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(54) Title: GLYCOSYLTRANSFERASE GENE AND USE THEREOF

(57) Abrégé/Abstract:
A polynucleotide is provided that encodes a protein having activity that transfers a sugar to the hydroxyl group at the 4'-position of a flavone. The polynucleotide is selected from the group consisting of: (a) a polynucleotide composed of the base sequence of SEQ ID NO: 1; (b) a polynucleotide that hybridizes under stringent conditions with a polynucleotide composed of a base sequence complementary to the base sequence of SEQ ID NO: 1, and encodes a protein having activity that transfers a sugar to the hydroxyl group at the 4'-position of a flavone; (c) a polynucleotide encoding a protein composed of the amino acid sequence of SEQ ID NO: 2; and, (d) a polynucleotide encoding a protein composed of an amino acid sequence in which one or a plurality of amino acids have been deleted, substituted, inserted and/or added in the amino acid sequence of SEQ ID NO: 2, and having activity that transfers a sugar to the hydroxyl group at the 4'-position of a flavone.

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ABSTRACT

A polynucleotide is provided that encodes a protein having activity that transfers a sugar to the hydroxyl group at the 4'-position of a flavone. The polynucleotide is selected from the group consisting of: (a) a polynucleotide composed of the base sequence of SEQ ID NO: 1; (b) a polynucleotide that hybridizes under stringent conditions with a polynucleotide composed of a base sequence complementary to the base sequence of SEQ ID NO: 1, and encodes a protein having activity that transfers a sugar to the hydroxyl group at the 4'-position of a flavone; (c) a polynucleotide encoding a protein composed of the amino acid sequence of SEQ ID NO: 2; and, (d) a polynucleotide encoding a protein composed of an amino acid sequence in which one or a plurality of amino acids have been deleted, substituted, inserted and/or added in the amino acid sequence of SEQ ID NO: 2, and having activity that transfers a sugar to the hydroxyl group at the 4'-position of a flavone.

DESCRIPTION

GLYCOSYLTRANSFERASE GENE AND USE THEREOF

TECHNICAL FIELD

[0001]

The present invention relates to a polynucleotide that encodes a protein having activity that transfers a sugar to the hydroxyl group at the 4'-position of a flavone.

BACKGROUND ART

[0002]

Flowers having new traits are always valued in the flower industry. In particular, the development of plants in which "color", which is considered to be the most important trait of a flower, has been altered is considered to be industrially important, and various flower colors have been developed through selective breeding using the classical method of crossbreeding. Although crossbreeding is an effective method for selective breeding, since there are genetic restrictions unique to plants, this method has the shortcoming of only being able to use genetic resources possessed by related species capable of crossbreeding. For example, despite many years of selective breeding, violet to blue-colored varieties of roses, carnations, chrysanthemums and lilies, vivid red-colored varieties of gentians and irises, and yellow varieties of morning glories have yet to be produced.

[0003]

Flower color originates in four types of pigments consisting of flavonoids, carotenoids, chlorophyll and betalain. Among these, flavonoids exhibit a diverse range of colors such as yellow, red, violet and blue. The group that exhibits red, violet and blue color is generically referred to as anthocyanins, and the diverse structure of anthocyanins is one of the causes of the diverse range of flower color. Anthocyanins can be

broadly divided into three groups according to their aglycon structure in consideration of the biosynthesis pathway thereof. Flowers having a vivid red color in the manner of carnations and geraniums contain a large amount of pelargonidin-based anthocyanins, while flowers having a blue or violet color contain a large amount of delphinidin-based anthocyanins. The reason for the absence of blue or violet varieties of roses, carnations, chrysanthemums and lilies is because these plants do not have the ability to synthesize delphinidin-based anthocyanins.

[0004]

In addition to the accumulation of delphinidin, any of (i) the modification of anthocyanin by one or a plurality of aromatic acyl groups, (ii) the presence of a co-pigment such as flavone or flavonol together with anthocyanin, (iii) the presence of iron ions or aluminum ions together with anthocyanin, (iv) a rise in the pH of vacuoles where anthocyanin is localized from neutral to weakly alkaline, or (v) the formation of a complex by anthocyanin, co-pigment and metal ions (and this type of anthocyanin is referred to as metalloanthocyanin) is thought to be required in order for flower color to become blue (see Non-Patent Document 1).

[0005]

Considerable research has been conducted on flavonoid and anthocyanin biosynthesis, and related biosynthetic enzymes and genes encoding those enzymes have been identified (see Non-Patent Document 2 and FIG. 1). For example, the gene of flavonoid 3',5'-hydroxylase (F3'5'H), which hydroxylates the B ring of flavonoids required for the biosynthesis of delphinidin, is obtained from numerous plants. In addition, transgenic plants that accumulate delphinidin in the petals thereof causing flower color to change to blue have been produced (see Non-Patent Document 4) by introducing these F3'5'H genes into carnations (see Patent Document 1), roses (see Non-

Patent Document 3 and Patent Documents 2 and 3) and chrysanthemums (see Patent Document 4). Such carnations and roses are available commercially.

[0006]

Flavone is a type of organic compound that is a cyclic ketone of a flavane derivative, and in the narrow sense, refers to the compound 2,3-dehydroflavan-4-one represented by the chemical formula $C_{15}H_{10}O_2$ and having a molecular weight of 222.24. In the broad sense, derivatives classified as flavones are generically referred to as "flavones". Flavone in the broad sense (flavones) refers to a category of flavonoids that are classified as having a flavone structure for the basic skeleton but not having a hydroxyl group at the 3-position. Examples of typical flavones include apigenin (4',5,7-trihydroxyflavone and luteolin (3',4',5,7-tetrahydroxyflavone). In the description of the present application, the term "flavone" refers to flavones in the broad sense, namely derivatives classified as flavones.

[0007]

Genes of flavone synthases (FNS) required for biosynthesis of flavone are obtained from numerous plants. Flavones are known to have the effect of changing the color of anthocyanins to a deep blue color when present with anthocyanins, and these FNS genes have attracted attention in the modification of flower color. Simultaneous to the accumulation of delphinidin in flower petals, flavones were also accumulated and flower color changed to an even deeper blue color as a result of introducing F3'5'H and FNS gene into roses not having the ability to synthesize flavones (see Patent Document 5). In addition, since flavones also absorb ultraviolet rays in addition to causing flower color to become blue, they protect plants from ultraviolet rays or function as a visual signal to insects in the case of insect-pollinated flowers. In addition, flavones are also involved in the interaction between plants and soil microbes. Moreover,

flavones are also used as an ingredient of foods and cosmetics as components beneficial to health. For example, flavones are said to have anticancer activity, and the ingestion of foods containing large amounts of flavones has been demonstrated to treat and prevent cancer.

[0008]

In addition, genes that modify anthocyanins and flavones have also been obtained from numerous plants. Although these include glycosyltransferases, acyltransferases and methyltransferases, the following provides a description of glycosyltransferases (GT) that catalyze glycosylation reactions. For example, a gene encoding a protein having activity that transfers glucose to the hydroxyl group at the 3-position of anthocyanin has been isolated from plants such as gentian, perilla, petunia, rose and snapdragon (see Non-Patent Documents 4 to 6 and Patent Document 6). A gene encoding a protein having activity that transfers glucose to the hydroxyl group at the 5-position of anthocyanin has been isolated from such plants as perilla, petunia, gentian, verbena or torenia (see Non-Patent Documents 5 to 7 and Patent Document 7). A gene encoding a protein having activity that transfers glucose to the hydroxyl group at the 7-position of a flavone has been isolated from thale cress (see Non-Patent Document 8). A gene encoding a protein having activity that transfers glucose to the hydroxyl group at the 7-position of baicalein has been isolated from Baikal skullcap, and protein expressed by this gene in Escherichia coli has been reported to catalyze a reaction demonstrating activity that transfers glucose to the hydroxyl group at the 7-position of flavonoids (see Non-Patent Document 9). A gene encoding a protein having activity that transfers glucose to the hydroxyl group at the 3'-position of anthocyanin has been isolated from gentian, butterfly pea and cineria (see Patent Document 8). In addition, a gene encoding a protein having

activity that successively transfers glucose to hydroxyl groups at two different locations of the A ring and C ring of anthocyanin has been isolated from rose (see Patent Document 9). A gene encoding a protein having activity that successively transfers glucose to hydroxyl groups at two different locations of the B ring of anthocyanin has been isolated from butterfly pea (see Patent Document 10).

[0009]

Although the aforementioned glycosyltransferases use UDP-glucose as a sugar donor, glycosyltranferases have recently been identified that use acyl-glucose as a sugar donor. A gene encoding a protein having activity that transfers glucose to the hydroxyl group at the 5-position of anthocyanin 3-glucoside has been isolated from carnation, while a gene encoding a protein having activity that transfers glucose to the hydroxyl group at the 7-position has been isolated from delphinium (see Non-Patent Document 10).

[0010]

In this manner, a large number of proteins exist as glycosyltransferases having activity that transfers glucose to various hydroxyl groups.

However, a large number of glycosyltransferases are thought to remain for which the function thereof has yet to be identified. For example, a gene encoding a protein having activity that transfers a sugar to the 4'-position of a flavonoid, and a gene encoding a protein having activity that successively transfers a sugar to hydroxyl groups at two locations of the A ring and B ring of a flavonoid have yet to be identified. Although a glycosyltransferase gene derived from Livingstone daisy has been reported to demonstrate activity that transfers glucose to one of the hydroxyl groups at the 4'-position or 7-position of a flavonoid in vitro, the inherent activity of this glycosyltransferase in plants transfers glucose to the hydroxyl group at the 5-position of

betanidine (see Non-Patent Document 11).

[0011]

However, metalloanthocyanins represented by the pigments of dayflower, cornflower, salvia and nemophila are composed of six anthocyanin molecules, six flavone molecules and two metal ion atoms, and each component is assembled to form a stable blue pigment (see FIG. 2 and Non-Patent Document 1). For example, the metalloanthocyanin of nemophila is formed from nemophilin (see FIG. 3), malonyl apigenin 4',7'-diglucoside (see FIG. 4), Mg^{2+} and Fe^{3+}. Salvia metalloanthocyanin is formed from cyanosalvianin (see FIG. 5), apigenin 4,7'-diglucoside (see FIG. 6) and Mg^{2+}. According to previous research, all blue flowers that form metalloanthocyanins biosynthesize a flavone in which a sugar is added to the hydroxyl groups at both the 4'-position and 7-position, and the sugar added to that flavone has been determined to play an important role in molecular recognition during metalloanthocyanin formation. The sugar coordinated at the 4'-position of a flavone is important in molecular recognition during formation, while the sugar at the 7-position has been indicated to be involved in the stability thereof (see Non-Patent Document 1). Only after these two sugars have been added to a flavone is a metalloanthocyanin formed which results in the expression of an attractive blue color. In addition, the petals of blue Dutch iris contain a flavone in which a sugar has been added to the 4'-position. In addition, since solubility increases and physical properties change as a result of adding two sugars to flavones, their applications to health foods, pharmaceuticals and cosmetic ingredients are expected to expand.

Prior Art Documents

Patent Documents

[0012]

Patent Document 1: International Publication No. WO 2006/105598

Patent Document 2: International Publication No. WO 2010/122849

Patent Document 3: International Publication No. WO 2005/017147

Patent Document 4: International Publication No. WO 2009/062253

Patent Document 5: International Publication No. WO 2008/156211

Patent Document 6: International Publication No. WO 2007/094521

Patent Document 7: International Publication No. WO 99/05287

Patent Document 8: International Publication No. WO 001/092509

Patent Document 9: Japanese Unexamined Patent Publication No. 2006-149293

Patent Document 10: Japanese Unexamined Patent Publication No. 2005-95005

Non-Patent Documents

[0013]

Non-Patent Document 1: Natural Product Reports (2009), 26, 884-915

Non-Patent Document 2: Biosci. Biotechnol. Biochem. (2010), 74(9), 1760-1769

Non-Patent Document 3: Plant Cell Physiol. (2007), 48(11), 1589-1600

Non-Patent Document 4: Plant Cell Physiol. (1996), 37(5), 711-716

Non-Patent Document 5: J. Biol. Chem. (1999), 274(11), 7405-7411

Non-Patent Document 6: Plant Molecular Biology (2002), 48, 401-411

Non-Patent Document 7: Journal of Experimental Botany (2008), 59(6), 1241-1252

Non-Patent Document 8: Biosci. Biotechnol. Biochem. (2006), 70(6), 1471-1477

Non-Patent Document 9: Planta (2010), 210, 1006-1013

Non-Patent Document 10: Plant Cell (2010), 22(10), 3374-89

Non-Patent Document 11: The Plant Journal (1999), 19(5), 509-519

DISCLOSURE OF THE INVENTION

Problems to be Solved by the Invention

[0014]

Alteration of the physical properties of flavones is required to change flower color and develop materials for foods, pharmaceuticals and cosmetics. For example, although carnations, roses and chrysanthemums that accumulate delphinidin have a bluish-violet color, research is being conducted to make this color even bluer.

With the foregoing in view, an object of the present invention is to provide a polynucleotide that encodes a protein having activity that transfers a sugar to the hydroxyl group at the 4'-position of a flavone, and the use thereof.

Means for Solving the Problems

[0015]

As a result of conducting extensive research and experimentation to solve the aforementioned problems, the present applicants isolated a polynucleotide that encodes a protein having activity that transfers a sugar to the hydroxyl group at the 4'-position of a flavone and confirmed the use thereof, thereby leading to completion of the present invention.

Namely, the present invention is as described below.

[0016]

[1] A polynucleotide selected from the group consisting of the following (a) to (e):

(a) a polynucleotide composed of the base sequence of SEQ ID NO: 1;

(b) a polynucleotide that hybridizes under stringent conditions with a polynucleotide composed of a base sequence complementary to the base sequence of SEQ ID NO:

1, and encodes a protein having activity that transfers a sugar to the hydroxyl group at the 4'-position of a flavone;

(c) a polynucleotide encoding a protein composed of the amino acid sequence of SEQ ID NO: 2;

(d) a polynucleotide encoding a protein composed of an amino acid sequence in which one or a plurality of amino acids have been deleted, substituted, inserted and/or added in the amino acid sequence of SEQ ID NO: 2, and having activity that transfers a sugar to the hydroxyl group at the 4'-position of a flavone; and,

(e) a polynucleotide encoding a protein having an amino acid sequence having identity of 90% or more with the amino acid sequence of SEQ ID NO: 2, and having activity that transfers a sugar to the hydroxyl group at the 4'-position of a flavone.

[0017]

[2] The polynucleotide described in [1] above, which is (a) a polynucleotide composed of the base sequence of SEQ ID NO: 1.

[0018]

[3] The polynucleotide described in [1] above, which is (c) a polynucleotide encoding a protein composed of the amino acid sequence of SEQ ID NO: 2.

[0019]

[4] The polynucleotide described in [1] above, which is (f) a polynucleotide encoding a protein having an amino acid sequence having identity of 95% or more with the amino acid sequence of SEQ ID NO: 2, and having activity that transfers a sugar to the hydroxyl group at the 4'-position of a flavone.

[0020]

[5] The polynucleotide described in [4] above, which is (g) a polynucleotide encoding a protein having an amino acid sequence having identity of 97% or more with the amino acid sequence of SEQ ID NO: 2, and having activity that transfers a sugar to the hydroxyl group at

the 4'-position of a flavone.

[0021]

[6] The polynucleotide described in [5] above, which is (h) a polynucleotide encoding a protein having an amino acid sequence having identity of 98% or more with the amino acid sequence of SEQ ID NO: 2, and having activity that transfers a sugar to the hydroxyl group at the 4'-position of a flavone.

[0022]

[7] The polynucleotide described in any of [1] to [6] above, which is DNA.

[0023]

[8] A protein encoded by the polynucleotide described in any of [1] to [7] above.

[0024]

[9] A vector containing the polynucleotide described in any of [1] to [7] above.

[0025]

[10] A non-human host introduced with the vector described in [9] above.

[0026]

[11] A method for adding a sugar to the hydroxyl group at the 4'-position of a flavone using the polynucleotide described in any of [1] to [7] above.

[0027]

[12] A plant, a progeny thereof, or a portion or tissue thereof, introduced with the polynucleotide described in any of [1] to [7] above and containing that polynucleotide.

[0028]

[13] A portion of the plant described in [12] above that is a cut flower.

[0029]

[14] A processed cut flower that uses the cut flower described in [13] above.

[0030]

[15] A method for producing a protein having

activity that transfers a sugar to the hydroxyl group at the 4'-position of a flavone, comprising the following steps:

culturing or growing the non-human host described in [10] above, and

harvesting protein from the non-human host that has activity that transfers a sugar to the hydroxyl group at the 4'-position of a flavone.

[0031]

[16] A method for producing a flavone in which a sugar has been added to the hydroxyl group at the 4'-position thereof, comprising the following steps:

culturing or growing the non-human host described in [10] above, and

harvesting a flavone from the non-human host in which a sugar has been added to the hydroxyl group at the 4'-position thereof.

[0032]

[17] A food containing a flavone produced according to the production method described in [16] above in which a sugar has been added to the hydroxyl group at the 4'-position thereof.

[0033]

[18] A pharmaceutical containing a flavone produced according to the production method described in [16] above in which a sugar has been added to the hydroxyl group at the 4'-position thereof.

[0034]

[19] A cosmetic containing a flavone produced according to the production method described in [16] above in which a sugar has been added to the hydroxyl group at the 4'-position thereof.

