134R/Q136G/Y434G	+	+
1945/1946	V134R/Q136G/Y434G/H442Y	+	+
1947/1948	P52G	+	+
1949/1950	P52G/H442Y	+	+++
1951/1952	A97T/E329Q/K724S	+	+
1953/1954	P52V/Y434G/S532Y	+	+
1955/1956	K41S/V134R/H442Y/S532Y	+	++
1957/1958	K41S/R44I/P52G/Y434G/H442Y/S532Y/K724S	+	+
1959/1960	A97T/V134R/Q136G/H442Y/S532Y	+	++
1961/1962	P52G/A97T/S532Y	+	+
1963/1964	P52G/S532Y	+	+
1965/1966	P52G/V553A	+	+
1967/1968	P52V/Y434G/H442Y/K724S	+	+
1969/1970	Q136G	+	++
1971/1972	R44I/P52G/V134R/Q136G/E329Q/Y434G/H442Y/S532Y	+	+
1973/1974	V134R/Q136G/H442Y/S532Y	+	++
1975/1976	A97T/V134R/Q136G/S532Y	+	+
1977/1978	W57C/A97T/Y434G/H442Y/K724S	+	+
1979/1980	P52V/V134R/H442Y/K724S	+	+
1981/1982	P52V/V134R/E329Q/S532Y	+	+
1983/1984	P52V/H442Y/S532Y/K724S	+	+
1985/1986	S532Y/K724S	+	+
1987/1988	K41S/P52G/V134R/H442Y/K724S	+	++

[a]Levels of increased production were determined relative to the reference polypeptide of SEQ ID NO: 1652, and defined as follows: "+" = production at least that of the reference but less than 1.15-fold reference polypeptide; "++" = at least 1.15-fold increased production but less than 1.3-fold increased production; and "+++" = at least 1.3-fold increased production relative to the reference polypeptide.

Table 24.2 SUS Saturation Mutagenesis Variants and RebM Levels			
SEQ ID NO: (nt/aa)	**Amino Acid Differences (Relative to SEQ ID NO: 1652)**	**Increased RebM[a]**	**Increased RebD[a]**
1989/1990	R38V	+++	+++
1991/1992	R38L	++	+
1993/1994	Y51H/L433P	++	++
1995/1996	R38N	++	+
1997/1998	E4V	++	+++
1999/2000	H22T	+	+
2001/2002	Y51A	+	++
2003/2004	P47S/D488N	+	+++
2005/2006	T708V	+	+
2007/2008	T34E	+	++
2009/2010	V62I	+	++
2011/2012	M75I/Q169A	+	+
2013/2014	L101V	+	+
2015/2016	R38W	+	+
2017/2018	H22L	+	+
2019/2020	S195K/A213T	+	+
2021/2022	L32S	+	-
2023/2024	Q169E	+	++
2025/2026	E3A	+	-
2027/2028	Y51T	+	++
2029/2030	A718H	+	-
2031/2032	Y51H	+	++
2033/2034	T34S	+	+++
[a]Levels of increased production were determined relative to the reference polypeptide of SEQ ID NO: 1652, and defined as follows: "-" = production less than 0.9-fold that of reference polypeptide; "+" = production at least 0.9-fold that of the reference but less than 1.1-fold reference polypeptide; "++" = at least 1.1-fold increased production but less than 1.2-fold increased production; and "+++" = at least 1.2-fold increased production relative to the reference polypeptide.			

Shake Flask Powder Characterization Assay and Analysis for Glucosyl Transfer from Sucrose to Rebaudioside A 60 to form Rebaudioside M

[0279] An experiment was performed to characterize the activity of these engineered SUS variants on sucrose and ADP to facilitate the formation of rebaudioside M from rebaudioside A 60%. Shake flask powder (SFP) was made up to 0.016-0.5 g/L in potassium phosphate buffer, pH 6 with and without 40 g/L RebA60, and the solutions with RebA60 were pre-incubated at 75 °C for 2 hours. 10 µL diluted, either pre-incubated or not pre-incubated SuS lysate, 0.08 g/L β1,2 GT SFP SEQ ID NO: 858, and 0.14 g/L β1,3GT SFP SEQ ID NO: 174, were used in 100 µL reaction volume with 20 g/L RebA60, 0.02 g/L ADP (Amresco, ultra pure grade), 30 g/L sucrose (cane sugar), and 7.2 g/L fructose. The following reaction conditions were used: 50 mM potassium phosphate buffer, pH 6, 60°C in a THERMOTRON ® titre-plate shaker with 300 RPM shaking for 16-18 h. The reaction described above was solubilized by diluting 20x in water, quenched by diluting 5x in acetonitrile with 0.2% formic acid, and precipitated by centrifugation. The supernatant was diluted 20x in water and steviol glycoside products were detected by RapidFire SPE-MS/MS with the instrument and parameters described in Table 5.1. To assess thermostability, reactions were also performed with 10 µL of crude clarified lysate that had been diluted 20x in 50 mM potassium phosphate buffer, pH 6 with 40 g/L RebA60, and incubated 24 h at 71.1 °C in 100 µL reaction volume under the same one-pot reaction conditions described above. The thermostability results and production levels of rebaudioside M in the one-pot reactions by these variants at 0.0125 g/L SFP loading are shown in Table 24.3.

Table 24.3 SUS SFP Variants and RebM Levels

SEQ ID NO: (nt/aa)	Amino Acid Differences (Relative to SEQ ID NO: 1652)	Increased RebM[a]	Increased RebM (pre-incubated)[a]	% Activity Remaining[b]
1835/1836	P52G/A97T/H442Y	+	+	++
1867/1868	R44I/P52V/A97T/H442Y/K724S	+	+	+++
1825/1826	A97T/H442Y	+	+	++
1821/1822	Y434G/H442Y	+	+	++

[a]Levels of increased production were determined relative to the reference polypeptide of SEQ ID NO: 1652, and defined as follows: "-" = production less than 0.9-fold that of the reference polypeptide; and "+" = production at least 0.9-fold that of the reference but less than 1.1-fold reference polypeptide.

[b]The percent of activity remaining for each variant was determined following 24 h pre-incubation at 71.1 °C, relative to the production from each variant following 24 h pre-incubation at 65 °C and is defined as follows: "+" = at least 15% but less than 20% of activity remained following 24 h pre-incubation at 71.1 °C; and "++" = at least 20% activity remained.

[0280] The four variants listed in Table 24.3 performed similarly to SEQ ID NO: 1652 in both activity assays and were more thermostable in the 24 h pre-incubation assay. One variant, SEQ ID NO: 1822 exhibited slightly higher activity in the one-pot assays, and it was selected as the starting point for further enzyme engineering.

EXAMPLE 25

Sucrose Synthase Variants of SEQ ID NO: 1822

[0281] Directed evolution of the sucrose synthase encoded by SEQ ID NO: 1821 was continued by constructing libraries of variant genes in which certain structural features of the enzyme were subjected to saturation mutagenesis and libraries in which mutations associated with improved production identified during the development of the present invention were recombined. These libraries were then plated, grown, and screened using the high-throughput (HTP) assay described below, to provide an additional round of 63 engineered SuS variant polypeptides with increased activity toward the generation of ADP-glucose.

HTP Coupled Assay for Glucose Transfer from Sucrose to ADP to Rebaudioside A 60 to Form Rebaudioside M

[0282] Libraries were screened using the following HTP enzyme-coupled assay. Pelleted *E. coli* cultures were lysed with 400 µL of Tris-HCl, pH 7.5 with 1 mM magnesium sulfate and 0.5 mg/mL lysozyme and polymyxin B sulfate (PMBS) and cleared by centrifugation. Lysate was diluted 90x into potassium phosphate buffer, pH 6.0 with 40 g/L RebA60 and pre-incubated for 2 hours at 75 °C. Saturation mutagenesis samples were retested with this pre-incubation condition and with 80 g/L RebA60 pre-incubation. Then, 10 µL diluted, pre-incubated SuS lysate, 0.04 g/L β1,2GT SFP SEQ ID NO: 994, and 0.08 g/L β1,3GT SFP SEQ ID NO: 350, were used in 100 µL reaction volume with 20 g/L RebA60, 0.02 g/L ADP (Amresco, ultra pure grade), 30 g/L sucrose (cane sugar), and 7.2 g/L fructose. The following reaction conditions were used: 50 mM potassium phosphate buffer, pH 6, 60°C in a THERMOTRON® titre-plate shaker with 300 RPM shaking for 16-18 h. The reactions described above were solubilized by diluting 20x in water, quenched by diluting 5x in acetonitrile with 0.2% formic acid, and precipitated by centrifugation. The supernatant was diluted 20x in water and steviol glycoside products were detected by RapidFire SPE-MS/MS with the instrument and parameters described in Table 5.1. The resulting engineered sucrose synthase variants that showed improved activity in the one-pot reaction are listed in Table 25.1 and 25.2. Shake-flask scale cultures were grown, lysed, and lyophilized to powder as described in Example 1 for variants listed in Table 25.3.

Table 25.1 SUS Combinatorial Variants and RebM Levels		
SEQ ID NO: (nt/aa)	**Amino Acid Differences (Relative to SEQ ID NO: 1822)**	**Increased RebM[a]**
2035/2036	V62I/T708V	+++
2037/2038	Q169A/T708V	+++
2039/2040	E4V/T708V	+++
2041/2042	E4V/L433P	+++
2043/2044	H22L/T34S/Q169A/T708V	+++
2045/2046	E4V/H22L/Y51A/T708V	++
2047/2048	H22T/T34E/L101V/Q169E	++
2049/2050	T34S/R38L/V62I	++
2051/2052	E4V/H22T/T34E/R38N	++
2053/2054	E4V/H22L/L101V	++
2055/2056	E4V/H22L/T34E	++
2057/2058	E4V/T34E/T708V	++
2059/2060	E4V/Q169A	++
2061/2062	E4V/V62I/L433P/T708V	++
2063/2064	H22T/T34E/L101V/Q169E/S195K	+
2065/2066	H22T/P47S/Q169A/L433P	+
2067/2068	E4V/H22T	+
2069/2070	M75I/Q169A	+
2071/2072	T34E/L101V	+
2073/2074	E4V/H22T/T34E/R38V/Q169A/T708V	+
2075/2076	H22L/M75I	+
2077/2078	E4V/Q169A/T708V	+
2079/2080	E4V/T34E/R38N/L101V	+
2081/2082	T34E/V62I/Q169E/L433P	+
2083/2084	S195K/T708V	+
2085/2086	E4V/H22T/T34E/P47S/Y51H/Q169A/A213T	+
2087/2088	H22L/T34E	+
2089/2090	H22T/T34E/R38V	+
2091/2092	E4V/Q169A/L433P	+

[a]Levels of increased production were determined relative to the reference polypeptide of SEQ ID NO: 1822, and defined as follows: "+" = production at least that of the reference but less than 1.19-fold reference polypeptide; "++" = at least 1.19-fold increased production but less than 1.22-fold increased production; and "+++" = at least 1.22-fold increased production relative to the reference polypeptide.

