



(86) Date de dépôt PCT/PCT Filing Date: 2012/01/11
(87) Date publication PCT/PCT Publication Date: 2012/07/19
(85) Entrée phase nationale/National Entry: 2013/07/12
(86) N° demande PCT/PCT Application No.: JP 2012/050379
(87) N° publication PCT/PCT Publication No.: 2012/096307
(30) Priorité/Priority: 2011/01/14 (JP2011-006317)

(51) Cl.Int./Int.Cl. *C12N 15/09* (2006.01),
A01H 5/00 (2006.01), *A23L 1/272* (2006.01),
A23L 1/30 (2006.01), *A61K 31/7048* (2006.01),
A61K 8/60 (2006.01), *C12N 1/15* (2006.01),
C12N 1/19 (2006.01), *C12N 1/21* (2006.01),
C12N 5/10 (2006.01), *C12N 9/10* (2006.01),
C12P 19/02 (2006.01)

(71) Demandeur/Applicant:
SUNTORY HOLDINGS LIMITED, JP

(72) Inventeurs/Inventors:
TANAKA, YOSHIKAZU, JP;
OKITSU, NAOKO, JP;
MATSUI, KEISUKE, JP

(74) Agent: NORTON ROSE FULBRIGHT CANADA

(54) Titre : NOUVEAU GENE DE GLYCOSYLTRANSFERASE ET UTILISATION DE CELUI-CI

(54) Title: NOVEL GLYCOSYLTRANSFERASE GENE AND USE THEREOF

(57) **Abrégé/Abstract:**

Provided is a polynucleotide encoding a protein having an activity to transfer a sugar to the hydroxy groups at the 4'- and 7-positions of a flavone. The polynucleotide is selected from the group consisting of: (a) a polynucleotide which comprises a base sequence represented by SEQ ID NO: 1, 3, or 12; (b) a polynucleotide which hybridizes to a polynucleotide comprising a base sequence complementary to a base sequence represented by SEQ ID NO: 1, 3, or 12 under high stringency conditions, and encodes a protein having an activity to transfer a sugar to the hydroxy groups at the 4'- and 7-positions of a flavone; (c) a polynucleotide which encodes a protein comprising an amino acid sequence represented by SEQ ID NO: 2, 4, or 13; (d) a polynucleotide which encodes a protein comprising an amino acid sequence in which one or more amino acids have been deleted, substituted, inserted, and/or added in an amino acid sequence represented by SEQ ID NO: 2, 4, or 13 and having an activity to transfer a sugar to the hydroxy groups at the 4'- and 7-positions of a flavone; etc.



(74) **Agent(suite/continued):** LLP/S.E.N.C.R.L., S.R.L.

ABSTRACT

Provided is a polynucleotide encoding a protein having an activity to transfer a sugar to the hydroxy groups at the 4'- and 7-positions of a flavone. The polynucleotide is selected from the group consisting of: (a) a polynucleotide which comprises a base sequence represented by SEQ ID NO: 1, 3, or 12; (b) a polynucleotide which hybridizes to a polynucleotide comprising a base sequence complementary to a base sequence represented by SEQ ID NO: 1, 3, or 12 under high stringency conditions, and encodes a protein having an activity to transfer a sugar to the hydroxy groups at the 4'- and 7-positions of a flavone; (c) a polynucleotide which encodes a protein comprising an amino acid sequence represented by SEQ ID NO: 2, 4, or 13; (d) a polynucleotide which encodes a protein comprising an amino acid sequence in which one or more amino acids have been deleted, substituted, inserted, and/or added in an amino acid sequence represented by SEQ ID NO: 2, 4, or 13 and having an activity to transfer a sugar to the hydroxy groups at the 4'- and 7-positions of a flavone; etc.

DESCRIPTION

NOVEL GLYCOSYLTRANSFERASE GENE AND USE THEREOF

5 [TECHNICAL FIELD]

[0001]

The present invention relates to a polynucleotide which encodes a protein having an activity of transferring a glycosyl to both of the hydroxyl groups at the 4'- and 7-positions of a flavone, as well as uses thereof.

[BACKGROUND ART]

[0002]

15 In the flower industry, flowers having a new trait are always highly valued. In particular, the development of plants having a different "color", the most important trait of flowers, is industrially very important, and so far flowers of a variety of colors have been developed by cultivar improvement using classical breeding methods. With these methods are effective in cultivar improvement, there are restrictions on the gene pool inherent to each plant, and thus the methods can be applied to gene resources owned by closely related species that are amenable to breeding. For example, despite long years of breeding efforts, no purple to blue varieties for roses, carnations, chrysanthemums or lilies, no bright red varieties for gentians or irises, and no yellow varieties for geranium or morning glories have been created.

30 [0003]

Flower color results from four types of pigments, i.e., flavonoids, carotenoids, chlorophylls, and betalains. Among them, flavonoids contribute to a variety of colors such as yellow, red, purple and blue. The group of pigments that develop red, purple and blue colors is collectively termed anthocyanins, and the diversity of anthocyanin structures is one reason for the

- 2 -

wide variety of flower colors. Considering the biosynthetic pathway, anthocyanins are roughly divided into three groups depending on the aglycon structure. Bright red-colored flowers such as carnation and geranium often contain pelargonidin-type anthocyanins, and blue- and purple-colored flowers often contain delphinidin-type anthocyanins. The absence of blue or purple varieties of roses, carnations, chrysanthemums and lilies is because they have no ability of synthesizing the delphinidin-type anthocyanins.

[0004]

In order for flowers to have a blue color, in addition to the accumulation of delphinidins, it has been thought that either one of the following is required: (i) the modification of anthocyanins with one or a plurality of aromatic acyl group(s), (ii) coexistence of anthocyanins with copigments such as flavones and flavonols, (iii) coexistence of anthocyanins with iron ions or aluminum ions, (iv) the increase in pH of anthocyanin-localized vacuoles from neutral to weak alkali, and (v) complex formation by anthocyanins, copigments and metal ions (such anthocyanins are termed metalloanthocyanins) (Non-patent document 1 below).

[0005]

Biosynthesis of flavonoids and anthocyanins has been well studied, and relevant biosynthetic enzymes and genes encoding them have been identified (see Non-patent document 2, Fig. 1 below). For example, genes of flavonoid 3',5'-hydroxylase (F3'5'H), which hydroxylates the B ring of flavonoids required in delphinidin biosynthesis, have been obtained from many plants. Also, by introducing these F3'5'H genes into carnations (see Patent document 1 below), roses (see Non-patent document 3 and Patent documents 2 and 3 below), and chrysanthemums (see Patent document 4 below), gene recombinant plants in which delphinidins are accumulated in petals and flower color is changed to blue have been generated (see Non-

- 3 -

patent document 4 below). Such carnations and roses are commercially available.

[0006]

5 Flavones, a family of organic compounds, are cyclic ketones of flavane derivatives. In a narrower sense, it indicates 2,3-didehydroflavan-4-one, a compound having a chemical formula $C_{15}H_{10}O_2$ and a molecular weight of 222.24. In a broader sense, derivatives belonging to flavanes are termed "flavone". Flavones as defined in the broader
10 sense (flavones) constitute one category of flavonoids. Those flavonoids that have the flavone structure as the basic skeleton and have no hydroxyl groups at the 3-position are classified into "flavones". Representative examples of "flavones" include apigenin (4',5,7-
15 trihydroxyflavone) and luteolin (3',4',5,7-tetrahydroxyflavone). As used herein the term "flavone" refers to a flavone as defined in the broader sense, i.e., a derivative belonging to flavone.

[0007]

20 Genes of flavone synthase (FNS) required for flavone biosynthesis have also been obtained from many plants. Flavone, when coexistent with anthocyanin, is known to have an effect of making the color of anthocyanin bluer, and these FNS genes attracted attention in the
25 modification of flower colors. By introducing the FNS gene together with F3'5'H into roses having no ability of synthesizing flavones, the flower petals accumulated delphinidin simultaneously with the accumulation of flavone, making flower color bluer (see Patent document 5
30 below). Since flavone absorbs an ultraviolet ray in addition to making flower color bluer, it protects plants against the ultraviolet ray or serves as a signal to vision of insects in insect-pollinated flowers. Flavone is also involved in interaction with soil microorganisms.
35 Furthermore, flavone is used in materials for foods or cosmetics as ingredients good for health. For example, flavone is said to have an anti-cancer effect, and it has

also been demonstrated that by taking flavone-rich food materials, cancer can be treated or prevented.

[0008]

Genes that modify anthocyanin and flavone have also
5 been obtained from many plants. There are
glycosyltransferase, acyl transferase, methyl transferase
etc., and, among them, glycosyltransferase (GT) that
catalyzes glycosylation is described herein. For
example, genes encoding a protein having an activity of
10 transferring glucose to the hydroxyl group at the 3-
position of anthocyanin have been isolated from gentian,
perilla, petunia, rose, antirrhinum and the like (see
Non-patent documents 4 to 6 and Patent Document 6).
Genes encoding a protein having an activity of
15 transferring glucose to a hydroxyl group at the 5-
position of anthocyanin have been isolated from perilla,
petunia, rose, gentian, verbena, torenia and the like
(see Non-patent documents 5 to 7, and Patent document 7
below). A gene encoding a protein having an activity of
20 transferring glucose to the hydroxyl group at the 7-
position of flavone has been isolated from arabidopsis
(see Non-patent document 8 below). A gene encoding a
protein having an activity of transferring glucose to the
hydroxyl group at the 7-position of baicalin has been
25 isolated from *Scutellaria baicalensis*, and it is also
reported that a protein obtained by expressing the gene
in *Escherichia coli* catalyzes a reaction that exhibits an
activity of transferring glucose to the hydroxyl group at
the 7-position of flavonoid (see Non-patent document 9
30 below). A gene encoding a protein having an activity of
transferring glucose to the hydroxyl group at the 3'-
position of anthocyanin has been isolated from gentian,
butterfly pea, and cineraria (see Patent document 8
below). Also, a gene encoding a protein having an
35 activity of transferring glucose to hydroxyl groups at
two different positions on the A and C rings of
anthocyanin has been isolated from rose (see Patent

- 5 -

document 9 below). A gene encoding a protein having an activity of transferring glucose to hydroxyl groups at two different positions of the B ring of anthocyanin has been isolated from butterfly pea (see Patent document 10 below).

[0009]

While the glycosyltransferases mentioned above rely on UDP-glucose as a glycosyl donor, a glycosyltransferase whose glycosyl donor is acyl glucose has been identified recently. A gene encoding a protein having an activity of transferring glucose to the hydroxyl group at the 5-position of anthocyanin-3 glucoside has been isolated from carnation, and a gene encoding a protein having an activity of transferring glucose to a hydroxyl group at the 7-position has been isolated from delphinium (see, Non-patent document 10 below).

[0010]

Thus, a multitude of proteins having an activity of transferring glucose to various hydroxyl groups are known as glycosyltransferases.

However, it is believed that there are still many glycosyltransferases of which functions have not been identified. For example, a gene encoding a protein having an activity of transferring a glycosyl to the 4'-position of a flavonoid, or a gene encoding a protein having an activity of transferring glycosyl sequentially to hydroxyl groups at two sites on the A and B rings of a flavonoid has not been identified yet. It is reported that a protein obtained by expressing a glycosyltransferase gene derived from Livingstone daisy in *Escherichia coli* exhibits an activity of transferring glucose to either one of the hydroxyl groups at the 4'-position and the 7-position of a flavonoid, but the original activity of the glycosyltransferase in plants is to transfer glucose to the hydroxyl group at the 5-position of betanidine (see Non-patent document 11 below).

[0011]

A metalloanthocyanins, which is represented by pigments of Commelina, Centaurea, Salvia, and Nemophila, is composed of six molecules of anthocyanin, six molecules of flavone, and two metal atoms, which components aggregate to form a stable blue pigment (see Fig. 2, Non-patent document 1). For example, anthocyanin of Nemophila is composed of nemophilin (see Fig. 3), malonyl apigenin 4',7-diglucoside (see Fig. 4), Mg^{2+} and Fe^{3+} . Metalloanthocyanin of Salvia is composed of cyanosalvianin (see Fig. 5), and apigenin 4',7-diglucoside (see Fig. 6) and Mg^{2+} . Studies so far have demonstrated that in all blue flowers forming metalloanthocyanins, flavone in which a glycosyl has been added to both of the hydroxyl groups at the 4'-position and the 7-position, and the glycosyl added to the flavone has been shown to play an important role in molecular recognition in metalloanthocyanin formation. The glycosyl coordinated at the 4'-position of a flavone is important in molecular recognition during the formation, and the glycosyl at the 7-position has been indicated to contribute to its stability (see Non-patent document 1 below). Only after the addition of these two glycosyls, metalloanthocyanin is formed thereby expressing a beautiful blue color. In Dutch iris petals, flavone in which a glycosyl has been added to the 4'-position is contained. Since the addition of two glycosyls to a flavone leads to increased solubility and altered properties, the expansion of uses as materials for health food products, pharmaceutical products and cosmetic products can be expected.

[CITATION LIST]

[PATENT DOCUMENTS]

[0012]

Patent document 1: WO2006/105598A

Patent document 2: WO2010/122849A

- 7 -

Patent document 3: WO2005/017147A
Patent document 4: WO2009/062253A
Patent document 5: WO2008/156211A
Patent document 6: WO2007/094521A
5 Patent document 7: WO99/05287A
Patent document 8: WO01/092509A
Patent document 9: JP2006-149293A
Patent document 10: JP2005-095005A

10 [NON-PATENT DOCUMENTS]

[0013]

Non-patent document 1: Natural Product Reports
(2009), 26, 884-915

15 Non-patent document 2: Bioscience, Biotechnology,
and Biochemistry (2010), 74(9), 1760-1769

Non-patent document 3: Plant Cell Physiology (2007),
48(11), 1589-1600

Non-patent document 4: Plant Cell Physiology (1996),
37(5), 711-716

20 Non-patent document 5: The Journal of Biological
Chemistry (1999), 274(11), 7405-7411

Non-patent document 6: Plant Molecular Biology
(2002), 48, 401-411

25 Non-patent document 7: Journal of Experimental
Botany (2008), 59(6), 1241-1252

Non-patent document 8: Bioscience, Biotechnology,
and Biochemistry (2006), 70(6), 1471-1477

Non-patent document 9: Planta (2000), 210, 1006-1013

30 Non-patent document 10: Plant Cell (2010), 22(10),
3374-89

Non-patent document 11: The Plant Journal (1999),
19(5), 509-519

[SUMMARY OF INVENTION]

35 [PROBLEMS TO BE SOLVED BY THE INVENTION]

[0014]

Changing the properties of flavone is necessary to

change flower colors and to develop materials for food products, pharmaceutical products and cosmetic products. For example, while the color of carnation, rose and chrysanthemum in which delphinidin is accumulated is blue
5 purple, research is going on to make the color further bluer.

Under such circumstances, the problem the present invention intends to solve is to provide a polynucleotide encoding a protein having an activity of transferring a
10 glycosyl to both of the hydroxyl groups at the 4'-position and 7-position of a flavone, and use thereof.