Effects of the Invention

[0035]

The polynucleotide of the present invention enables the production of a protein having activity that specifically transfers a sugar to the hydroxyl group at

the 4'-position of a flavone as a result of being expressed in suitable host cells. According to the present invention, the present invention can be used to alter flower color by constitutively or tissue-specifically expressing in a plant a protein having activity that transfers a sugar to the hydroxyl group at the 4'-position of a flavone.

[0036]

A flavone in which a sugar has been added to both of the hydroxyl groups at the 4'-position and 7-position is formed by introducing a protein having activity that transfers a sugar to the hydroxyl group at the 4'-position of a flavone into a plant inherently having activity that transfers a sugar to the hydroxyl group at the 7-position of a flavone in the manner of rose, for example. Alternatively, a flavone in which a sugar has been added to both of the hydroxyl groups at the 4'-position and 7-position is formed by expressing in a plant a protein having activity that transfers a sugar to the hydroxyl group at the 4'-position of a flavone together with a protein having activity that transfers a sugar to the hydroxyl group at the 7-position of a flavone.

In addition, according to the present invention, a method for producing a flavone in which a sugar has been added to the hydroxyl group at the 4'position, and a food, pharmaceutical or cosmetic and the like obtained according to that production method, are also provided.

BRIEF DESCRIPTION OF THE DRAWINGS

[0037]

FIG. 1 is a drawing for explaining the biosynthesis pathway of anthocyanins.

FIG. 2 is a schematic diagram of the structure of metalloanthocyanin.

FIG. 3 shows the structural formula of a nemophila-derived anthocyanin (nemophilin).

FIG. 4 shows the structural formula of a nemophila-

derived flavone (malonyl apigenin 4',7'-diglucoside).

FIG. 5 shows the structural formula of salvia-derived flavone (cyanosalvianin).

FIG. 6 shows the structural formula of a salvia-derived flavone (apigenin 4',7-diglucoside).

FIG. 7 is a high-performance liquid chromatogram obtained following an enzyme reaction between a flower petal extract and apigenin.

FIG. 8 is a drawing for explaining the biosynthesis pathway of apigenin 4',7-diglucoside.

FIG. 9 is a high-performance liquid chromatogram obtained following an enzyme reaction between NmGT8 protein solution and apigenin.

FIG. 10 is a high-performance liquid chromatogram obtained following an enzyme reaction between NmGT8 protein solution and apigenin 7-glucoside.

FIG. 11 is a drawing summarizing the reactivities of NnGT8 protein solution and enzyme in which a sugar has been added to the 5-position of baicalein on various flavonoid substrates.

FIG. 12-1 is an alignment diagram comparing the amino acid sequences of NmGT8 and NmG13.

FIG. 12-2 is an alignment diagram comparing the amino acid sequences of NmGT8 and NmGT4.

FIG. 12-3 is an alignment diagram comparing the amino acid sequences of NmGT8, NmGT3 and NmGT4.

FIG. 13 is an alignment diagram comparing the amino acid sequences of NmGT8 and an enzyme in which a sugar has been added to the 4'-position of snapdragon chalcone.

FIG. 14 is an alignment diagram comparing the amino acid sequences of NmGT8 and an enzyme in which sugars have been added to the 3-position and 5-position of rose anthocyanidin.

FIG. 15 is a phylogenetic tree indicating the relationships between the NmGT8 of the present invention and the aforementioned enzymes.

FIG. 16 shows a construct containing NmGT8

introduced into torenia (pSPB4583).

FIG. 17 shows constructs containing NmGT8 introduced into petunia (pSPB5424 and pSPB5428).

FIG. 18 is a high-performance liquid chromatogram of a flower petal extract of recombinant petunia introduced with NmGT8.

FIG. 19 is an extracted positive ion gas chromatogram of a flower petal extract (250 m/z to 1250 m/z) of recombinant petunia introduced with NmGT8.

FIG. 20 shows a construct containing NmGT8 introduced into carnation (pSPB5433).

FIG. 21 shows constructs containing NmGT8 introduced into rose (pSPB4577, pSPB4578, pSPB5437 and pSPB5440).

FIG. 22 is a high-performance liquid chromatogram of a flower petal extract of recombinant rose introduced with NmGT8.

FIG. 23 is an extracted positive ion mass chromatogram of a flower petal extract (250 m/z to 1250 m/z) of recombinant rose introduced with NmGT8.

BEST MODE FOR CARRYING OUT THE INVENTION

[0038]

The present invention relates to a polynucleotide selected from the group consisting of the following (a) to (e):

(a) a polynucleotide composed of the base sequence of SEQ ID NO: 1;

(b) a polynucleotide that hybridizes under stringent conditions with a polynucleotide composed of a base sequence complementary to the base sequence of SEQ ID NO: 1, and encodes a protein having activity that transfers a sugar to the hydroxyl group at the 4'-position of a flavone;

(c) a polynucleotide encoding a protein composed of the amino acid sequence of SEQ ID NO: 2;

(d) a polynucleotide encoding a protein composed of an amino acid sequence in which one or a plurality of amino acids have been deleted, substituted, inserted

and/or added in the amino acid sequence of SEQ ID NO: 2, and having activity that transfers a sugar to the hydroxyl group at the 4'-position of a flavone; and,

(e) a polynucleotide encoding a protein having an amino acid sequence having identity of 90% or more with the amino acid sequence of SEQ ID NO: 2 and having activity that transfers a sugar to the hydroxyl group at the 4'-position of a flavone.

[0039]

In the present description, the term "polynucleotide" refers to DNA or RNA.

In the present description, the term "stringent conditions" refers to conditions under which a polynucleotide or oligonucleotide and genomic DNA bind selectively and specifically so as to be able to be detected. Stringent conditions are defined by a suitable combination of salt concentration, organic solvent (such as formaldehyde) concentration, temperature and other known conditions. Namely, stringency is increased by reducing salt concentration, increasing organic solvent concentration or raising hybridization temperature. Moreover, washing conditions following hybridization also influence stringency. These washing conditions are also defined by salt concentration and temperature, and washing stringency increases by reducing salt concentration or raising temperature. Thus, the term "stringent conditions" refers to conditions under which specific hybridization occurs only between base sequences having high homology such that the degree of identity or homology between each base sequence is, for example, an average of about 80% or more, preferably about 90% or more, more preferably about 95% or more, even more preferably about 97% or more, and most preferably 98% or more. An example of "stringent conditions" consists of a sodium concentration of 150 mM to 900 mM, and preferably 600 mM to 900 mM and pH of 6 to 8 at a temperature of 60°C to 68°C, and more specifically, consists of carrying out

hybridization under conditions consisting of 5 × SSC (750 mM NaCl, 75 mM trisodium citrate), 1% SDS, 5 × Denhardt's solution, 50% formaldehyde and 42°C, followed by washing under conditions consisting of 0.1 × SSC (15 mM NaCl, 1.5 mM trisodium citrate), 0.1% SDS and 55°C.

[0040]

Hybridization can be carried out in accordance with a method known in the art or a method complying therewith, such as the method described in Current Protocols in Molecular Biology (edited by Frederick M. Ausubel, et al., 1987). In addition, in the case of using a commercially available library, hybridization can be carried out in accordance with the method described in the manual provided therewith. Genes selected using such hybridization may be naturally-derived genes such as those derived from plants or those not derived from plants. In addition, genes selected by hybridization may be cDNA, genomic DNA or chemically synthesized DNA.

[0041]

The aforementioned "amino acid sequence in which one or a plurality of amino acids have been deleted, substituted, inserted and/or added" refers to an amino acid sequence in which an arbitrary number of amino acids, such as 1 to 20, preferably 1 to 5 and even more preferably 1 to 3, have been deleted, substituted, inserted and/or added. A genetic engineering technique in the form of site-specific mutagenesis is useful since it allows the introduction of a specific mutation at a specific site, and can be carried out in compliance with a method such as that described in Molecular Cloning: A Laboratory Manual, 2nd ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., 1989. By expressing this mutant DNA using a suitable expression system, a protein can be obtained that is composed of an amino acid sequence in which one or a plurality of amino acids have been deleted, substituted, inserted and/or added.

In addition, the DNA according to the present invention can be obtained by a method known among persons with ordinary skill in the art, such as a method consisting of chemical synthesis according to the phosphoamide method, or a nucleic acid amplification method using a nucleic acid sample from a plant as template and using primers designed based on the nucleotide sequence of a target gene.

[0042]

In the present description, the terms "identity" and "homology" refer to a quantity (number) that makes it possible to determine that corresponding amino acid residues or bases composing two chains in a polypeptide sequence (or amino acid sequence) or polynucleotide sequence (or base sequence) are identical in terms of the relationship of mutual compatibility between the two chains, and "identity" and "homology" can easily be calculated. Numerous methods are known for measuring homology between two polynucleotide sequences or polypeptide sequences, and the terms "identity" and "homology" are widely known among persons with ordinary skill in the art (see, for example, Lesk, A.M. (ed.), Computational Molecular Biology, Oxford University Press, New York (1988); Smith, D.W. (ed.), Biocomputing: Informatics and Genome Projects, Academic Press, New York (1993); Grifin, A.M. & Grifin, H.G. (ed.), Computer Analysis of Sequence Data: Part I, Human Press, New Jersey (1994); von Heinje, G., Sequence Analysis in Molecular Biology, Academic Press, New York (1987); and Gribskov, M. & Devereux, J. (ed.), Sequence Analysis Primer, M-Stockton Press, New York (1991)).

[0043]

In addition, although the values of "identity" and "homology" described in the present description may be values calculated using a homology search program known among persons with ordinary skill in the art unless specifically indicated otherwise, they are preferably

values calculated using the Clustal W Program of the MacVector Application (Version 9.5, Oxford Molecular Ltd., Oxford, England).

[0044]

The polynucleotide (nucleic acid or gene) of the present invention is that which "encodes" a protein of interest. Here, "encodes" refers to expressing a protein of interest in a state of retaining the activity thereof. In addition, "encodes" includes both the meaning of encoding in the form of a constituent sequence (exon) in which the protein of interest is contiguous, and encoding through an intermediary sequence (intron).

[0045]

As will be described in the examples to be subsequently described, a gene having a naturally-occurring base sequence is obtained by, for example, analysis with a DNA sequencer. In addition, DNA encoding an enzyme having a modified amino acid sequence can be synthesized using ordinary site-specific mutagenesis or PCR using DNA having a naturally-occurring sequence as a base. For example, after obtaining a DNA fragment desired to be modified by treatment with cDNA or genomic DNA restrictase, site-specific mutagenesis or PCR is carried out using the DNA fragment as a template and using primers introduced with a desired mutation to obtain a desired modified DNA fragment. Subsequently, the DNA fragment introduced with this mutation is linked to a DNA fragment encoding another segment of a target enzyme.

Alternatively, in order to obtain DNA encoding enzyme composed of a shortened amino acid sequence, DNA encoding an amino acid sequence longer than the target amino acid sequence, such as DNA encoding the full-length amino acid sequence, is cleaved with a restrictase, and in the case the resulting DNA fragment does not encode the entire target amino acid sequence, a DNA fragment composed of the insufficient portion of the sequence is

synthesized and linked thereto.

[0046]

In addition, by measuring enzyme activity after expressing the resulting polynucleotide in Escherichia coli or yeast using a gene expression system, the resulting polynucleotide can be confirmed to encode a protein having activity that transfers a sugar to the hydroxyl group at the 4'-position of a flavone. Moreover, the polynucleotide product in the form of a protein having activity that transfers a sugar to the hydroxyl group at the 4'-position of a flavone can be obtained by expressing this polynucleotide. Alternatively, a protein having activity that transfers a sugar to the hydroxyl group at the 4'-position of a flavone can also be acquired by using an antibody to a polypeptide composed of the amino acid sequence described in SEQ ID NO: 2, and a polynucleotide encoding a protein having activity that transfers a sugar to the hydroxyl group at the 4'-position of a flavone originating in another organism can also be cloned using this antibody.

[0047]

The present invention also relates to a (recombinant) vector containing the aforementioned polynucleotide, and particularly an expression vector, as well as a host transformed by that vector.

Prokaryotes or eukaryotes can be used for the host. Examples of prokaryotes include commonly used prokaryotic hosts such as bacteria, including bacteria belonging to the genus Escherichia such as Escherichia coli, and bacteria belonging to the genus Bacillus such as Bacillus subtilis. Examples of eukaryotes that can be used include lower eukaryotes such as eukaryotic organisms in the manner of fungi such as yeast or filamentous fungi.

[0048]

Examples of yeast include Saccharomyces species such as Saccharomyces cerevisiae, and examples of filamentous fungi include Aspergillus species, such as Aspergillus

oryzae or Aspergillus niger, and Penicillium species. Animal cells or plant cells can also be used for the host, examples of animal cell systems used include mouse, hamster, monkey or human cells, and insect cells such as silkworm cells or silkworm adults per se are also used as hosts.

[0049]

The expression vector of the present invention contains an expression regulatory region, such as a promoter, terminator or replication point, dependent on the type of host into which it is introduced. A commonly used promoter such as a trc promoter, a tac promoter or an lac promoter is used as a promoter of a bacterial expression vector, a glyceraldehyde-3-phosphate dehydrogenase promoter or a PH05 promoter, for example, is used as a yeast promoter, and an amylase promoter or trpC promoter, for example, is used as a filamentous fungi promoter. In addition, examples of promoters for animal cell hosts include viral promoters such as an SV40 early promoter or SV40 late promoter.

Examples of promoters that constitutively express polynucleotide in plant cells include cauliflower mosaic virus 35S RNA promoter, rd29A gene promoter, rbcS promoter and mac-1 promoter. In addition, a promoter of a gene that specifically expresses in that tissue can be used for tissue-specific gene expression.

An expression vector can be produced in accordance with ordinary methods using restrictases, ligases and the like. In addition, transformation of a host with an expression vector can also be carried out in accordance with ordinary methods.

[0050]

A target protein can be obtained by culturing, cultivating or growing a host transformed by the aforementioned expression vector, and recovering and purifying from the culture or medium in accordance with ordinary methods such as filtration, centrifugal

separation, cell disruption, gel filtration chromatography or ion exchange chromatography.

Although the present description describes a gene encoding a protein having activity that transfers a sugar to the hydroxyl group at the 4'-position of a flavone derived from nemophila, the polynucleotide according to the present invention is not limited to a gene derived from nemophila, but rather can be used to alter flower color in a plant regardless of whether the origin of the gene encoding a protein having activity that transfers a sugar to the hydroxyl group at the 4'-position of a flavone is a plant, animal or microorganism provided it has activity that transfers a sugar to the hydroxyl group at the 4'-position of a flavone.

[0051]

The present invention also relates to a plant, a progeny thereof, or a portion or tissue thereof, obtained by introducing an exogenous polynucleotide encoding a protein having activity that transfers a sugar to the hydroxyl group at the 4'-position of a flavone, and causing that exogenous polynucleotide to be contained in the plant. An example of the form of the portion or tissue is a cut flower. The 4'-position of a flavone can be glycosylated or glycosylation of the 4'-position of a flavone can be inhibited by using the polynucleotide according to the present invention that encodes a protein having activity that transfers a sugar to the hydroxyl group at the 4'-position of a flavone, and flower color in a plant can be altered as a result thereof.

[0052]

At the present level of technology, technology can be used that enables a polynucleotide to be introduced into a plant, and enables that polynucleotide to be expressed constitutively or tissue-specifically. Introduction of DNA into a plant can be carried out by a method known among persons with ordinary skill in the art, such as the Agrobacterium method, binary vector

method, electroporation method, PEG method or particle gun method.

[0053]

Examples of plants able to be transformed include, but are not limited to, rose, carnation, chrysanthemum, snapdragon, cyclamen, orchid, bluebell, freesia, Transvaal daisy, gladiola, baby's breath, kalanchoe, lily, pelargonium, geranium, petunia, torenia, tulip, anthurium, moth orchid, rice, barley, wheat, rape, potato, tomato, poplar, banana, eucalyptus, sweet potato, soybean, alfalfa, rubin basil, corn, cauliflower and dahlia.

[0054]

The present invention also relates to a processed product using the aforementioned cut flower (processed cut flower). Here, examples of processed cut flowers include, but are not limited to, pressed flowers, preserved flowers, dry flowers and resin-sealed flowers using these cut flowers.

In addition, a flavone produced according to the production method of the present invention in which a sugar has been added to the hydroxyl group at the 4'-position thereof can be used in applications such as the production methods of foods, pharmaceuticals or cosmetics.

[0055]

In the present invention, expression of a target gene in a plant can be suppressed by an antisense method, co-suppression method or RNAi method. Although suppression of the expression of a target gene can be carried out in accordance with a method known among persons with ordinary skill in the art, examples thereof include the antisense RNA/DNA technique (Bioscience and Industry, 50, 322 (1992); Chemistry, 46, 681 (1991); Biotechnology, 9, 358 (1992); Trends in Biotechnology, 10, 152 (1992); Cellular Engineering, 16, 1463 (1997)), and the triple helix technique (Trends in Biotechnology,

10, 132 (1992)). For example, suppression of gene expression is carried out using a single-stranded nucleic acid molecule comprising all or a portion of a nucleotide sequence identical to the antisense strand of the gene according to the present invention. This type of method is known as the antisense method. In the antisense method, expression of a target gene is suppressed by expressing a high level of RNA having a sequence complementary to the gene for which expression is desired to be suppressed. In this method, single-stranded RNA can be used that comprises the entirety of a nucleotide sequence identical to the antisense strand of the polynucleotide (gene) according to the present invention. In addition, in the aforementioned method, single-stranded RNA can also be used that comprises a portion of a nucleotide sequence identical to the antisense strand of the gene according to the present invention. Although this type of partial single-stranded RNA can be suitably designed by a person with ordinary skill in the art provided it is able to suppress expression of the gene according to the present invention, it is preferably specific for the gene according to the present invention, and the length thereof is preferably 5 nucleotides to 100 nucleotides, more preferably 5 nucleotides to 50 nucleotides, and even more preferably 10 nucleotides to 20 nucleotides.