Table 25.2 SUS Saturation Mutagenesis Variants and RebM Levels			
SEQ ID NO: (nt/aa)	**Amino Acid Differences (Relative to SEQ ID NO: 1822)**	**Increased RebM, 40 g/L RebA60 preincubated[a]**	**Increased RebM, 80 g/L RebA60 preincubated[a]**
2093/2094	V602P	+	+++
2095/2096	S585A	++	+++
2097/2098	M565V	+	+++
2099/2100	H623K	+	+++
2101/2102	R518S	+	+++
2147/2148	L433P	+	+++
2149/2150	Q169A	+	++
2151/2152	E4V	++	++
2103/2104	R557P	+	++
2105/2106	D731Q	+	++
2107/2108	D441A	+	++
2109/2110	D731T	+	++
2111/2112	E121D	+	++
2113/2114	D441L	++	++
2115/2116	T526H	+	++
2153/2154	T708V	+	++
2117/2778	E528Q	-	++
2119/2120	N604A	+	++
2155/2156	H22L	-	++
2157/2158	T34E	+	+
2121/2122	D731M	+	+
2159/2160	H22T	+	+
2123/2124	D731A	+	+
2125/2126	Q558G	+	+
2127/2128	V770T	+	+
2129/2130	P341N	-	+
2131/2132	H623Q	+	+
2133/2134	T526V	+	+
2135/2136	E527Q	-	+
2137/2138	L411I	+	+
2139/2140	H623A	-	+

Table 25.2 SUS Saturation Mutagenesis Variants and RebM Levels			
SEQ ID NO: (nt/aa)	**Amino Acid Differences (Relative to SEQ ID NO: 1822)**	**Increased RebM, 40 g/L RebA60 preincubated[a]**	**Increased RebM, 80 g/L RebA60 preincubated[a]**
2141/2142	E527A	-	+
2143/2144	R544H	+	+
2145/2146	H623R	++	+
[a]Levels of increased production were determined relative to the reference polypeptide of SEQ ID NO: 1822, and defined as follows: "-" = production less than 0.9-fold that of the reference; "+" = production at least 0.9-fold that of the reference but less than 1.1-fold reference polypeptide; "++" = at least 1.1-fold increased production but less than 1.2-fold increased production; and "+++" = at least 1.2-fold increased production relative to the reference polypeptide.			

Shake Flask Powder Characterization Assay and Analysis for Glucosyl Transfer from Sucrose to Rebaudioside A 60 to form Rebaudioside M

[0283] An experiment was performed to characterize the activity of some of these engineered SUS variants on sucrose and ADP to facilitate the formation of rebaudioside M from rebaudioside A 60%. Shake flask powder (SFP) was made up to 0.016-0.5 g/L in potassium phosphate buffer, pH 6 with and without 80 g/L RebA60, and the solutions with RebA60 were pre-incubated at 75 °C for 2 hours. 10 µL diluted, either pre-incubated or not pre-incubated SuS lysate, 0.04 g/L β1,2 GT SFP SEQ ID NO: 994, and 0.08 g/L β1,3GT SFP SEQ ID NO: 350 were used in 100 µL reaction volume with 20 g/L RebA60, 0.02 g/L ADP (Amresco, ultra pure grade), 30 g/L sucrose (cane sugar), and 7.2 g/L fructose. The following reaction conditions were used: 50 mM potassium phosphate buffer, pH 6, 60°C in a THERMOTRON® titre-plate shaker with 300 RPM shaking for 16-18 h. The reaction described above was solubilized by diluting 20x in water, quenched by diluting 5x in acetonitrile with 0.2% formic acid, and precipitated by centrifugation. The supernatant was diluted 20x in water and steviol glycoside products were detected by RapidFire SPE-MS/MS with the instrument and parameters described in Table 5.1. To assess thermostability, reactions were also performed with 10 µL of crude clarified lysate that had been diluted 200x in 50 mM potassium phosphate buffer, pH 6 with 40 g/L RebA60, and incubated 24 h at 71.1 °C in 100 µL reaction volume under the same one-pot reaction conditions described above. The thermostability results and production levels of rebaudioside M in the one-pot reactions by these variants at 0.0125 g/L SFP loading are shown in Table 25.3.

Table 25.3 SUS SFP Variants and RebM Levels				
SEQ ID NO: (nt/aa)	**Amino Acid Differences (Relative to SEQ ID NO: 1822)**	**Increased RebM[a]**	**Increased RebM (pre-incubated)[a]**	**% Activity Remaining[b]**
2151/2152	E4V	+	++	++
2035/2036	V62I/T708V	+	+	++
2051/2052	E4V/H22T/T34E/R38N	+	+	+
2091/2092	E4V/Q169A/L433P	+	+++	++
2041/2042	E4V/L433P	+	+++	++
2037/2038	Q169A/T708V	+	+	+
2059/2060	E4V/Q169A	++	+	++

[a]Levels of increased production were determined relative to the reference polypeptide of SEQ ID NO: 1822, and defined as follows: "+" = production at least that of the reference but less than 1.1-fold reference polypeptide; "++" = production at least 1.1-fold but less than 1.25-fold; and "+++" = production at least 1.25-fold increased relative to that of the reference polypeptide.

[b]The percent of activity remaining for each variant was determined following 24 h pre-incubation at 71.1 °C, relative to the production from each variant following 24 h pre-incubation at 65 °C and is defined as follows: "+" = at least 25% but less than 30% of activity remained following 24 h pre-incubation at 71.1 °C; and "++" = at least 30% activity remained.

[0284] All 7 variants listed in Table 25.3 performed similarly or better than SEQ ID NO: 1822 in both activity assays and in the 24 h pre-incubation assay. The most active variant following 2 h pre-incubation at 75 °C in 80 g/L rebaudioside A 60%, SEQ ID NO: 2092, was selected as the starting point for further enzyme engineering.

EXAMPLE 26

Sucrose Synthase Variants of SEQ ID NO: 2092

[0285] Directed evolution of the sucrose synthase encoded by SEQ ID NO: 2092 was continued by constructing libraries of variant genes in which certain structural features of the enzyme were subjected to saturation mutagenesis and libraries in which mutations associated with improved production identified during the development of the present invention were recombined. These libraries were then plated, grown, and screened using the high-throughput (HTP) assay described below, to provide another round of 74 engineered SuS variant polypeptides with increased activity toward the generation of ADP-glucose.

HTP Coupled Assay for Glucose Transfer from Sucrose to ADP to Rebaudioside A 60 to Form Rebaudioside M

[0286] Libraries were screened using the following HTP enzyme-coupled assay. Pelleted *E. coli* cultures were lysed with 400 µL of Tris-HCl, pH 7.5 with 1 mM magnesium sulfate and 0.5 mg/mL lysozyme and polymyxin B sulfate (PMBS) and cleared by centrifugation. Lysate was diluted 50-100x into potassium phosphate buffer, pH 6.0 with 100 g/L RebA60 and pre-incubated for 2 hours at 75 °C. Then, 10 µL diluted, pre-incubated SuS lysate, 0.04 g/L β1,2GT SFP SEQ ID NO: 1080, and 0.2 g/L β1,3GT SFP SEQ ID NO: 440, were used in 100 µL reaction volume with 30 g/L RebA60, 0.02 g/L ADP (Amresco, ultra pure grade), 30 g/L sucrose (cane sugar), and 7.2 g/L fructose. The saturation mutagenesis library was also retested with 10 µL diluted, pre-incubated SuS lysate, 0.2 g/L β1,2 GT SFP SEQ ID NO: 1080, and 0.43 g/L β1,3GT SFP SEQ ID NO: 440 in 100 µL reaction volume with 50 g/L RebA60, 0.01 g/L ADP (Amresco, ultra pure grade), 75 g/L sucrose (cane sugar), and 18 g/L fructose. The following reaction conditions were used: 50 mM potassium phosphate buffer, pH 6, 60°C in a THERMOTRON® titre-plate shaker with 300 RPM shaking for 16-18 h. The reactions described above were solubilized by diluting to 1-1.5 g/L starting RebA60 in water, quenched by diluting 5x in acetonitrile with 0.2% formic acid, and precipitated by centrifugation. The supernatant was diluted 20x in water and steviol glycoside products were detected by RapidFire SPE-MS/MS with the instrument and parameters described in Table 5.1. The resulting engineered sucrose synthase variants that showed improved activity in the one-pot reaction are listed in Table 26.1 and 26.2. Shake-flask scale cultures were grown, lysed, and lyophilized to powder as described in Example 1 for variants listed in Table 26.3.

Table 26.1 SUS Combinatorial Variants and RebM Levels

SEQ ID NO: (nt/aa)	Amino Acid Differences (Relative to SEQ ID NO: 2092)	Increased RebM[a]
2161/2162	T34E/V62I/D441L/R557P/H623K	+++
2163/2164	T34E/D441L/S585A/H623K/T708V/D731Q	+++
2165/2166	D441L/S585A/V602P/N604A/H623R/T708V/D731Q	+++
2167/2168	D441L/R518S/R557P/M565V/S585A	+++
2169/2170	D441L/R518S/T526H/R557P/M565V/V602P/N604A/H623K/T708V	+++
2171/2172	L411I/D441L/R518S/R557P/H623K	+++
2173/2174	T34E/V62I/H623K/D731Q	+++
2175/2176	T34E/V62I/L411I/R557P/V602P/N604A	+++
2177/2178	V62I/E121D/D441L/M565V/S585A	+++
2179/2180	T34E/V62I/R518S/H623K	+++
2181/2182	D441L/R518S/T526H/R557P/S585A/N604A/H623R/T708V	+++
2183/2184	L411I/D441L/N604A/H623K/T708V/D731Q	++

Table 26.1 SUS Combinatorial Variants and RebM Levels		
SEQ ID NO: (nt/aa)	**Amino Acid Differences (Relative to SEQ ID NO: 2092)**	**Increased RebM[a]**
2185/2186	T34E/D441L/R518S/T526H/S585A/N604A/D731Q	++
2187/2188	V62I/D441L/H623R/T708V/V770T	++
2189/2190	E121D/N604A/T708V/D731Q/V770T	++
2191/2192	T34E/D441L/T526H/M565V/T708V/V770T	++
2192/2194	D441L/R518S/T526H/N604A/H623R	++
2195/2196	V62I/L411I/T526H/M565V/N604A/H623K	++
2197/2198	R557P/N604A	++
2199/2200	E121D/D441L/R518S/T526H/V602P/N604A	++
2201/2202	L411I/R518S/T526H/N604A/H623K/D731Q	++
2203/2204	T34E/D441L/R544H/R557P/S585A/V602P/H623K	++
2205/2206	L411I/S585A/H623K	++
2207/2208	V62I/E121D/L411I/D441L/R518S/R544H/R557P/S585A/N604A/H623K	++
2209/2210	V62I/E121D/D441L/R518S/T526H/H623R/V770T	++
2211/2212	V62I/L411I/S585A/D731Q	++
2213/2214	T34E/E121D/T526H/N604A/D731Q	++
2215/2216	L411I/M565V/N604A/H623R	+
2217/2218	D441L/R518S/M565V/H623R/D731Q/V770T	+
2219/2220	T34E/V62I/L411I/T526H/R557P	+
2221/2222	T34E/L411I/D441L	+
2223/2224	V62I/E121D/R518S/R557P/S585A/N604A/T708V/V770T	+
2225/2226	E121D/D441L/R518S/T526H/H623K/T708V	+
2227/2228	V62I/D441L/R518S/R557P/N604A/H623K/T708V/D731Q	+
2229/2230	V62I/D441L/V770T	+
2231/2232	D441L/R518S/S585A	+
2233/2234	R557P/V602P/N604A	+
2235/2236	D441L/S585A	+
2237/2238	T34E/L411I/D441L/R518S/R544H/R557P/M565V/T708V/D731Q	+
2239/2240	H623R	+
2241/2242	T34E/L411I/D441L/R518S/S585A/N604A/V770T	+
2243/2244	T34E/E121D/D441L/R544H/N604A/H623K	+
2245/2246	V62I/E121D/E329Q/R518S/R557P/M565V/H623R/T708V	+
2247/2248	E121D/D441L/T526H/R557P/M565V/T708V	+

Table 26.1 SUS Combinatorial Variants and RebM Levels		
SEQ ID NO: (nt/aa)	**Amino Acid Differences (Relative to SEQ ID NO: 2092)**	**Increased RebM[a]**
2249/2250	D441L/T708V/D731Q	+
2251/2252	T34E/T526H/S585A/H623K	+
2253/2254	S585A/H623R/T708V	+
2255/2256	T34E/L411I/D441L/R518S/T526H/R557P/M565V/D731Q	+
2257/2258	L411I/D441L/R518S/T708V	+

[a]Levels of increased production were determined relative to the reference polypeptide of SEQ ID NO: 2092, and defined as follows: "+" = production at least 1.3-fold that of the reference but less than 1.47-fold reference polypeptide; "++" = at least 1.47-fold increased production but less than 1.7-fold increased production; and "+++" = at least 1.7-fold increased production relative to the reference polypeptide.