[MEANS TO SOLVE THE PROBLEMS]

[0015]

15 After intensive and extensive research to solve the above problem, the applicants of the present application have confirmed that a polynucleotide encoding a protein having an activity of transferring a glycosyl to both of the hydroxyl groups at the 4'-position and 7-position of
20 a flavone can be isolated and used, and therefore have completed the present invention.

Thus, the present invention is as follows:

[0016]

25 [1] A polynucleotide selected from the group consisting of:

- (a) a polynucleotide comprising a base sequence defined in SEQ ID NO: 1, 3 or 12;
- (b) a polynucleotide which hybridizes with a polynucleotide comprising a base sequence complementary
30 to a base sequence defined in SEQ ID NO: 1, 3 or 12 under a stringent condition and encodes a protein having an activity of transferring a glycosyl to both of the hydroxyl groups at the 4'- and 7-positions of a flavone;
- (c) a polynucleotide which encodes a protein comprising
35 an amino acid sequence defined in SEQ ID NO: 2, 4 or 13;
- (d) a polynucleotide which encodes a protein comprising an amino acid sequence in which one or several amino

acids have been deleted, substituted, inserted, and/or added in an amino acid sequence defined in SEQ ID NO: 2, 4 or 13 and having an activity of transferring a glycosyl to both of the hydroxyl groups at the 4'- and 7-positions of a flavone; and

(e) a polynucleotide which encodes a protein having an amino acid sequence which has an identity of 90% or more to an amino acid sequence defined in SEQ ID NO: 2, 4 or 13 and having an activity of transferring a glycosyl to both of the hydroxyl groups at the 4'- and 7-positions of a flavone.

[0017]

[2] The polynucleotide according to the above [1], which is (a) a polynucleotide comprising a base sequence defined in SEQ ID NO: 1, 3 or 12.

[0018]

[3] The polynucleotide according to the above [1], which is (c) a polynucleotide which encodes a protein comprising an amino acid sequence defined in SEQ ID NO: 2, 4 or 13.

[0019]

[4] The polynucleotide according to the above [1], which is (f) a polynucleotide which encodes a protein having an amino acid sequence which has an identity of 95% or more to an amino acid sequence defined in SEQ ID NO: 2, 4 or 13 and having an activity of transferring a glycosyl to both of the hydroxyl groups at the 4'- and 7-positions of a flavone.

[0020]

[5] The polynucleotide according to the above [4], which is (g) a polynucleotide which encodes a protein having an amino acid sequence which has an identity of 97% or more to an amino acid sequence defined in SEQ ID NO: 2, 4 or 13 and having an activity of transferring a glycosyl to both of the hydroxyl groups at the 4'- and 7-positions of a flavone.

[0021]

- 10 -

[6] The polynucleotide according to the above [5], which is (h) a polynucleotide which encodes a protein having an amino acid sequence which has an identity of 98% or more to an amino acid sequence defined in SEQ ID NO: 2, 4 or 13 and having an activity of transferring a glycosyl to both of the hydroxyl groups at the 4'- and 7-positions of a flavone.

[0022]

[7] The polynucleotide according to any one of the above [1] to [6], which is DNA.

[0023]

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

[0024]

[9]

A vector comprising the polynucleotide according to any one of the above [1] to [7].

[0025]

[10] A non-human host according to the above [9] into which the vector according to the above [9] has been introduced.

[0026]

[11] A method of adding a glycosyl to both of the hydroxyl groups at the 4'- and 7-positions of a flavone using the polynucleotide according to any one of the above [1] to [7].

[0027]

[12] A plant or a progeny thereof, or a part or tissue thereof, into which the polynucleotide according to any one of the above [1] to [7] has been introduced and which contains the polynucleotide.

[0028]

[13] The part of a plant according to the above [12], which is a cut flower.

[0029]

[14] A cut flower artifact using the cut flower according to the above [13].

[0030]

[15] A process of producing a protein having an activity of transferring a glycosyl to both of the hydroxyl groups at the 4'- and 7-positions of a flavone, comprising the steps of:

culturing or growing the non-human host according to the above [10]; and

collecting a protein having an activity of transferring a glycosyl to both of the hydroxyl groups at the 4'- and 7-positions of a flavone from the non-human host.

[0031]

[16] A process of producing a flavone in which a glycosyl has been added to both of the hydroxyl groups at 4'- and 7-positions thereof, comprising the steps of:

culturing or growing the non-human host according to the above [10]; and

collecting a flavone in which a glycosyl has been added to both of the hydroxyl groups at 4'- and 7-positions thereof from the non-human host.

[0032]

[17] A food product comprising a flavone which has been produced by the process according to the above [16] and in which a glycosyl has been added to both of the hydroxyl groups at 4'- and 7-positions thereof.

[0033]

[18] A pharmaceutical product comprising a flavone which has been produced by the process according to the above [16] and in which a glycosyl has been added to both of the hydroxyl groups at 4'- and 7-positions thereof.

[0034]

[19] A cosmetic product comprising a flavone which has been produced by the process according to the above [16] and in which a glycosyl has been added to both of the hydroxyl groups at 4'- and 7-positions thereof.

[EFFECTS OF THE INVENTION]

[0035]

By expressing the polynucleotide of the present invention in a suitable host cell, a protein having an activity of specifically transferring a glycosyl to both of the hydroxyl groups at the 4'- and 7-positions of a flavone can be produced. In accordance with the present invention, a protein having an activity of specifically transferring a glycosyl to both of the hydroxyl groups at the 4'- and 7-positions of a flavone can be used in altering flower color by expressing it in a constitutive and tissue-specific manner in plants. Also, in accordance with the present invention, there are provided a method for producing a flavone in which a glycosyl has been added to both of the hydroxyl groups at 4'- and 7-positions thereof, and foods, pharmaceuticals, and cosmetics obtained by the production method.

[BRIEF DESCRIPTION OF DRAWINGS]**[0036]**

[Fig. 1]

A drawing that explains the biosynthetic pathway of anthocyanin.

[Fig. 2]

A schematic diagram of the structure of metalloanthocyanin.

[Fig. 3]

A structural formula of an anthocyanin (nemophilin) derived from *Nemophila*.

[Fig. 4]

A structural formula of a flavone (malonyl apigenin 4',7-diglucoside) derived from *Nemophila*.

[Fig. 5]

A structural formula of an anthocyanin (cyanosalvianin) derived from *Salvia*.

[Fig. 6]

A structural formula of a flavone (apigenin 4',7-diglucoside) derived from *Salvia*.

[Fig. 7]

A high performance liquid chromatogram of a reaction mixture in which a petal extract and apigenin were enzymatically reacted.

5 [Fig. 8]

A drawing that explains the biosynthetic pathway of apigenin 4',7-diglucoside.

[Fig. 9]

10 A high performance liquid chromatogram of a reaction mixture in which an NmGT3 protein solution and apigenin were enzymatically reacted.

[Fig. 10]

15 A high performance liquid chromatogram of a reaction mixture in which an NmGT4 protein solution and apigenin were enzymatically reacted.

[Fig. 11]

A high performance liquid chromatogram of a reaction mixture in which an NmGT3 protein solution and apigenin 7-glucoside were enzymatically reacted.

20 [Fig. 12]

A high performance liquid chromatogram of a reaction mixture in which an NmGT4 protein solution and apigenin 7-glucoside were enzymatically reacted.

[Fig. 13]

25 A drawing that summarizes the reactivity of an enzyme that adds a glycosyl to the 5-gene therapy of the NmGT3 protein, the NmGT4 protein, the SuGT5 protein, and baicalin for various flavonoids.

[Fig. 14]

30 An alignment diagram that compares the amino acid sequences of NmGT3 and NmGT4 (an identity of 31%, an identity on the nucleic acid level of 51%).

[Fig. 15]

35 An alignment diagram that compares the amino acid sequences of NmGT3 and an enzyme that adds a glycosyl to the 2'-position of chalcononaringenin (an identity of 32%, an identity on the nucleic acid level of 47%).

[Fig. 16]

An alignment diagram that compares the amino acid sequences of NmGT4 and an enzyme that adds a glycosyl to the 7-position of flavonoid (an identity of 52%, an identity on the nucleic acid level of 60%).

[Fig. 17]

A construct (pSPB4584 through 4587) comprising NmGT3 and NmGT4 introduced into a torenia.

[Fig. 18]

A construct (pSPB5414 and 5427) comprising NmGT3 and NmGT4 introduced into a petunia.

[Fig. 19]

A construct (pSPB5433) comprising NmGT3 introduced into a carnation.

[Fig. 20]

A construct (pSPB4581, 4582, 5437, and 5440) comprising NmGT3 introduced into a rose.

[Fig. 21]

A high performance liquid chromatogram of a reaction mixture in which a SuGT5 protein solution and apigenin were enzymatically reacted.

[Fig. 22]

A high performance liquid chromatogram of a reaction mixture in which a SuGT5 protein solution and apigenin 7-glucoside were enzymatically reacted.

[Fig. 23]

An alignment diagram that compares the amino acid sequences of SuGT5 and NmGT3 (an identity of 38%, an identity on the nucleic acid level of 47%).

[Fig. 24]

An alignment diagram that compares the amino acid sequences of SuGT5 and NmGT4 (an identity of 51%, an identity on the nucleic acid level of 58%).

[DESCRIPTION OF EMBODIMENTS]

[0037]

The present invention relates to a polynucleotide

- 15 -

selected from the group consisting of:

(a) a polynucleotide comprising a base sequence defined in SEQ ID NO: 1, 3 or 12;

5 (b) a polynucleotide which hybridizes with a polynucleotide comprising a base sequence complementary to a base sequence defined in SEQ ID NO: 1, 3 or 12 under a stringent condition and encodes a protein having an activity of transferring a glycosyl to both of the hydroxyl groups at the 4'- and 7-positions of a flavone;

10 (c) a polynucleotide which encodes a protein comprising an amino acid sequence defined in SEQ ID NO: 2, 4 or 13;

(d) a polynucleotide which encodes a protein comprising an amino acid sequence in which one or several amino acids have been deleted, substituted, inserted, and/or
15 added in an amino acid sequence defined in SEQ ID NO: 2, 4 or 13 and having an activity of transferring a glycosyl to both of the hydroxyl groups at the 4'- and 7-positions of a flavone; and

(e) a polynucleotide which encodes a protein having an
20 amino acid sequence which has an identity of 90% or more to an amino acid sequence defined in SEQ ID NO: 2, 4 or 13 and having an activity of transferring a glycosyl to both of the hydroxyl groups at the 4'- and 7-positions of a flavone.

25 **[0038]**

As used herein the term "polynucleotide" means DNA or RNA.

As used herein the term "stringent condition" means a condition that permits a selective and detectable
30 specific bonding between a polynucleotide or an oligonucleotide and genomic DNA. The stringent condition may be defined by the suitable combination of salt concentration, organic solvent (e.g., formamide), temperature, and other known conditions. Thus, by
35 reducing the salt concentration, increasing the organic solvent concentration, or increasing the hybridization temperature, stringency can be increased. Furthermore,

- 16 -

the washing condition after hybridization can also affect stringency. The washing condition also can be defined by salt concentration and temperature, and by reducing the salt concentration and increasing the temperature, the stringency of washing can be increased. Thus, the term "stringent condition" means a condition under which specific hybridization only occurs between highly homologous base sequences as in cases where the degree of "identity" or "homology" between the base sequences is on the average about 80% or more, preferably about 90% or more, more preferably about 95% or more, still more preferably 97% or more, and most preferably 98% or more. As a "stringent condition", for example, there can be mentioned conditions in which, at a temperature of 60°C to 68°C, the sodium concentration is 150 to 900 mM, preferably 600 to 900 mM, and pH 6 to 8. As a specific example, there can be mentioned a case wherein hybridization is performed at a condition of 5xSSC (750 mM NaCl, 75 mM trisodium citrate), 1% SDS, 5x Denhardt's solution, 50% formaldehyde, and 42°C, followed by washing at a condition of 0.1xSSC (15 mM NaCl, 1.5 mM trisodium citrate), 0.1% SDS and 55°C.

[0039]

Hybridization may be performed according to, for example, a method described in Current Protocols in Molecular Biology (edited by Frederick M. Ausubel et al., 1987)), a method known in the art, or an equivalent method. When a commercially available library is used, hybridization may be performed according to a method described in the attached instruction for use. A gene selected for such hybridization may be of natural origin, such as derived from plants, or from other than plants. Also a gene selected by hybridization may be cDNA, genomic DNA, or a chemically synthesized DNA.

[0040]

The statement "amino acid sequence in which one or

- 17 -

several amino acids have been deleted, substituted, inserted, and/or added" above means an amino acid sequence in which 1 to 20, preferably 1 to 5, and more preferably 1 to 3 amino acids have been deleted, substituted, inserted, and/or added. Since site-directed mutagenesis, which is one of the gene engineering methods, can introduce specific mutation into a specific site, it is useful, and may be performed according to a method described in Molecular Cloning: A Laboratory Manual, 2nd Ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., 1989, etc. By allowing this mutated DNA to be expressed using a suitable expression system, a protein consisting of an amino acid sequence in which one or several amino acids have been deleted, substituted, inserted, and/or added can be obtained.

DNA according to the present invention can be obtained by any method known to a person skilled in the art, such as chemical synthesis using, e.g., a phosphoramidite method, or a nucleic acid amplification using a primer designed based on the nucleotide sequence of the gene of interest and also using a nucleic acid sample of a plant as a template.

[0041]

As used herein the terms "identity" and "homology" mean that, in two chains of polypeptide sequences (or amino acid sequences) or of polynucleotide sequences (or base sequences), the quantities (numbers) of respective amino acid residues or respective bases constituting the chains are such that they may be determined to be identical in the compatibility of the two chains, and mean the degree of sequence correlation between two polypeptide sequences or two polynucleotide sequences. The "identity" and "homology" can be easily calculated. Many methods for determining homology between two polynucleotide sequences or polypeptide sequences are known, and the terms "identity" and "homology" are well known to a person skilled 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); Griffin, A.M. & Griffin,
5 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); Gribskov, M. & Devereux, J. (Ed.), Sequence
Analysis Primer, M-Stockton Press, New York, (1991),
10 etc.).

[0042]

While, unless otherwise specified, the numerical
values of "identity" and "homology" described herein may
be values calculated using a homology search program
15 known to a person skilled in the art, they may preferably
be numerical values calculated using the Clustal W
program of MacVector application (version 9.5, Oxford
Molecular Ltd., Oxford, England).

[0043]

20 The polynucleotide (nucleic acid, gene) of the
present invention may "encode" a protein of interest. As
used herein "encode" means that the protein of interest
may be expressed in a form in which it retains the
activity. Also "encode" has the meaning of both of
25 encoding the protein of interest as a contiguous
structural sequence (exon) or encoding it via intervening
sequences (introns).

[0044]

A gene having the original base sequence can be
30 obtained using, for example, analysis with a DNA
sequencer as described in the Examples below. DNA
encoding an enzyme having a modified amino acid sequence
can also be synthesized using a common site-directed
mutagenesis or a PCR method based on the DNA having the
35 original base sequence. For example, a DNA fragment
desired to be modified may be obtained by restriction
treatment of the original cDNA or genomic DNA, and with

this fragment as the template, site-directed mutagenesis or a PCR reaction may be performed using primers in which the desired mutation has been introduced to obtain the desired modified DNA fragment. Thereafter, this
5 mutation-introduced DNA fragment may be linked to a DNA fragment encoding the other part of the enzyme of interest.