[0056]

Suppression of gene expression is carried out using a single-stranded nucleic acid molecule comprising all or a portion of a nucleotide sequence identical to a sense strand of the gene according to the present invention. Namely, this sense single-stranded nucleic acid can be used to suppress expression of the gene according to the present invention in the same manner as the aforementioned antisense single-stranded nucleic acid. In this method, single-stranded RNA can be used that comprises the entirety of a nucleotide sequence identical

to a sense strand of the gene according to the present invention. In addition, in the aforementioned method, single-stranded RNA can be used that comprises a portion of a nucleotide sequence identical to a sense strand of a gene. Although this type of partial single-stranded RNA can be suitably designed by a person with ordinary skill in the art provided it is able to suppress expression of the gene according to the present invention, it preferably specific for the gene according to the present invention, and the length thereof is preferably 5 nucleotides to 100 nucleotides, more preferably 5 nucleotides to 50 nucleotides and even more preferably 10 nucleotides to 20 nucleotides.

[0057]

Moreover, suppression of gene expression is carried out using a double-stranded nucleic acid molecule comprising all or a portion of a nucleotide sequence identical to the gene according to the present invention. For example, an antisense or sense single-stranded nucleic acid of a gene according to the present invention can be suppressed in an angiosperm by using this double-stranded nucleic acid molecule. The double-stranded nucleic acid molecule according to the present invention is preferably DNA, and the strand length and specific nucleotide sequence thereof correspond to the strand length and nucleotide sequence of the target single-stranded nucleic acid molecule. For example, in the case of expressing the aforementioned antisense single-stranded nucleic acid, the double-stranded nucleic acid molecule according to the present invention contains an antisense strand of the gene according to the present invention as the coding strand. In addition, in the case of expressing the aforementioned sense single-stranded nucleic acid, the double-stranded nucleic acid according to the present invention contains a sense strand of the gene according to the present invention as the coding strand.

[0058]

A double-stranded nucleic acid molecule can be expressed in a plant using a method known among persons with ordinary skill in the art. For example, a double-stranded nucleic acid molecule can be expressed by introducing an expression vector containing a promoter, the double-stranded nucleic acid molecule according to the present invention, and a transcription terminator into a target plant followed by cultivating the resulting plant body. Introduction of the expression vector into the plant can be carried out according to a method known among persons with ordinary skill in the art, such as the Agrobacterium method, binary vector method, electroporation method, PEG method or particle gun method.

[0059]

Another example of a method for suppressing gene expression using the nucleic acid molecule according to the present invention is the co-suppression method. In this method, sense double-stranded DNA having the complete nucleotide sequence of the gene according to the present invention is introduced into a target plant. As a result, sense single-stranded RNA according to the present invention is expressed, and gene expression is suppressed considerably by this RNA (Plant Cell, 9: 1357-1368, 1997).

Examples

[0060]

The following provides a detailed explanation of the invention according to examples thereof.

[Example 1: Detection of Activity that Transfers a Sugar to the Hydroxyl Group at the 4'-Position of Flavone in Nemophila Flower Petals]

Flower petals of nemophila (Nemophila menziesii) were collected by dividing into developmental stages defined in the manner indicated below followed by freezing with liquid nitrogen and storing in a freezer at

-80°C.

Stage 1: Uncolored, tightly closed bud (approx. 2 to 5 mm)

Stage 2: Colored, tightly closed bud (approx. 2 to 5 mm)

Stage 3: Colored, closed bud with calyx about to open (approx. 5 to 10 mm)

Stage 4: Bud with flower petals about to open (approx. 10 to 15 mm)

Stage 5: Completely open flower

[0061]

<Preparation of Nemophila Flower Petal Extract>

Flavone glycosyltransferase activity is expected to be detected in flower petals in stage 1 and stage 2 prior to biosynthesis of anthocyanin. Therefore, flower petal extracts were prepared using flower petals in stages 1 and 2. 500 mg of sample flower petals (250 mg each of flower petals in stages 1 and 2 stored at -80°C) were crushed with a mortar and pestle while cooling with liquid nitrogen followed by dissolving in 1.5 ml of extraction buffer (composition: potassium phosphate buffer (pH 7.5): 100 mM, dithiothreitol (DTT): 1 mM, polyvinylpyrrolidone 40: 50 mg/ml, sucrose: 100 mg/ml). The resulting protein solution was centrifuged (10,000 rpm, 4°C, 10 minutes), and ammonium sulfate was added to the resulting supernatant to a saturated concentration of 30%. After stirring for 1 hour at 4°C, the solution was centrifuged (10,000 rpm, 4°C, 10 minutes) followed by recovery of the supernatant. Ammonium sulfate was added to the resulting supernatant to a saturated concentration of 70%, and after stirring for 1 hour at 4°C, the solution was centrifuged (10,000 rpm, 4°C, 10 minutes) to obtain a precipitate. This precipitate was dissolved in 500 µl of elution buffer (composition: Tris HCl (pH 7.5): 2.5 mM, DTT: 1 mM, amidinophenylmethanesulfonyl fluoride hydrochloride (APMSF): 10 µM) followed by column

purification using NAP-5 Columns Sephadex™ G-25 DNA Grade (GE Healthcare Inc.) and removal of the ammonium sulfate. This liquid was then used as "flower petal extract". The Avanti HP-26XP (rotor: JA-2) (Beckman Coulter Inc.) was used for centrifugation.

[0062]

<Measurement of Enzyme Activity>

A reaction liquid prepared by mixing 40 µl of flower petal extract, 20 µl of 5 mM UDP-glucose, 20 µl of 1 M Tris HCl (pH 7.5) and 1 µl of 500 ng/µl apigenin on ice and bringing to a volume of 200 µl with water was held for 1 hour at 30°C. Subsequently, 200 µl of stop buffer (90% aqueous acetonitrile solution containing 0.1% TFA) were added to stop the reaction followed by analyzing the reaction liquid by high-performance liquid chromatography (Prominence (Shimadzu Corp.)). The Shimadzu PDA SPD-M10AVP was used for the detector and flavone was detected at 330 nm. The Shim-Pack ODS 150 mm × 4.6 mm column (Shimadzu Corp.) was used for the column. A Solution A (0.1% aqueous TFA solution) and a Solution B (90% aqueous acetonitrile solution containing 0.1% TFA) were used for elution. Elution was carried out using a linear concentration gradient from an 8:2 mixture of the two solutions to a 3:7 mixture of the two solutions over the course of 10 minutes followed by a 3:7 mixture for 5 minutes. The flow rate was 0.6 ml/min. A reaction liquid obtained by allowing enzyme to react under the same conditions using a flower petal extract obtained by heat-treating the flower petal extract for 20 minutes at 100°C was used as a control.

As a result, flavone demonstrating the same retention time and absorption maximum as purified apigenin 4',7-diglucoside and apigenin 7-glucoside standard was biosynthesized in addition to flavone demonstrating retention time close to that of apigenin 7-glucoside (see FIG. 7). Neither of these products was

formed when the enzyme was allowed to react without adding UDP-glucose.

[0063]

[Example 2: Determination of Retention Time and Absorption Maximum of Apigenin 4'-Glucoside]

In consideration of the biosynthesis pathway of apigenin 4',7-diglucoside in nemophila flower petals, apigenin 4'-glucoside and apigenin 7-glucoside ought to be formed as intermediate products during course of biosynthesis of apigenin 4',7-diglucoside (see FIG. 8). On the basis thereof, the flavone demonstrating retention time close to that of apigenin 7-glucoside detected in Example 1 was judged to be apigenin 4'-glucoside (see FIG. 7). The retention time and absorption maximum of apigenin 4'-glucoside were able to be determined.

On the basis of these results, a protein having activity that transfers a sugar to the hydroxyl groups at the 4'-position and 7-position of a flavone that is dependent on UDP-glucose was clearly determined to be present in the flower petals of nemophila. Two possibilities were considered for the glycosylation of the hydroxyl groups at the 4'-position and 7-position, one possibility being that a single enzyme carries out glycosylation of both hydroxyl groups, and the other possibility being that glycosylation of the hydroxyl groups at the 4'-position and 7-position is respectively carried out by different enzymes.

[0064]

[Example 3: Acquisition of Candidate Genes for Gene Encoding Protein having Activity that Transfers a Sugar to the Hydroxyl Group at the 4'-Position of Flavone]

<Isolation of Total RNA>

Total RNA was isolated from stage 1 and stage 2 flower petals of nemophila using the Plant RNAeasy Kit (Qiagen Corp.) in accordance with the protocol recommended by the manufacturer.

<Analysis of Expression of cDNA Derived from Nemophila Flower Petals>

A reverse transcription reaction was carried out on 30 μg of total RNA derived from Nemophila flower petals followed by the production of a normalized cDNA library. After amplifying the resulting library for each clone by emulsion PCR, the base sequences were determined using an FLX Genome Sequencer (Roche Diagnostics K.K.). In addition, the resulting sequence data was translated to amino acid sequences followed by extraction of those sequences that demonstrated homology with the amino acid sequence of snapdragon anthocyanin 3'-glycosyltransferase. These sequences were then assembled to obtain candidate genes encoding glycosyltransferase.

[0065]

[Example 4: Acquisition of Full-Length cDNA Sequence of Candidate Genes Encoding Protein having Activity that Transfers a Sugar to the Hydroxyl Group at the 4'-Position of Flavone]

25 types of sequences of glycosyltransferase gene were obtained in Example 3. Experiments were conducted to acquire full-length cDNA sequences for 10 of those sequences (NmGT0 to NmGT9).

Acquisition of full-length cDNA was carried out using GeneRacer (Invitrogen Corp.) in accordance with the protocol recommended by the manufacturer. Regions specific to the clones were selected from the cDNA partial sequences obtained in Example 3 and RACE primers were designed based on the sequences of these regions to obtain 5'- and 3'-terminal sequences by RACE PCR. Primers for amplifying the full-length cDNA sequence were designed based on these sequences, and a PCR reaction was carried out on 50 μl using nemophila cDNA as template and using KOD-Plus Polymerase (Toyobo Co., Ltd.) in accordance with the protocol recommended by the manufacturer (consisting of repeating 30 cycles of

holding at 94°C for 2 minutes, 94°C for 15 seconds, 55°C for 30 seconds and 68°C for 2 minutes followed by holding at 4°C). Nemophila cDNA was synthesized using the total RNA isolated in Example 2 using SuperScript™ II Reverse Transcriptase (Invitrogen Corp.) in accordance with the protocol recommended by the manufacturer. The primers were designed so as to contain restrictase sites on both ends of the full-length cDNA so as to enable the insertion of NmGT0 to NmGT9 genes into E. coli expression vector pET15b (Novagen Inc.). Plasmids containing the full length of the NmGT genes (pTOPO-NmGT0 to pTOPO-NmGT9) were acquired using the Zero Blunt TOPO PCR Cloning Kit for Sequencing (Invitrogen Corp.) in accordance with the protocol recommended by the manufacturer. Base sequences inserted with the plasmids were analyzed to acquire a full-length cDNA sequence (NmGT8: SEQ ID NO: 1) from among candidate genes (NmGT0 to NmGT9) for a gene encoding a protein having activity that transfers a sugar to the hydroxyl group at the 4'-position of a flavone.

[0066]

[Example 5: Experiment on Measurement of Enzyme Activity of Protein Candidates having Activity that Transfers a Sugar to the Hydroxyl Group at the 4'-Position of Flavone (Case of Using Crude Enzyme)]

<Preparation of E. Coli Expression Constructs>

3 μg aliquots of each of pTOPO-NmGT0 to pTOPO-NmGT9 were treated with their corresponding restrictases followed by recovery of the resulting approximately 1.5 kb DNA fragments. 2 μg of vector pET15b was also treated with restrictase and ligated with the resulting DNA fragments to prepare E. coli expression constructs (pET-NmGT0 to pET-NmGT9).

[0067]

<Expression of Glycosyltransferase in E. Coli>

pET-NmGT0 to pET-NmGT9 were introduced into E. coli

strain BL2 using One Shot BL21 (DE3) (Invitrogen Corp.) in accordance with the protocol recommended by the manufacturer to acquire transformed E. coli. This E. coli was then cultured using the Overnight Express Autoinduction System 1 (Novagen Inc.) in accordance with the protocol recommended by the manufacturer. The transformed E. coli were cultured at 37°C to an OD600 value of 0.5 in 2 ml of the prepared culture liquid (approx. 4 hours). This E. coli culture liquid was then used as a pre-culture liquid and added to 50 ml of culture liquid followed by final culturing overnight at 27°C.

Following final culturing overnight, the E. coli culture liquid was centrifuged (3000 rpm, 4°C, 15 minutes), the collected cells were suspended in 5 ml of sonic buffer (composition: Tris HCl (pH 7.0): 2.5 mM, dithiothreitol (DTT): 1 mM, amidinophenylmethanesulfonyl fluoride hydrochloride (APMSF): 10 μM), and after crushing the E. coli by ultrasonic treatment, the crushed cells were centrifuged (15,000 rpm, 4°C, 10 minutes) followed by recovery of the supernatant. This supernatant was used as a crude enzyme liquid. The Avanti HP-26XP (rotor: JA-2) (Beckman Coulter Inc.) was used for centrifugation.

[0068]

<Measurement of Enzyme Activity>

A reaction liquid prepared by mixing 80 μl of crude enzyme liquid, 20 μl of 5 mM UDP-glucose, 20 μl of 1 M Tris HCl (pH 7.5) and 1 μl of 500 ng/μl apigenin on ice and bringing to a volume of 200 μl with water was held for 1 hour at 30°C. Subsequently, 200 μl of stop buffer (90% aqueous acetonitrile solution containing 0.1% TFA) were added to stop the reaction followed by analyzing the reaction liquid by high-performance liquid chromatography (Prominence (Shimadzu Corp.)). The Shimadzu PDA SPD-

M10AVP was used for the detector and flavone was detected at 330 nm. The Shim-Pack ODS 150 mm × 4.6 mm column (Shimadzu Corp.) was used for the column. A Solution A (0.1% aqueous TFA solution) and a Solution B (90% aqueous acetonitrile solution containing 0.1% TFA) were used for elution. Elution was carried out using a linear concentration gradient from an 8:2 mixture of the two solutions to a 3:7 mixture of the two solutions over the course of 10 minutes followed by a 3:7 mixture for 5 minutes. The flow rate was 0.6 ml/min. A reaction liquid obtained by allowing enzyme to react under the same conditions using a crude enzyme liquid of E. coli introduced with a pET vector not inserted with an insert was used as a control.

As a result, a peak other than that of the substrate was observed for NmGT8.

Descriptions starting with that of Example 6 are described with respect to NmGT8 (SEQ ID NO: 1).

[0069]

[Example 6: Experiment on Measurement of Enzyme Activity of Protein having Activity that Transfers a Sugar to the Hydroxyl Group at the 4'-Position of Flavone (Case of Using His-Tag-Added Protein] <Expression of Glycosyltransferase in E. coli and Protein Purification>

E. coli strain BL2 introduced with the pET-NmGT8 described in Example 5 was cultured using the Overnight Express Autoinduction System 1 (Novagen Inc.) in accordance with the protocol recommended by the manufacturer. The transformed E. coli were cultured at 37°C to an OD600 value of 0.5 in 8 ml of the prepared culture liquid (approx. 4 hours). This E. coli culture liquid was then used as a pre-culture liquid and added to 200 ml of culture liquid followed by final culturing overnight at 25°C.

Following final culturing overnight, the E. coli

culture liquid was centrifuged (1000 × g, 4°C, 10 minutes), the collected cells were suspended in 20 ml of extract (composition: buffer (KCl: 300 mM, KH_2PO_4: 50 mM, imidazole: 5 mm) (pH 8.0), amidinophenylmethanesulfonyl fluoride hydrochloride (APMSF): 10 μM), and after crushing the E. coli by ultrasonic treatment, the crushed cells were centrifuged (1400 × g, 4°C, 20 minutes) followed by recovery of the supernatant. This supernatant was passed through a 0.45 μm filter and purified by His-Tag protein purification using Profinia (Bio-Rad Laboratories, Inc.) in accordance with the protocol recommended by the manufacturer. The resulting purified protein solution was centrifuged (7500 × g, 4°C, 15 minutes) using centrifugal filters (Ultracel™-10K, Amicon Corp.) and the concentrated protein solution was designated as "NmGT8 Protein Solution". The Avanti HP-26XP (rotor: JA-2) (Beckman Coulter Inc.) was used for centrifugation.

[0070]

<Measurement of Enzyme Activity>

A reaction liquid prepared by mixing 10 μl of protein solution, 2 μl of 50 mM UDP-glucose, 10 μl of 1 M Tris HCl (pH 7.5) and 5 μl of 1 mM apigenin on ice and bringing to a volume of 100 μl with water was held for 20 minutes at 30°C. Subsequently, 100 μl of stop buffer (90% aqueous acetonitrile solution containing 0.1% TFA) were added to stop the reaction followed by analyzing the reaction liquid by high-performance liquid chromatography (Prominence (Shimadzu Corp.)). The Shimadzu PDA SPD-M10AVP was used for the detector and flavone was detected at 330 nm. The Shim-Pack ODS 150 mm × 4.6 mm column (Shimadzu Corp.) was used for the column. A Solution A (0.1% aqueous TFA solution) and a Solution B (90% aqueous methanol solution containing 0.1% TFA) were used for elution. Elution was carried out using a linear

concentration gradient from an 8:2 mixture of the two solutions to a 3:7 mixture of the two solutions over the course of 10 minutes followed by a 3:7 mixture for 6 minutes. The flow rate was 0.6 ml/min.