Table 26.2 SUS Saturation Mutagenesis Variants and RebM Levels			
SEQ ID NO: (nt/aa)	**Amino Acid Differences (Relative to SEQ ID NO: 2092)**	**Increased RebM, 30 g/L RebA60 preincubated[a]**	**Increased RebM, 50 g/L RebA60 preincubated[a]**
2269/2270	T640A	+++	+++
2271/2272	T640N	++	+++
2273/2274	T640R	+++	+++
2275/2276	T65C	+++	++
2277/2278	A63T	-	++
2279/2280	A63G	+	++
2281/2282	T640E	+++	++
2283/2284	T640H	+++	++
2285/2286	A323G	+++	++
2287/2288	N406G	+	+
2289/2290	T640W	-	+
2291/2292	L416F	+	+
2293/2294	V511L	-	+
2295/2296	A323S	+	+
2297/2298	I269V	-	+
2299/2300	A63S	+++	+
2301/2302	T640V	-	+
2303/2304	A323T	++	+

Table 26.2 SUS Saturation Mutagenesis Variants and RebM Levels			
SEQ ID NO: (nt/aa)	**Amino Acid Differences (Relative to SEQ ID NO: 2092)**	**Increased RebM, 30 g/L RebA60 preincubated[a]**	**Increased RebM, 50 g/L RebA60 preincubated[a]**
2305/2306	T65L	+	+
2307/2308	T469S	-	+
[a]Levels of increased production were determined relative to the reference polypeptide of SEQ ID NO: 2092, and defined as follows: "-" = production less than 0.9-fold that of the reference; "+" = production at least 0.9-fold but less than 1.1-fold that of the reference; "++" = at least 1.1-fold but less than 1.2-fold increased production; and "+++" = at least 1.2-fold increased production relative to the reference polypeptide.			

Shake Flask Powder Characterization Assay and Analysis for Glucosyl Transfer from Sucrose to Rebaudioside A 60 to form Rebaudioside M

[0287] An experiment was performed to characterize the activity of some of the engineered SUS variants on sucrose and ADP to facilitate the formation of rebaudioside M from rebaudioside A 60%. Shake flask powder (SFP) was made up to 0.016-0.5 g/L in potassium phosphate buffer, pH 6 with and without 100 g/L RebA60, and the solutions with RebA60 were pre-incubated at 75 °C for 2 hours. 10 µL diluted, either pre-incubated or not pre-incubated SuS lysate, 0.04 g/L β1,2 GT SFP SEQ ID NO: 1080, and 0.2 g/L β1,3GT SFP SEQ ID NO: 440 were used in 100 µL reaction volume with 30 g/L RebA60, 0.02 g/L ADP (Amresco, ultra pure grade), 30 g/L sucrose (cane sugar), and 7.2 g/L fructose. The following reaction conditions were used: 50 mM potassium phosphate buffer, pH 6, 60°C in a THERMOTRON® titre-plate shaker with 300 RPM shaking for 16-18 h. The reaction described above was solubilized by diluting 30x in water, quenched by diluting 5x in acetonitrile with 0.2% formic acid, and precipitated by centrifugation. The supernatant was diluted 20x in water and steviol glycoside products were detected by RapidFire SPE-MS/MS with the instrument and parameters described in Table 5.1. To assess thermostability, reactions were also performed with 10 µL of crude clarified lysate that had been diluted 100x in 50 mM potassium phosphate buffer, pH 6 with 100 g/L RebA60, and incubated 24 h at 71.1 °C in 100 µL reaction volume under the same one-pot reaction conditions described above. The thermostability results and production levels of rebaudioside M in the one-pot reactions by these variants at 0.0125 g/L SFP loading are shown in Table 26.3.

Table 26.3 SUS SFP Variants and RebM Levels				
SEQ ID NO: (nt/aa)	**Amino Acid Differences (Relative to SEQ ID NO: 2092)**	**Increased RebM[a]**	**Increased RebM (pre-incubated)[a]**	**% Activity Remaining[b]**
2163/2664	T34E/D441L/S585A/H623K/T708V/D731Q	+	++	+
2167/2168	D441L/R518S/R557P/M565V/S585A	++	++	+
2161/2162	T34E/V62I/D441L/R557P/H623K	+	+	+
2165/2166	D441L/S585A/V602P/N604A/H623R/T708V/D731Q	+	+	+
2171/2172	L411I/D441L/R518S/R557P/H623K	++	+	+
2183/2184	L411I/D441L/N604A/H623K/T708V/D731Q	++	++	++
2181/2182	D441L/R518S/T526H/R557P/S585A/N604A/H623R/T708V	+	++	+++
2187/2188	V62I/D441L/H623R/T708V/V770T	++	+	+

[a]Levels of increased production were determined relative to the reference polypeptide of SEQ ID NO: 2092, and defined as follows: "-" = production less than 0.9-fold that of the reference polypeptide; "+" = production at least 0.9-fold that of the reference but less than 1.1-fold reference polypeptide; and "++" = production at least 1.1-fold increased relative to that of the reference polypeptide.

[b]The percent of activity remaining for each variant was determined following 24 h pre-incubation at 71.1 °C, relative to the production from each variant following 24 h pre-incubation at 65 °C and is defined as follows: "+" = at least 14% but less than 18% of activity remained following 24 h pre-incubation at 71.1 °C; "++" = at least 18% but less than 30% activity remained; and "+++" = at least 30% activity remained.

[0288] All 8 variants listed in Table 26.3 performed similarly or better than SEQ ID NO: 2092 in both activity assays and in the 24 h pre-incubation assay. The most thermostable variant following 24 h pre-incubation, SEQ ID NO: 2182, was selected as the starting point for further enzyme engineering.

EXAMPLE 27

Sucrose Synthase Variants of SEQ ID NO: 2182

[0289] Directed evolution of the sucrose synthase encoded by SEQ ID NO: 2182 was continued by constructing libraries of variant genes in which certain structural features of the enzyme were

subjected to saturation mutagenesis and libraries in which mutations associated with improved production identified during the development of the present invention were recombined. These libraries were then plated, grown, and screened using the high-throughput (HTP) assay described below, to provide another round of 80 engineered SuS variant polypeptides with increased activity toward the generation of ADP-glucose.

HTP Coupled Assay for Glucose Transfer from Sucrose to ADP to Rebaudioside A 60 to Form Rebaudioside M

[0290] Libraries were screened using the following HTP enzyme-coupled assay. Pelleted *E. coli* cultures were lysed with 400 µL of Tris-HCl, pH 7.5 with 1 mM magnesium sulfate and 0.5 mg/mL lysozyme and polymyxin B sulfate (PMBS) and cleared by centrifugation. Lysate was diluted 75-100x into potassium phosphate buffer, pH 6.0 with 100 g/L RebA60 and pre-incubated for 2 hour at 75 °C. Then, 10 µL diluted, pre-incubated SuS lysate, 0.2 g/L β1,2GT SFP SEQ ID NO: 1216, and 0.43 g/L β1,3GT SFP SEQ ID NO: 520, were used in 100 µL reaction volume with 50 g/L RebA60, 0.01 g/L ADP (Amresco, ultra pure grade), 75 g/L sucrose (cane sugar), and 18 g/L fructose. The following reaction conditions were used: 50 mM potassium phosphate buffer, pH 6, 60°C in a THERMOTRON® titre-plate shaker with 300 RPM shaking for 16-18 h. The reactions described above were solubilized by diluting 50x in water, quenched by diluting 5x in acetonitrile with 0.2% formic acid, and precipitated by centrifugation. The supernatant was diluted 20x in water and steviol glycoside products were detected by RapidFire SPE-MS/MS with the instrument and parameters described in Table 5.1. The resulting engineered sucrose synthase variants that showed improved activity in the one-pot reaction are listed in Table 27.1 and 99.2. Shake-flask scale cultures were grown, lysed, and lyophilized to powder as described in Example 1 for variants listed in Table 27.3.

Table 27.1 SUS Combinatorial Variants and RebM Levels

SEQ ID NO: (nt/aa)	Amino Acid Differences (Relative to SEQ ID NO: 2182)	Increased RebM[a]
2309/2310	T34E/A323G/T640A	++
2311/2312	T34E/V62I	++
2313/2314	N406G/T640R	++
2315/2316	T34E/T65C	++
2317/2318	T34E/T640A	++
2319/2320	A323G/T640A/D731Q	++
2321/2322	R38H/T640R	++
2323/2324	V62I/A63S/T65C/E528D/T640A	++
2325/2326	A323G/H526T/E528D/T640R	++
2327/2328	A63T/T65C/N406G/D731Q	++

Table 27.1 SUS Combinatorial Variants and RebM Levels		
SEQ ID NO: (nt/aa)	**Amino Acid Differences (Relative to SEQ ID NO: 2182)**	**Increased RebM[a]**
2329/2330	A63T/T65C/A323G/N406G/H526T/E528D/T640N/D731Q	++
2331/2332	T34E/A63S/N406G/E528D/T640N/D731Q	+
2333/2334	A63S/D731Q	+
2335/2336	A63S/T65C/T640R	+
2337/2338	T34E/T65C/E528D/T640R	+
2339/2340	T34E/A63S/A323G/H526T/E528D/T640R	+
2341/2342	A63S/N406G	+
2343/2344	D731Q	+
2345/2346	A63S/A323G/N406G/T640A	+
2347/2348	A63S/H526T/E528D/T640R/D731Q	+
2349/2350	T34E/A323G/N406G/T640A	+
2351/2352	T34E/A63T/T65C/A323G/E528D/T640R	+
2353/2354	H526T/E528D/T640A/D731Q	+
2355/2356	T34E/V62I/T65C/T640R	+
2357/2358	V62I/A63S/H526T/T640R/D731Q	+
2359/2360	A323G/N406G/T640N	+
2361/2362	V62I/A63T/I69F/A323G/N406G/T640R	+
2363/2364	A63T/N406G/T640A	+
2365/2366	T34E/A63S/T65C/N406G/E528D/T640R/A713V	+
2367/2368	V62I/A323G/T640R	+
2369/2370	A323G/N406G/T640R/D731Q	+
2371/2372	T34E/T640R	+
2373/2374	A63T/T65C/E528D/T640A/D731Q	+
2375/2376	T34E/V62I/A323G	+
2377/2378	A323G/H526T/T640R	+
2379/2380	V62I/A63T	+
2381/2382	T34E	+

[a]Levels of increased production were determined relative to the reference polypeptide of SEQ ID NO: 2182, and defined as follows: "+" = production at least that of the reference but less than 1.1-fold reference polypeptide; and "++" = at least 1.1-fold increased production relative to the reference polypeptide.