Alternatively, in order to obtain a DNA encoding an enzyme comprising a shortened amino acid sequence, a DNA
10 encoding an amino acid sequence longer than the amino acid sequence of interest, such as DNA encoding the full-length amino acid sequence, may be cleaved with the desired restriction enzyme, and when the resulting DNA fragment does not encode the entire amino acid sequence
15 of interest, a DNA fragment comprising the sequence of the lacking part may be synthesized and linked.

[0045]

Also, by expressing the polynucleotide obtained using an expression system in *Escherichia coli* and yeast
20 and determining the enzyme activity, the polynucleotide obtained can be confirmed to encode a protein having an activity of transferring a glycosyl to both of the hydroxyl groups at the 4'-position and 7-position of a flavone. Furthermore, by expressing this polynucleotide,
25 a polynucleotide product, a protein having an activity of transferring a glycosyl to both of the hydroxyl groups at the 4'-position and 7-position of a flavone, can be obtained. Alternatively, a protein having an activity of transferring a glycosyl to both of the hydroxyl groups at
30 the 4'-position and 7-position of a flavone can also be obtained using an antibody against a polypeptide comprising an amino acid sequence described in SEQ ID NO: 2, 4 or 13, and, using such an antibody, a polynucleotide encoding a protein having an activity of transferring a
35 glycosyl to both of the hydroxyl groups at the 4'-position and 7-position of a flavone derived from another organism can be cloned.

[0046]

The present invention also relates to a (recombinant) vector, specifically an expression vector, comprising the above-mentioned polynucleotide, and
5 furthermore to a host transformed with the vector.

As the host, a prokaryotic or a eukaryotic organism can be used. As the prokaryotic organism, bacteria, for example, commonly used hosts such as bacteria belonging to genus *Escherichia* such as *Escherichia coli* and
10 bacteria belonging to genus *Bacillus* such as *Bacillus subtilis* can be used. As the eukaryotic organism, lower eukaryotic organisms such as eukaryotic microorganisms including, for example, yeast or fungi can be used.

[0047]

15 Examples of yeast include microorganisms belonging to genus *Saccharomyces* such as *Saccharomyces cereviceae*, and examples of fungi include microorganisms belonging to genus *Aspergillus* such as *Aspergillus oryzae* and *Aspergillus niger*, and microorganisms belonging to genus
20 *Penicillium*. Animal cells or plant cells also can be used as a host. Examples of animal cells that can be used include cell lines of mouse, hamster, monkey, human, etc., as well as insect cells such as silkworm cells and adult silkworm per se.

[0048]

25 Depending on the type of a host into which the expression vector of the present invention is introduced, the vector may contain expression regulatory regions such as a promoter, a terminator, and an origin of
30 replication. As the promoter for bacterial expression vectors, commonly used promoters such as the *trc* promoter, the *tac* promoter and the *lac* promoter may be used. As the promoter for yeasts, glyceraldehyde-3-phosphate dehydrogenase promoter, the *PH05* promoter etc.
35 may be used. As the promoter for fungi, the *amylase* promoter, the *trpC* promoter etc. may be used. As the promoter for animal cell hosts, viral promoters such as

the SV40 early promoter, the SV40 late promoter etc. may be used. As examples of promoters for constitutively expressing a polynucleotide in plant cells, there can be mentioned ,for example, the 35S RNA promoter of
5 cauliflower mosaic virus, the rd29A gene promoter, the rbcS promoter, the mac-1 promoter, etc.. Also, for tissue-specific gene expression, the promoter of the gene to be specifically expressed in the tissue can be used.

Construction of an expression vector may be
10 performed using a restriction enzyme, ligase etc. according to a standard method. The transformation of a host with an expression vector can also be performed according to a standard method.

[0049]

15 A protein of interest can be obtained by culturing, cultivating or growing a host transformed with the above expression vector, and then by recovering and/or purifying the culture or the culture medium according to a standard method, such as filtration, centrifugation,
20 cell disruption, gel filtration chromatography, ion exchange chromatography and the like.

Herein, a gene encoding a protein having an activity of transferring a glycosyl to both of the hydroxyl groups at the 4'-position and 7-position of a flavone derived
25 from Nemophila or Salvia may be mentioned, but the polynucleotide claimed in the present invention is not limited to gene derived from Nemophila or Salvia. The origin of the gene encoding a protein having an activity of transferring a glycosyl to both of the hydroxyl groups
30 at the 4'-position and 7-position of a flavone may be a plant, an animal or a microorganism, and as long as it has an activity of transferring a glycosyl to both of the hydroxyl groups at the 4'-position and 7-position of a flavone, it can be used in changing flower colors of
35 plants, regardless of the origin of the gene.

[0050]

The present invention also relates to a plant or a

progeny thereof, or a part or tissue thereof obtained by introducing an exogenous polynucleotide encoding a protein having an activity of transferring a glycosyl to both of the hydroxyl groups at the 4'-position and 7-position of a flavone into the plant, and by allowing it to be contained in said plant. The form of the above part or tissue may be a cut flower. By using a polynucleotide encoding a protein having an activity of transferring a glycosyl to both of the hydroxyl groups at the 4'-position and 7-position of a flavone, both of the 4'-position and 7-position of a flavone can be glycosylated or the glycosylation of both of the 4'-position and 7-position of a flavone can be suppressed, with a result that the flower color of the plant can be changed.

[0051]

According to today's state of the art, a technology of introducing a polynucleotide into a plant and allowing the polynucleotide to be expressed in a constitutive or tissue-specific manner can be used. The introduction of DNA into a plant can be performed according to a method known to a person skilled in the art, such as an Agrobacterium method, a binary vector method, an electroporation method, a PEG method, a particle gun method and the like.

[0052]

As examples of plants that can be transformed, there can be mentioned, but not limited to, rose, carnation, chrysanthemum, snapdragon, cyclamen, orchids, Turkey homecoming, freesia, gerbera, gladiolus, baby's breath, kalanchoe, lily, Pelargonium, geranium, petunia, torenia, tulip, anthurium, phalaenopsis, rice, barley, wheat, rapeseed, potato, tomato, poplar, banana, eucalyptus, sweet potato, soybean, Arufarusa, Rubin, corn, cauliflower, dahlia etc.

[0053]

The present invention also relates to processed

products (processed cut flowers) using the above-mentioned cut flowers. Here, the processed cut flowers include, but not limited to, pressed flowers, preserved flowers, dried flowers, resin-sealed flowers etc.

5 **[0054]**

A flavone, produced by a production method of the present invention, having a glycosyl added to the 4'-position and 7-position thereof can be used in applications such as food products, pharmaceutical
10 products, cosmetic products, and the like.

[0055]

In accordance with the present invention, the expression of a gene of interest in a plant can also be suppressed by, e.g., an antisense method, a cosuppression
15 method, or an RNAi method. The method of suppressing the expression of a gene of interest can be performed by a method known to a person skilled in the art, such as an antisense RNA/DNA technology [Biotechnology, 9, 358 (1992), Trends in Biotechnology, 10, 87 (1992), Trends in
20 Biotechnology, 10, 152 (1992)], and a triple helix technology [Trends in Biotechnology, 10, 132 (1992)]. For example, the suppression of gene expression can be performed using a single stranded nucleic acid molecule comprising all or part of a nucleotide sequence identical
25 with the antisense chain of the gene of the present invention. Such a method is known as an antisense method. In the antisense method, RNA having a sequence complementary to the gene of which expression is desired to be suppressed may be expressed at a high level in
30 order to suppress the expression of the target gene. In this method, a single stranded RNA comprising all of a nucleotide sequence identical with the antisense chain of the polynucleotide (gene) of the present invention can be used. Also, in the above method, a single stranded RNA
35 comprising a part of a nucleotide sequence identical with the antisense chain of the polynucleotide (gene) of the present invention can be used. Such a partial single

stranded RNA may be any RNA that can suppress the expression of the gene of the present invention. While it can be designed as appropriate by a person skilled in the art, it may preferably be specific to the gene of the present invention, and the chain length thereof may preferably be 5 to 100 nucleotides, more preferably 5 to 50 nucleotides, and still more preferably 10 to 20 nucleotides.

[0056]

The suppression of gene expression may be performed using a single stranded nucleic acid molecule comprising all or a part of a nucleotide sequence identical with the sense chain of the gene of the present invention. Thus, this sense single stranded nucleic acid, similarly to the above antisense single stranded nucleic acid, can be used in suppressing the expression of the gene of the present invention. In this method, a single stranded RNA comprising all of a nucleotide sequence identical with the sense chain of the gene of the present invention can be used. Also, in the above method, a single stranded RNA comprising part of a nucleotide sequence identical with the sense chain of the gene of the present invention can be used. Such a partial single stranded RNA may be any RNA that can suppress the expression of the gene of the present invention. While it can be designed as appropriate by a person skilled in the art, it may preferably be specific to the gene of the present invention, and the chain length thereof may preferably be 5 to 100 nucleotides, more preferably 5 to 50 nucleotides, and still more preferably 10 to 20 nucleotides.

[0057]

Furthermore, the suppression of gene expression may be performed using a double stranded nucleic acid molecule comprising all or a part of a nucleotide sequence identical with the gene of the present invention. For example, by using this double stranded

- 25 -

nucleic acid molecule, an antisense or a sense single stranded nucleic acid of the present invention can be expressed in angiosperms. The double stranded nucleic acid molecule of the present invention may preferably be DNA, and its chain length and a specific nucleotide sequence should correspond to the chain length and the nucleotide sequence of the single stranded nucleic acid molecule of interest. For example, when the above antisense single stranded nucleic acid is to be expressed, the double stranded nucleic acid molecule of the present invention should contain the antisense chain of the gene of the present invention as a coding chain. Also, when the above sense single stranded nucleic acid is to be expressed, the double stranded nucleic acid molecule of the present invention should contain the sense chain of the gene of the present invention as a coding chain.

[0058]

A double stranded nucleic acid molecule can be expressed in plants using a method known to a person skilled in the art. For example, by introducing an expression vector comprising a promoter, a double stranded nucleic acid molecule of the present invention, and a transcription terminator etc. into a plant of interest and cultivating the plant obtained, the double stranded nucleic acid molecule can be expressed. The introduction of an expression vector into a plant can be performed according to a method known to a person skilled in the art, such as an Agrobacterium method, a binary vector method, an electroporation method, a PEG method, a particle gun method and the like.

[0059]

As other examples for suppressing gene expression using the nucleic acid molecule of the present invention, the cosuppression method can be mentioned. In this method, a sense double stranded DNA having the entire nucleotide sequence of the gene of the present invention

may be introduced into a plant of interest. By so doing, a sense single stranded RNA of the present invention may be expressed, and thus the expression of this RNA may be extremely suppressed (Plant Cell 9: 1357-1368, 1997).

5

[EXAMPLES]

[0060]

The present invention will now be explained specifically with reference to examples.

10 [Example 1: Detection of an activity of transferring a glycosyl to hydroxyl groups at the 4'-position and 7-position of a flavone in *Nemophila* petals]

Petals of *Nemophila menziessi* were collected at each development stage defined below, frozen in liquid
15 nitrogen, and stored in a -80°C freezer:

Stage 1: Uncolored, hard and folded buds (about 2-5 mm):

Stage 2: Colored, hard and folded buds (about 2-5 mm):

20 Stage 3: Colored and folded buds, and sepals are about to unfold (about 5-10 mm);

Stage 4: Buds of which petals are about to unfold (about 10-15 mm);

Stage 5: Completely unfolded flowers

25 **[0061]**

<Preparation of *Nemophila* petal extract>

In petals at stages 1 and 2 before the biosynthesis of anthocyanin, the detection of the glycosyltransferase activity of a flavone can be expected. Thus, using
30 petals at stages 1 and 2, petal extracts were prepared. 500 mg of petal samples (250 mg each of samples of stages 1 and 2 that had been stored at -80°C) were mashed in a mortar while cooling in liquid nitrogen, and dissolved in 1.5 ml of an extraction buffer (composition: potassium
35 phosphate buffer (pH 7.5): 100 mM, dithiothreitol (DTT); 1 mM, polyvinyl pyrrolidone 40; 50 mg/ml, sucrose; 10 mg/ml). The protein solution obtained was centrifuged

- 27 -

(10,000 rpm, 4°C, 10 minutes), and to the supernatant collected, ammonium sulfate was added to a 30% saturation concentration. After stirring at 4°C for 1 hour, it was centrifuged (10,000 rpm, 4°C, 10 minutes) to collect the supernatant. To the supernatant collected, ammonium sulfate was added to a 70% saturation concentration, which was then stirred at 4°C for 1 hour, and then centrifuged (10,000 rpm, 4°C, 10 minutes) to collect the precipitate. The precipitate was dissolved in 500 µl of an elution buffer (composition: Tris HCl (pH 7.5): 2.5 mM, DTT: 1 mM, amidinophenyl methanesulfonyl fluoride hydrochloride (APMSF): 10 µM), and column-purified using the NAP-5 Columns Sephadex G-25 DNA Grade (GE Healthcare) to remove ammonium sulfate. This solution was set as a "petal extract". For centrifugation, the Avanti HP-26 XP (rotor: JA-2) was used (BECKMAN COULTER).

[0062]

<Determination of enzyme activity>

40 µl of the petal extract, 20 µl of 5 mM UDP-glucose, 20 µl of 1M Tris HCl (pH 7.5), and 1 µl of 500 ng/µl apigenin were mixed and prepared to 200 µl in water to obtain a reaction mixture, and the reaction mixture was maintained at 30°C for 1 hour. Subsequently, 200 µl of a stopping buffer (a 90% acetonitrile aqueous solution containing 0.1% TFA) was added to stop the reaction, and the reaction mixture was analyzed with a high performance liquid chromatography (Prominence (Shimadzu)). The detector used is Shimadzu PDA SPD-M10AVP, and the flavone was detected at 330 nm. The column used is Shim-Pack ODS 150 mmx4.6 mm (Shimadzu). In elution, solution A (0.1% TFA aqueous solution) and solution B (a 90% acetonitrile aqueous solution containing 0.1% TFA) were used. A linear gradient from a 8:2 mixture of the two solutions to a 3:7 mixture over 10 minutes and then a 3:7 mixture over 5 minutes were used in elution. The flow rate was

- 28 -

set at 0.6 ml/minute. As a control, a reaction mixture obtained by heat treating the petal extract and then subjecting it to an enzyme reaction under the same condition was used.

5 As a result, a flavone that exhibits the same retention time and the same absorption maximum as the purified apigenin 4',7-diglucoside product was biosynthesized (see Fig. 7). When UDP-glucose was not added in the enzyme reaction, none was biosynthesized.
10 These results revealed the UDP-glucose-dependent presence of a protein having an activity of transferring a glycosyl to the hydroxyl groups of the 4'-position and 7-position of a flavone.

[0063]

15 [Example 2: Determination of the retention time and absorption maximum of apigenin 4'-glucoside]

 In order to clarify the biosynthetic pathway of flavone 4',7-diglucoside, the retention time and absorption maximum of apigenin 4'-glucoside were
20 determined.