[0071]

As a result, flavone demonstrating the same retention time and absorption maximum as purified apigenin 4'-glucoside was biosynthesized (see FIG. 9). Luteolin 4'-glucoside was biosynthesized in the case of carrying out an enzyme reaction under the same conditions by replacing the substrate with another flavone in the form of luteolin (see FIG. 11). On the other hand, apigenin 4',7-diglucoside was not biosynthesized in the case of carrying out the enzyme reaction under the same conditions by replacing the substrate with 1 mM apigenin 7-glucoside (see FIG. 10). Similarly, luteolin 4',7-diglucoside was also not biosynthesized in the case of carrying out the enzyme reaction under the same conditions by replacing the substrate with 1 mM luteolin 7-glucoside (see FIG. 11). On the basis thereof, NmGT8 protein was clearly determined to carry out glycosylation of the 4'-position prior to glycosylation of the 7-position of apigenin and luteolin (see FIG. 8). Moreover, when reactivity to the various types of flavone compounds described in FIG. 11 and betanidine was investigated, NmGT8 protein was clearly determined to demonstrate high substrate specificity and selectively glycosylate the 4'-position of flavones in the manner of apigenin and luteolin (see FIG. 11).

[0072]

Furthermore, although glycosyltransferase gene derived from Livingstone daisy (Dbs5GT) transfers glucose to the hydroxyl group at the 5-position of betanidine, it has also been reported to demonstrate activity in vitro that transfers glucose to the hydroxyl group at one of either the 4'-position or 7-position of a flavonoid. This glycosyltransferase derived from Livingstone daisy

was clearly determined to differ considerably from the NmGT8 protein of the present invention with respect to reactivity to flavonoid compounds and betanidine (see FIG. 11).

[0073]

Amino acid sequence identities between NmGT8 and NmGT3 and between NmGT8 and NmGT4 were 32% and 32%, respectively (see FIGS. 12-1 to 12-3). The Clustal W Program of the MacVector Application (Version 11.02, Oxford Molecular Ltd., Oxford, England) was used for this analysis. Among previously identified glycosyltransferases, the amino acid sequence having the highest identity with NmGT8 was that of an enzyme that adds a sugar to the 3-position and 5-position of rose anthocyanidin (GenBank Accession No. Q4R119), and demonstrated amino acid sequence identity of 52% (see FIG. 14). Next, an amino acid sequence having high identity with NmGT8 was that of an enzyme that adds a sugar to the 4'-position of snapdragon chalcone (described in PCT/JP2004/019461), and this demonstrated identity of 51% (see FIG. 13).

[0074]

In addition, FIG. 15 shows a phylogenetic tree indicating the relationships between the NmGT8 of the present invention and the aforementioned enzymes.

[0075]

[Example 7: Expression of Gene Encoding Peptide Having Activity that Transfers a Sugar to the Hydroxyl Group at the 4'-Position of Flavone in Torenia]

A binary vector pSPB4583 for expressing NmGT8 was constructed and introduced into torenia (Summer Wave) in order to confirm whether or not the NmGT8 gene of the present invention translates a protein having activity that transfers a sugar to the hydroxyl group at the 4'-position of a flavone in plants. Details of the introduced construct are described below (see FIG. 16).

[0076]

<Preparation of Construct>

The binary vector pBINPLUS for introduction into plants (van Engel, et al., Transgenic Research, 4, p. 288) was used for the basic skeleton of pSPB4583, and EI235S promoter having two repetitions of an enhancer sequence upstream from cauliflower mosaic virus 35S promoter (Mitsuhara, et al., (1996) Plant Cell Physiol., 37, p. 49), full-length cDNA NmGT8 and mas terminator were contained therein.

[0077]

<Gene Expression Analysis>

Gene expression analysis was carried out by forming chutes in selective media containing kanamycin, acclimating individuals in which rooting was observed, and using the petals of buds of each recombinant torenia in which the calyx had not yet split. Isolation of total RNA was carried out in the same manner as that described in Example 3, and synthesis of cDNA was carried out in the same manner as the method described in Example 4. A reverse transcription PCR reaction was carried out on 30 µl using the cDNA as template and using ExTaq™ Polymerase (Takara Co., Ltd.) in accordance with the protocol recommended by the manufacturer (consisting of repeating 25 cycles of holding at 94°C for 2 minutes, 94°C for 1 minute, 55°C for 1 minute and 72°C for 2 minutes followed by holding at 4°C). Buffers were designed so as to specifically amplify each full-length cDNA. As a result, NmGT8 was confirmed to be transcribed in Torenia.

[0078]

[Example 8: Expression of Gene Encoding Protein Having Activity that Transfers a Sugar to the Hydroxyl Group at the 4'-Position of Flavone in Petunia]

Binary vectors pSPB5424 and pSBP5428 for expressing NmGT8 were constructed and introduced into petunia

(Surfinia Bouquet Red). Since petunia does not biosynthesize flavones naturally, the vectors were introduced together with torenia flavone synthase and evaluated as to whether or not NmGT8 has activity that transfers a sugar to the hydroxyl group at the 4'-position of a flavone in petunia. Details of the introduced constructs are described below (see FIG. 17).

[0079]

<Preparation of Constructs>

pBINPLUS was used for the basic skeleton of pSPB5424, and three expression cassettes were contained therein (consisting of 1: EI235S promoter, full-length cDNA pansy F3'5'H (described in PCT/JP2004/011958, SEQ ID NO: 3) and HSP terminator (Plant Cell Physiology (2010), 51, 328-332); 2: EI235S promoter, full-length cDNA torenia flavone synthase and HSP terminator; and 3: EI235S promoter, full-length cDNA NmGT8 and HSP terminator).

pBINPLUS was also used for the basic skeleton of pSPB5428, and two expression cassettes were contained therein (consisting of 1: EI235S promoter, full-length cDNA torenia flavone synthase and HSP terminator; and 2: EI235S promoter, full-length cDNA NmGT8 and HSP terminator).

[0080]

<Gene Expression Analysis>

Gene expression analysis was carried out in the same manner as the method described in Example 7 by forming chutes in selective media containing kanamycin, acclimating individuals in which rooting was observed, and using the petals of flowers of each recombinant petunia that had completely opened. As a result, NmGT8 was confirmed to be transcribed in petunia.

[0081]

<Flower Petal Pigment Analysis>

Pigments in the flower petals were analyzed for full-length cDNA torenia flavone synthase and full-length

cDNA NmGT8 in those stains in which transcription products were confirmed. 0.2 g of completely open flower petals were freeze-dried for 24 hours or more and finely crushed with a spatula, followed by the addition of 4 ml of extract buffer (composition: 50% aqueous acetonitrile solution containing 0.1% TFA) and subjecting to ultrasonic treatment for 20 minutes. The flower petal extract was analyzed by high-performance liquid chromatography (Prominence (Shimadzu Corp.)). Analyses were carried out under the conditions and in the same manner as in Example 5. Non-recombinant petunia not introduced with genes and recombinant petunia introduced only with torenia flavone synthase that biosynthesizes flavone were analyzed in the same manner as controls (FIG. 18). Moreover, flower petal extract diluted 50-fold was also analyzed with a high-performance liquid chromatograph (Shimadzu Corp.). The Shimadzu LCMS-IF-TOF was used for the detector and flavones were detected at 433.1057 ([Api-Glc+H]) and 449.1084 ([Lut-Glc+H]). The Inertsil ODS-4 (250 × 4.6 mm, 5 μm, Shimadzu Corp.) was used for the column. A Solution A (0.1% aqueous formic acid solution) and a Solution B (90% aqueous acetonitrile solution containing 0.1% formic acid) were used for elution. Elution was carried out using a linear concentration gradient from a 9:1 mixture of the two solutions to an 11:9 mixture of the two solutions over the course of 35 minutes and a linear concentration gradient from an 11:9 mixture of the two solutions to a 0:10 mixture of the two solutions over the course of 10 minutes followed by a 0:10 mixture for 5 minutes. The flow rate was 0.6 ml/min (FIG. 19).

[0082]

As a result, apigenin 4'-glucoside and luteolin 4'-glucoside were detected in the recombinant petunia introduced with flavone synthase and NmGT8 (FIG. 18), and flavone 4'-glucoside was determined to account for 95.6% of the biosynthesized flavones. The remaining 4.4% was

determined to consist of flavone 7-glucoside biosynthesized by the intrinsic activity of petunia that transfers a sugar to the hydroxyl group at the 7-position of a flavone (FIG. 19). On the other hand, flavones were not detected in the non-recombinant petunia, and flavone 7-glucoside was determined to account for 82.8% of the biosynthesized flavones in the recombinant petunia introduced only with torenia flavone synthase. On the basis thereof, NmGT8 was clearly demonstrated to be a protein having activity that transfers a sugar to the hydroxyl group at the 4'-position of a flavone that functions preferentially over protein having activity that transfers a sugar to the hydroxyl group at the 7-position of a flavone that is intrinsic to petunia. The use of NmGT8 makes it possible to efficiently biosynthesize flavone 4'-glucoside in petunia.

[0083]

[Example 9: Expression of Gene Encoding Protein Having Activity that Transfers a Sugar to the Hydroxyl Group at the 4'-Position of Flavone in Carnation]

A binary vector pSPB5433 for expressing NmGT8 was constructed and introduced into carnation (Cream Cinderella). Since carnation does not biosynthesize flavones naturally, the vector was introduced together with torenia flavone synthase and evaluated as to whether or not NmGT8 has activity that transfers a sugar to the hydroxyl group at the 4'-position of a flavone in carnation. Details of the introduced construct are described below (see FIG. 20).

The binary vector pWTT2132 for introduction into plants (DNA Plant Technologies, USA = DNAP) was used for the basic skeleton of pSPB5433, and four expression cassettes were contained therein (consisting of 1: snapdragon chalcone synthase promoter (described in PCT/AU94/00265), full-length cDNA pansy F3'5'H and HSP terminator; 2: snapdragon chalcone synthase promoter,

full-length cDNA torenia flavone synthase and HSP terminator; 3: carnation anthocyanin synthase promoter (described in PCT/AU/2009/001659), full-length cDNA NmGT8 and HSP terminator; and, 4: carnation anthocyanin synthase promoter, full-length cDNA NmGT3 (Japanese Patent Application No. 2011-006317) and HSP terminator).

[0084]

[Example 10: Expression of Gene Encoding Protein Having Activity that Transfers a Sugar to the Hydroxyl Group at the 4'-Position of Flavone in Rose]

Binary vectors pSPB4577, pSBP4578, pSBP5437 and pSBP5440 for expressing NmGT8 were constructed and introduced into rose (Noblesse, Ritapa Humera). Since rose does not biosynthesize flavones naturally, the vectors were introduced together with torenia flavone synthase and evaluated as to whether or not NmGT8 has activity that transfers a sugar to the hydroxyl group at the 4'-position of a flavone in rose. Details of the introduced constructs are described below (see FIG. 21).

pBINPLUS was used for the basic skeleton of pSPB5477, and three expression cassettes were contained therein (consisting of 1: EI235S promoter, full-length cDNA pansy F3'5'H and mas terminator; 2: EI235S promoter, full-length cDNA torenia flavone synthase and mas terminator; and, 3: EI235S promoter, full-length cDNA NmGT8 and mas terminator).

pBINPLUS was used for the basic skeleton of pSPB4578, and three expression cassettes were contained therein (consisting of 1: perilla anthocyanin 3-acyltransferase promoter (described in PCT/JP2010/053909), full-length cDNA pansy F3'5'H and mas terminator; 2: EI235S promoter, full-length cDNA torenia flavone synthase and mas terminator; and, 3: EI235S promoter, full-length cDNA NmGT8 and mas terminator).

[0085]

pBINPLUS was used for the basic skeleton of

pSPB5437, and five expression cassettes were contained therein (consisting of 1: EI235S promoter, full-length cDNA pansy F3'5'H and HSP terminator; 2: perilla anthocyanin 3-acyltransferase chromogene (described in PCT/JP2010/053886, see SEQ ID NO: 7); 3: EI235S promoter, full-length cDNA torenia flavone synthase and HSP terminator; 4: EI235S promoter, full-length cDNA NmGT8 and HSP terminator); and, 5: EI235S promoter, full-length cDNA NmGT3 and HSP terminator).

pBINPLUS was used for the basic skeleton of pSPB5440, and five expression cassettes were contained therein (consisting of 1: EI235S promoter, full-length cDNA pansy F3'5'H and HSP terminator; 2: EI235S promoter, cDNA lavender anthocyanin 3-acyltransferase (described in PCT/JP1996/000348, see SEQ ID NO: 8); 3: EI235S promoter, full-length cDNA torenia flavone synthase and HSP terminator; 4: EI235S promoter, full-length cDNA NmGT8 and HSP terminator); and, 5: EI235S promoter, full-length cDNA NmGT3 and HSP terminator).

[0086]

<Gene Expression Analysis>

Gene expression analysis was carried out in the same manner as the method described in Example 7 by forming chutes in selective media containing kanamycin, acclimating individuals in which rooting was observed, and using the petals of flowers of each recombinant rose that had completely opened. As a result, NmGT8 was confirmed to be transcribed in rose.

[0087]

<Flower Petal Pigment Analysis>

Pigments in the flower petals were analyzed for full-length cDNA torenia flavone synthase and full-length cDNA NmGT8 in those strains in which transcription products were confirmed. 0.2 g of completely open flower petals were freeze-dried for 24 hours or more and finely crushed with a spatula, followed by the addition of 4 ml of extract buffer (composition: 50% aqueous acetonitrile

solution containing 0.1% TFA) and subjecting to ultrasonic treatment for 20 minutes. The flower petal extract was analyzed by high-performance liquid chromatography (Prominence (Shimadzu Corp.)). Analyses were carried out under the conditions and in the same manner as in Example 5. Non-recombinant rose not introduced with the genes was analyzed in the same manner as a control (FIG. 22). Flower petal extract diluted 50-fold was also analyzed with a high-performance liquid chromatograph (Shimadzu Corp.). The Shimadzu LCMS-IF-TOF was used for the detector and flavones were detected at 433.1057 nm ([Api-Glc+H]) and 449.1084 nm ([Lut-Glc+H]). The Inertsil ODS-4 (250 × 4.6 mm, 5 μm, Shimadzu Corp.) was used for the column. A Solution A (0.1% aqueous formic acid solution) and a Solution B (90% aqueous acetonitrile solution containing 0.1% formic acid) were used for elution. Elution was carried out using a linear concentration gradient from a 9:1 mixture of the two solutions to an 11:9 mixture of the two solutions over the course of 35 minutes and a linear concentration gradient from an 11:9 mixture of the two solutions to a 0:10 mixture of the two solutions over the course of 10 minutes followed by a 0:10 mixture for 5 minutes. The flow rate was 0.6 ml/min (FIG. 23).

[0088]

As a result, apigenin 4'-glucoside and luteolin 4'-glucoside were detected in the recombinant rose introduced with flavone synthase and NmGT8 (FIG. 22), and flavone 4'-glucoside was determined to account for 97.0% of the biosynthesized flavones. The remaining 3.0% was determined to consist of flavone 7-glucoside biosynthesized by the intrinsic activity of rose that transfers a sugar to the hydroxyl group at the 7-position of a flavone (FIG. 23). On the other hand, flavones were not detected in the non-recombinant rose. On the basis thereof, NmGT8 was clearly demonstrated to be a protein having activity that transfers a sugar to the hydroxyl

group at the 4'-position of a flavone that functions preferentially over protein having activity that transfers a sugar to the hydroxyl group at the 7-position of a flavone that is intrinsic to rose. The use of NmGT8 makes it possible to efficiently biosynthesize flavone 4'-glucoside in rose.

INDUSTRIAL APPLICABILITY

[0089]

In the present invention, a polynucleotide encoding a protein having activity that transfers a sugar to the hydroxyl group at the 4'-position of a flavone was identified for the first time. Expression of the polynucleotide of the present invention in suitable host cells makes it possible to produce a protein having activity that specifically transfers a sugar to the hydroxyl group at the 4'-position of a flavone. According to the present invention, constitutively or tissue-specifically expressing a protein having activity that transfers a sugar to the hydroxyl group at the 4'-position of a flavone in a plant can be used to alter flower color. In addition, according to the present invention, a method for producing a flavone in which a sugar has been added to the hydroxyl group at the 4'-position thereof, and a food, pharmaceutical or cosmetic obtained according to that production method, are provided.

Claims

1. A polynucleotide selected from the group consisting of the following (a) to (e):

(a) a polynucleotide comprising the base sequence of SEQ ID NO: 1;

(b) a polynucleotide that hybridizes under stringent conditions with a polynucleotide comprising a base sequence complementary to the base sequence of SEQ ID NO: 1, and encodes a protein having activity that transfers a sugar to the hydroxyl group at the 4'-position of a flavone, wherein the stringent conditions are for carrying out hybridization under conditions consisting of 5 × SSC, 1% SDS, 5 × Denhardt's solution, 50% formaldehyde and 42°C, followed by washing under conditions consisting of 0.1 × SSC (15 mM NaCl, 1.5 mM trisodium citrate), 0.1% SDS and 55°C;

(c) a polynucleotide encoding a protein comprising the amino acid sequence of SEQ ID NO: 2;

(d) a polynucleotide encoding a protein comprising an amino acid sequence in which 1 to 20 amino acids have been deleted, substituted, inserted and/or added in the amino acid sequence of SEQ ID NO: 2, and having activity that transfers a sugar to the hydroxyl group at the 4'-position of a flavone; and,

(e) a polynucleotide encoding a protein comprising an amino acid sequence having identity of 90% or more with the amino acid sequence of SEQ ID NO: 2 and having activity that transfers a sugar to the hydroxyl group at the 4'-position of a flavone.