Table 27.2 SUS Saturation Mutagenesis Variants and RebM Levels		
SEQ ID NO: (nt/aa)	**Amino Acid Differences (Relative to SEQ ID NO: 2182)**	**Increased RebM[a]**
2383/2384	S102T	++
2385/2386	S30A	++
2387/2388	Q108R	++
2389/2390	R683V	++
2391/2392	L206I	++
2393/2394	P546N	++
2395/2396	A59T	++
2397/2398	R158A	++
2399/2400	Q37L	++
2401/2402	N183P	++
2403/2404	R158K	+
2405/2406	N307H	+
2407/2408	A164G	+
2409/2410	A409S	+
2411/2412	Q37M	+
2413/2414	E710S	+
2415/2416	Q311S	+
2417/2418	R28H	+
2419/2420	T191L	+
2421/2422	R683L	+
2423/2424	V235A	+
2425/2426	A164T	+
2427/2428	T419V	+
2429/2430	S30M	+
2431/2432	E710A	+
2433/2434	S30H/R158H	+
2435/2436	Q311L	+
2437/2438	S30Q	+
2439/2440	T419L	+
2441/2442	P546Q	+
2443/2444	E710V	+
2445/2446	E793G	+
2447/2448	G543A	+

Table 27.2 SUS Saturation Mutagenesis Variants and RebM Levels		
SEQ ID NO: (nt/aa)	**Amino Acid Differences (Relative to SEQ ID NO: 2182)**	**Increased RebM[a]**
2449/2450	N307E	+
2451/2452	L533R	+
2453/2454	K559R	+
2455/2456	A164Q	+
2457/2458	T752R	+
2459/2460	E710G	+
2461/2462	A164S	+
2463/2464	S102N	+
2465/2466	L533K	+
2467/2468	P546T	+
[a]Levels of increased production were determined relative to the reference polypeptide of SEQ ID NO: 2182, and defined as follows: "+" = production at least 0.99-fold that of the reference but less than 1.1-fold reference polypeptide; and "++" = at least 1.1-fold increased production relative to the reference polypeptide.		

Shake Flask Powder Characterization Assay and Analysis for Glucosyl Transfer from Sucrose to Rebaudioside A 60 to form Rebaudioside M

[0291] An experiment was performed to characterize the activity of some of the engineered SUS variants on sucrose and ADP to facilitate the formation of rebaudioside M from rebaudioside A 60%. Shake flask powder (SFP) was made up to 0.016-0.5 g/L in potassium phosphate buffer, pH 6 with and without 100 g/L RebA60, and the solutions with RebA60 were pre-incubated at 75 °C for 2 hours. 10 µL diluted, either pre-incubated or not pre-incubated SuS lysate, 0.2 g/L β1,2 GT SFP SEQ ID NO: 1216, and 0.43 g/L β1,3GT SFP SEQ ID NO: 520, were used in 100 µL reaction volume with 50 g/L RebA60, 0.01 g/L ADP (Amresco, ultra pure grade), 75 g/L sucrose (cane sugar), and 18 g/L fructose. The following reaction conditions were used: 50 mM potassium phosphate buffer, pH 6, 60°C in a THERMOTRON® titre-plate shaker with 300 RPM shaking for 16-18 h. The reaction described above was solubilized by diluting 50x in water, quenched by diluting 5x in acetonitrile with 0.2% formic acid, and precipitated by centrifugation. The supernatant was diluted 20x in water and steviol glycoside products were detected by RapidFire SPE-MS/MS with the instrument and parameters described in Table 5.1. To assess thermostability, reactions were also performed with 10 µL of shake flask powders that had been dissolved at 0.2 g/L in 50 mM potassium phosphate buffer, pH 6 with 100 g/L RebA60, and incubated 24 h at 71.1 °C and then assayed in 100 µL reaction volume under the same one-pot reaction conditions described above. The thermostability results and

production levels of rebaudioside M in the one-pot reactions by these variants at 0.0125 g/L SFP loading are shown in Table 27.3.

Table 27.3 SUS SFP Variants and RebM Levels

SEQ ID NO: (nt/aa)	Amino Acid Differences (Relative to SEQ ID NO: 2182)	Increased RebM[a]	Increased RebM (pre-incubated)[a]	% Activity Remaining[b]
2317/2318	T34E/T640A	+	++	+
2315/2316	T34E/T65C	+	+	-
2327/2328	A63T/T65C/N406G/D731Q	+	+	++
2321/2322	R38H/T640R	+	+	+
2311/2312	T34E/V62I	+	+	+
2309/2310	T34E/A323G/T640A	-	+	++

[a]Levels of increased production were determined relative to the reference polypeptide of SEQ ID NO: 2182, and defined as follows: "-" = production less than 0.9-fold that of the reference polypeptide; "+" = production at least 0.9-fold that of the reference but less than 1.1-fold reference polypeptide; and "++" = production at least 1.1-fold increased relative to that of the reference polypeptide.

[b]The percent of activity remaining for each variant was determined following 24 h pre-incubation at 71.1°C, relative to the production from each variant following 24 h pre-incubation at 65 °C and is defined as follows: "-" = less than 16% of activity remained following 24 h pre-incubation at 71.1 °C; "+" = at least 16% but less than 26% activity remained; and "++" = at least 26% activity remained.

[0292] All 6 variants listed in Table 27.3 performed similarly or better than SEQ ID NO: 2182 in both activity assays and in the 24 h pre-incubation assay. The most active variant in the one-pot assay without 2 h pre-incubation, SEQ ID NO: 2322, was selected as the starting point for further enzyme engineering.

EXAMPLE 28

Sucrose Synthase Variants of SEQ ID NO: 2322

[0293] Directed evolution of the sucrose synthase encoded by SEQ ID NO: 2321 was continued by constructing libraries of variant genes in which certain structural features of the enzyme were subjected to saturation mutagenesis and libraries in which mutations associated with improved production identified during the development of the present invention were recombined. These libraries were then plated, grown, and screened using the high-throughput (HTP) assay described

below, to provide an additional round of 18 engineered SuS variant polypeptides with increased activity toward the generation of ADP-glucose.

HTP Coupled Assay for Glucose Transfer from Sucrose to ADP to Rebaudioside A 60 to Form Rebaudioside M

[0294] Libraries were screened using the following HTP enzyme-coupled assay. Pelleted *E. coli* cultures were lysed with 400 µL of Tris-HCl, pH 7.5 with 1 mM magnesium sulfate and 0.5 mg/mL lysozyme and polymyxin B sulfate (PMBS) and cleared by centrifugation. Lysate was diluted 100x into potassium phosphate buffer, pH 6.0 with 100 g/L RebA60 and pre-incubated for 2 hour at 75 °C. Then, 10 µL diluted, pre-incubated SuS lysate, 0.4 g/L β1,2GT SFP SEQ ID NO: 1516, and 0.9 g/L β1,3GT SFP SEQ ID NO: 678, were used in 100 µL reaction volume with 100 g/L RebA60, 0.05 g/L ADP (Amresco, ultra pure grade), 150 g/L sucrose (cane sugar), and 36 g/L fructose. The following reaction conditions were used: 50 mM potassium phosphate buffer, pH 6, 60°C in a THERMOTRON® titre-plate shaker with 300 RPM shaking for 16-18 h. The reactions described above were solubilized by diluting 100x in water, quenched by diluting 5x in acetonitrile with 0.2% formic acid, and precipitated by centrifugation. The supernatant was diluted 20x in water and steviol glycoside products were detected by RapidFire SPE-MS/MS with the instrument and parameters described in Table 5.1. The resulting engineered sucrose synthase variants that showed improved activity in the one-pot reaction are listed in Table 28.1. Shake-flask scale cultures were grown, lysed, and lyophilized to powder as described in Example 1 for variants listed in Table 28.2.

Table 28.1 SUS Variants and RebM Levels

SEQ ID NO: (nt/aa)	Amino Acid Differences (Relative to SEQ ID NO: 2322)	Increased RebM[a]
2469/2470	V62I/T65C/A323G/N406G/M565V/D731Q	+
2471/2472	A63T/T65C/A323G/N406G/M565V/D731Q	+
2473/2474	T34E/T65C/A323G/N406G/L411I/M565V	++
2475/2476	T34E/A323G/N406G/M565V/D731Q	+
2477/2478	V62I/T65C/N406G/L411I/M565V/D731Q	+++
2479/2480	A323G/L411I/M565V	++
2481/2482	A63T/T65C/N406G/L411I/M565V	+
2483/2484	A323G/N406G/L411I/M565V	++
2495/2486	V62I/A323G/L411I/M565V	+++
2487/2488	V62I/A323G/N406G/L411I/M565V	++
2489/2490	T34E/A323G/N406G/L411I/M565V	+++
2491/2492	Q636H	+
2493/2494	Q636N	+

Table 28.1 SUS Variants and RebM Levels		
SEQ ID NO: (nt/aa)	**Amino Acid Differences (Relative to SEQ ID NO: 2322)**	**Increased RebM[a]**
2495/2496	Q636A	+
2497/2498	V62I/T65C/A323G/N406G/M565V	+++
2499/2500	V62I/A323G/N406G/L411I	++
2501/2502	V62I/T65C/A323G/L411I/M565V	+
2503/2504	T65C/A323G/N406G/M565V	+
[a]Levels of increased production were determined relative to the reference polypeptide of SEQ ID NO: 2322, and defined as follows: "+" = production at least 1.05-fold that of the reference but less than 1.34-fold reference polypeptide; "++" = at least 1.34-fold increased production but less than 1.39-fold increased production; and "+++" = at least 1.39-fold increased production relative to the reference polypeptide.		

Shake Flask Powder Characterization Assay and Analysis for Glucosyl Transfer from Sucrose to Rebaudioside A 60 to form Rebaudioside M

[0295] An experiment was performed to characterize the activity of some of the engineered SUS variants on sucrose and ADP to facilitate the formation of rebaudioside M from rebaudioside A 60%. Shake flask powder (SFP) was made up to 0.125-2 g/L in potassium phosphate buffer, pH 6 with and without 100 g/L RebA60, and the solutions with RebA60 were pre-incubated at 75 °C for 2 hours. 10 µL diluted, either pre-incubated or not pre-incubated SuS lysate, 0.4 g/L β1,2 GT SFP SEQ ID NO: 1516, and 0.9 g/L β1,3GT SFP SEQ ID NO: 678**,** were used in 100 µL reaction volume with 100 g/L RebA60, 0.05 g/L ADP (Amresco, ultra pure grade), 150 g/L sucrose (cane sugar), and 36 g/L fructose. The following reaction conditions were used: 50 mM potassium phosphate buffer, pH 6, 60°C in a THERMOTRON® titre-plate shaker with 300 RPM shaking for 16-18 h. The reaction described above was solubilized by diluting 100x in water, quenched by diluting 5x in acetonitrile with 0.2% formic acid, and precipitated by centrifugation. The supernatant was diluted 20x in water and steviol glycoside products were detected by RapidFire SPE-MS/MS with the instrument and parameters described in Table 5.1. To assess thermostability, reactions were also performed with 10 µL of crude clarified lysate that had been diluted 25x in 50 mM potassium phosphate buffer, pH 6 with 100 g/L RebA60, and incubated 24 h at 71.1 °C and was then assayed in 100 µL reaction volume under the same one-pot reaction conditions described above. The thermostability results and production levels of rebaudioside M in the one-pot reactions by these variants at 0.025 g/L SFP loading are shown in Table 28.2.

Table 28.2 SUS SFP Variants and RebM Levels				
SEQ ID NO: (nt/aa)	**Amino Acid Differences (Relative to SEQ ID NO: 2322)**	**Increased RebM[a]**	**Increased RebM (pre-incubated)[a]**	**% Activity Remaining[b]**
2473/2474	T34E/T65C/A323G/N406G/L411I/M565V	+	+++	+
2477/2478	V62I/T65C/N406G/L411I/M565V/D731Q	+	+++	++
2485/2486	V62I/A323G/L411I/M565V	++	++	-
2487/2488	V62I/A323G/N406G/L411I/M565V	+	+++	+
2489/2490	T34E/A323G/N406G/L411I/M565V	+	+++	-
2497/2498	V62I/T65C/A323G/N406G/M565V	++	++	-
2499/2500	V62I/A323G/N406G/L411I	+	++	+
2505/2506	V62I/T65C/N406G/L411I/M565V/Q636H/D731Q	+	+	++

[a]Levels of increased production were determined relative to the reference polypeptide of SEQ ID NO: 2322, and defined as follows: "+" = production at least 1.15-fold that of the reference but less than 1.35-fold reference polypeptide; "++" = production at least 1.15-fold but less than 1.35-fold; and "+++" = production at least 1.35-fold increased relative to that of the reference polypeptide.
[b]The percent of activity remaining for each variant was determined following 24 h pre-incubation at 71.1 °C, relative to the production from each variant following 24 h pre-incubation at 65 °C and is defined as follows: "-" = less than 44% of activity remained following 24 h pre-incubation at 71.1 °C; "+" = at least 44% but less than 54% activity remained; and "++" = at least 54% activity remained.