 During the process of biosynthesis of apigenin 4',7-diglucoside in Example 1, apigenin 4'-glucoside and apigenin 7-glucoside should be biosynthesized as intermediate products (see Fig. 8). In the result of
25 analysis of Example 1, the appearance of peaks of a product other than apigenin 7-glucoside and apigenin 4',7-diglucoside that are samples was expected.

 As a result, a flavone exhibiting a retention time close to that of apigenin 7-glucoside was found to have
30 been biosynthesized, which was judged to be apigenin 4'-glucoside (see Fig. 7). Thus the retention time and absorption maximum of apigenin 4'-glucoside was determined.

[0064]

35 [Example 3: Acquisition of candidate genes of a gene encoding a protein having an activity of transferring a glycosyl to both of the hydroxyl groups at the 4'- and 7-

positions of a flavone]

<Isolation of total RNA>

Using Plant RNAeasy Kit (QIAGEN), total RNA was isolated from the petals of stages 1 and 2 of *Nemophila* according to a protocol recommended by the manufacturer.
5 <Expression analysis of cDNA derived from *Nemophila* petals>

After 30 µg of total RNA derived from *Nemophila* petals was subjected to a reverse transcription reaction, a uniform cDNA library was generated. The library generated was amplified clone by clone by an emulsion PCR, and then the base sequence was determined using the Genome Sequencer FLX (Roche Diagnostics Japan). The sequence data obtained were translated into amino acid sequences, and the sequences exhibiting homology with the amino acid sequence of anthocyanin 3'-glycosyltransferase of gentian were extracted. These sequences were assembled to obtain candidate genes encoding the glycosyltransferase.

20 **[0065]**

[Example 4: Preparation of full-length cDNA of candidate genes of a gene encoding a protein having an activity of transferring a glycosyl to both of the hydroxyl groups at the 4'- and 7-positions of a flavone]

25 In Example 3, 25 sequences of glycosyltransferase were obtained. Ten genes (NmGT0 through NmGT9) among them were subjected to experiments for obtaining full-length cDNA sequences.

A full-length cDNA sequence was obtained using the GeneRacer Kit (Invitrogen) according to a protocol recommended by the manufacturer. From among the cDNA partial sequences obtained in Example 3, regions specific to the clones were selected, and based on the sequences of the regions RACE primers were designed to obtain 5',3' end sequences by RACE PCR. Based on the sequences, primers for amplifying the full-length cDNA sequences were designed, and a PCR reaction was performed on 50 µl

- 30 -

with *Nemophila* cDNA as the template using the KOD-plus polymerase (TOYOBO) according to a protocol recommended by the manufacturer (94°C is maintained for 2 minutes, and a cycle comprising 94°C for 15 seconds, 55°C for 30 seconds and 68°C for 2 minutes was repeated for 30 cycles, and then maintained at 4°C). *Nemophila* cDNA was synthesized using the SuperScript II Reverse Transcriptase (Invitrogen) with the total RNA isolated in Example 2 as the template according to a protocol recommended by the manufacturer. The primers were designed so that restriction sites can be included on both ends of the full-length cDNA in order to insert the NmGT0 through NmGT9 genes into an *Escherichia coli* expression vector pET15b (Novagen). Using the PCR product, plasmids (pTOPO-NmGT0 through pTOPO-NmGT9) containing the full-length NmGT gene were obtained using the Zero Blunt TOPO PCR Cloning kit for sequencing (Invitrogen) according to a protocol recommended by the manufacturer. By analyzing the base sequences inserted into the plasmids, the full-length cDNA sequences of the candidate genes (NmGT0 through NmGT9) encoding a protein having an activity of transferring a glycosyl to both of the hydroxyl groups at the 4'-position and 7-position of a flavone were obtained (NmGT3: SEQ ID NO: 1, NmGT4: SEQ ID NO: 3).

[0066]

[Example 5: Experiment of determining the enzyme activity of candidate proteins having an activity of transferring a glycosyl to both of the hydroxyl groups at the 4'-position and 7-position of a flavone (when crude enzyme was used)]

<Creation of an *Escherichia coli* expression construct>

3 µg each of pTOPO-NmGT0 through pTOPO-NmGT9 was treated with the corresponding restriction enzymes, and about 1.5 kb DNA fragments obtained were collected. 2 µg of the vector pET15b was also treated with the

- 31 -

restriction enzymes, and was ligated to the DNA fragments obtained to create *Escherichia coli* expression constructs (pET-NmGT0 through pET-NmGT9).

[0067]

5 <Expression of glycosyltransferase in *Escherichia coli*>

pET-NmGT0 through pET-NmGT9 were introduced into *Escherichia coli* strain BL2 using the One Shot BL21 (DE3) (Invitrogen) according to a protocol recommended by the manufacturer to obtain transformed *Escherichia coli*. The
10 *Escherichia coli* was cultured, using the Overnight Express Autoinduction System 1 (Novagen) according to a protocol recommended by the manufacturer. With 2 ml of the culture liquid prepared, the transformed *Escherichia coli* was cultured at 37°C (about 4 hours) until the OD600
15 value reached 0.5. To this *Escherichia coli* solution as the preculture, 50 ml of the culture liquid was added and then subjected to the main culture overnight at 27°C.

The overnight-cultured *Escherichia coli* solution was centrifuged (3000 rpm, 4°C, 15 minutes), and the cells
20 collected were suspended in 5 ml of a sonic buffer (composition: Tris HCl (pH 7.0): 2.5 mM, dithiothreitol (DTT): 1 mM, amidinophenyl methanesulfonyl fluoride hydrochloride (APMSF): 10 µM), and *Escherichia coli* was disrupted by sonication. The cells were then centrifuged
25 (1500 rpm, 4°C, 10 minutes), and the supernatant was collected. The supernatant was used as a crude enzyme solution. For centrifugation, the Avanti HP-26 XP (rotor: JA-2) was used (BECKMAN COULTER).

[0068]

30 <Determination of enzyme activity>

80 µl of the crude enzyme solution, 20 µl of 5 mM UDP-glucose, 20 µl of 1M Tris HCl (pH 7.5), and 1 µl of 500 ng/µl apigenin were prepared on ice to 200 µl in water, and the reaction mixture obtained was maintained
35 at 30°C for 1 hour. Subsequently, 200 µl of the stopping buffer (a 90% acetonitrile aqueous solution containing

0.1% TFA) was added to stop the reaction, and analyzed with a high performance liquid chromatography (Prominence (Shimadzu)). The detector used is Shimadzu PDA SPD-M10AVP, and the flavone was detected at 330 nm. The
5 column used is Shim-Pack ODS 150 mmx4.6 mm (Shimadzu). In elution, solution A (a 0.1% TFA aqueous solution) and solution B (a 90% acetonitrile aqueous solution containing 0.1% TFA) were used. A linear gradient from a
10 8:2 mixture of the two solutions to a 3:7 mixture over 10 minutes and then a 3:7 mixture over 5 minutes were used in elution. The flow rate was set at 0.6 ml/minute. As a control, the crude enzyme solution of Escherichia coli in which no insert-inserted pET vector was introduced and was subjected to an enzyme reaction under the same
15 condition was used.

As a result, peaks other than that of the substrate were observed for NmGT3 and NmGT4. NmGT3 and NmGT4 were contained in the 7,3' GT cluster.

Examples 6 to 10 below will describe NmGT3 and NmGT4
20 (SEQ ID NO: 1 and 3, respectively).

[0069]

[Example 6: Experiment of determining the enzyme activity of a protein having an activity of transferring a glycosyl to both of the hydroxyl groups at the 4'-
25 position and 7-position of a flavone (when the His-Tag-added protein was purified)]

<Expression of glycosyltransferase in Escherichia coli and protein purification>

The Escherichia coli strain BL2 in which pET-NmGT3
30 or pET-NmGT4 described in Example 5 was introduced was cultured, using the Overnight Express Autoinduction System 1 (Novagen) according to a protocol recommended by the manufacturer. With 8 ml of the culture liquid prepared, the transformed Escherichia coli was cultured
35 at 37°C (about 4 hours) until the OD600 value reached 0.5. To this Escherichia coli solution as the preculture, 200 ml of the culture liquid was added and then subjected to

the main culture overnight at 25°C.

The overnight-cultured *Escherichia coli* solution was centrifuged (1000xg, 4°C, 10 minutes), and the cells collected were suspended in 20 ml of a buffer (composition: NaCl: 0.5 M, Tris HCl (pH 7.9): 20 mM, imidazole: 5 mM, amidinophenyl methanesulfonyl fluoride hydrochloride (APMSF): 10 µM), and *Escherichia coli* was disrupted by sonication. The cells were then centrifuged (1400xg, 4°C, 20 minutes), and the supernatant was collected. The supernatant was passed through a 0.45 µm filter to His-Tag-purify using the Profinia (Bio-Rad) according to a protocol recommended by the manufacturer. The purified protein solution thus obtained was centrifuged (7500xg, 4°C, 15 minutes) using the Centrifugal filters (Ultracel-10K) (Amicon Ultra), and the concentrated protein solution was termed as "NmGT3 protein solution" and "NmGT4 protein solution". For centrifugation, the Avanti HP-26 XP (rotor: JA-2) was used (BECKMAN COULTER).

20 [0070]

<Determination of enzyme activity>

20 µl of the protein solution, 20 µl of 5 mM UDP-glucose, 20 µl of 1M Tris HCl (pH 7.5), and 1 µl of 500 ng/µl apigenin were mixed and prepared on ice to 200 µl in water, and the reaction mixture obtained was maintained at 30°C for 20 minutes. Subsequently, 200µl of the stopping buffer (a 90% acetonitrile aqueous solution containing 0.1% TFA) was added to stop the reaction, and analyzed with a high performance liquid chromatography (Prominence (Shimadzu)). The detector used is Shimadzu PDA SPD-M10AVP, and the flavone was detected at 330 nm. The column used is Shim-Pack ODS 150 mmx4.6 mm (Shimadzu). In elution, solution A (a 0.1% TFA aqueous solution) and solution B (a 90% methanol aqueous solution containing 0.1% TFA) were used. A linear

gradient from a 8:2 mixture of the two solutions to a 3:7 mixture over 10 minutes and then a 3:7 mixture over 6 minutes were used in elution. The flow rate was set at 0.6 ml/minute.

5 **[0071]**

As a result, a flavone exhibiting the same retention time and the same absorption maximum as the purified apigenin 4',7-diglucoside product had been biosynthesized (see Figs. 9 and 10). Even if the substrate was replaced with 50 ng/ μ l apigenin 7-glucoside and subjected to an enzyme reaction under the same condition, a flavone exhibiting the same retention time and the same absorption maximum as the purified apigenin 4',7-diglucoside product was biosynthesized (see Figs. 11 and 12). Furthermore, even if the substrate was replaced with apigenin 4'-glucoside and subjected to an enzyme reaction under the same condition, a flavone exhibiting the same retention time and the same absorption maximum as the apigenin 4',7-diglucoside purified product was biosynthesized (not shown). These results demonstrated that the NmGT3 protein solution and the NmGT4 protein solution are proteins having an activity of transferring a glycosyl to both of the hydroxyl groups at the 4'-position and 7-position of a flavone capable of biosynthesizing apigenin 4',7-diglucoside using apigenin, apigenin 4'-glucoside or apigenin 7-glucoside as the substrate. Furthermore, when the reactivity to various flavonoid compounds and betanidine was examined, as shown in Fig. 13, the NmGT3 and NmGT4 proteins were found to have an activity for not only apigenin and its glycosides but for luteolin and its glycosides and flavonol and its glycosides, and to glycosylate them.

25 **[0072]**

The glycosyltransferase gene (Dbs5GT; betanidin 5GT) derived from Livingstone daisy originally transfers glucose to a hydroxyl group at the 5-position of betanidine, but it is reported that it has an in vitro

35

activity of transferring glucose to either one of the hydroxyl groups at the 4'-position or 7-position of a flavonoid. It was revealed that this glycosyltransferase gene derived from Livingstone daisy has a great different reactivity from the NmGT3 and 4 proteins and flavonoid compounds and betanidine (see Fig. 13).

[0073]

The identity and the homology of the amino acid sequences (SEQ ID NO: 2 and 4, respectively) of NmGT3 and NmGT4 were 31% and 47% (see Fig. 14). In this analysis, the Clustal W program of the MacVector application (version 9.5, Oxford Molecular Ltd., Oxford, England) was used. The identity of NmGT3 and NmGT4 on the nucleic acid level was 51%.

Among the glycosyltransferases already identified, the amino acid sequence having the highest identity with NmGT3 was an enzyme (GenBank Accession No. BAD52006) that adds a glycosyl to the 2'-position of chalcononaringenin of carnation. The identity of NmGT3 and an enzyme that adds a glycosyl to the 2'-position of chalcononaringenin of carnation was 32% (see Fig. 15). The identity of NmGT3 and an enzyme that adds a glycosyl to the 2'-position of chalcononaringenin of carnation on the nucleic acid level was 47%.

Among the glycosyltransferases already identified, the amino acid sequence having the highest identity with NmGT4 was an enzyme (described in Non-patent document 9) that adds a glycosyl to the 7-position of a flavonoid of *Scutellaria baicalensis*. The identity of NmGT4 and an enzyme that adds a glycosyl to the 7-position of a flavonoid of *Scutellaria baicalensis* was 52% (see Fig. 16). The identity of NmGT4 and an enzyme that adds a glycosyl to the 7-position of a flavonoid of *Scutellaria baicalensis* on the nucleic acid level was 60%.

[0074]

[Example 7: Expression of a gene encoding a protein having an activity of transferring a glycosyl to both of

the hydroxyl groups at the 4'- and 7-positions of a flavone in torenia]

In order to confirm whether or not the NmGT3 gene and the NmGT4 gene translate a protein having an activity of transferring a glycosyl to both of the hydroxyl groups at the 4'- and 7-positions of a flavone in plants, binary vectors pSPB4584 through 4587 for expressing NmGT3 and NmGT4 were constructed, and introduced into torenia (Summer Wave). Details of the constructs introduced are shown below (see Fig. 17).

<Preparation of constructs>

pSPB4584 has the basic skeleton of a binary vector pBINPLUS (vanEngel et al., Transgenic Research 4, p288) for introduction into plants, and comprises the E1235S promoter (Mitsuhara et al., (1996) Plant Cell Physiol. 37, p49) having two repeats of an enhancer sequence upstream to the cauliflower mosaic virus 35S promoter, the full-length cDNA NmGT3, and the mas terminator.

pSPB4585 has the basic skeleton of pBINPLUS, and comprises the E1235S promoter, the full-length cDNA NmGT4, and the mas terminator.

pSPB4586 has the basic skeleton of pBINPLUS, and comprises two expression cassettes (1. the E1235S promoter, the full-length cDNA NmGT8, and the mas terminator, 2. the E1235S promoter, the full-length cDNA NmGT3, and the mas terminator).

pSPB4587 has the basic skeleton of pBINPLUS, and comprises two expression cassettes (1. the E1235S promoter, the full-length cDNA NmGT8, and the mas terminator, 2. the E1235S promoter, the full-length cDNA NmGT4, and the mas terminator).