2. The polynucleotide according to claim 1, which is (a) a polynucleotide comprising the base sequence of SEQ ID NO: 1.

3. The polynucleotide according to claim 1, which is (c) a polynucleotide encoding a protein comprising the amino acid sequence of SEQ ID NO: 2.

4. The polynucleotide according to claim 1, which is (f) a polynucleotide encoding a protein comprising an amino acid sequence having identity of 95% or more with the amino acid sequence of SEQ ID NO: 2, and having activity that transfers a sugar to the hydroxyl group at the 4'-position of a flavone.

5. The polynucleotide according to claim 4, which is (g) a polynucleotide encoding a protein comprising an amino acid sequence having identity of 97% or more with the amino

acid sequence of SEQ ID NO: 2, and having activity that transfers a sugar to the hydroxyl group at the 4'-position of a flavone.

6. The polynucleotide according to claim 5, which is (h) a polynucleotide encoding a protein comprising an amino acid sequence having identity of 98% or more with the amino acid sequence of SEQ ID NO: 2, and having activity that transfers a sugar to the hydroxyl group at the 4'-position of a flavone.

7. The polynucleotide according to any one of claims 1 to 6, which is DNA.

8. A protein encoded by the polynucleotide according to any one of claims 1 to 7.

9. A vector containing the polynucleotide according to any one of claims 1 to 7.

10. An isolated cell introduced with the vector according to claim 9.

11. A method for adding a sugar to the hydroxyl group at the 4'-position of a flavone in the production of a transgenic plant, comprising the step of introducing the polynucleotide according to any of claims 1 to 7 into a host plant.

12. A method for producing a recombinant plant, a progeny thereof, or a portion or tissue thereof, comprising the step of introducing the polynucleotide according to any one of claims 1 to 7 into a host plant.

13. The method according to claim 12 wherein the portion of the plant is a cut flower.

14. A method for producing a protein having activity that transfers a sugar to the hydroxyl group at the 4'-position of a flavone, comprising the following steps:

culturing or growing the isolated cell according to claim 10, and

harvesting from the isolated cell the protein having activity that transfers a sugar to the hydroxyl group at the 4'-position of a flavone.

15. A method for producing a flavone in which a sugar has been added to the hydroxyl group at the 4'-position thereof, comprising the following steps:

culturing or growing the isolated cell according to claim 10, and

harvesting a flavone from the isolated cell in which a sugar has been added to the hydroxyl group at the 4'-position thereof.

FIG. 1

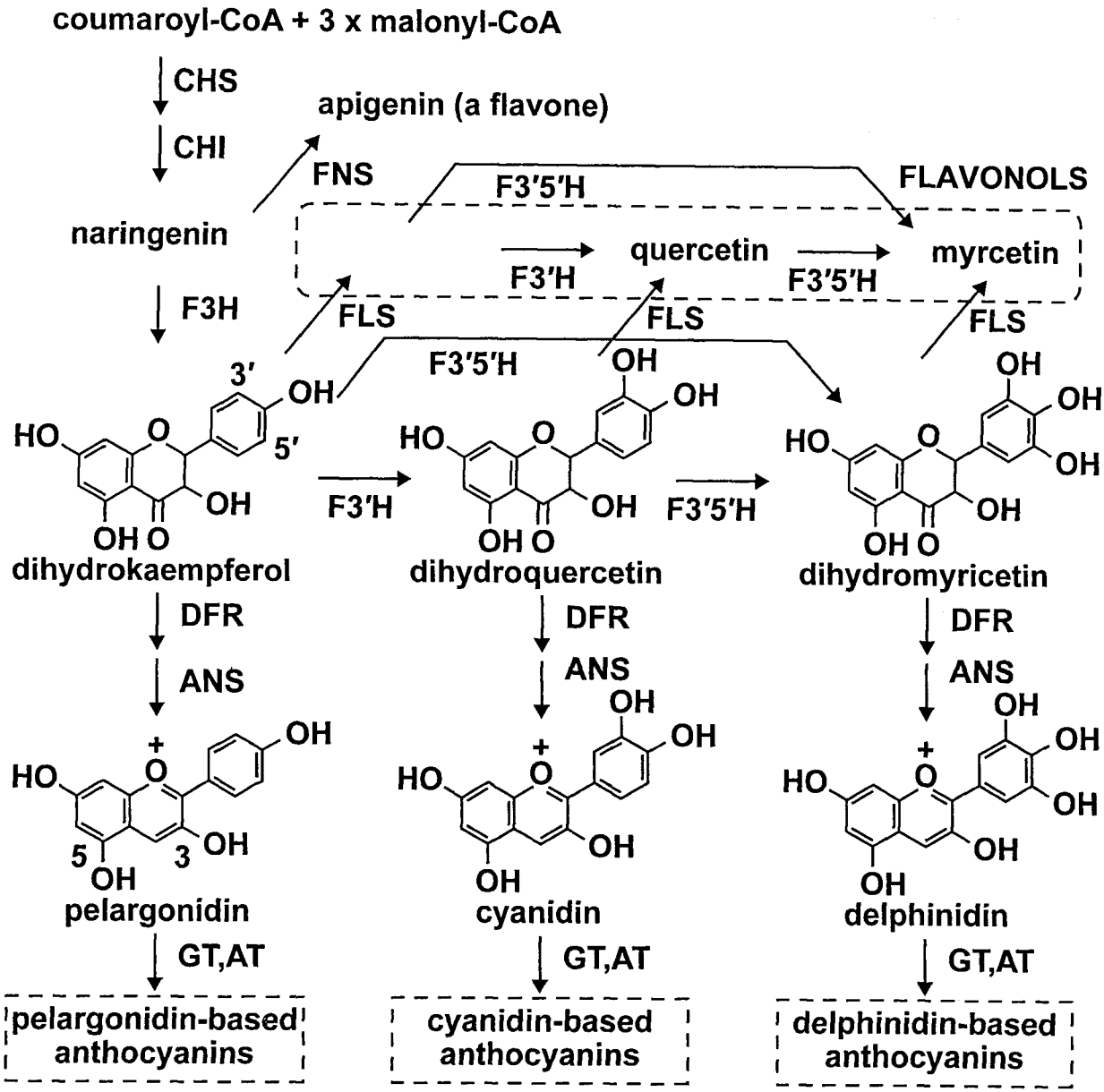
coumaroyl-CoA + 3 x malonyl-CoA
CHS
CHI
naringenin
F3H
apigenin (a flavone)
FNS
FLS
F3'5'H
FLAVONOLS
quercetin
myrcetin
F3'H
F3'5'H
FLS
FLS
FLS
F3'5'H
3'
5'
OH
HO
O
OH O
dihydrokaempferol
F3'H
dihydroquercetin
F3'5'H
dihydromyricetin
DFR
ANS
DFR
ANS
DFR
ANS
+
5
3
pelargonidin
cyanidin
delphinidin
GT,AT
GT,AT
GT,AT
pelargonidin-based anthocyanins
cyanidin-based anthocyanins
delphinidin-based anthocyanins

FIG. 2

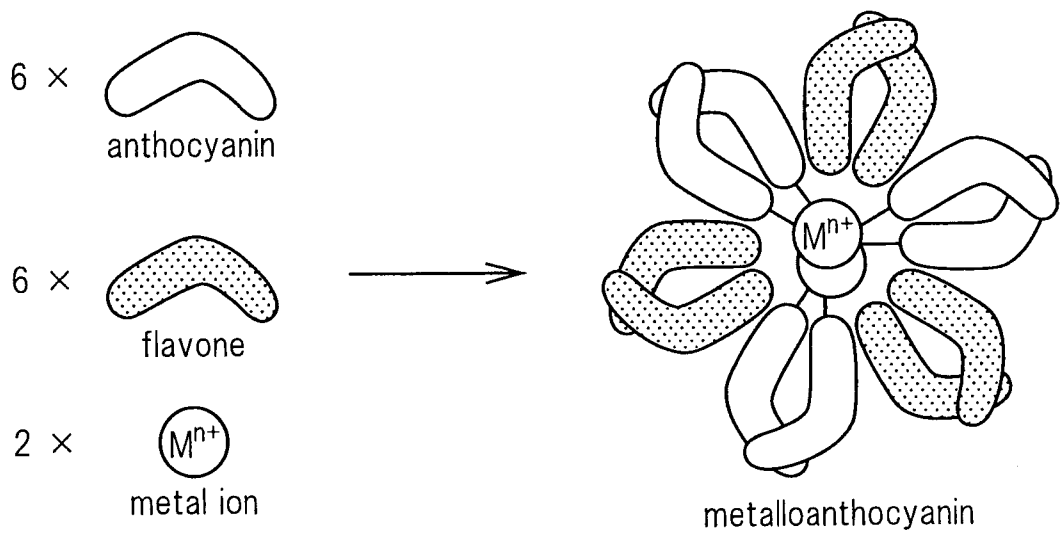
6 ×
anthocyanin
6 ×
flavone
2 ×
M^{n+}
metal ion
M^{n+}
metalloanthocyanin

FIG. 3

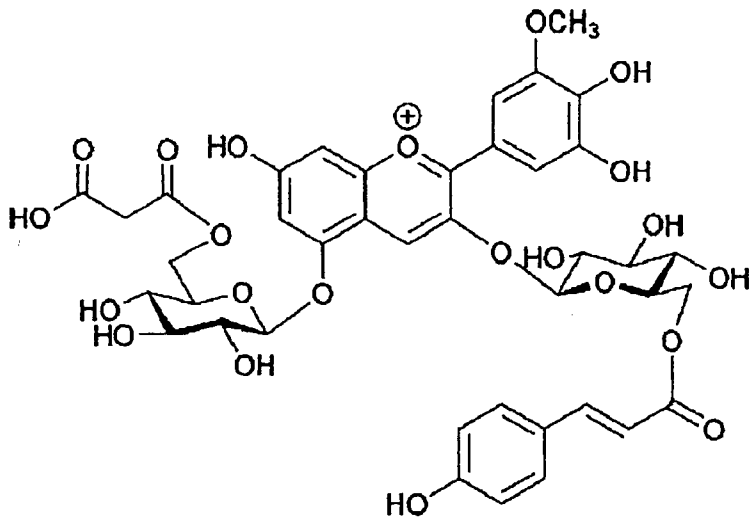
OCH3
OH
OH
HO
O
HO
O
O
O
HO
HO
OH
O
HO
HO
OH
O
O
O
HO

FIG. 4

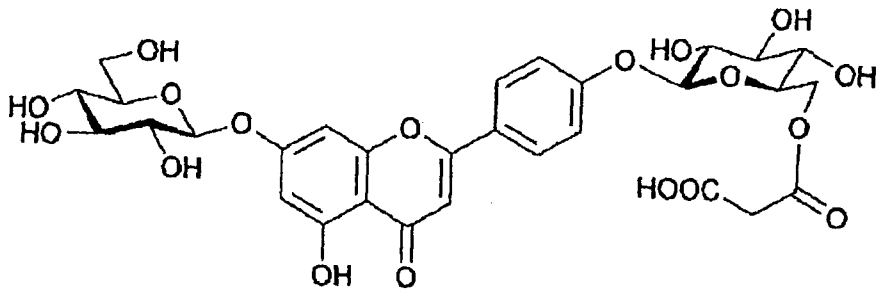

FIG. 5

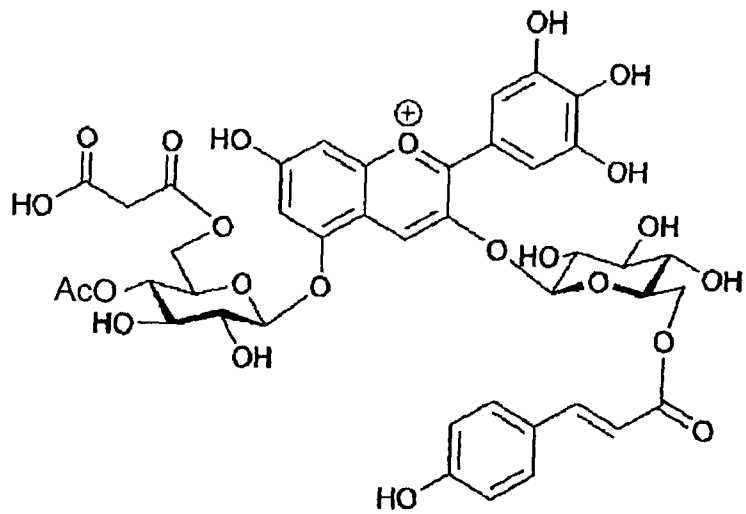

FIG. 6

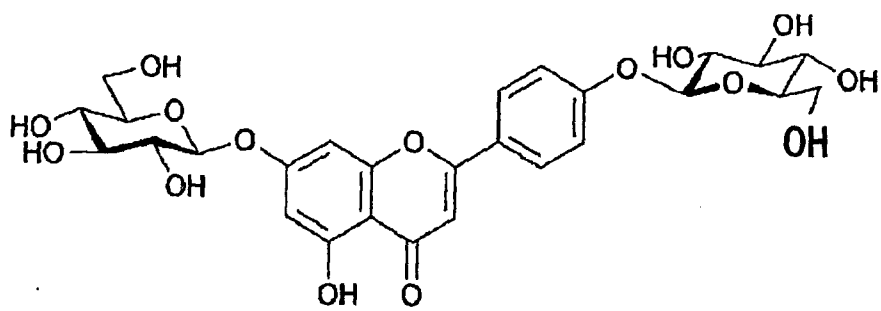

FIG. 7

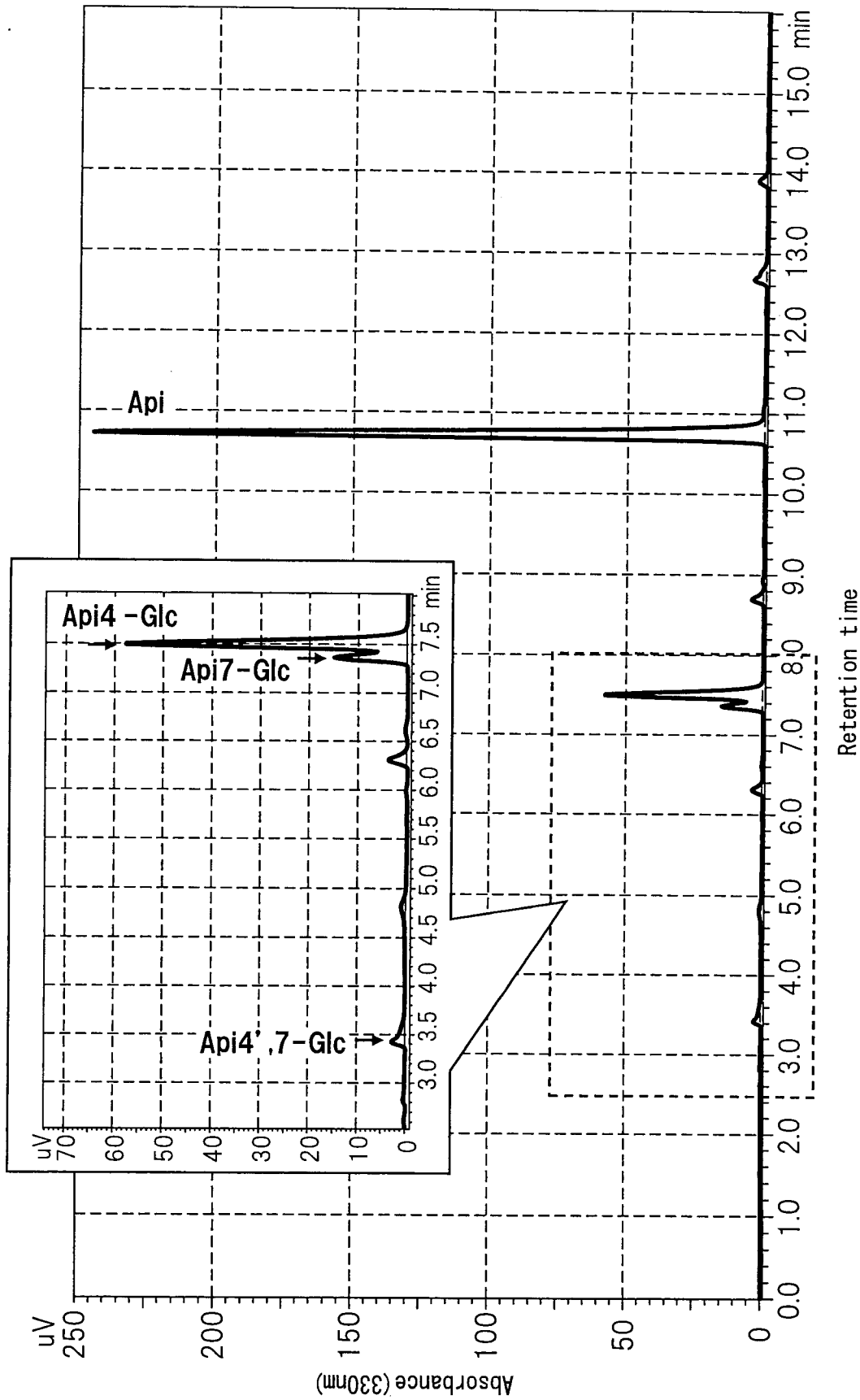
Api
Api4 -Glc
Api7-Glc
Api4',7-Glc
Absorbance (330nm)
uV
250
200
150
100
50
0
0.0
1.0
2.0
3.0
4.0
5.0
6.0
7.0
8.0
9.0
10.0
11.0
12.0
13.0
14.0
15.0 min
Retention time
uV
70
60
50
40
30
20
10
0
3.0
3.5
4.0
4.5
5.0
5.5
6.0
6.5
7.0
7.5 min