[0296] All 8 variants listed in Table 28.2 performed similarly or better than SEQ ID NO: 2322 in both activity assays and 3 of these were also more stable after 24 h pre-incubation at 71.1 °C in 100 g/L RebA60. Of the three more thermostable variants, the most active variant in the one-pot assays with and without 2 h pre-incubation was SEQ ID NO: 2478. This variant, SEQ ID NO: 2478, was further engineered by introducing the amino acid mutation Q636H to produce SEQ ID NO: 2505/2506. This enzyme, SEQ ID NO: 2506 was selected as the best enzyme for the catalysis of glycosyltransfer from sucrose to ADP to regenerate ADP-glucose in the context of a one-pot reaction with a β-1,2-glycosyltransferase and a β-1,3-glycosyltransferase and for formation of rebaudioside M from steviol glycosides (e.g., rebaudioside A 60% or steviol glycosides 95% or steviol glycosides 85%).

[0297] All publications, patents, patent applications and other documents cited in this application are hereby incorporated by reference in their entireties for all purposes to the same extent as if each individual publication, patent, patent application or other document were individually indicated to be incorporated by reference for all purposes.

[0298] While various specific embodiments have been illustrated and described, it will be appreciated that various changes can be made without departing from the spirit and scope of the invention.

CLAIMS

We claim:

1. An engineered glycosyltransferase comprising a polypeptide sequence that has at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or more sequence identity to SEQ ID NO: 2.

2. The engineered glycosyltransferase of Claim 1, wherein said engineered glycosyltransferase comprising a polypeptide that is at least 95%, 96%, 97%, 98%, 99%, or more sequence identity to SEQ ID NO: 20.

3. The engineered glycosyltransferase of Claim 2, wherein said polypeptide sequence of said engineered glycosyltransferase comprises at least one mutation or mutation set at one or more positions selected from 41/72/233/338, 41/72/338, 41/144/233, 41/233, 41/233/338, 61, 61/87/91/107, 61/87/91/259, 61/91/431, 61/107, 61/259/428, 61/407/428, 61/411, 72, 72/76, 72/76/163/197, 72/76/195/233, 72/76/197/204, 72/76/207/233, 72/76/207/338, 72/81, 72/81/195/233, 72/139/195/204, 72/144/338, 72/200/204/207, 72/207, 76/144/197/200, 76/195/197/204/207/233, 76/197/207/233, 76/233, 81/139/144/195/200/204/207/233, 81/144/233, 81/197/200/207/233/338, 81/233/338, 81/338, 107, 107/259, 139/144/233, 144/233, 144/233/338, 156/407, 163/233/338, 200/204/207/233, 233/338, and 259, wherein said positions are numbered with reference to SEQ ID NO: 20.

4. The engineered glycosyltransferase of Claim 2, wherein said polypeptide sequence of said engineered glycosyltransferase comprises at least one mutation or mutation set at one or more positions selected from 71, 80, 81, 81/270, 83, 85, 97, 124, 263, 286, 334, 402, 420, and 456, wherein said positions are numbered with reference to SEQ ID NO: 20.

5. The engineered glycosyltransferase of Claim 2, wherein said polypeptide sequence of said engineered glycosyltransferase comprises at least one mutation or mutation set at one or more positions selected from 41/72/233/338, 41/72/338, 61, 61/91/431, 61/259/428, 61/407/428, 81/139/144/195/200/204/207/233, and 81/197/200/207/233/338, wherein said positions are numbered with reference to SEQ ID NO: 20.

6. The engineered glycosyltransferase of Claim 2, wherein said polypeptide sequence of said engineered glycosyltransferase comprises at least one mutation or mutation set at one or more positions selected from 73/81/144/207/259, 73/81/144/207/285/451, 73/81/259/285/426/451,

73/81/259/451, 73/111/285/451, 73/144/207/252/426/451, 73/144/207/259/285/426/451, 73/144/207/259/285/451, 73/144/207/259/426/451, 73/144/259/285, 73/259/426/451, 73/259/451, 81/111/144/207/285/451, 81/111/259/451, 81/144/207/252/285/426, 81/144/207/259/451, 81/144/252/259/285/451, 81/144/259, 81/144/259/285, 81/144/259/451, 81/207/252/259/451, 81/207/285/426/451, 81/285, 81/285/451, 111/144/207/252/285/426/451, 111/144/252/259/285/451, 111/144/259/285/426/451, 111/207/285, 144/207/252/259, 144/207/259, 144/207/259/285/451, 144/207/259/426/451, 144/207/285/426, 144/207/451, 144/252/259/285, 144/252/259/285/451, 144/252/259/426, 144/252/259/426/451, 144/259/285, 144/285, 144/285/451, 144/451, 207, 207/252/259/451, 207/252/451, 207/259, 207/259/426/451, 207/285/426/451, 207/451, 252/259, 259, 259/285/451, 285, and 285/451, wherein said positions are numbered with reference to SEQ ID NO: 36.

7. The engineered glycosyltransferase of Claim 2, wherein said polypeptide sequence of said engineered glycosyltransferase comprises at least one mutation or mutation set at one or more positions selected from 81/111/144/207/285/451, 144/207/252/259, 144/207/259/285/451, 144/252/259/426, 144/252/259/426/451, and 144/285/451, wherein said positions are numbered with reference to SEQ ID NO: 36.

8. The engineered glycosyltransferase of Claim 2, wherein said polypeptide sequence of said engineered glycosyltransferase comprises at least one mutation or mutation set at one or more positions selected from 80/81, 80/81/85, 80/81/85/238, 80/81/85/251/259, 80/81/85/252/256/259, 80/81/85/259, 80/81/251/252/259, 80/85, 80/85/238, 80/85/238/251/259, 80/85/251, 80/85/251/252, 80/85/251/252/256/259, 80/85/251/252/259, 80/85/251/259, 80/85/259, 80/259, 81/85, 81/85/251/252, 81/85/259, 85, 85/238, 85/251, 85/251/252/256, 85/251/259, 85/256/259, 175/402, 251/252/259, 259, 270/402, and 420, wherein said positions are numbered with reference to SEQ ID NO: 174.

9. The engineered glycosyltransferase of Claim 2, wherein said polypeptide sequence of said engineered glycosyltransferase comprises at least one mutation or mutation set at one or more positions selected from 3, 5, 7, 99, 153, 232/317, 252, 273, 299, 326, 393, 404, 409, 422, 443, 451, and 455, wherein said positions are numbered with reference to SEQ ID NO: 174.

10. The engineered glycosyltransferase of Claim 2, wherein said polypeptide sequence of said engineered glycosyltransferase comprises at least one mutation or mutation set at one or more positions selected from 80/81/85, 80/85, 80/85/251/259, 85, 85/251/252/256, 175/402, and 270/402, wherein said positions are numbered with reference to SEQ ID NO: 174.

11. The engineered glycosyltransferase of Claim 2, wherein said polypeptide sequence of said engineered glycosyltransferase comprises at least one mutation or mutation set at one or more positions selected from 81/85, 81/85/175/259/402, 81/85/251, 81/85/251/259, 81/85/259, 81/85/259/402, 85, 85/175, 85/175/251, 85/175/251/259/402, 85/175/259, 85/175/259/402, 85/175/402, 85/259, 85/259/402, and 85/402, wherein said positions are numbered with reference to SEQ ID NO: 406.

12. The engineered glycosyltransferase of Claim 2, wherein said polypeptide sequence of said engineered glycosyltransferase comprises at least one mutation or mutation set at one or more positions selected from 153, 153/326, 153/326/443, 153/326/443/455, 232, 232/273/299, 232/393/451, 299/451, 326, 404, and 451, wherein said positions are numbered with reference to SEQ ID NO: 408.

13. The engineered glycosyltransferase of Claim 2, wherein said polypeptide sequence of said engineered glycosyltransferase comprises at least one mutation or mutation set at one or more positions selected from 25, 116, 146, 170, 173, 227, 296, 300, 315, 327, 330, 361, 408, 412, 438, 448, and 449, wherein said positions are numbered with reference to SEQ ID NO: 408.

14. The engineered glycosyltransferase of Claim 2, wherein said polypeptide sequence of said engineered glycosyltransferase comprises at least one mutation or mutation set at one or more positions selected from 153, 232, 232/393/451, and 451, wherein said positions are numbered with reference to SEQ ID NO: 408.

15. The engineered glycosyltransferase of Claim 2, wherein said polypeptide sequence of said engineered glycosyltransferase comprises at least one mutation or mutation set at one or more positions selected from 146/170/196, 146/170/196/232, 146/170/196/232/423, 146/170/196/232/451/455, 146/170/196/408/451, 146/170/232, 146/170/232/423, 146/170/232/423/448/451/455, 146/170/259, 146/196, 146/196/232, 146/196/232/259/448/451, 146/196/455, 146/232/259/455, 146/232/315, 146/232/315/423/451/455, 146/232/326, 146/232/448, 146/232/451, and 146/413/451/455, wherein said positions are numbered with reference to SEQ ID NO: 440.

16. The engineered glycosyltransferase of Claim 2, wherein said polypeptide sequence of said engineered glycosyltransferase comprises at least one mutation or mutation set at one or more positions selected from 9, 12, 107, 131, 156, 161, 169, 199, 204, 209, 233, 262, 289, 337, and 417, wherein said positions are numbered with reference to SEQ ID NO: 440.

17. The engineered glycosyltransferase of Claim 2, wherein said polypeptide sequence of said engineered glycosyltransferase comprises at least one mutation or mutation set at one or more positions selected from 146/170/196, 146/170/196/232, 146/170/196/232/451/455, 146/170/232, 146/170/232/423/448/451/455, 146/196/232, 146/196/232/259/448/451, and 146/232/448, wherein said positions are numbered with reference to SEQ ID NO: 440.

18. The engineered glycosyltransferase of Claim 2, wherein said polypeptide sequence of said engineered glycosyltransferase comprises at least one mutation or mutation set at one or more positions selected from 9/12/107/161/199/209/337, 9/12/161/169/199/209/337, 9/12/199/204/209/337/455, 9/12/199/209/337/451/455, 9/107/131/204/259/417/451, 9/107/156/161/199/204/417/455, 9/107/161/209/259/289/451/455, 9/131/156/209/289/337, 9/131/204/337/451, 9/156/169/204/337, 9/169/204/289/337/451/455, 9/204, 9/204/259/289, 9/337, 12/14/107/204/289/455, 12/107/131/204/289/337/417/451/455, 12/107/156/209/289/455, 107/161/169/199/204/259/451, 107/199/204/289, 107/199/209/259/451/455, 107/204, 156/169/199/204/209/259/289, 161/204/417, 204/451/455, 289, and 451/455, wherein said positions are numbered with reference to SEQ ID NO: 520.

19. The engineered glycosyltransferase of Claim 2, wherein said polypeptide sequence of said engineered glycosyltransferase comprises at least one mutation or mutation set at one or more positions selected from 16, 53, 54, 78, 80, 95, 111, 221, 257, 336, 349, 391, 410, 413, 426, and 430, wherein said positions are numbered with reference to SEQ ID NO: 520.