[0075]

<Tissue-specific expression analysis>

In a selective medium containing kanamycin, shoots were formed, and plants in which rooting was noted were acclimated. Using petals of buds that are not calyx-splitting of each transformant, gene expression analysis

- 37 -

was performed. Total RNA was isolated in a method similar to that described in Example 3, and cDNA was synthesized in a method similar to that described in Example 4. The reverse transcription PCR reaction was performed with cDNA as the template using the ExTaq polymerase (Takara) according to a protocol recommended by the manufacturer on 30 μ l (94°C is maintained for 2 minutes, and a cycle comprising 94°C for 1 minute, 55°C for 1 minute and 72°C for 2 minutes was repeated for 25 cycles, and then maintained at 4°C). Primers were designed so that each full-length cDNA can be specifically amplified. As a result, the transcription of NmGT3 and NmGT4 in torenia was confirmed.

[0076]

[Example 8: Expression of a gene encoding a protein having an activity of transferring a glycosyl to both of the hydroxyl groups at the 4'- and 7-positions of a flavone in petunia]

Binary vectors pSPB5414 and 5427 for expressing NmGT3 were constructed, and introduced into petunia (*Surfinia bouquet red*). Details of the constructs introduced are shown below (see Fig. 18).

<Preparation of constructs>

pSPB5414 has the basic skeleton of pBINPLUS, and comprises four expression cassettes (1. the E1235S promoter, the full-length cDNA pansy F3'5'H (described in PCT/JP2004/011958, see SEQ ID NO: 5) and a heat shock protein terminator (HSP terminator) that is very useful in the expression of foreign genes in plants (Plant Cell Physiol (2010) 51, 328-332), 2. the E1235S promoter, the full-length cDNA torenia flavone synthase (described in PCT/JP2008/061600, see SEQ ID NO: 7) and the HSP terminator, 3. the E1235S promoter, the full-length cDNA NmGT8 and the HSP terminator, 4. the E1235S promoter, the full-length cDNA NmGT3 and the HSP terminator).

pSPB5427 has the basic skeleton of pBINPLUS, and

- 38 -

comprises three expression cassettes (1. the E1235S promoter, the full-length cDNA torenia flavone synthase and the HSP terminator, 2. the E1235S promoter, the full-length cDNA NmGT8 and the HSP terminator, 3. the E1235S promoter, the full-length cDNA NmGT3 and the HSP terminator).

[0077]

<Tissue-specific expression analysis>

In a selective medium containing kanamycin, shoots were formed, and plants in which rooting was noted were acclimated. Using leaves of each transformant, gene expression analysis was performed in a manner similar to that described in Example 7. The result confirmed the transcription of NmGT3 and NmGT4 in petunia.

[0078]

[Example 9: Expression of a gene encoding a protein having an activity of transferring a glycosyl to both of the hydroxyl groups at the 4'- and 7-positions of a flavone in carnation]

A binary vector pSPB5433 for expressing NmGT3 was constructed, and introduced into carnation (Cream Cinderella). Details of the construct introduced are shown below (see Fig. 19).

pSPB5433 has the basic skeleton of pWTT2132 (DNA Plant Technologies, USA=DNAP), a binary vector for introduction into plants, and comprises four expression cassettes (1. the chalcone synthase promoter of *Antirrhinum majus* (described in PCT/AU94/00265), the full-length cDNA pansy F3'5'H and the HSP terminator, 2. the chalcone synthase promoter of *Antirrhinum majus*, the full-length cDNA torenia flavone synthase and the HSP terminator, 3. carnation anthocyanin synthase promoter (described in PCT/AU/2009/001659), the full-length cDNA NmGT8 and the HSP terminator, 4. carnation anthocyanin synthase promoter, the full-length cDNA NmGT3 and the HSP terminator).

[0079]

[Example 10: Expression of a gene encoding a protein having an activity of transferring a glycosyl to both of the hydroxyl groups at the 4'- and 7-positions of a flavone in rose]

5 Binary vectors pSPB4581, 4582, 5437 and 5440 for expressing NmGT3 were constructed, and introduced into Rose (Noblesse, Ritapahyamera). Details of the constructs introduced are shown below (see Fig. 20).

10 pSPB4581 has the basic skeleton of pBINPLUS, and comprises four expression cassettes (1. the perilla anthocyanin 3-acyl transferase promoter (described in PCT/JP2010/053909), the full-length cDNA pansy F3'5'H and the mas terminator, 2. the E1235S promoter, the full-length cDNA torenia flavone synthase and the mas
15 terminator, 3. the E1235S promoter, the full-length cDNA NmGT8 and the mas terminator, 4. the E1235S promoter, the full-length cDNA NmGT3 and the mas terminator).

 pSPB4582 has the basic skeleton of pBINPLUS, and comprises four expression cassettes (1. the pansy F3'5'H
20 promoter (described in PCT/JP2010/053909), the full-length cDNA pansy F3'5'H and the mas terminator, 2. the E1235S promoter, the full-length cDNA torenia flavone synthase and the mas terminator, 3. the E1235S promoter, the full-length cDNA NmGT8 and the mas terminator, 4. the
25 E1235S promoter, the full-length cDNA NmGT3 and the mas terminator).

 pSPB5437 has the basic skeleton of pBINPLUS, and comprises five expression cassettes (1. the E1235S
30 promoter, the full-length cDNA pansy F3'5'H and the HSP terminator, 2. the perilla anthocyanin 3-acyl transferase promoter chromosome gene (described in PCT/JP2010/053909, see SEQ ID NO: 9), 3. the E1235S promoter and the full-length cDNA torenia flavone synthase and the HSP
terminator, 4. the E1235S promoter, the full-length cDNA NmGT8 and the HSP terminator, 5. E1235S promoter and the
35 full-length cDNA NmGT3 and the HSP terminator).

 pSPB5440 has the basic skeleton of pBINPLUS, and

comprises five expression cassettes (1. the E1235S promoter, the full-length cDNA pansy F3'5'H and the HSP terminator, 2. the E1235S promoter, cDNA lavender anthocyanin 3-acyl transferase (described in PCT/JP/1996/000348, see SEQ ID NO: 10) and the HSP terminator, 3. the E1235S promoter, the full-length cDNA torenia flavone synthase and the HSP terminator, 4. the E1235S promoter, the full-length cDNA NmGT8 and the HSP terminator, 5. the E1235S promoter, the full-length cDNA NmGT3 and the HSP terminator).

[0080]

[Example 11: Acquisition of candidate genes of a gene encoding a protein having an activity of transferring a glycosyl to both of the hydroxyl groups at the 4'- and 7-positions of a flavone derived from *Salvia uliginosa*]

Petals of *Salvia uliginosa* contains apigenin 4',7-diglucoside (see Fig. 6) as a major flavone. Thus, *Salvia uliginosa* is expected to have a gene encoding a protein having an activity of transferring a glycosyl to both of the hydroxyl groups at the 4'- and 7-positions of a flavone. Thus, petals were obtained from buds of *Salvia uliginosa*, and a cDNA library was generated in a manner similar to that described in PCT/JP2003/010500, and screened for candidate genes of a gene encoding a protein having an activity of transferring a glycosyl to both of the hydroxyl groups at the 4'- and 7-positions of a flavone. After the base sequences of 24 positive clones were determined, three different cDNA sequences (SuGT2, 5, 10) contained in the 7, 3' GT cluster were obtained. For these genes, in a manner similar to that described in Example 4, plasmids (pTOPO-SuGT2, 5, 10) containing cDNA full-length were constructed. The base sequences inserted into the plasmids were analyzed, and the full-length cDNA sequence of the candidate genes (SuGT2, 5, 10) of a gene encoding a protein having an activity of transferring a glycosyl to both of the hydroxyl groups at the 4'- and 7-positions of a flavone

derived from *Salvia uliginosa* were obtained (SuGT5, see SEQ ID NO: 12).

[0081]

5 [SEQ ID NO: 12: Experiment of determining the enzyme activity of protein candidates having an activity of transferring a glycosyl to both of the hydroxyl groups at the 4'-position and 7-position of a flavone derived from *Salvia uliginosa* (when the His-Tag-added protein was purified)]

10 <Preparation of an *Escherichia coli* expression construct>

Escherichia coli expression constructs (pET-SuGT2, 5, 10) were created in a manner similar to that described in Example 5.

15 <Expression of glycosyltransferase in *Escherichia coli* and protein purification>

In a manner similar to that described in Example 5, "SuGT2 protein solution", "SuGT5 protein solution" and "SuGT10 protein solution" were prepared.

[0082]

20 <Determination of enzyme activity>

20 μ l of the protein solution, 20 μ l of 5 mM UDP-glucose, 20 μ l of 1M Tris HCl (pH 7.5), and 1 μ l of 500 ng/ μ g apigenin were mixed and prepared on ice to 200 μ l in water, and the reaction mixture obtained was
25 maintained at 30°C for 90 minutes. Subsequently, 200 μ l of the stopping buffer (a 90% acetonitrile aqueous solution containing 0.1% TFA) was added to stop the reaction, and analyzed with a high performance liquid chromatography (Prominence (Shimadzu)). The detector
30 used is Shimadzu PDA SPD-M10AVP, and the flavone was detected at 330 nm. The column used is Shim-Pack ODS 150 mmx4.6 mm (Shimadzu). In elution, solution A (a 0.1% TFA aqueous solution) and solution B (a 90% methanol aqueous solution containing 0.1% TFA) were used. A linear
35 gradient from a 8:2 mixture of the two solutions to a 3:7 mixture over 10 minutes and then a 3:7 mixture over 6

- 42 -

minutes were used in elution. The flow rate was set at 0.6 ml/minute.

[0083]

As a result, when the SuGT5 protein solution was used, a flavone exhibiting the same retention time and the same absorption maximum as the purified apigenin 4',7-diglucoside product had been biosynthesized (see Figs. 21). Even if the substrate was replaced with 500 ng/ μ g apigenin 7-glucoside and subjected to an enzyme reaction under the same condition, a flavone exhibiting the same retention time and the same absorption maximum as the purified apigenin 4',7-diglucoside product was biosynthesized (see Figs. 22). These results demonstrated that the SuGT5 protein solution is a protein having an activity of transferring a glycosyl to both of the hydroxyl groups at the 4'-position and 7-position of a flavone capable of biosynthesizing apigenin 4',7-diglucoside using apigenin or apigenin 7-glucoside as the substrate.

[0084]

Similarly to the above-mentioned NmGT3 and NmGT4 proteins, SuGT5 was found to have an activity for not only apigenin and its glycosides but for luteolin and its glycosides and flavonol and its glycosides, and to glycosylate them. On the other hand, its reactivity for flavonoid compounds and betanidine was greatly different from that of the glycosyltransferase derived from Livingstone daisy (see Fig. 13).

[0085]

The identity and homology of the amino acid sequences (SEQ ID NO: 2 and 6, respectively) of SuGT5 and NmGT3 were 38% and 47% (see Fig. 23). In this analysis, the Clustal W program of the MacVector application (version 9.5, Oxford Molecular Ltd., Oxford, England) was used. The identity of SuGT5 and NmGT3 on the nucleic acid level was 47%.

The amino acid sequences (SEQ ID NO: 4 and 6,

respectively) of SuGT5 and NmGT4 had an identity of were 51% and a homology of 66% (see Fig. 24). The identity of SuGT5 and NmGT4 on the nucleic acid level was 58%.

5 [INDUSTRIAL APPLICABILITY]

[0086]

In accordance with the present invention, a polynucleotide encoding a protein having an activity of transferring a glycosyl to both of the hydroxyl groups at the 4'-position and 7-position of a flavone was identified for the first time. By expressing the polynucleotide of the present invention in a suitable host cell, a protein having an activity of transferring a glycosyl to both of the hydroxyl groups at the 4'-position and 7-position of a flavone can be produced. In accordance with the present invention, a protein having an activity of specifically transferring a glycosyl to both of the hydroxyl groups at the 4'- and 7-positions of a flavone can be used in altering flower color by expressing it in a constitutive and tissue-specific manner in a plant. Also, in accordance with the present invention, there are provided a method for producing a flavone in which a glycosyl has been added to both of the hydroxyl groups at 4'- and 7-positions thereof, and food products, pharmaceutical products, and cosmetic products obtained by this production method.

CLAIMS

1. A polynucleotide selected from the group consisting of:

5 (a) a polynucleotide comprising a base sequence defined in SEQ ID NO: 1, 3 or 12;

(b) a polynucleotide which hybridizes with a polynucleotide comprising a base sequence complementary to a base sequence defined in SEQ ID NO: 1, 3 or 12 under a stringent condition and encodes a protein having an activity of transferring a glycosyl to both of the hydroxyl groups at 4'- and 7-positions of a flavone;

10 (c) a polynucleotide which encodes a protein comprising an amino acid sequence defined in SEQ ID NO: 2, 4 or 13;

15 (d) a polynucleotide which encodes a protein comprising an amino acid sequence in which one or several amino acids have been deleted, substituted, inserted, and/or added in an amino acid sequence defined in SEQ ID NO: 2, 4 or 13 and having an activity of transferring a glycosyl to both of the hydroxyl groups at 4'- and 7-positions of a flavone; and

20 (e) a polynucleotide which encodes a protein having an amino acid sequence which has an identity of 90% or more to an amino acid sequence defined in SEQ ID NO: 2, 4 or 13 and having an activity of transferring a glycosyl to both of the hydroxyl groups at 4'- and 7-positions of a flavone.

25 2. The polynucleotide according to claim 1, which is (a) a polynucleotide comprising a base sequence defined in SEQ ID NO: 1, 3 or 12.

30 3. The polynucleotide according to claim 1, which is (c) a polynucleotide which encodes a protein comprising an amino acid sequence defined in SEQ ID NO: 2, 4 or 13.

35 4. The polynucleotide according to claim 1, which is (f) a polynucleotide which encodes a protein having an amino acid sequence which has an identity of 95% or more

- 45 -

to an amino acid sequence defined in SEQ ID NO: 2, 4 or 13 and having an activity of transferring a glycosyl to both of the hydroxyl groups at 4'- and 7-positions of a flavone.

5 5. The polynucleotide according to claim 4, which is (g) a polynucleotide which encodes a protein having an amino acid sequence which has an identity of 97% or more to an amino acid sequence defined in SEQ ID NO: 2, 4 or 13 and having an activity of transferring a glycosyl to
10 both of the hydroxyl groups at 4'- and 7-positions of a flavone.

 6. The polynucleotide according to claim 5, which is (h) a polynucleotide which encodes a protein having an amino acid sequence which has an identity of 98% or more
15 to an amino acid sequence defined in SEQ ID NO: 2, 4 or 13 and having an activity of transferring a glycosyl to both of the hydroxyl groups at 4'- and 7-positions of a flavone.

 7. The polynucleotide according to any one of
20 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 comprising the polynucleotide according to any one of claims 1 to 7.

25 10. A non-human host into which the vector according to claim 9 has been introduced.

 11. A method of adding a glycosyl to both of the hydroxyl groups at 4'- and 7-positions of a flavone using the polynucleotide according to any one of claims 1 to 7.

30 12. A plant or a progeny thereof, or a part or tissue thereof, into which the polynucleotide according to claim 9 has been introduced and which contains the polynucleotide.