FIG. 8

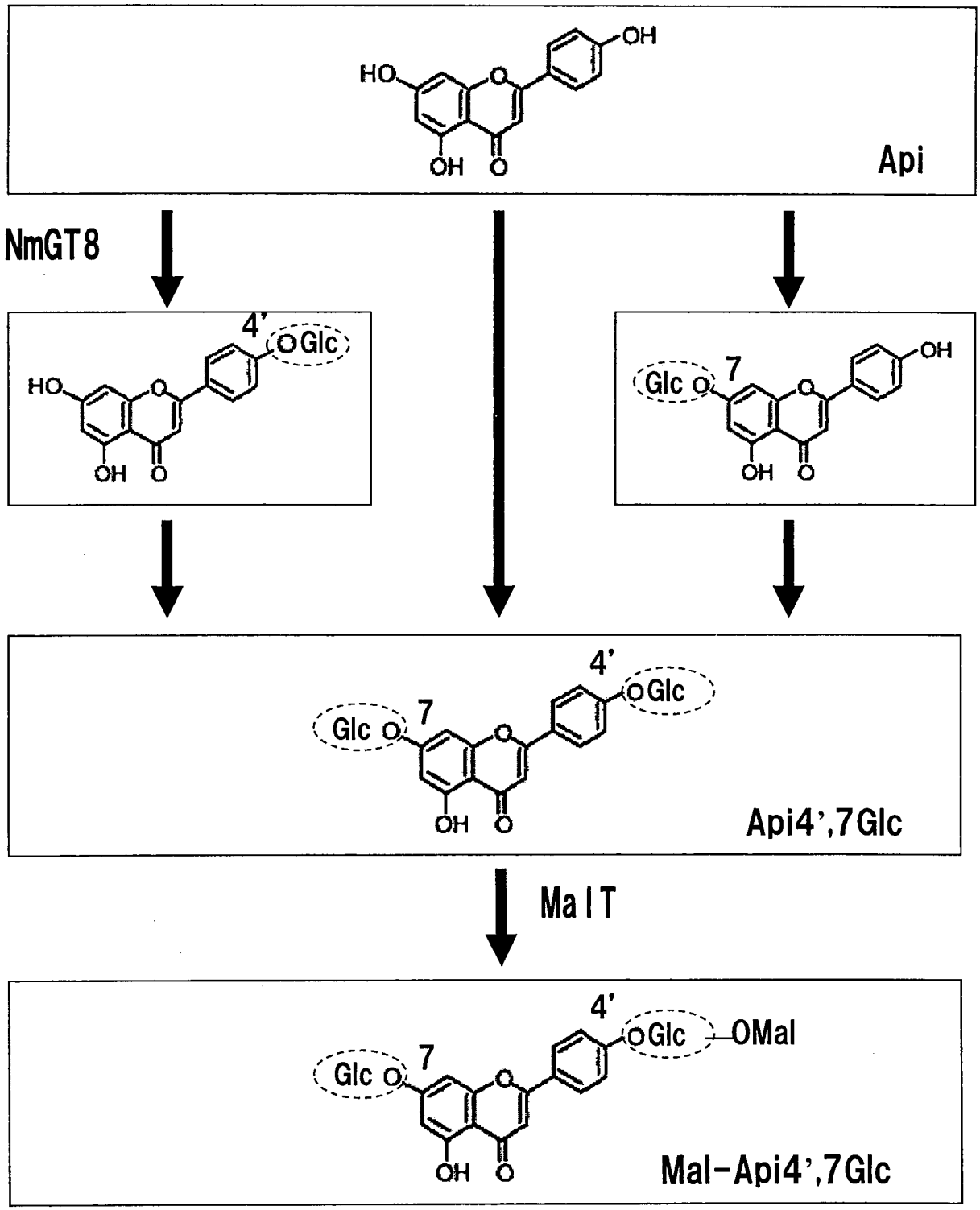
Api
NmGT8
4'
Glc
HO
OH
O
Glc
7
OH
4'
Glc
Glc
7
OH
Api4',7Glc
MalT
4'
Glc
OMal
Glc
7
OH
Mal-Api4',7Glc

FIG. 9

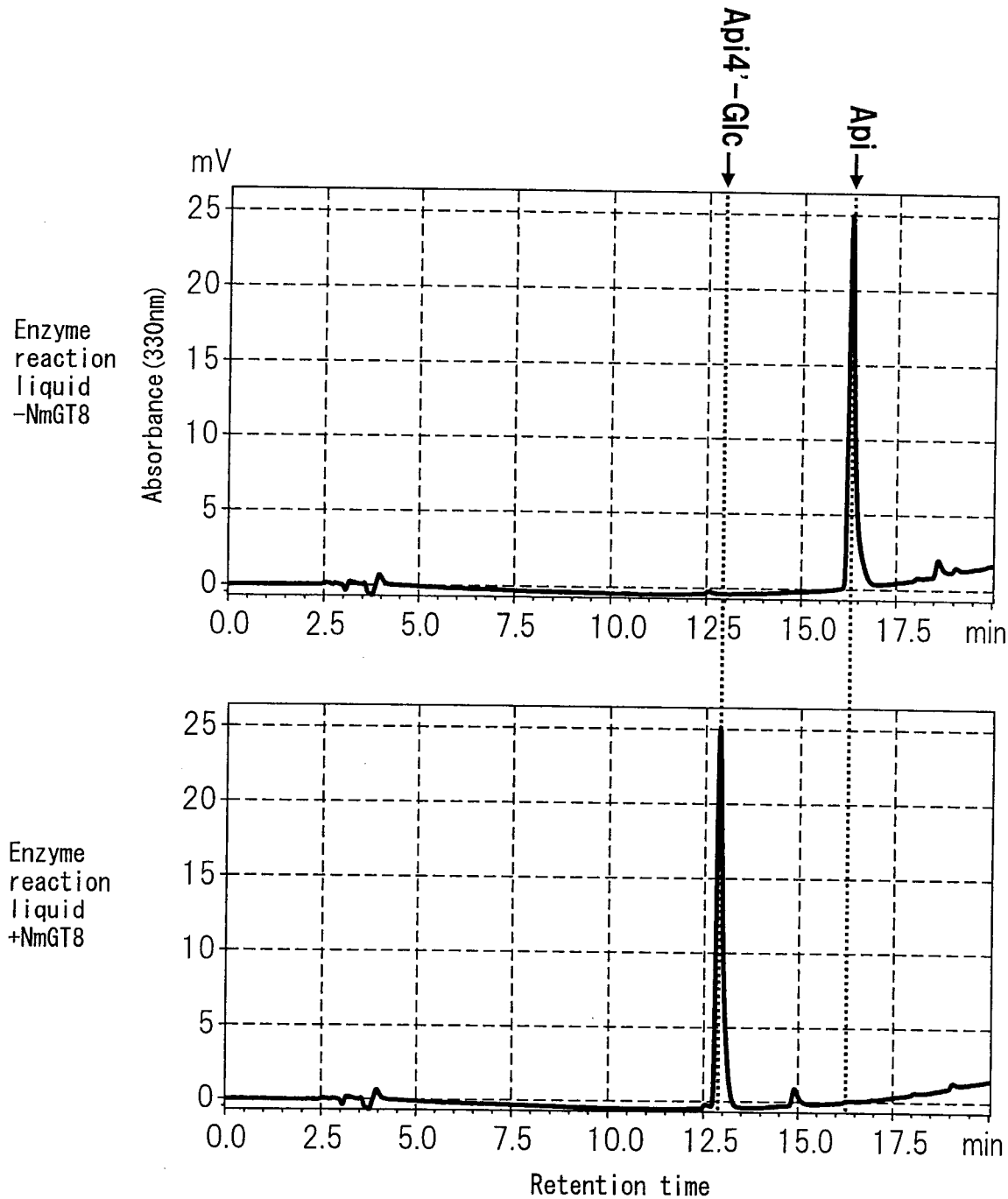
Api4'-Glc
Api
mV
25
20
15
10
5
0
Absorbance (330nm)
Enzyme reaction liquid -NmGT8
0.0
2.5
5.0
7.5
10.0
12.5
15.0
17.5
min
Enzyme reaction liquid +NmGT8
Retention time

FIG. 10

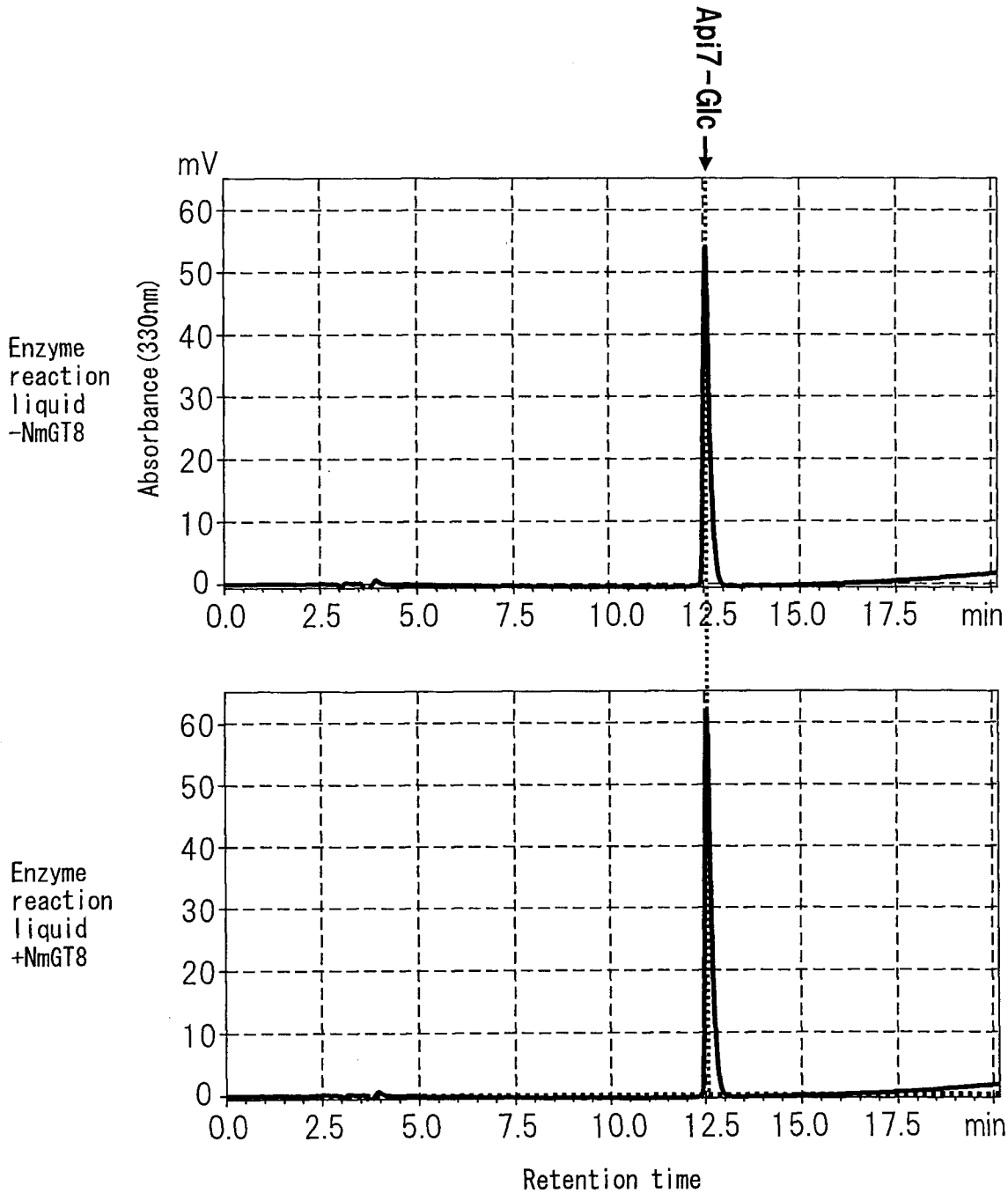
Api7-Glc
mV
60
50
40
30
20
10
0
Absorbance (330nm)
Enzyme reaction liquid -NmGT8
0.0
2.5
5.0
7.5
10.0
12.5
15.0
17.5
min
Enzyme reaction liquid +NmGT8
Retention time

FIG. 11

	NmGT8	Dbs5GT
Pel	×	(×)
Pel3Glc	×	
Cya	×	△(Cya4'Glc)
Cya3Glc	×	
Del	×	
Del3Glc	×	
Pet	×	
Mal	×	
Api	◎(Api4'Glc)	(×)
Api7Glc	×	
Lut	◎(Lut4'Glc)	△(Lut4'Glc,Lut7Glc)
Lut7Glc	×	
Lut4'Glc	×	
Tri	×	
K	○(K3Glc)	(×)(K4'Glc)
K3Glc	×	
Q	○	○(Q4'Glc,Q7Glc)
Q3Glc	×	
M	×	△(M4'Glc,M7Glc)
Narigenin	×	
betanidin	×	○(betanidin5Glc)

↑Source : Thesis Database(Thomas Vogt, 1997, 1999)

Enzyme reaction process
◎ : Reacts strongly and completely uses substrate
○ : Reacts
△ : Reacts, but hardly any product formed
× : Does not react at all
(×) : Hardly reacts at all

() : Reaction biosynthesis product
Cya4'Glc : Cyanidin 4'-glucoside
Api4'Glc : Apigenin 4'-glucoside
Api7Glc : Apigenin 7-glucoside
Api4',7Glc : Apigenin 4',7-diglucoside
Lut4'Glc : Luteolin 4'-glucoside
Lut7Glc : Luteolin 7-glucoside
Lut4',7Glc : Luteolin 4',7-diglucoside
K3Glc : Kaempferol 3-glucoside
K4'Glc : Kaempferol 4'-glucoside
Q3Glc : Quercetin 3-glucoside
Q4'Glc : Quercetin 4'-glucoside
Q7Glc : Quercetin 7-glucoside
M4'Glc : Myricetin 4'-glucoside
M7Glc : Myricetin 7-glucoside
betanidin5Glc : Betanidin 5-glucoside