20. The engineered glycosyltransferase of Claim 2, wherein said polypeptide sequence of said engineered glycosyltransferase comprises at least one mutation or mutation set at one or more positions selected from 9/12/199/204/209/337/455, 9/131/156/209/289/337, 9/204, 12/14/107/204/289/455, 107/161/169/199/204/259/451, 107/199/204/289, and 204/451/455, wherein said positions are numbered with reference to SEQ ID NO: 520.

21. The engineered glycosyltransferase of Claim 2, wherein said polypeptide sequence of said engineered glycosyltransferase comprises at least one mutation or mutation set at one or more positions selected from 16, 16/59/80/413, 16/80, 16/80/111, 16/80/111/257, 16/80/221/257/336/410, 16/80/221/336/410, 16/80/257, 16/111/221, 16/111/221/257/391, 16/221, 16/221/257, 16/221/257/336/391, 16/221/410, 16/257, 16/257/336/413/420, 80/111/221/257/410, 111/221/257/336/391, 111/221/257/391, 221, and 221/257, wherein said positions are numbered with reference to SEQ ID NO: 626.

22. The engineered glycosyltransferase of Claim 2, wherein said polypeptide sequence of said engineered glycosyltransferase comprises at least one mutation or mutation set at one or more positions selected from 5, 7, 14, 65, 91, 99, 102, 118, 138, 194, 254, 286, 416, 418, and 420, wherein said positions are numbered with reference to SEQ ID NO: 626.

23. The engineered glycosyltransferase of Claim 2, wherein said polypeptide sequence of said engineered glycosyltransferase comprises at least one mutation or mutation set at one or more positions selected from 16/80/221/257/336/410, 16/111/221, 16/221/257, 16/221/410, 16/257, 80/111/221/257/410, and 221, wherein said positions are numbered with reference to SEQ ID NO: 626.

24. The engineered glycosyltransferase of Claim 2, wherein said polypeptide sequence of said engineered glycosyltransferase comprises at least one mutation or mutation set at one or more positions selected from 5, 5/14/99, 5/14/416, 5/91, 5/91/102/118, 5/91/102/254/418, 5/91/106/254/286, 5/91/118/194/254/286, 5/91/118/194/254/418, 5/91/118/194/418, 5/91/118/254/286/418, 5/91/118/286, 5/91/194/254, 5/91/194/418, 5/91/254, 5/91/254/286, 5/102/418, 5/118/194/286/418, 5/118/254, 5/118/286, 5/194, 5/254/286, 5/418, 7/14, 7/14/65/99, 7/14/99/416, 14, 14/65/99/416, 14/99, 14/99/254, 14/99/254/416, 14/99/254/416/455, 14/99/416, 14/167, 14/254, 14/455, 91, 91/102/194, 91/118, 91/118/194, 91/118/194/286, 91/118/194/418, 91/118/286, 91/194/254/286, 91/286, and 118/286/418, wherein said positions are numbered with reference to SEQ ID NO: 678.

25. The engineered glycosyltransferase of Claim 2, wherein said polypeptide sequence of said engineered glycosyltransferase comprises at least one mutation or mutation set at one or more positions selected from 5/91/106/254/286, 5/91/118/286, 5/91/254/286, 14, 14/65/99/416, 14/99, and 14/99/254, wherein said positions are numbered with reference to SEQ ID NO: 678.

26. An engineered glycosyltransferase comprising a polypeptide sequence that has at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or more sequence identity to SEQ ID NO: 12.

27. The engineered glycosyltransferase of Claim 26, wherein said polypeptide sequence of said engineered glycosyltransferase comprises at least one mutation or mutation set at one or more positions selected from 16, 16/127/169, 16/134, 16/143/423, 16/169, 16/169/398/399, 16/169/423, 16/398, 16/398/399, 16/398/427, 16/423, 16/423/427, 134, 143, 399/423, and 423/427, wherein said positions are numbered with reference to SEQ ID NO: 24.

28. The engineered glycosyltransferase of Claim 26, wherein said polypeptide sequence of said engineered glycosyltransferase comprises at least one mutation or mutation set at one or more positions selected from 3, 8, 11, 41, 44, 56, 60, 122, 135, 137, 138, 139, 158, 164, 176, 221, 232, 233, 235, 248, 249, 281, 284, 285, 301, 322, 372, 392, 400, 421, 426, 427, 433, 440, 443, and 446, wherein said positions are numbered with reference to SEQ ID NO: 24.

29. The engineered glycosyltransferase of Claim 26, wherein said polypeptide sequence of said engineered glycosyltransferase comprises at least one mutation or mutation set at one or more positions selected from 16, 16/127/169, and 16/169, wherein said positions are numbered with reference to SEQ ID NO: 24.

30. The engineered glycosyltransferase of Claim 26, wherein said polypeptide sequence of said engineered glycosyltransferase comprises at least one mutation or mutation set at one or more positions selected from 3/22/198/421, 3/22/421, 3/201/248/322, 3/201/248/322/392, 13/233/331/440, 13/440/443, 22/32/56/60/198/248/322/392/421, 22/56, 22/56/137/198/201/248/322/392, 22/56/137/198/248/392/421, 22/56/137/322, 22/56/137/322/392/423, 22/56/198/202/248, 22/56/198/248/322/392/421, 22/56/198/248/421/423, 22/56/421/423, 22/60/392/421, 22/137, 22/137/198/202/248, 22/198/202/248/392, 22/248/392, 22/392, 22/421, 22/421/423, 22/423, 44/164/233, 44/164/233/329/331, 44/331, 125, 125/233/443/446, 164/221/233/331/440/446, 164/233/331/440, 164/233/446, 164/331, 164/440, 164/443, 198/202/392, 221/329/331, 233, 233/446, 329, 421/423, and 443/446, wherein said positions are numbered with reference to SEQ ID NO: 858.

31. The engineered glycosyltransferase of Claim 26, wherein said polypeptide sequence of said engineered glycosyltransferase comprises at least one mutation or mutation set at one or more positions selected from 3/22/198/421, 13/440/443, 22/56/198/202/248, 22/137, 22/198/202/248/392, 164/221/233/331/440/446, 164/233/331/440, and 233/446, wherein said positions are numbered with reference to SEQ ID NO: 858.

32. The engineered glycosyltransferase of Claim 26, wherein said polypeptide sequence of said engineered glycosyltransferase comprises at least one mutation or mutation set at one or more positions selected from 83/202/233, 164, 164/202, 164/202/233/331, 164/202/248/272, 164/202/331, 164/202/331/423, 164/423, 202/233, 202/233/248, 202/233/248/423, 202/248, 202/331, 202/421/423, 202/423, 202/446, 233, 248, and 423, wherein said positions are numbered with reference to SEQ ID NO: 994.

33. The engineered glycosyltransferase of Claim 26, wherein said polypeptide sequence of said engineered glycosyltransferase comprises at least one mutation or mutation set at one or more

positions selected from 7, 9, 10, 54, 73, 84, 106, 115, 116, 132, 165, 286, 309, 389, 406, 422, and 438, wherein said positions are numbered with reference to SEQ ID NO: 994.

34. The engineered glycosyltransferase of Claim 26, wherein said polypeptide sequence of said engineered glycosyltransferase comprises at least one mutation or mutation set at one or more positions selected from 83/202/233, 164, 164/202, 164/423, 202/233/248, and 233, wherein said positions are numbered with reference to SEQ ID NO: 994.

35. The engineered glycosyltransferase of Claim 26, wherein said polypeptide sequence of said engineered glycosyltransferase comprises at least one mutation or mutation set at one or more positions selected from 7/9/73/165/286, 7/9/165/286, 7/9/422, 7/116/165/286, 7/165, 9/73/116/165/286/422, 9/286/389, 54, 54/84, 54/406, 73, 73/116/165/286/389, 73/116/286, 73/116/286/422, 73/165/286, 73/286/389, 73/286/422, 73/422, 84, 115, 116, 116/165, 116/165/286/422, 116/389, 165, 165/286, 165/389, 165/389/422, 286, 286/422, 389/422, and 422, wherein said positions are numbered with reference to SEQ ID NO: 1080.

36. The engineered glycosyltransferase of Claim 26, wherein said polypeptide sequence of said engineered glycosyltransferase comprises at least one mutation or mutation set at one or more positions selected from 185/190, 219, 220, 255, 257, 302, 385, 395, 395/437, 399, 401, 409, 412, 416, 434, 441, 445, 447, and 449, wherein said positions are numbered with reference to SEQ ID NO: 1080.

37. The engineered glycosyltransferase of Claim 26, wherein said polypeptide sequence of said engineered glycosyltransferase comprises at least one mutation or mutation set at one or more positions selected from 9/73/116/165/286/422, 54, 54/84, 54/406, 73/116/286/422, 116/165/286/422, and 286/422, wherein said positions are numbered with reference to SEQ ID NO: 1080.

38. The engineered glycosyltransferase of Claim 26, wherein said polypeptide sequence of said engineered glycosyltransferase comprises at least one mutation or mutation set at one or more positions selected from 54, 54/185/190, 54/185/190/219/257/302/385/389/395/445/447/449, 54/185/190/219/385, 54/185/190/219/385/389/395/399/441/445/447/449, 54/185/190/219/385/395/399/441/445/447/449, 54/185/190/219/385/445/447, 54/185/190/219/389/395/399/441/445/447/449, 54/185/190/302, 54/185/190/302/395/399, 54/185/190/385/447/449, 54/185/190/389/395, 54/185/190/389/441/445/447/449, 54/185/190/389/445, 54/185/190/395/399/441/445/447/449, 54/185/219/389, 54/185/257/389/441/445/447/449, 54/185/302/385/399/445/447, 54/185/385/389/395/441/445/449, 54/185/385/395/399/445/447/449, 54/185/389/395/445/447, 54/190/219/257/302,

54/190/219/257/395/445/447/449, 54/190/257, 54/190/257/302/399, 54/190/257/309/385/389/395/399/445, 54/190/257/385/389, 54/190/257/395/445/449, 54/190/302/389, 54/190/302/389/395/399/445/447, 54/190/385/389/395/441/445, 54/190/385/395, 54/190/385/445/447/449, 54/190/395, 54/190/395/445/447, 54/219/302/395/441/445/447, 54/219/385/389/395/441/445/447, 54/257, 54/257/385/449, 54/257/389, 54/257/399, 54/257/441/447, 54/257/441/449, 54/302/385/399/441/445/447, 54/302/385/399/441/445/449, 54/385, 54/385/389/441/445/447/449, 54/389/395/399/447/449, 185/190/219/257/389/395/445/447, 185/190/257/302/395/441/445/447, 185/190/257/385/389/399, 185/190/257/385/399/445/447, 185/190/257/389/395, 185/190/257/389/395/399/441/447/449, 185/190/385/389/441/445/447, 185/190/389/447/449, 185/190/395/399, 185/219/257/399/445/447, 185/257/385/395/399/445/447, 185/302/395/399/441/445/447/449, 185/395/399/441/445/447, 190/219/257/385/389/441/445/447/449, 190/219/302/385/399/445, 190/257/302/385/399, 190/257/385/389/399, 190/257/385/389/445/447, 190/257/385/395, 190/257/385/441/445/447/449, 190/257/389/395/441/445/447, 190/257/399/447/449, 190/302/385/389/395/399/441/445/447, 190/389, 219/257/385/389/395/441/447/449, 219/257/395, 219/385/389/399/445/449, 219/395, 257/385/389/399, 257/389/395/399/445/447, 257/389/395/399/445/449, 257/389/399, 302/389/395/445, 385/389, 385/395, 389/395/445/447, 395/399, and 399 wherein said positions are numbered with reference to SEQ ID NO: 1216.

39. The engineered glycosyltransferase of Claim 26, wherein said polypeptide sequence of said engineered glycosyltransferase comprises at least one mutation or mutation set at one or more positions selected from 5, 14, 96, 108, 157, 181, 188, 278, 293, and 341, wherein said positions are numbered with reference to SEQ ID NO: 1216.