 13. The part of a plant according to claim 12,
35 which is a cut flower.

 14. A cut flower artifact using the cut flower according to claim 13.

15. A process of producing a protein having an activity of transferring a glycosyl to both of the hydroxyl groups at 4'- and 7-positions of a flavone, comprising the steps of:

5 culturing or growing the non-human host according to claim 10; and

 collecting a protein having an activity of transferring a glycosyl to both of the hydroxyl groups at 4'- and 7-positions of a flavone from the non-human host.

10 16. A process of producing a flavone in which a glycosyl has been added to both of the hydroxyl groups at 4'- and 7-positions thereof, comprising the steps of:

 culturing or growing the non-human host according to claim 10; and

15 collecting a flavone in which a glycosyl has been added to both of the hydroxyl groups at 4'- and 7-positions thereof from the non-human host.

 17. A food product comprising a flavone which has been produced by the process according to claim 16 and in which a glycosyl has been added to both of the hydroxyl groups at 4'- and 7-positions thereof.

 18. A pharmaceutical product comprising a flavone which has been produced by the process according to claim 16 and in which a glycosyl has been added to both of the hydroxyl groups at 4'- and 7-positions thereof.

25 19. A cosmetic product comprising a flavone which has been produced by the process according to claim 16 and in which a glycosyl has been added to both of the hydroxyl groups at 4'- and 7-positions thereof.

1/22

Fig.1

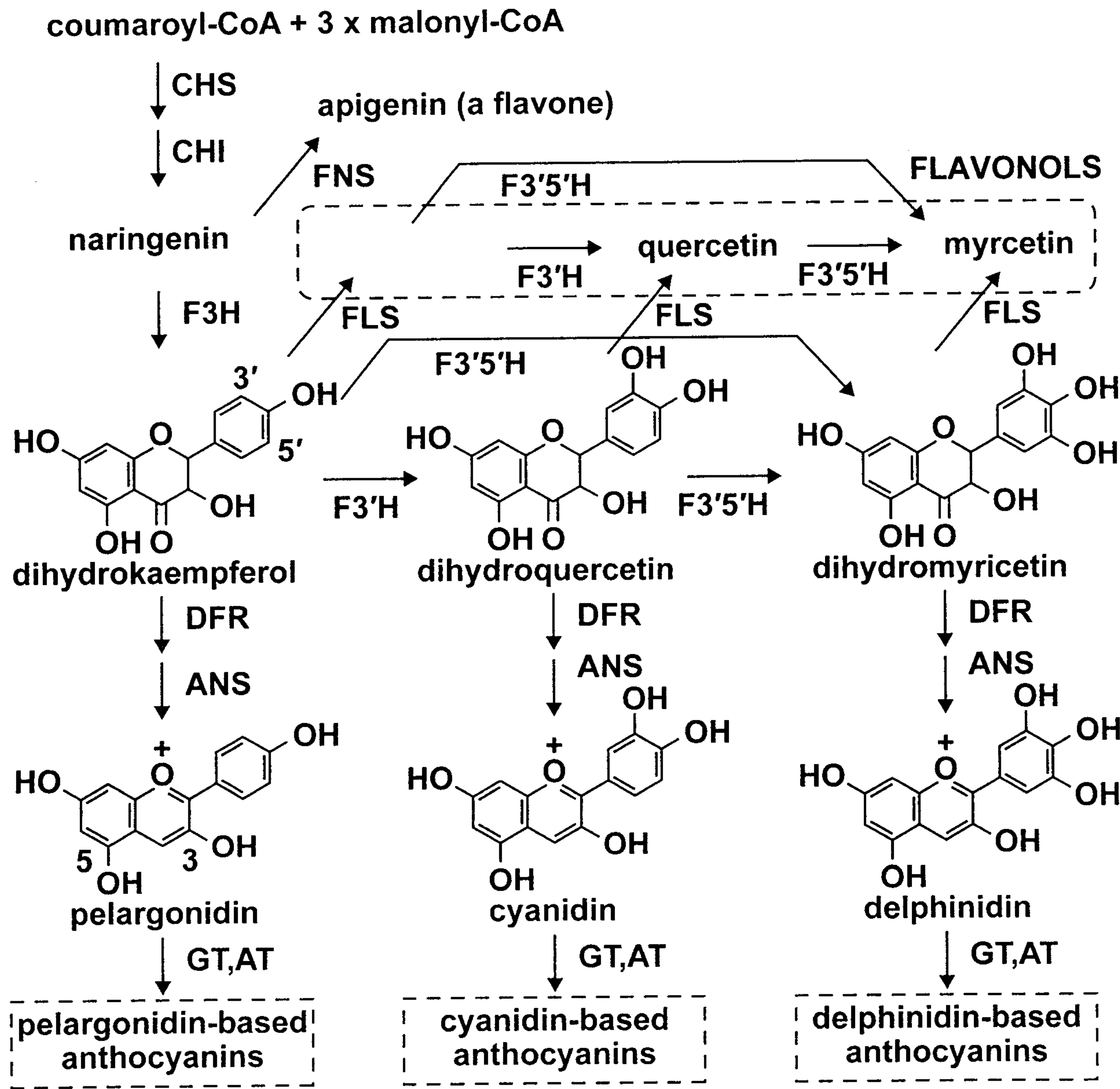
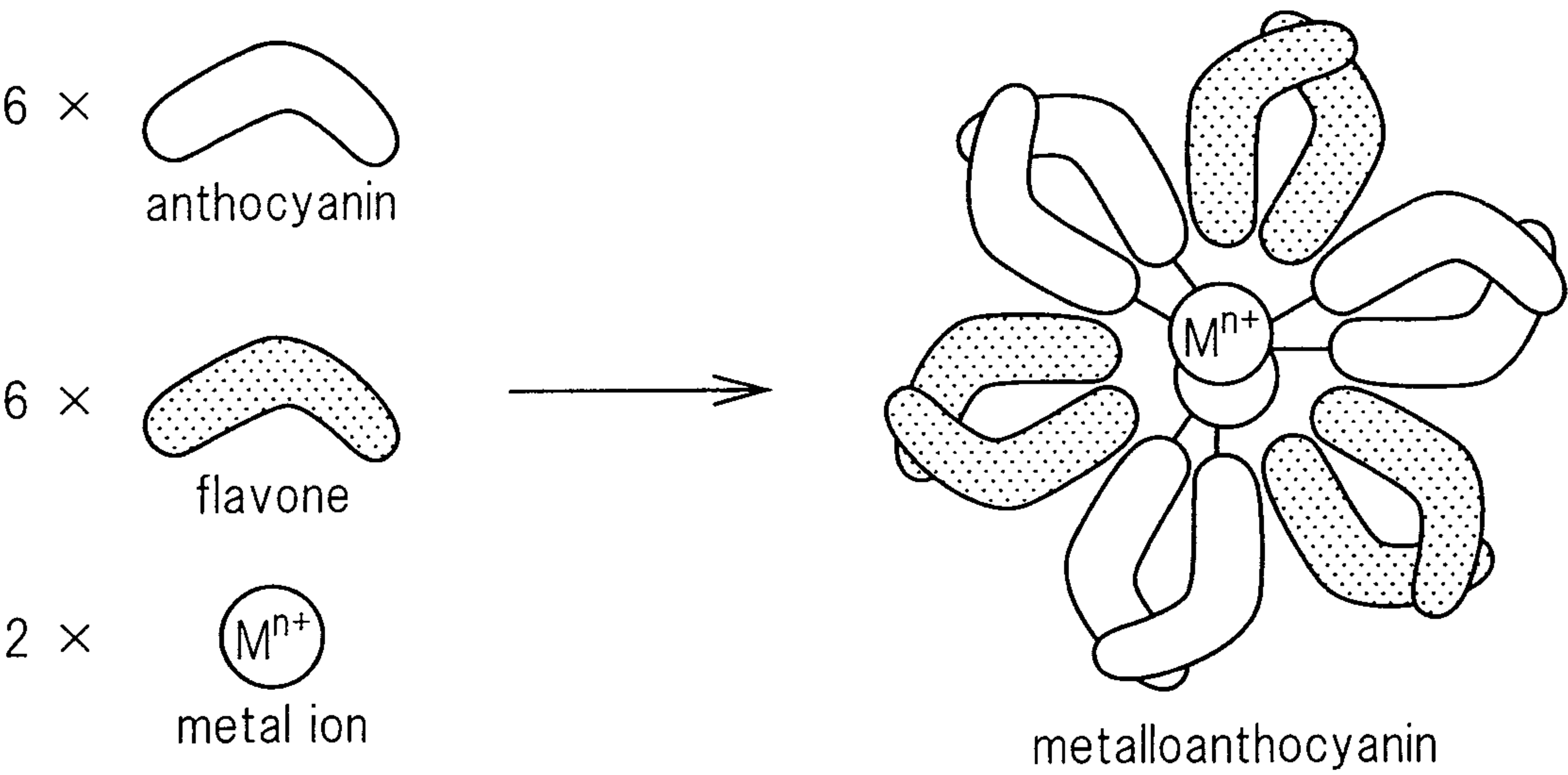


Fig.2



COc1cc(O)c(O)c(C2=CC(=C(C=C2)OC(=O)C=Cc3ccc(O)cc3)O2)c1O[C@H]3C[C@@H](OC(=O)CC(=O)O)[C@H](O)[C@H](O)[C@H]3O

The chemical structure shows a central coumarin ring system. At position 7 of the coumarin, there is a hydroxyl group (-OH). At position 2, there is a carbonyl group (=O). At position 3, there is a 4-phenoxyphenyl group (-C₆H₄-O-C₆H₄-). This 4-phenoxyphenyl group is further substituted at its para position with a 2,3,4,6-tetrahydroxyphenyl group (-C₆H₂(OH)₄-). Additionally, the 4-phenoxyphenyl group is substituted at its other para position with a 2-acetoxypropyl group (-CH₂-CH₂-O-C(=O)-CH₃).

4/22

Fig.5

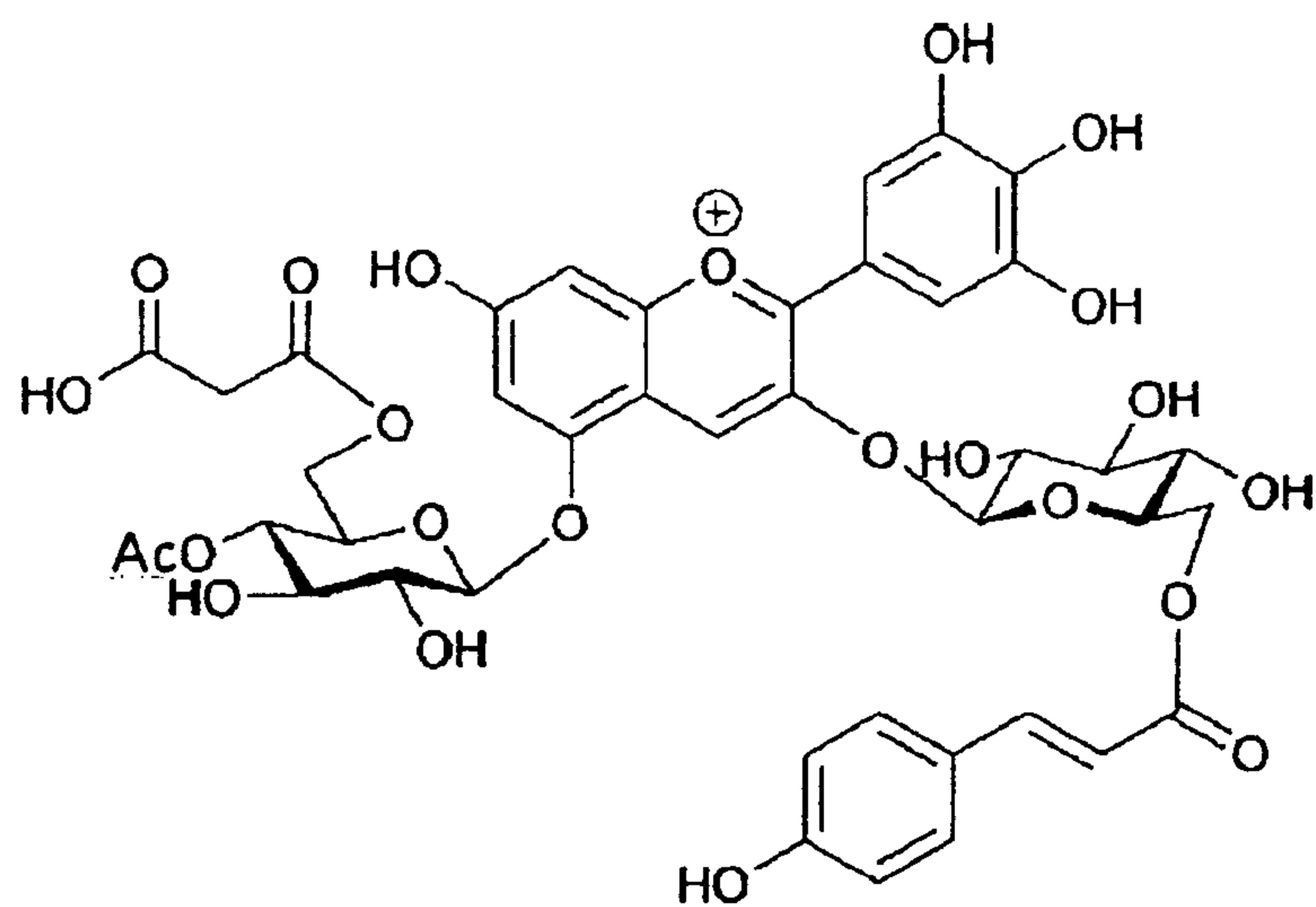
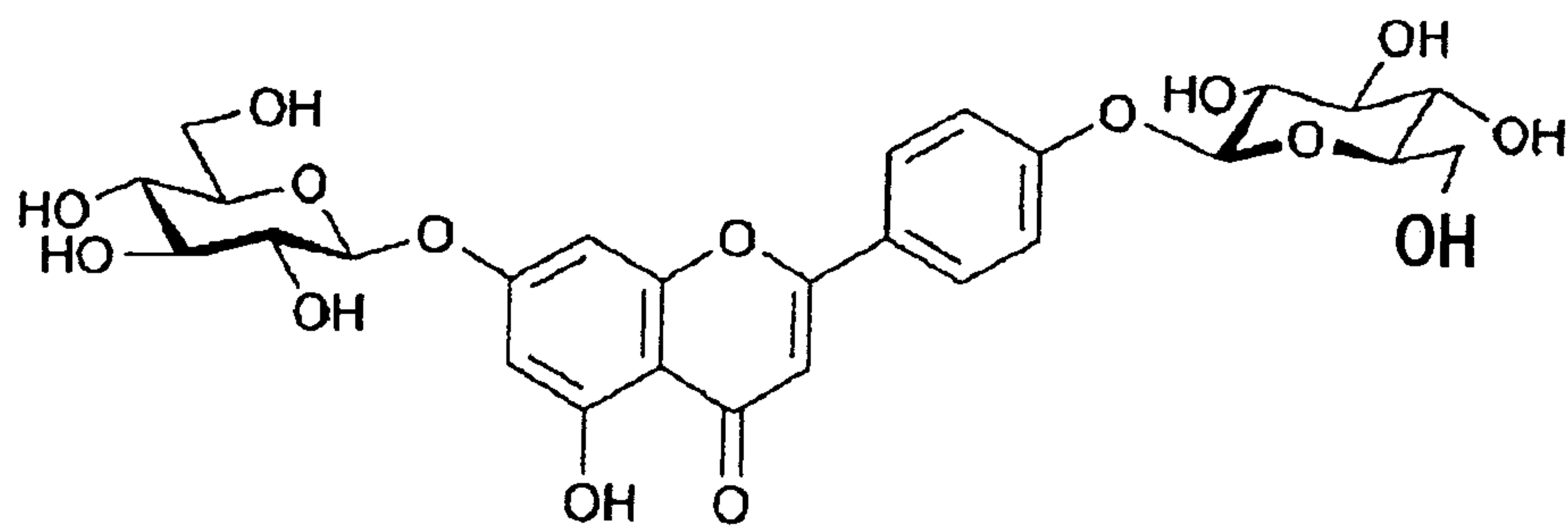
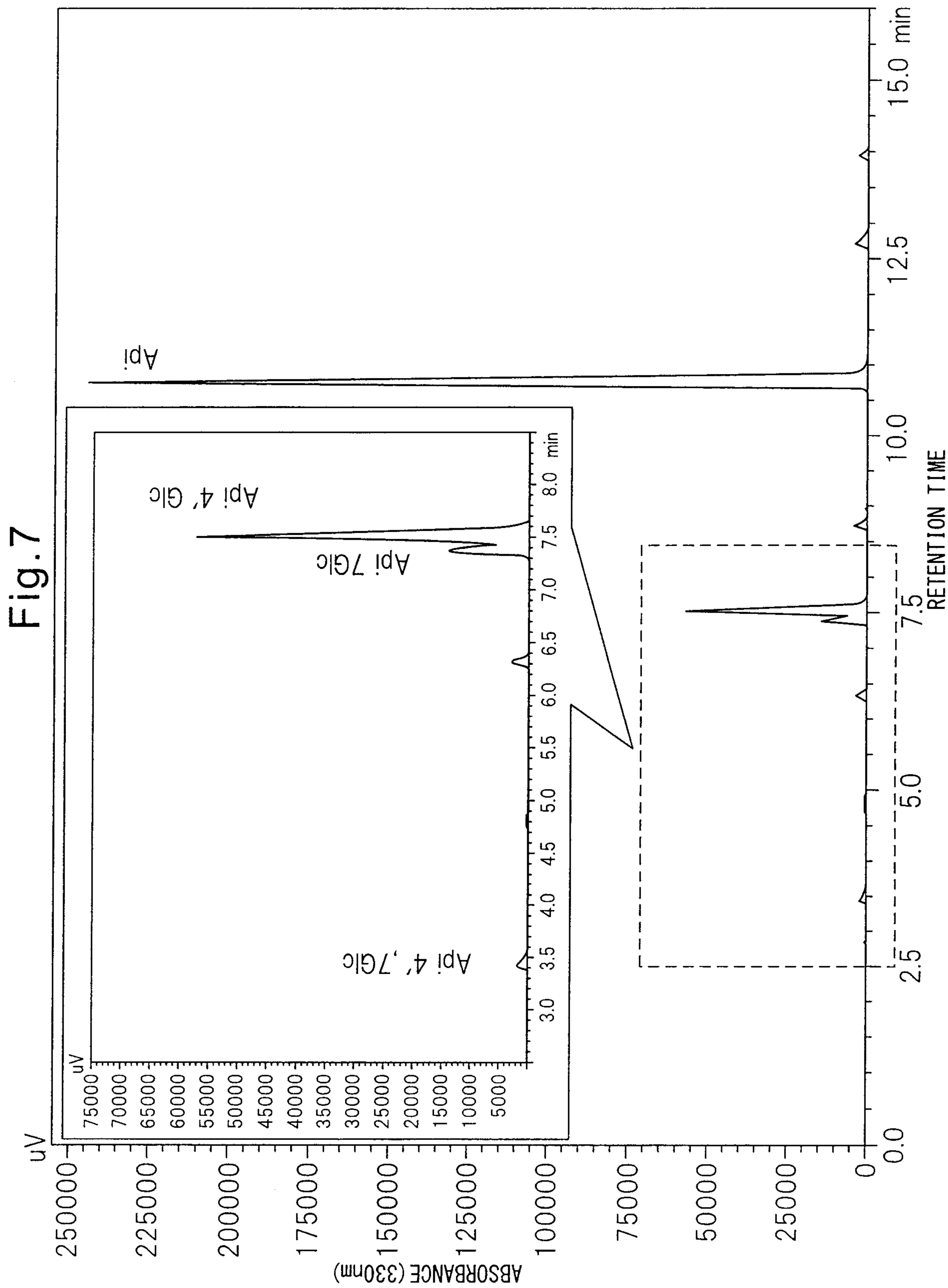