FIG. 12-1

FIG. 12-2

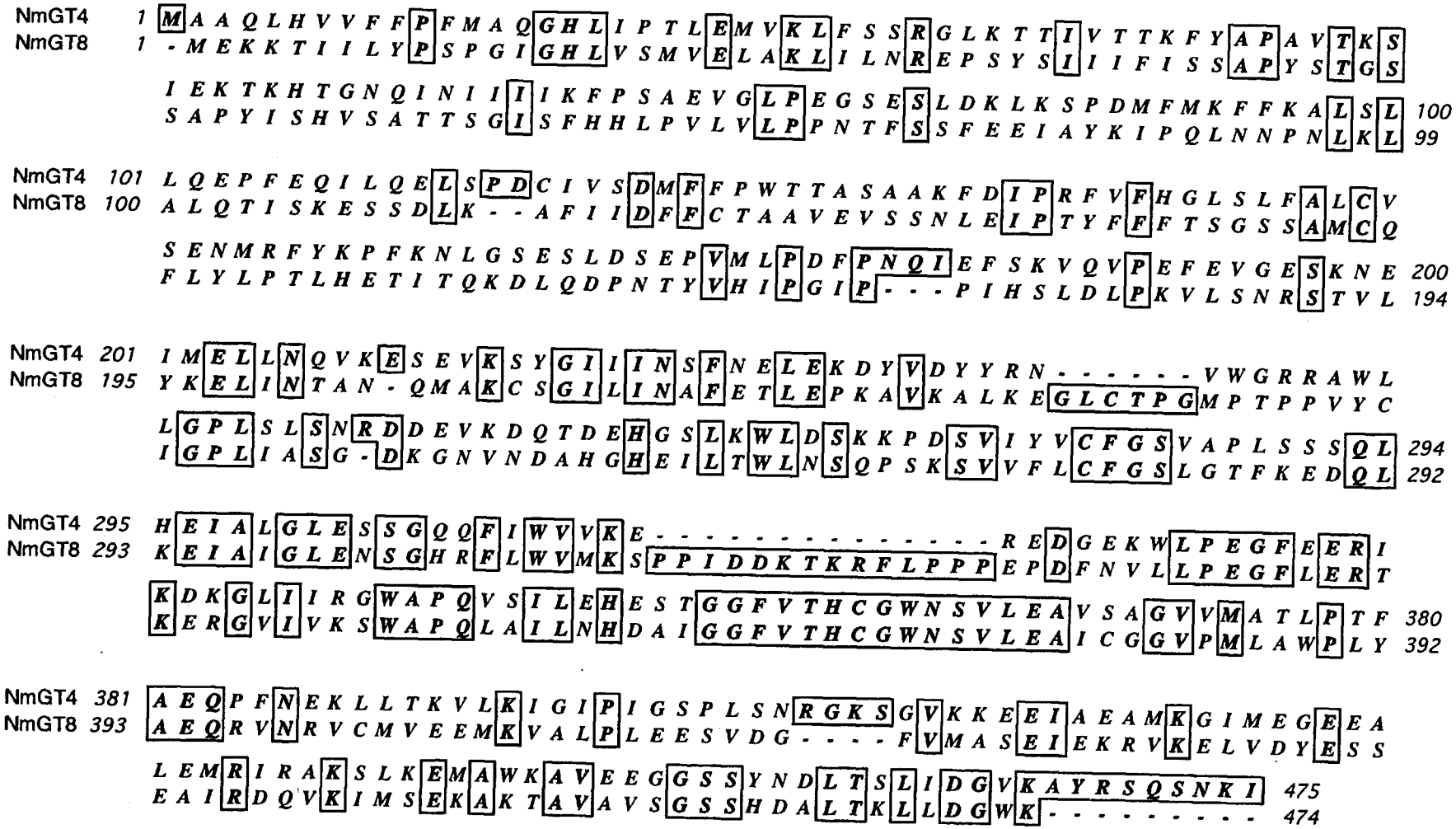

FIG. 12-3

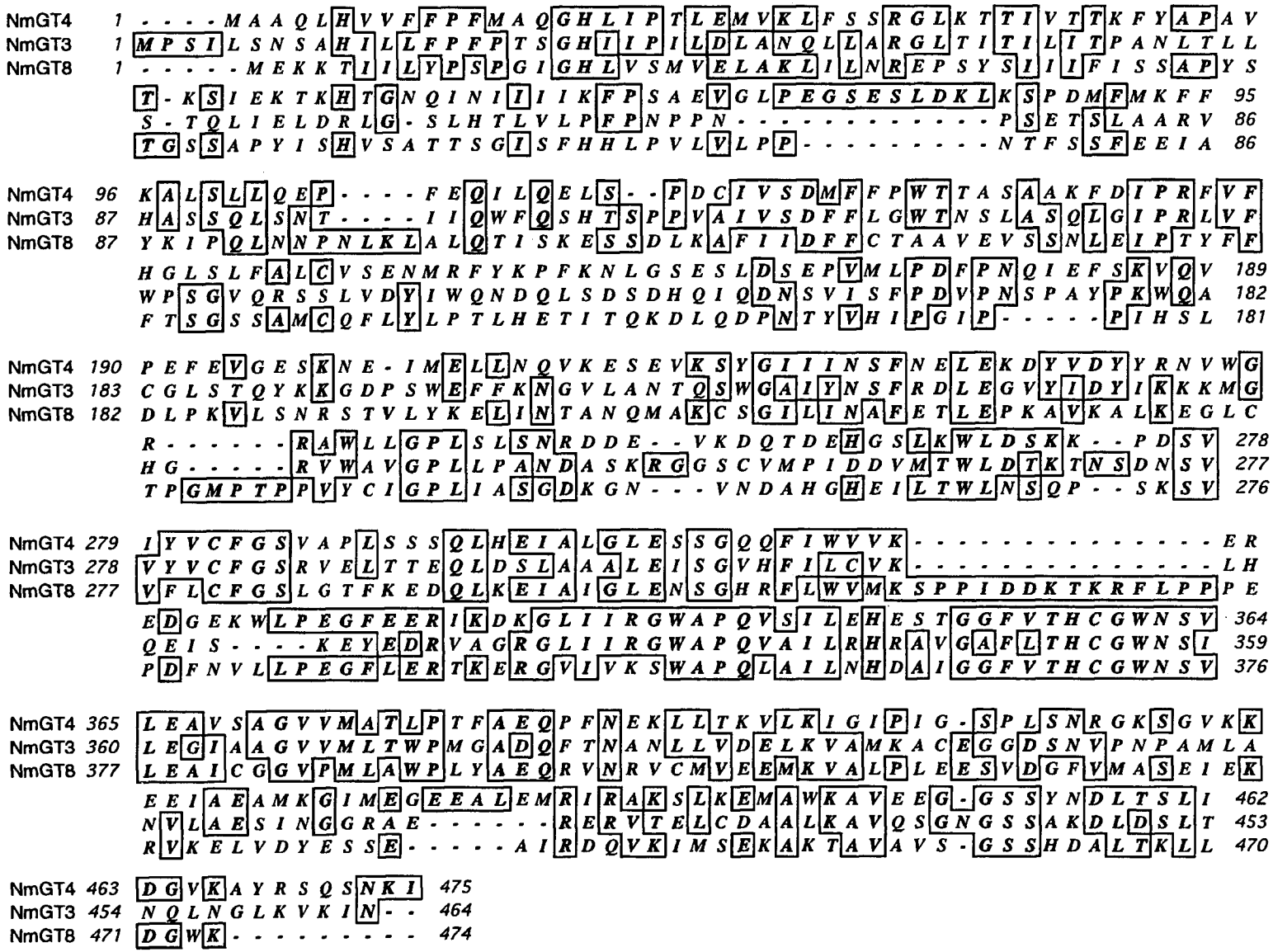

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FIG. 13

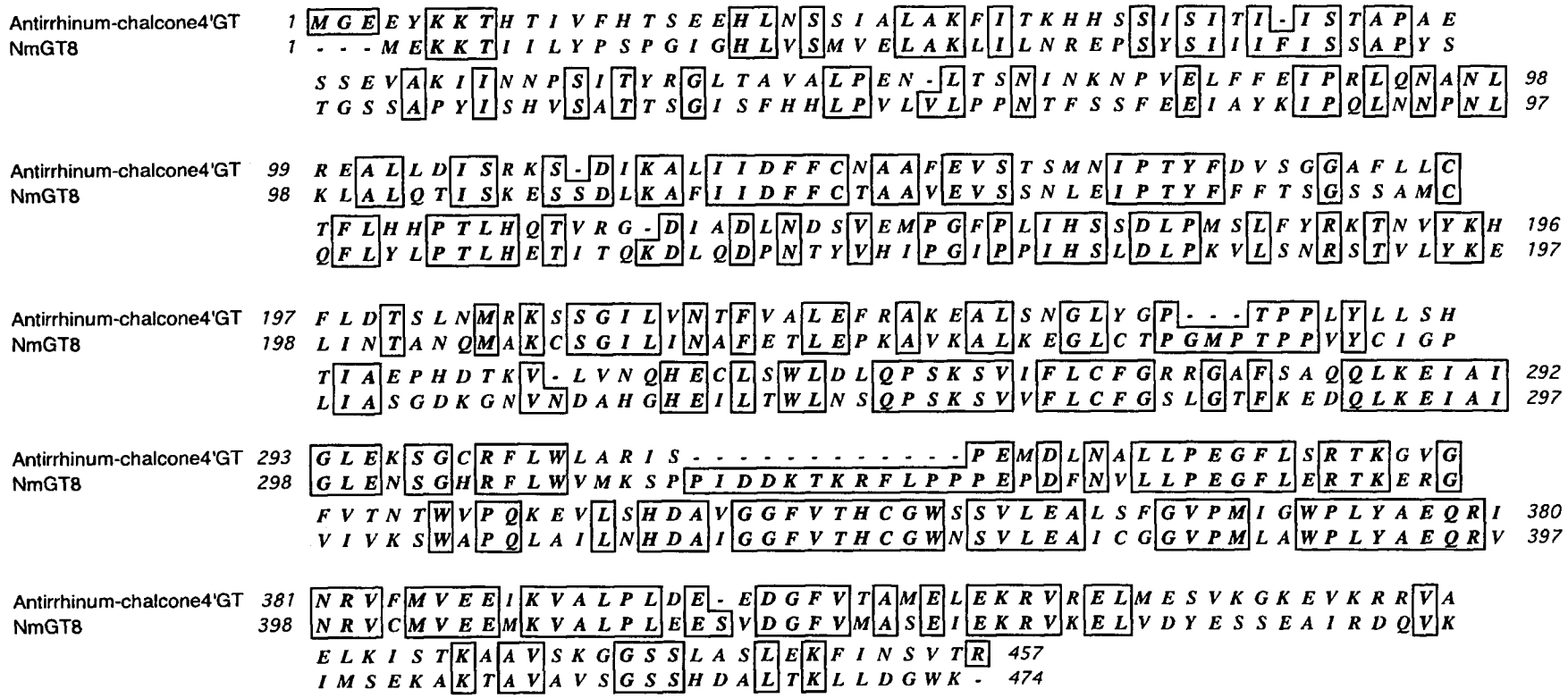

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FIG. 14

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FIG. 15

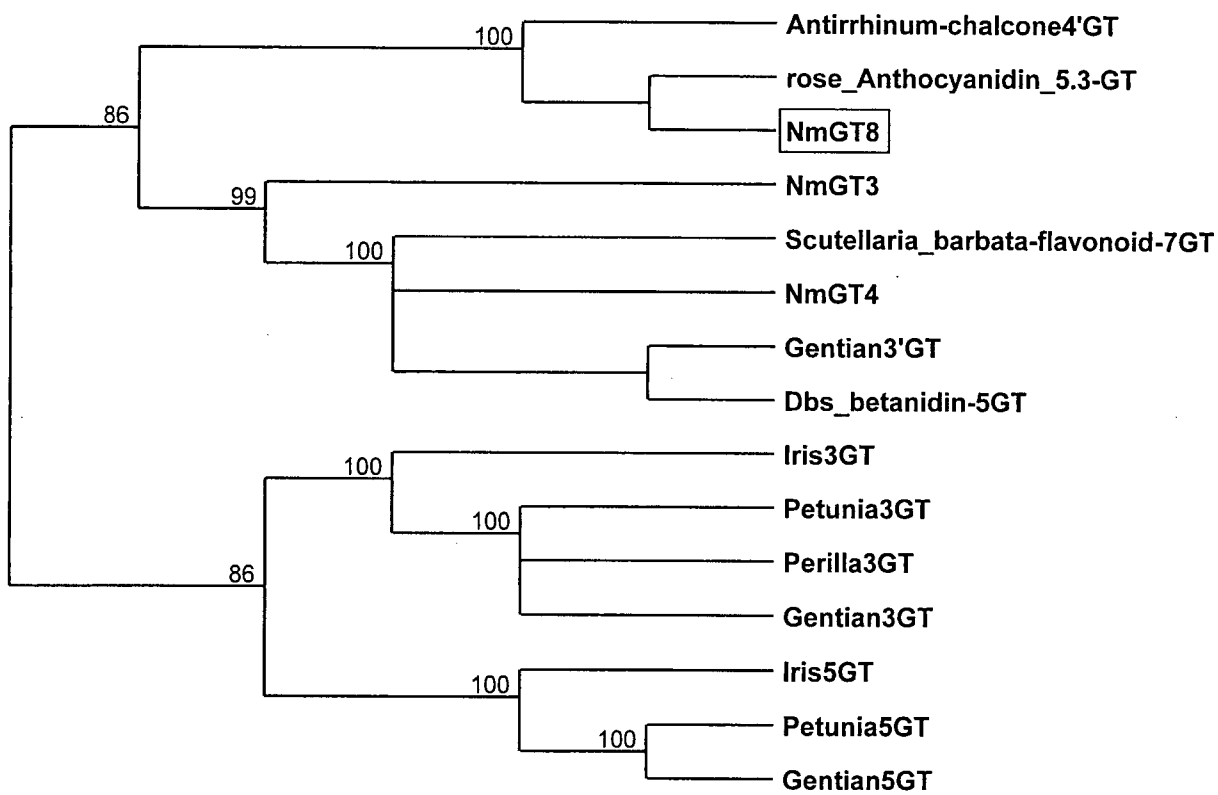

100
86
99
100
100
100
86
100
100
100
Antirrhinum-chalcone4'GT
rose_Anthocyanidin_5.3-GT
NmGT8
NmGT3
Scutellaria_barbata-flavonoid-7GT
NmGT4
Gentian3'GT
Dbs_betanidin-5GT
Iris3GT
Petunia3GT
Perilla3GT
Gentian3GT
Iris5GT
Petunia5GT
Gentian5GT

FIG. 16

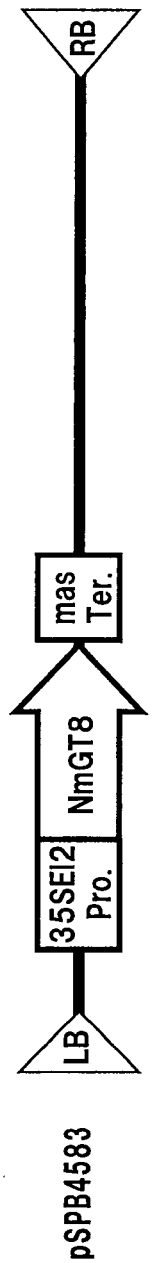


FIG. 17

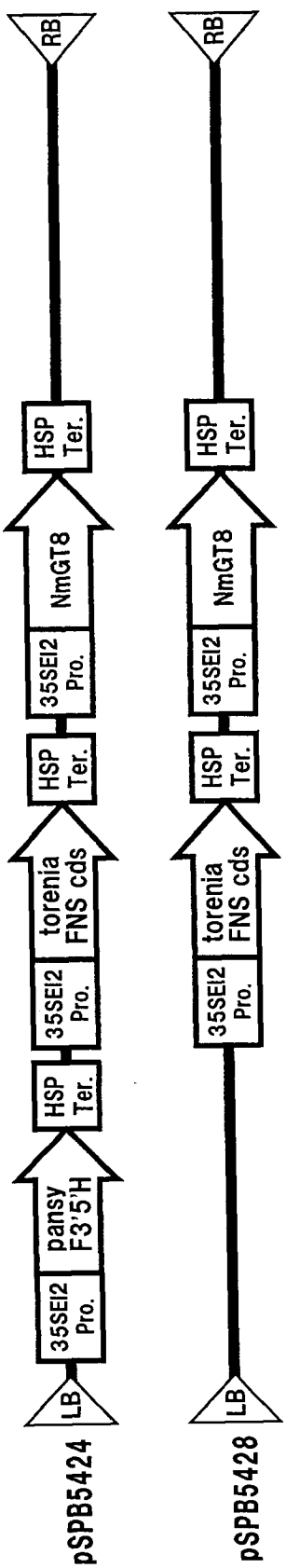

LB
35SEI2 Pro.
pansy F3'5'H
HSP Ter.
35SEI2 Pro.
torenia FNS cds
HSP Ter.
35SEI2 Pro.
NmGT8
HSP Ter.
RB
pSPB5424
LB
35SEI2 Pro.
torenia FNS cds
HSP Ter.
35SEI2 Pro.
NmGT8
HSP Ter.
RB
pSPB5428

FIG. 18

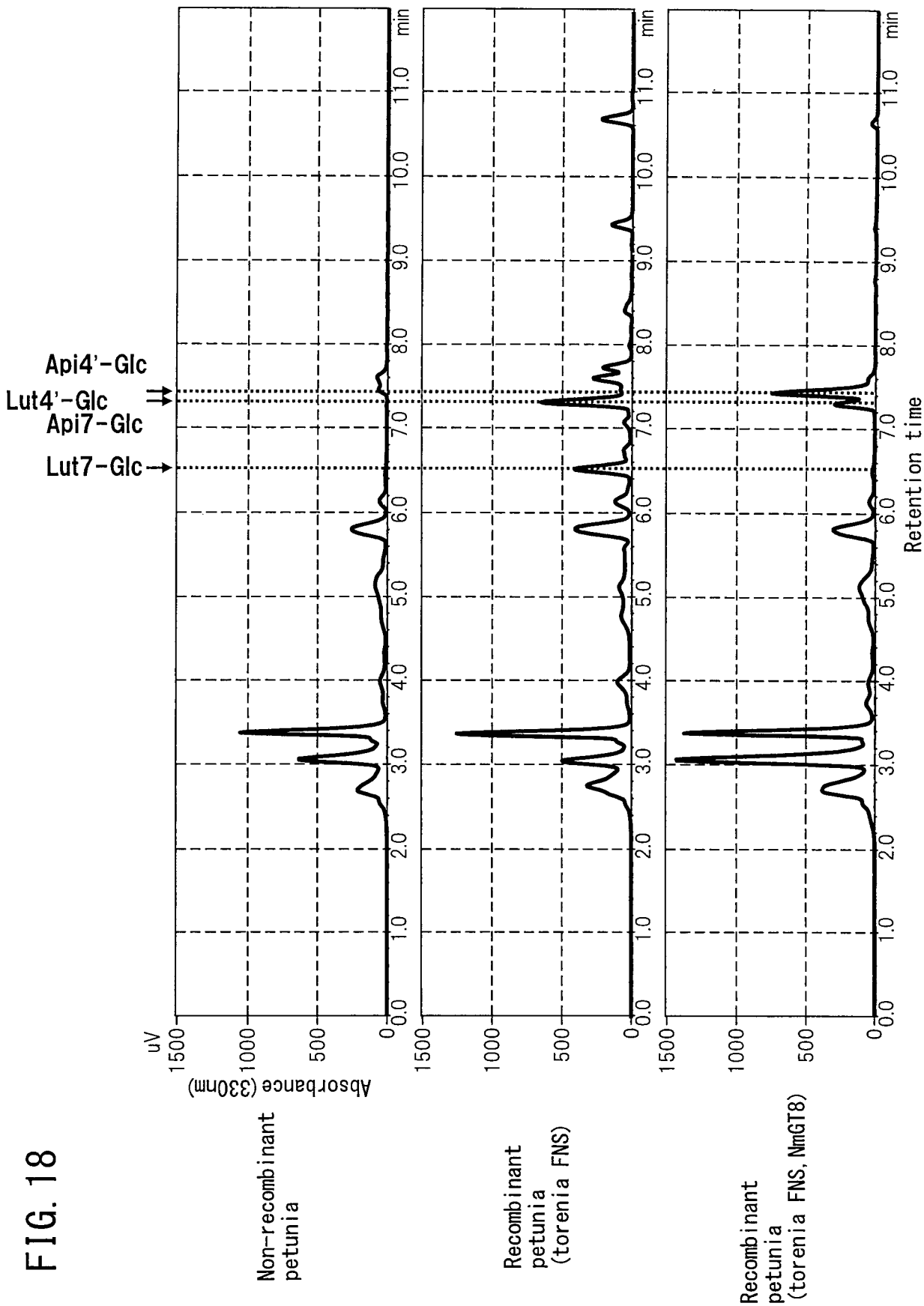

Api4'-Glc
Lut4'-Glc
Api7-Glc
Lut7-Glc
Absorbance (330nm)
uV
1500
1000
500
0
0.0
1.0
2.0
3.0
4.0
5.0
6.0
7.0
8.0
9.0
10.0
11.0
min
Retention time
Non-recombinant petunia
Recombinant petunia (torenia FNS)
Recombinant petunia (torenia FNS, NmGT8)