40. The engineered glycosyltransferase of Claim 26, wherein said polypeptide sequence of said engineered glycosyltransferase comprises at least one mutation or mutation set at one or more positions selected from 54/185/190/219/257/302/385/389/395/445/447/449, 54/185/190/385/447/449, 54/190/257/302/385/395/399, 54/190/302/389/395/399/445/447, 54/257/385/449, 54/257/389, and 54/389/395/399/447/449, wherein said positions are numbered with reference to SEQ ID NO: 1216.

41. The engineered glycosyltransferase of Claim 26, wherein said polypeptide sequence of said engineered glycosyltransferase comprises at least one mutation or mutation set at one or more positions selected from 14/42/51/108/181, 14/42/51/157/341, 14/42/341, 14/51/96/157, 14/51/96/157/341, 14/51/96/341, 14/51/108, 14/51/341, 14/96, 14/96/108/133, 14/96/108/181/341, 14/96/108/188, 14/96/341, 14/108/157, 14/108/157/188, 14/157/181/278, 14/278, 42/96/157/341, 42/188/341, 96/108/181/293, 96/108/188, 96/108/188/341, 96/188, 181, 188, and 293, wherein said positions are numbered with reference to SEQ ID NO: 1488.

42. The engineered glycosyltransferase of Claim 26, wherein said polypeptide sequence of said engineered glycosyltransferase comprises at least one mutation or mutation set at one or more positions selected from 122, 144, 147, 187, 196, 197, 198, 199, 201, 268, and 324, wherein said positions are numbered with reference to SEQ ID NO: 1488.

43. The engineered glycosyltransferase of Claim 26, wherein said polypeptide sequence of said engineered glycosyltransferase comprises at least one mutation or mutation set at one or more positions selected from 14/42/341, 14/96, 14/96/108/133, 14/96/108/181/341, and 188, wherein said positions are numbered with reference to SEQ ID NO: 1488.

44. The engineered glycosyltransferase of Claim 26, wherein said polypeptide sequence of said engineered glycosyltransferase comprises at least one mutation or mutation set at one or more positions selected from 147/188/196/201, 152/187/188/324, 152/188, 152/188/196/198/199/324, 152/188/196/199, 152/188/196/201/324, 152/188/324, 188, 188/196/198/199/201, 188/196/198/199/201/324, 188/196/198/201, 188/196/198/324, 188/196/201, 188/198/199/201/324, 188/198/199/268, 188/198/201/324, 188/198/324, 188/199/201, 188/201, 188/201/324, and 188/324, wherein said positions are numbered with reference to SEQ ID NO: 1516.

45. The engineered glycosyltransferase of Claim 26, wherein said polypeptide sequence of said engineered glycosyltransferase comprises at least one mutation or mutation set at one or more positions selected from 152/188/196/198/199/324, 188/196/198/199/201, 188/196/198/201, 188/196/201, 188/198/199/268, 188/201, and 188/324, wherein said positions are numbered with reference to SEQ ID NO: 1516.

46. The engineered glycosyltransferase of any of Claims 1-45, wherein said engineered glycosyltransferase is selected from beta-1,2-glycosyltransferases and beta-1,3-glycosyltransferases.

47. The engineered glycosyltransferase of any of Claims 1-46, wherein said engineered glycosyltransferase is an NDP-glycosyltransferase selected from ADP-glucose-dependent glycosyltransferases (AGTs), CDP-glucose-dependent glycosyltransferases (CGTs), GDP-glucose-dependent glycosyltransferase (GGTs), TDP-glucose-dependent glycosyltransferases (TGTs), and IDP-glucose-dependent glycosyltransferase (IGTs).

48. The engineered glycosyltransferase of any of Claims 1-47, wherein said engineered glycosyltransferase is an ADP-glucose-dependent glycosyltransferase.

49. The engineered glycosyltransferase of any of Claims 1-48, wherein said engineered glycosyltransferase preferentially uses a sugar donor other than uracil-diphosphate.

50. An engineered polynucleotide encoding at least one engineered glycosyltransferase polypeptide provided in any of Claims 1-48.

51. A vector comprising at least one engineered polynucleotide of Claim 50.

52. The vector of Claim 51, wherein said vector further comprises at least one control sequence.

53. A host cell comprising at least one engineered polynucleotide of Claim 50.

54. A host cell comprising at least one vector of Claim 51 and/or 52.

55. The host cell of Claim 53 or 54, wherein said host cell is selected from eukaryotic and prokaryotic organisms.

56. A method for producing at least one engineered glycosyltransferase provided in any of Claims 1-45, comprising culturing the host cell of any of Claims 53-55, under conditions such that said engineered glycosyltransferase is produced by said host cell.

57. The method of Claim 56, further comprising the step of recovering said engineered glycosyltransferase.

58. A composition comprising at least one engineered glycosyltransferase of any of Claims 1-45.

59. An engineered sucrose synthase comprising a polypeptide sequence that has at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or more sequence identity to SEQ ID NO: 6.

60. An engineered sucrose synthase comprising a polypeptide sequence that has at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or more sequence identity to SEQ ID NO: 22.

61. The engineered sucrose synthase of Claim 59, wherein said polypeptide sequence of said engineered sucrose synthase comprises at least one mutation or mutation set at one or more positions selected from 3/548, 4, 7, 12, 41, 42, 44, 47, 52, 57, 59, 71, 81, 85, 93, 97, 122, 129, 134, 136, 139, 154, 175, 215, 266, 270, 343, 358, 381, 388, 434, 442, 519, 524, 532, 536, 570, 589, 603, 606, 615, 635, 641, 652, 724, 727, and 738 wherein said positions are numbered with reference to SEQ ID NO: 22.

62. The engineered sucrose synthase of Claim 59, wherein said polypeptide sequence of said engineered sucrose synthase comprises at least one mutation or mutation set at one or more positions selected from 41, 52, 134, 381, 442, 519, 524, and 724, wherein said positions are numbered with reference to SEQ ID NO: 22.

63. The engineered sucrose synthase of Claim 59, wherein said polypeptide sequence of said engineered sucrose synthase comprises at least one mutation or mutation set at one or more positions selected from 25/44/52/134/434/724, 41/44/52/97/442/719/724, 41/44/52/134/442, 41/44/52/434/442/532/724, 41/44/52/434/724, 41/44/136/329, 41/44/136/442, 41/44/532, 41/52/97/434/442/532/724, 41/52/134/136/434, 41/52/134/442/724, 41/52/136, 41/52/434, 41/52/434/442, 41/52/434/442/724, 41/52/442, 41/97/134/434/442/532/553, 41/134/442/532, 41/329/442, 41/434/442/532, 41/434/442/532/724, 41/434/532, 41/532, 44/52/97/434/442/724, 44/52/97/442/724, 44/52/134/136/329/434/442/532, 44/52/134/434/532, 44/136/329/434/532, 44/136/532, 44/434/442/553, 52, 52/97, 52/97/434/442, 52/97/442, 52/97/532, 52/134, 52/134/136/434, 52/134/136/442, 52/134/329/434, 52/134/329/532, 52/134/434/442/532/553, 52/134/442/724, 52/136, 52/136/434, 52/136/434/442, 52/136/434/442/532, 52/136/434/724, 52/136/442, 52/434, 52/434/442/532, 52/434/442/724, 52/434/532, 52/442, 52/442/532/724, 52/442/553/724, 52/442/724, 52/532, 52/553, 57/97/434/442/724, 97/134/136/442/532, 97/134/136/532, 97/134/442, 97/136/434/442, 97/329/724, 97/442, 134, 134/136/434/442, 134/136/434/442/532/553/724, 134/136/434/442/553/724, 134/136/434/532/724, 134/136/442/532, 134/434/442/724, 136, 136/442, 136/442/724, 136/532/724, 434, 434/442, 442, 442/724, and 532/724, wherein said positions are numbered with reference to SEQ ID NO: 1652.

64. The engineered sucrose synthase of Claim 59, wherein said polypeptide sequence of said engineered sucrose synthase comprises at least one mutation or mutation set at one or more positions selected from 3, 4, 22, 32, 34, 38, 47/488, 51, 51/433, 62, 75/169, 101, 169, 195/213, 708, and 718, wherein said positions are numbered with reference to SEQ ID NO: 1652.

65. The engineered sucrose synthase of Claim 59, wherein said polypeptide sequence of said engineered sucrose synthase comprises at least one mutation or mutation set at one or more

positions selected from 44/52/97/442/724, 52/97/442, 97/442, and 434/442, wherein said positions are numbered with reference to SEQ ID NO: 1652.

66. The engineered sucrose synthase of Claim 59, wherein said polypeptide sequence of said engineered sucrose synthase comprises at least one mutation or mutation set at one or more positions selected from 4/22, 4/22/34, 4/22/34/38, 4/22/34/38/169/708, 4/22/34/47/51/169/213, 4/22/51/708, 4/22/101, 4/34/38/101, 4/34/708, 4/62/433/708, 4/169, 4/169/433, 4/169/708, 4/433, 4/708, 22/34, 22/34/38, 22/34/101/169, 22/34/101/169/195, 22/34/169/708, 22/47/169/433, 22/75, 34/38/62, 34/62/169/433, 34/101, 62/708, 75/169, 169/708, and 195/708, wherein said positions are numbered with reference to SEQ ID NO: 1822.

67. The engineered sucrose synthase of Claim 59, wherein said polypeptide sequence of said engineered sucrose synthase comprises at least one mutation or mutation set at one or more positions selected from 4, 22, 34, 121, 169, 341, 411, 433, 441, 518, 526, 527, 528, 544, 557, 558, 565, 585, 602, 604, 623, 708, 731, and 770, wherein said positions are numbered with reference to SEQ ID NO: 1822.

68. The engineered sucrose synthase of Claim 59, wherein said polypeptide sequence of said engineered sucrose synthase comprises at least one mutation or mutation set at one or more positions selected from 4, 4/22/34/38, 4/169, 4/169/433, 4/433, 62/708, and 169/708, wherein said positions are numbered with reference to SEQ ID NO: 1822.

69. The engineered sucrose synthase of Claim 59, wherein said polypeptide sequence of said engineered sucrose synthase comprises at least one mutation or mutation set at one or more positions selected from 34/62/411/526/557, 34/62/411/557/602/604, 34/62/441/557/623, 34/62/518/623, 34/62/623/731, 34/121/441/544/604/623, 34/121/526/604/731, 34/411/441, 34/411/441/518/526/557/565/731, 34/411/441/518/544/557/565/708/731, 34/411/441/518/585/604/770, 34/441/518/526/585/604/731, 34/441/526/565/708/770, 34/441/544/557/585/602/623, 34/441/585/623/708/731, 34/526/585/623, 62/121/329/518/557/565/623/708, 62/121/411/441/518/544/557/585/604/623, 62/121/441/518/526/623/770, 62/121/441/565/585, 62/121/518/557/585/604/708/770, 62/411/526/565/604/623, 62/411/585/731, 62/441/518/557/604/623/708/731, 62/441/623/708/770, 62/441/770, 121/441/518/526/602/604, 121/441/518/526/623/708, 121/441/526/557/565/708, 121/604/708/731/770, 411/441/518/557/623, 411/441/518/708, 411/441/604/623/708/731, 411/518/526/604/623/731, 411/565/604/623, 411/585/623, 441/518/526/557/565/602/604/623/708, 441/518/526/557/585/604/623/708, 441/518/526/604/623, 441/518/557/565/585, 441/518/565/623/731/770, 441/518/585, 441/585, 441/585/602/604/623/708/731, 441/708/731,

557/602/604, 557/604, 585/623/708, and 623, wherein said positions are numbered with reference to SEQ ID NO: 2092.