Fig.6

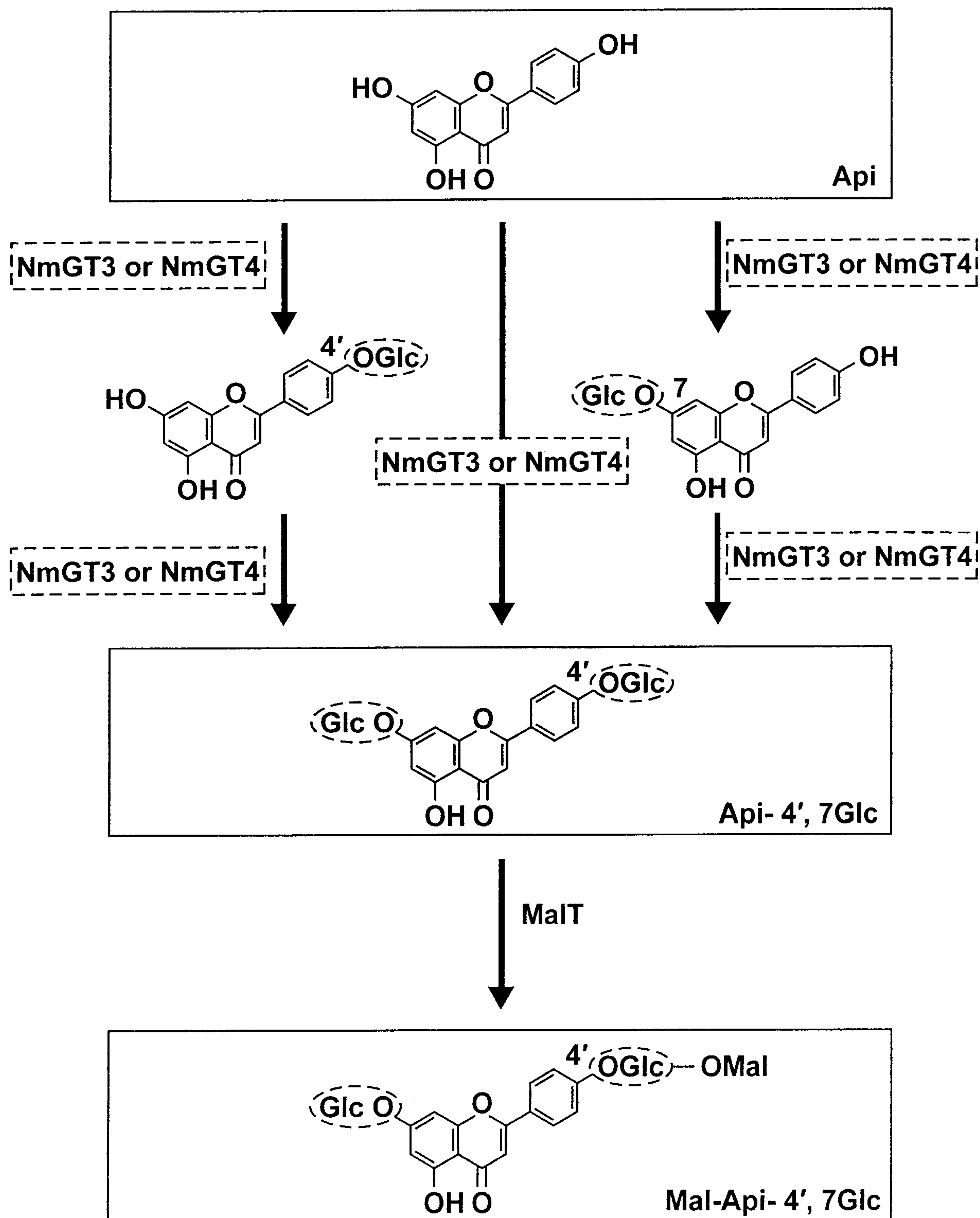


5/22



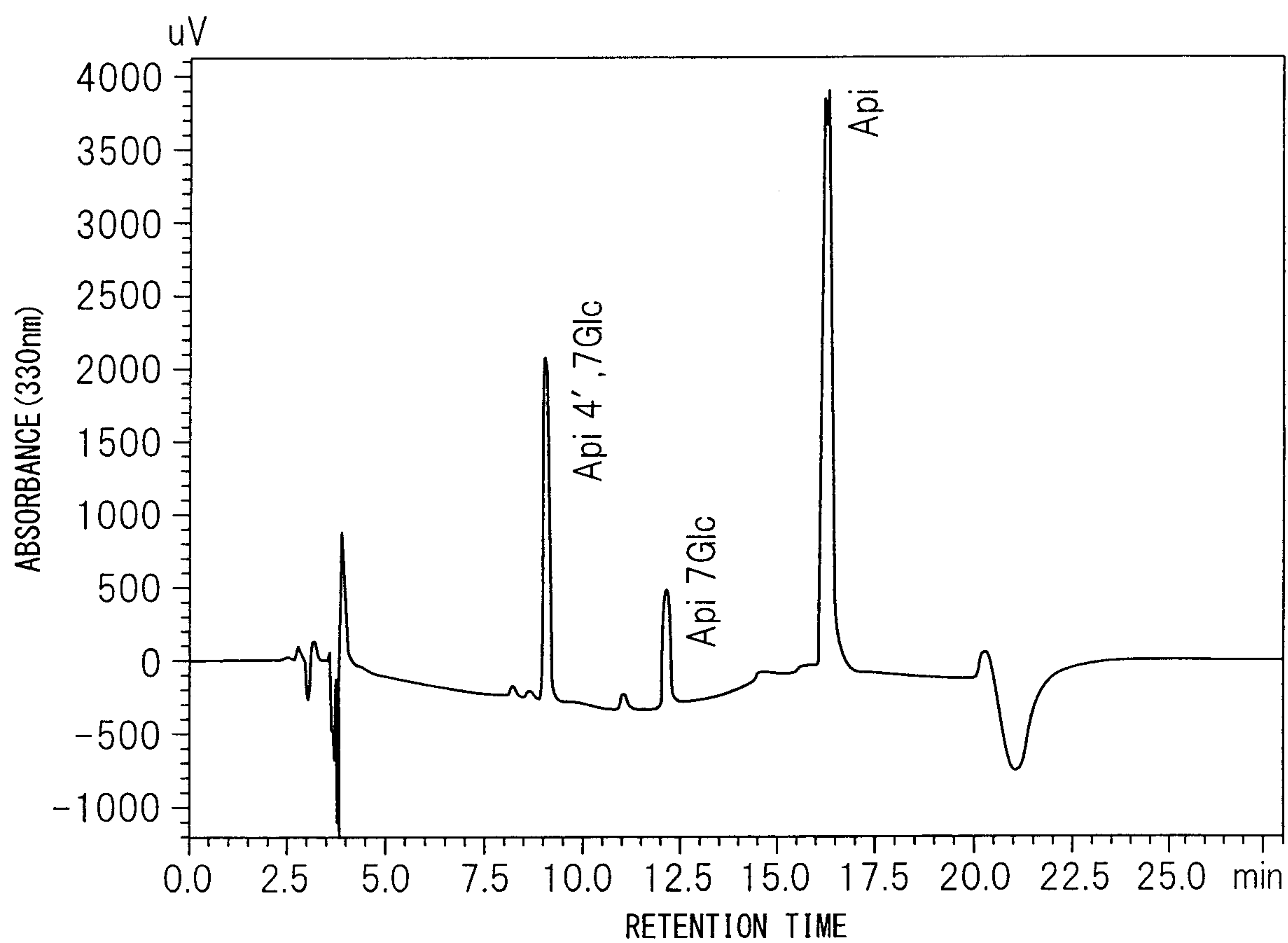
6/22

Fig.8



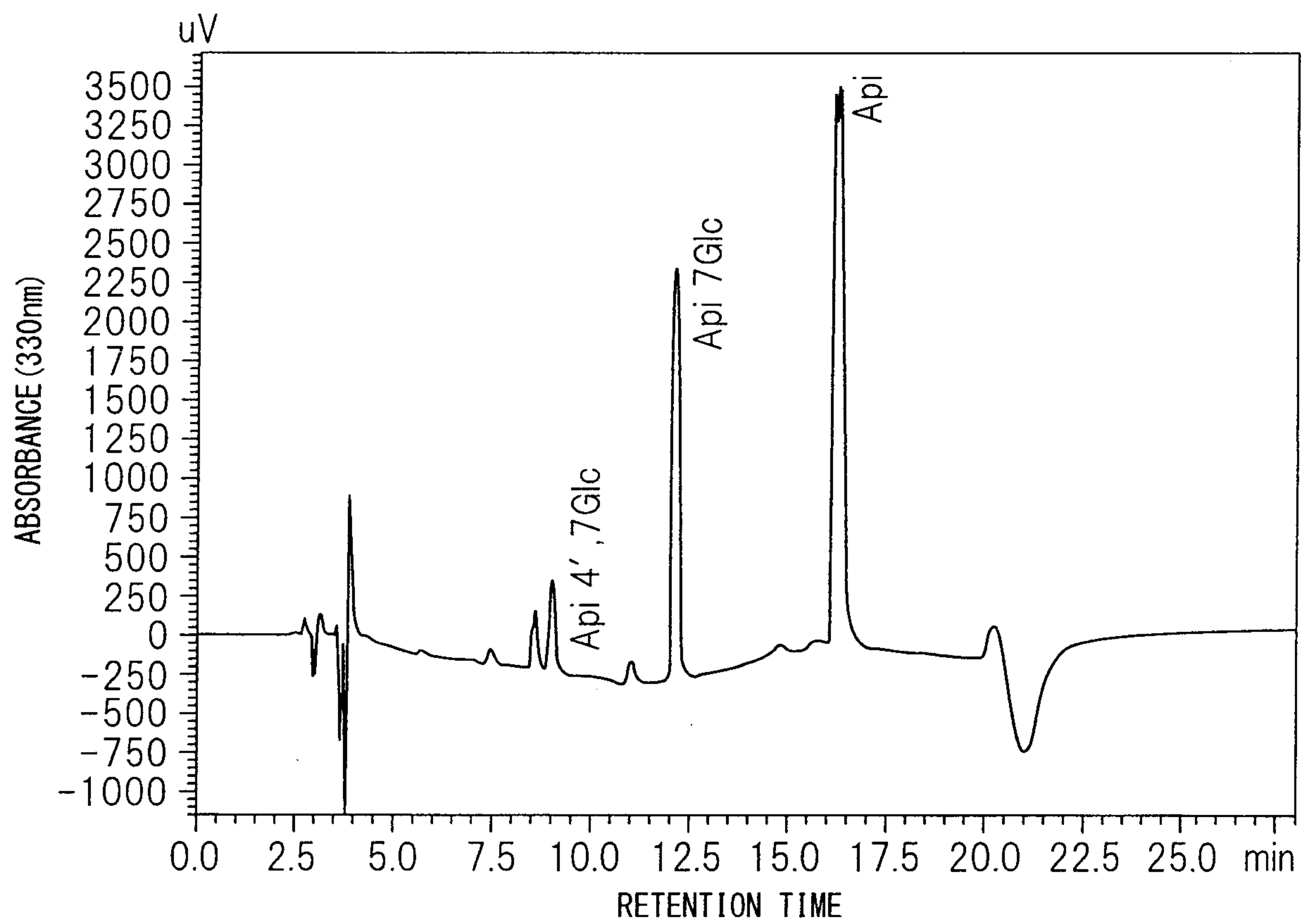
7/22

Fig.9



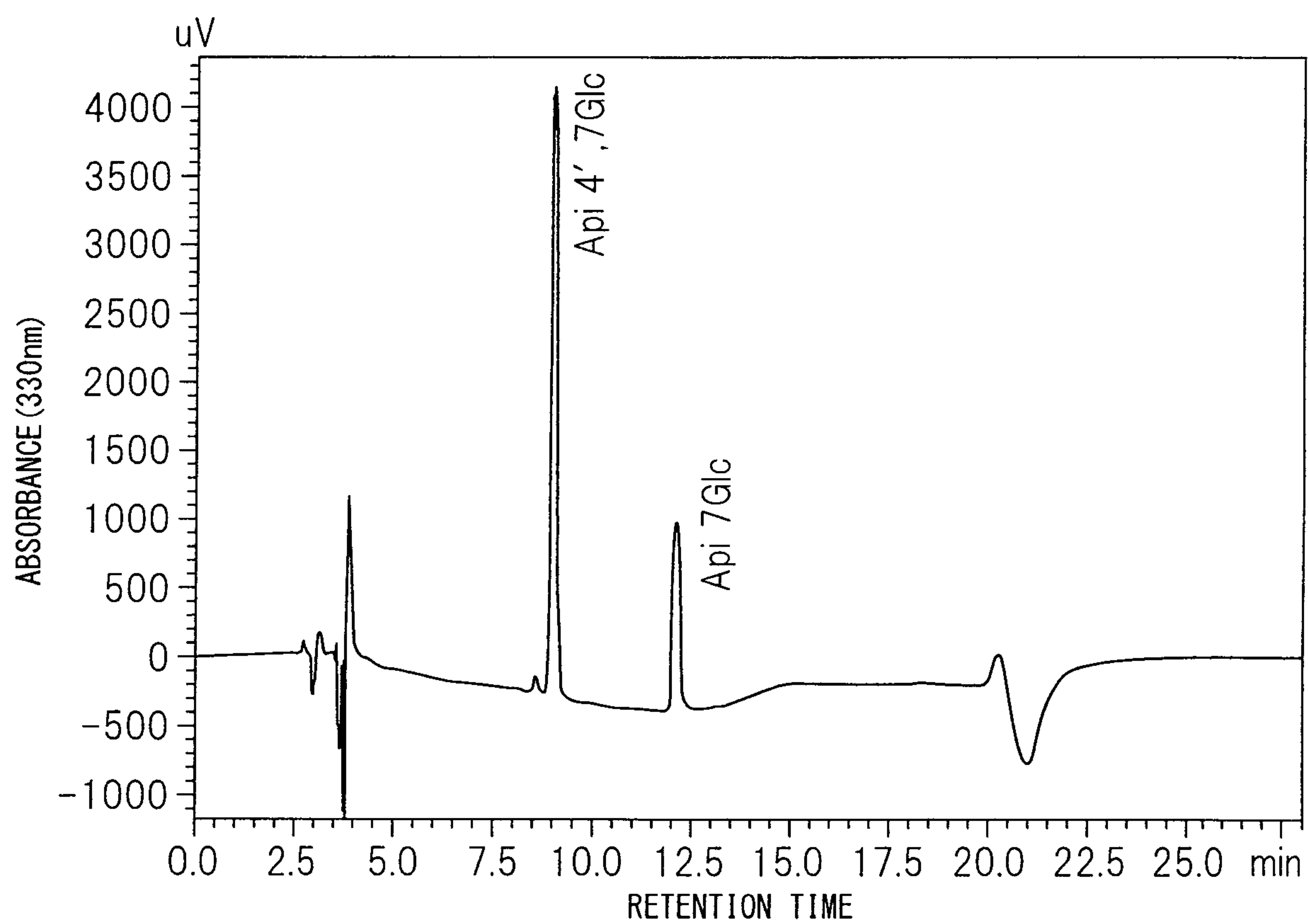
8/22

Fig.10



9/22

Fig.11



10/22

Fig.12

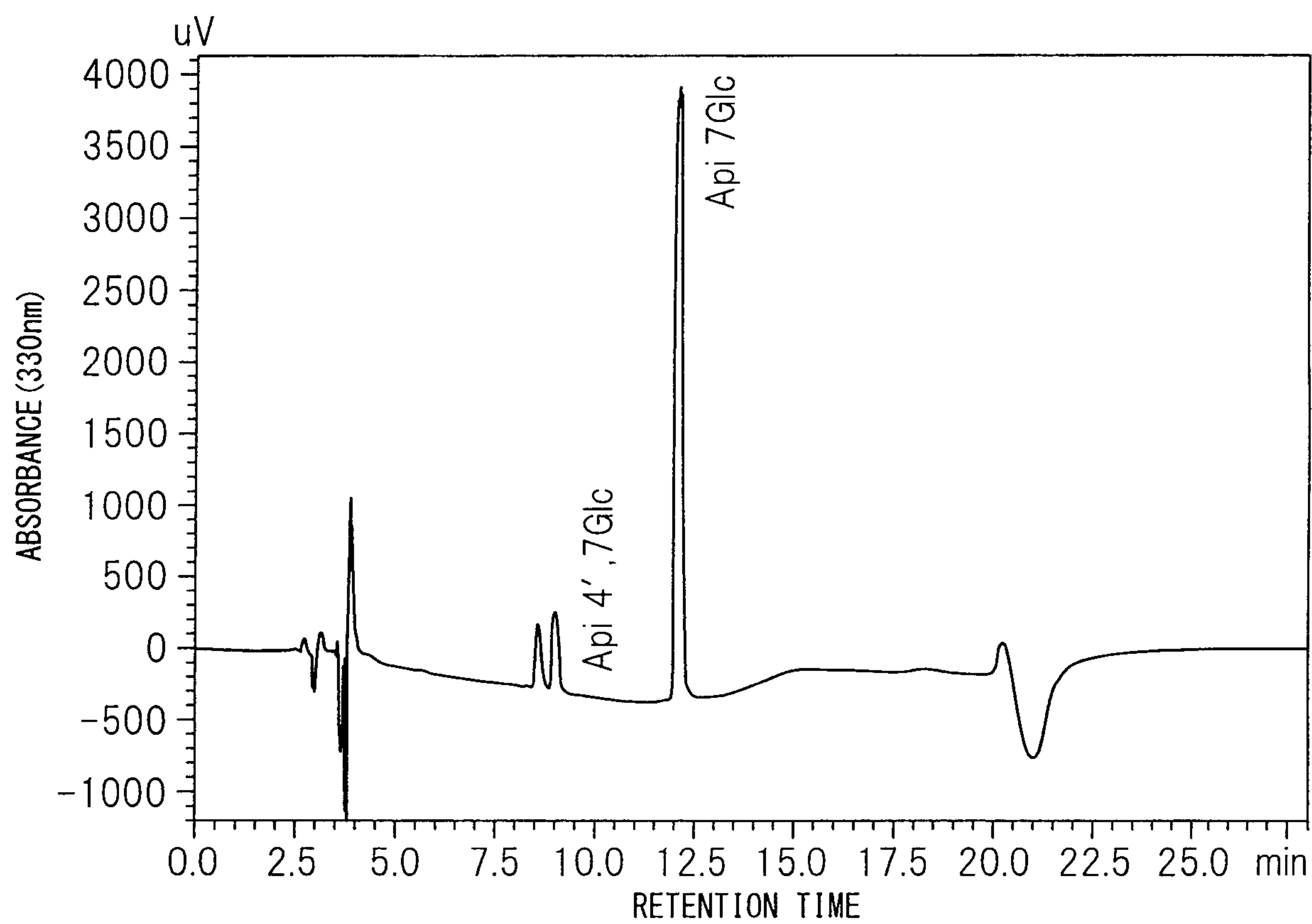


Fig.13

	NmGT3	NmGT4	SuGTAK5	betanidin5GT
Api	○(Api4'Glc,Api7Glc,Api4'7Glc)	○(Api4'Glc,Api7Glc,Api4'7Glc)	○(Api4'Glc,Api4'7Glc)	(x)
Api7Glc	△(Api4'7Glc)	△(Api4'7Glc)	△(Api4'7Glc)	
Lut	◎(Lut4'Glc,Lut7Glc,Lut4'7Glc)	◎(Lut4'Glc,Lut7Glc,Lut4'7Glc)	○(Lut4'Glc,Lut7Glc,Lut4'7Glc)	△(Lut4'Glc,Lut7Glc)
Lut7Glc	△(Lut4'7Glc)	△(Lut4'7Glc)	△(Lut4'7Glc)	
Lut4'Glc	◎(Lut4'7Glc)	◎(Lut4'7Glc)	◎(Lut4'7Glc)	
Tri	○	○	○	
K	◎(K3Glc)	◎(K3Glc)	○(K3Glc)	(x)(K4'Glc)
K3Glc	◎	◎	○	
Q	◎(Q3Glc)	◎(Q3Glc)	○(Q3Glc)	○(Q4'Glc,Q7Glc)
Q3Glc	◎	◎	○	
M	◎	◎	○	△(M4'Glc,M7Glc)
Narigenin	△	△	△	
betanidin	○	x	x	○(betanidin5Glc)

↑ From data in an article
(Thomas Vogt, 1997, 1999)

Degree of enzyme reaction

◎ : So vigorous as to use up the substrate

○ : Reactive

△ : Reactive, but few products

x : Completely nonreactive

(x) : Hardly reactive

○	: Biosynthetic reaction product
Api4' Glc	: Apigenin 4' -glucoside
Api7Glc	: Apigenin 7-glucoside
Api4' , 7Glc	: Apigenin 4' , 7-diglucoside
Lut4' Glc	: Luteonin 4' -glucoside
Lut7Glc	: Luteonin 7-glucoside
Lut4' , 7Glc	: Luteonin 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. 14

NmGT4	1	-	-	-	M	A	A	Q	L	H	V	V	F	P	F	M	A	Q	G	H	L	I	P	T	L	E	M	V	K	L	F	S	S	R	G	L	K	T	T	I	V	T	K	F	Y	A	P	A	V	T	
NmGT3	1	M	P	S	I	L	S	N	S	A	H	I	L	L	F	P	F	P	T	S	G	H	I	I	P	I	L	D	L	A	N	Q	L	L	A	R	G	L	T	I	L	I	T	P	A	N	L	T	L	L	S
	K	S	I	E	K	T	K	H	T	G	N	Q	I	N	I	I	I	I	K	F	P	S	A	E	V	G	L	P	E	G	S	E	S	L	D	K	L	K	S	P	D	M	F	M	K	F	F	K	96		
	T	Q	L	I	E	L	D	R	L	G	-	S	L	H	T	L	V	L	P	F	P	N	P	P	N	-	-	-	-	-	-	-	-	-	-	-	-	-	S	E	T	S	L	A	A	R	V	H	87		

NmGT4	97	A	L	S	L	L	Q	E	P	F	E	Q	I	L	Q	E	L	S	-	P	D	C	I	V	S	D	M	F	F	P	W	T	T	A	S	A	A	K	F	D	I	P	R	F	V	F	H	G	L	S	L	