FIG. 19

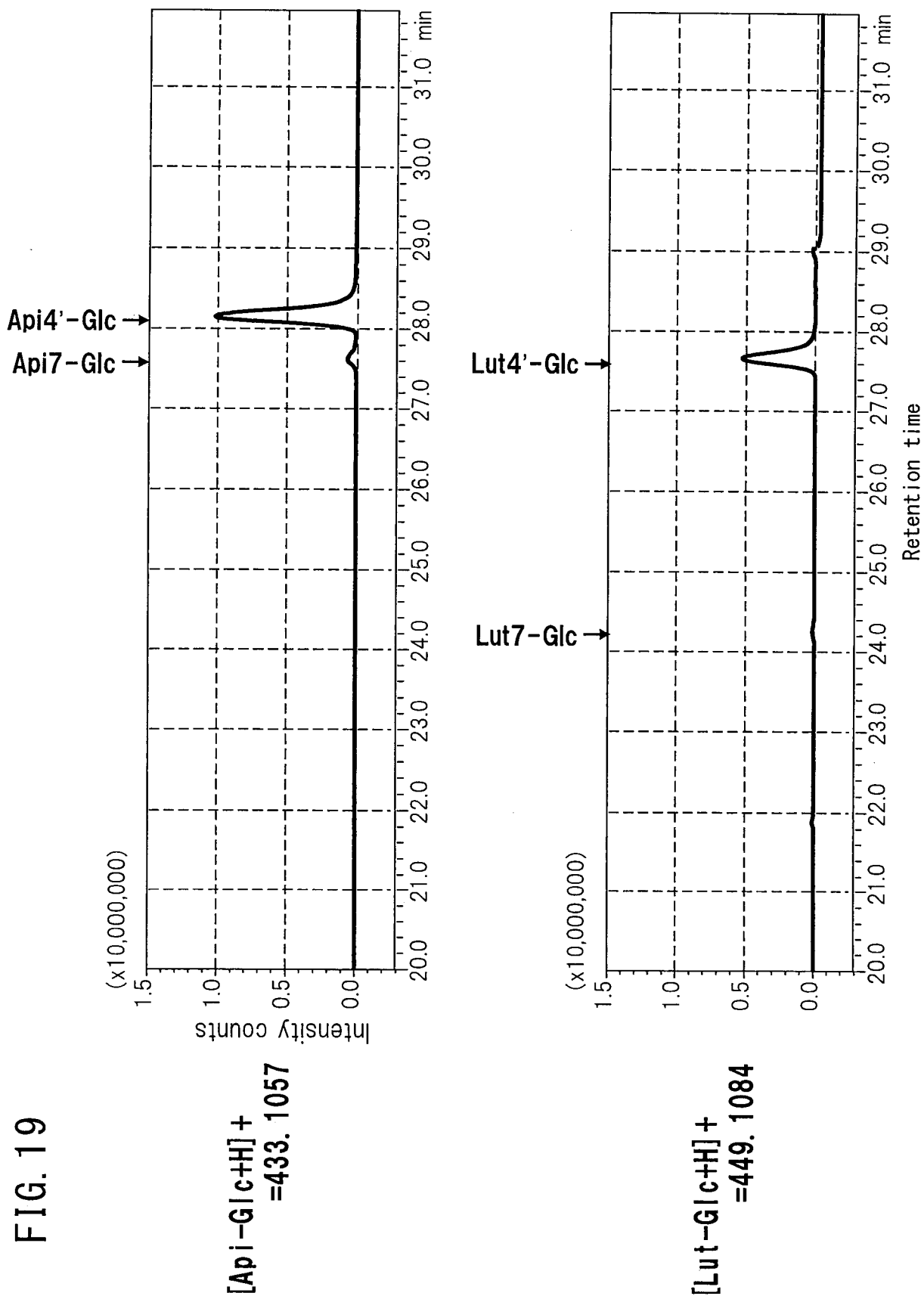

Api4'-Glc
Api7-Glc
Lut4'-Glc
Lut7-Glc
Intensity counts
(x10,000,000)
1.5
1.0
0.5
0.0
20.0
21.0
22.0
23.0
24.0
25.0
26.0
27.0
28.0
29.0
30.0
31.0 min
Retention time
[Api-Glc+H]+
=433.1057
[Lut-Glc+H]+
=449.1084

FIG. 20

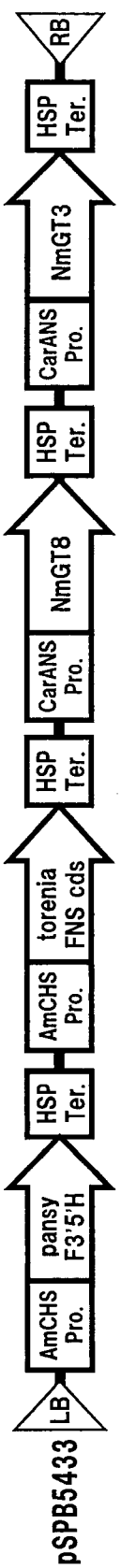

pSPB5433
LB
AmCHS Pro.
pansy F3'5'H
HSP Ter.
AmCHS Pro.
torenia FNS cds
HSP Ter.
CarANS Pro.
NmGT8
HSP Ter.
CarANS Pro.
NmGT3
HSP Ter.
RB

FIG. 21

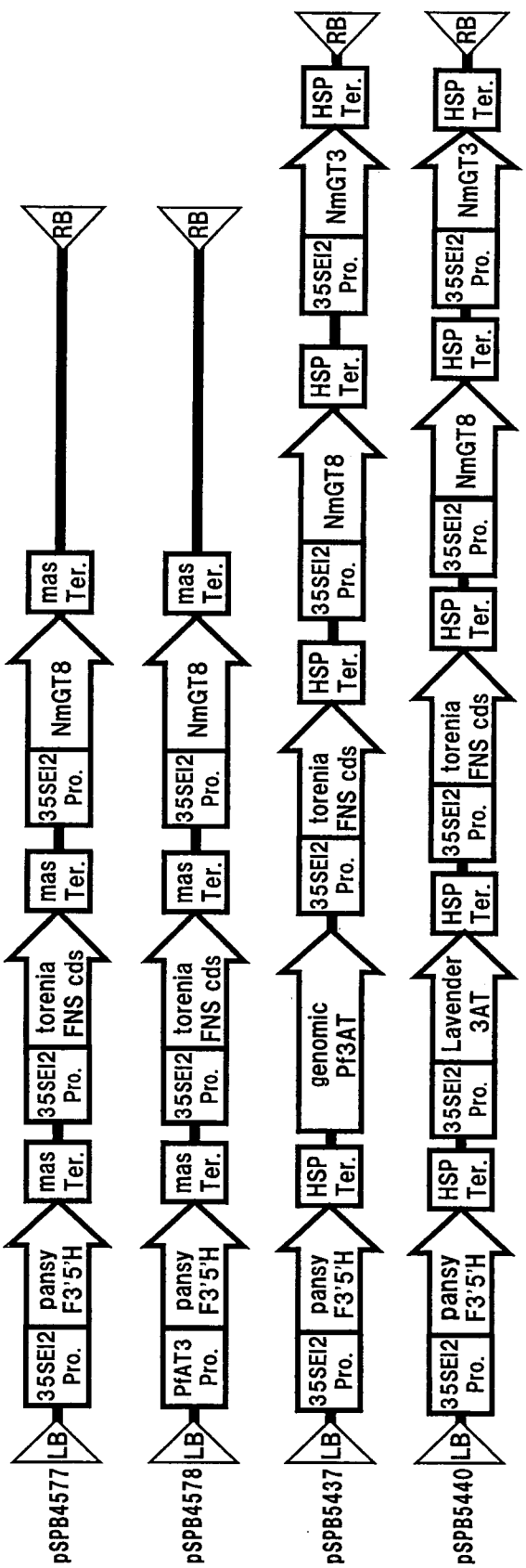

pSPB4577
LB
35SEI2 Pro.
pansy F3'5'H
mas Ter.
35SEI2 Pro.
torenia FNS cds
mas Ter.
35SEI2 Pro.
NmGT8
mas Ter.
RB
pSPB4578
LB
PfAT3 Pro.
pansy F3'5'H
mas Ter.
35SEI2 Pro.
torenia FNS cds
mas Ter.
35SEI2 Pro.
NmGT8
mas Ter.
RB
pSPB5437
LB
35SEI2 Pro.
pansy F3'5'H
HSP Ter.
genomic Pf3AT
35SEI2 Pro.
torenia FNS cds
HSP Ter.
35SEI2 Pro.
NmGT8
HSP Ter.
35SEI2 Pro.
NmGT3
HSP Ter.
RB
pSPB5440
LB
35SEI2 Pro.
pansy F3'5'H
HSP Ter.
35SEI2 Pro.
Lavender 3AT
HSP Ter.
35SEI2 Pro.
torenia FNS cds
HSP Ter.
35SEI2 Pro.
NmGT8
HSP Ter.
35SEI2 Pro.
NmGT3
HSP Ter.
RB

FIG. 22

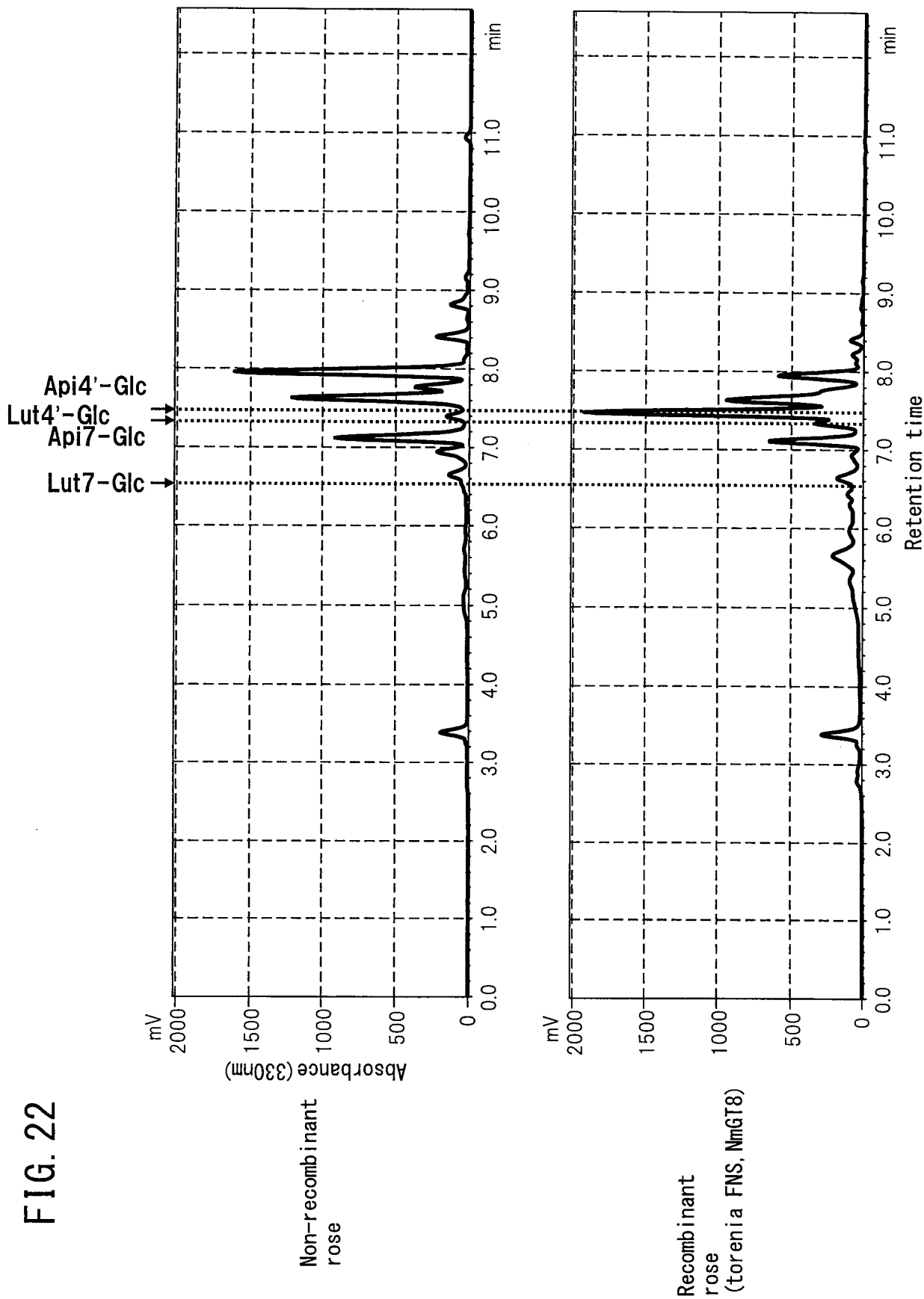
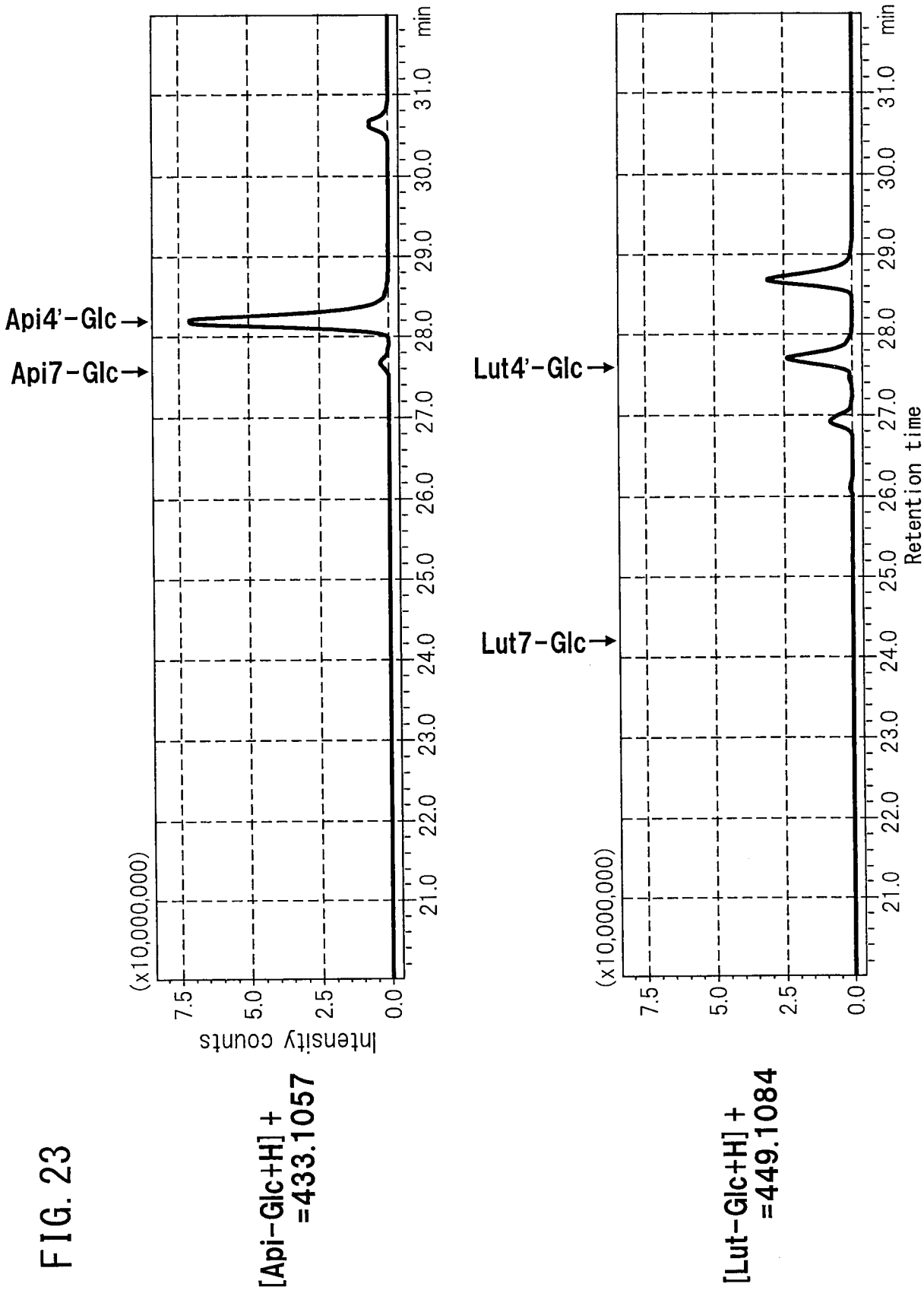

Api4'-Glc
Lut4'-Glc
Api7-Glc
Lut7-Glc
Absorbance (330nm)
mV
2000
1500
1000
500
0
0.0
1.0
2.0
3.0
4.0
5.0
6.0
7.0
8.0
9.0
10.0
11.0
min
Retention time
Non-recombinant rose
Recombinant rose (torenia FNS, NmGT8)

FIG. 23


Api4'-Glc
Api7-Glc
Lut4'-Glc
Lut7-Glc
Intensity counts
(x10,000,000)
7.5
5.0
2.5
0.0
21.0
22.0
23.0
24.0
25.0
26.0
27.0
28.0
29.0
30.0
31.0
min
Retention time
[Api-Glc+H] +
=433.1057
[Lut-Glc+H] +
=449.1084