70. The engineered sucrose synthase of Claim 59, wherein said polypeptide sequence of said engineered sucrose synthase comprises at least one mutation or mutation set at one or more positions selected from 63, 65, 269, 323, 406, 416, 469, 511, and 640, wherein said positions are numbered with reference to SEQ ID NO: 2092.

71. The engineered sucrose synthase of Claim 59, wherein said polypeptide sequence of said engineered sucrose synthase comprises at least one mutation or mutation set at one or more positions selected from 34/62/441/557/623, 34/441/585/623/708/731, 62/441/623/708/770, 411/441/518/557/623, 411/441/604/623/708/731, 441/518/526/557/585/604/623/708, 441/518/557/565/585, and 441/585/602/604/623/708/731, wherein said positions are numbered with reference to SEQ ID NO: 2092.

72. The engineered sucrose synthase of Claim 59, wherein said polypeptide sequence of said engineered sucrose synthase comprises at least one mutation or mutation set at one or more positions selected from 34, 34/62, 34/62/65/640, 34/62/323, 34/63/65/323/528/640, 34/63/65/406/528/640/713, 34/63/323/526/528/640, 34/63/406/528/640/731, 34/65, 34/65/528/640, 34/323/406/640, 34/323/640, 34/640, 38/640, 62/63, 62/63/65/528/640, 62/63/69/323/406/640, 62/63/526/640/731, 62/323/640, 63/65/323/406/526/528/640/731, 63/65/406/731, 63/65/528/640/731, 63/65/640, 63/323/406/640, 63/406, 63/406/640, 63/526/528/640/731, 63/731, 323/406/640, 323/406/640/731, 323/526/528/640, 323/526/640, 323/640/731, 406/640, 526/528/640/731, and 731, wherein said positions are numbered with reference to SEQ ID NO: 2182.

73. The engineered sucrose synthase of Claim 59, wherein said polypeptide sequence of said engineered sucrose synthase comprises at least one mutation or mutation set at one or more positions selected from 28, 30, 30/158, 37, 59, 102, 108, 158, 164, 183, 191, 206, 235, 307, 311, 409, 419, 533, 543, 546, 559, 683, 710, 752, and 793, wherein said positions are numbered with reference to SEQ ID NO: 2182.

74. The engineered sucrose synthase of Claim 59, wherein said polypeptide sequence of said engineered sucrose synthase comprises at least one mutation or mutation set at one or more positions selected from 34/62, 34/65, 34/323/640, 34/640, 38/640, and 63/65/406/731, wherein said positions are numbered with reference to SEQ ID NO: 2182.

75. The engineered sucrose synthase of Claim 59, wherein said polypeptide sequence of said engineered sucrose synthase comprises at least one mutation or mutation set at one or more positions selected from 34/65/323/406/411/565, 34/323/406/411/565, 34/323/406/565/731, 62/65/323/406/565, 62/65/323/406/565/731, 62/65/323/411/565, 62/65/406/411/565/731, 62/323/406/411, 62/323/406/411/565, 62/323/411/565, 63/65/323/406/565/731, 63/65/406/411/565, 65/323/406/565, 323/406/411/565, 323/411/565, and 636, wherein said positions are numbered with reference to SEQ ID NO: 2322.

76. The engineered sucrose synthase of Claim 59, wherein said polypeptide sequence of said engineered sucrose synthase comprises at least one mutation or mutation set at one or more positions selected from 34/65/323/406/411/565, 34/323/406/411/565, 62/65/323/406/565, 62/65/406/411/565/636/731, 62/65/406/411/565/731, 62/323/406/411, 62/323/406/411/565, and 62/323/411/565, wherein said positions are numbered with reference to SEQ ID NO: 2322.

77. An engineered polynucleotide encoding at least one engineered sucrose synthase polypeptide provided in any of Claims 59-76.

78. A vector comprising at least one engineered polynucleotide of Claim 77.

79. The vector of Claim 78, further comprising at least one control sequence.

80. A host cell comprising at least one engineered polynucleotide of Claim 78.

81. A host cell comprising the vector of Claim 78 or Claim 79.

82. The host cell of Claim 80 and/or 81, wherein said host cell is selected from eukaryotic and prokaryotic organisms.

83. A method for producing at least one engineered sucrose synthase variant provided of Claims 59-76, comprising culturing the host cell of any of Claims 80-82, under conditions such that said engineered sucrose synthase variant is produced by said host cell.

84. The method of Claim 83, further comprising the step of recovering said engineered sucrose synthase variant.

85. A composition comprising at least one engineered sucrose synthase variant of any of Claims 60-75.

86. A method for glycosylation of a substrate comprising providing at least one substrate, at least one engineered glycosyl transferase of any of Claims 1-45, and contacting said substrate with said glycosyltransferase under conditions such that said substrate is glycosylated to produce at least one glycosylated product.

87. The method of Claim 86, wherein said substrate comprises at least one steviol glycoside.

88. The method of Claim 86, wherein said glycosylated product comprises at least one mono-glycosylated and/or polyglycosylated product.

89. A method for producing rebaudioside M, comprising providing a rebaudioside D and/or rebaudioside I substrate, NDP-glucose, and a least one engineered glycosyltransferase of Claims 1-45, combining said rebaudioside D and rebaudioside I substrate, NDP-glucose, and said glycosyltransferase under conditions such that rebaudioside M is produced.

90. A method for producing rebaudioside A and/or rebaudioside I, comprising providing a stevioside substrate, NDP-glucose, and at least one engineered glycosyltransferase of Claims 1-45, combining said stevioside substrate, NDP-glucose, and glycosyltransferase under conditions such that rebaudioside A and/or rebaudioside I is produced.

91. A method for producing rebaudioside D, comprising providing a stevioside substrate, NDP-glucose, and at least one engineered glycosyltransferase of Claims 1-45, combining said stevioside substrate, NDP-glucose, and glycosyltransferase under conditions such that rebaudioside D is produced.

92. The method of any of Claims 86-91, wherein said NDP-glucose is selected from ADP-glucose, CDP-glucose, TDP-glucose, GDP-glucose, and/or IDT-glucose.

93. The method of any of Claims 86-92, wherein said NDP-glucose is not UDP-glucose.

94. The method of any of Claims 86-93, wherein said glycosyltransferase comprises at least one the glycosyltransferase of any of Claims 1-25 and/or Claims 26-45.

95. A method for producing rebaudioside M comprising providing a rebaudioside D and/or rebaudioside I substrate, ADP-glucose, and at least one engineered glycosyltransferase of

Claims 1-45, combining said rebaudioside D and/or rebaudioside I substrate, ADP-glucose, and glycosyltransferase under conditions such that rebaudioside M is produced.

96. A method for producing rebaudioside A and/or rebaudioside I, comprising providing a stevioside substrate, ADP-glucose, and at least one engineered ADP-glycosyltransferase of Claims 1-45, combining said stevioside substrate, ADP-glucose, and glycosyltransferase under conditions such that rebaudioside A and/or rebaudioside I is produced.

97. A method for producing rebaudioside D, comprising providing a stevioside substrate, ADP-glucose, and at least one engineered glycosyltransferase of Claims 1-45, combining said stevioside substrate, ADP-glucose, and glycosyltransferase under conditions such that rebaudioside D is produced.

98. A method for producing rebaudioside M comprising providing a rebaudioside D and/or rebaudioside I substrate, NDP, sucrose, a sucrose synthase, and at least one engineered glycosyltransferase of Claims 1-45, combining said rebaudioside D substrate, NDP, sucrose, sucrose synthase, and glycosyltransferase under conditions such that rebaudioside M is produced.

99. A method for producing rebaudioside A and/or rebaudioside I comprising providing a stevioside substrate, NDP, sucrose, a sucrose synthase, and at least one engineered glycosyltransferase of Claims 1-45, combining said stevioside substrate, NDP, sucrose, sucrose synthase, and glycosyltransferase under conditions such that rebaudioside A and/or rebaudioside I is produced.

100. A method for producing rebaudioside D, comprising providing a stevioside substrate, NDP, sucrose, a sucrose synthase, and at least one engineered glycosyltransferase of Claims 1-45, combining said stevioside substrate, NDP, sucrose, sucrose synthase, and glycosyltransferase under conditions such that rebaudioside D is produced.

101. A method for producing rebaudioside M, comprising providing a stevioside substrate comprising at least one stevioside and/or a mixture of steviosides and rebA, NDP, sucrose, a sucrose synthase, and at least one engineered glycosyltransferase of Claims 1-45, combining said stevioside substrate, NDP, sucrose, sucrose synthase, and glycosyltransferase under conditions such that rebaudioside M is produced.

102. A method of producing rebaudioside M, comprising providing a stevioside substrate, NDP, sucrose, at least one sucrose synthase, and at least one engineered glycosyltransferase of Claims 1-45, combining said stevioside substrate, NDP, and glycosyltransferase under conditions such that

rebaudioside A is first produced, rebaudioside D and/or rebaudioside I is then produced, and rebaudioside M finally produced.

103. The method of any of Claims 98-102, wherein said sucrose synthase is an engineered sucrose synthase provided in any of Claims 59-76.

104. The method of any of Claims 86-103, wherein said method is conducted as a one-pot reaction.

105. The method of any of Claims 86-104, wherein said method is conducted sequentially.

106. The method of any of Claims 86-104, further comprising repeating the steps of the methods.

107. The method of Claim 106, wherein said sucrose is recycled during repeated steps.

108. The method of Claim 106 and/or 101, wherein the engineered glycosyltransferase and/or other reaction components are recycled.

109. The method of any of Claims 86-108, wherein said stevioside substrate is extracted from *Stevia rebaudiana*.

110. The method of any of Claims 86-108, wherein said stevioside substrate is synthetically produced.

111. The method of any of Claims 86-110, wherein said glycosyltransferase and/or said sucrose synthase is immobilized.

112. The method of any of Claims 86-110, wherein the method produces a reaction product that includes fructose.

113. The method of Claim 112, wherein said fructose is removed from said reaction product.

114. The method of any of Claims 86-113, wherein the method further comprises a washing step.

115. The method of any of Claims 86-114, wherein the method further comprises at least one column chromatography step.

116. The method of any of Claims 86-115, wherein said at least one engineered glycosyltransferase is a beta-1,2 glycosyltransferase selected from the glycosyltransferase of any of Claims 26-45.

117. The method of any of Claims 86-116, wherein said at least one engineered glycosyltransferase is a beta-1,3 glycosyltransferase selected from the glycosyltransferase of any of Claims 1-25.

118. The method of any of Claims 86-117, wherein said at least one engineered glycosyltransferase is a beta-1,2 glycosyltransferase selected from the glycosyltransferase of any of Claims 26-45, and further comprising at least one engineered glycosyltransferase is a beta-1,3 glycosyltransferase selected from the glycosyltransferase of any of Claims 1-25.

119. The method of Claims 86-118, further comprising at least one engineered sucrose synthase of any of Claims 59-76.

120. At least one rebaudioside produced according to the methods of any of Claims 86-119.

121. A composition comprising the rebaudioside of Claim 120.

122. Rebaudioside M produced according to the methods of any of Claims 86-89, 92-95, 98, and/or 101-121.

123. A composition comprising the rebaudioside M of Claim 122.

124. Rebaudioside A produced according to the methods of any of Claims 86-94, 96, 99, and/or 101-121.

125. A composition comprising the rebaudioside A of Claim 124.

126. Rebaudioside I produced according to the methods of any of Claims 86-94, 96, 99, and/or 101-121.

127. A composition comprising the rebaudioside I of Claim 126.

128. Rebaudioside D produced according to the methods of any of Claims 86-94, 97, and/or 100-121.

129. A composition comprising the rebaudioside D of Claim 128.

130. A composition comprising at least two rebaudiosides provided in Claims 122, 124, 126, and/or 128.

131. A composition comprising a mixture of at least two compositions provided in Claims 123, 125, 127, and/or 129.