NmGT3	88	A	S	S	Q	L	S	N	T	I	I	Q	W	F	Q	S	H	T	S	P	P	V	A	I	V	S	D	F	F	L	G	W	T	N	S	L	A	S	Q	L	G	I	P	R	L	V	F	W	P	S	G	V
		F	A	L	C	V	S	E	N	M	R	F	Y	K	P	F	K	N	L	G	S	E	S	L	D	S	E	P	V	M	L	P	D	F	P	N	Q	I	E	F	S	K	V	Q	V	P	E	F	E	V	194	
		Q	R	S	S	L	V	D	Y	I	W	Q	N	D	Q	L	S	D	S	D	H	Q	I	Q	D	N	S	V	I	S	F	P	D	V	P	N	S	P	A	Y	P	K	W	Q	A	C	G	L	S	T	187	

NmGT4	195	G	E	S	K	N	E	-	I	M	E	L	L	N	Q	V	K	E	S	E	V	K	S	Y	G	I	I	I	N	S	F	N	E	L	E	K	D	Y	V	D	Y	Y	R	N	V	W	G	R	-
NmGT3	188	Q	Y	K	K	G	D	P	S	W	E	F	F	K	N	G	V	L	A	N	T	Q	S	W	G	A	I	Y	N	S	F	R	D	L	E	G	V	Y	I	D	Y	I	K	K	M	G	H	G	

NmGT4	289	L S S	S Q L	H E	I A L G L E	S S G	Q Q F I	W V	V K E	R E D	G E K W L P E G	F F E R I	K D	K G L I I R G W A P Q V
NmGT3	288	L T T E Q L	S Q L	H E	L A A A L E	I S G	V H F I	L C	V K L	H Q E	S - - K E	Y E D R V A G	R G L I I R G W A P Q V	
		S I L E H E	R R A V	T G G F V T H C G W N	S V L E A V	S A G V V M A T	L P T F A E Q	P F N	E K	388				
		A I L R H E	R R A V	G A F L T H C G W N	S I L E G I A	A G V V M L T W	P P M G A D Q	F T N	A N	383				

NmGT4	389	L L	T K V	L K I G	I P I	G - S P L	S N R G K S	G V K K E E	I A E	A M K G	I M E G E A L E M R
NmGT3	384	L L	V D E	L K V A M K A	C E G G D	S N V P N P A	M L A N V L A E	S I N G G	R A E - - - - R		
		I R A K S	L K E M A	W K A V E E G	- G S S Y N D L	T S L I D G V	K A Y R S	Q S N K I	475		
		E R V T E	L C D A A	L K A V Q S G	N G S S A K D L	T N Q L N G	V K I N -	464			

13/22

Fig.15

Dianthus_caryophyllus-chalcononaringenin_2'-GT NmGT3	1 - M G S E H Q Q L H V A F F P F M A H G H M I P T F D L A K L F A G R D V K T T I I T T P M N A H A F A 1 M P S I L S N S A H I L L F P F P T S G H I I P I L D L A N Q L L A R G L T I T I L I T P A N L T L L S K T N V P M N . . . - L E I F T F P A Q E A G L P E N C E N L E Q A M S I G L L P A F I K A S A 95 T Q L I E L D R L G S L H T L V L P F P N P P N P S E T S L A A R V H A S S 90
Dianthus_caryophyllus-chalcononaringenin_2'-GT NmGT3	96 M L C D Q L E R F L E . . R S Q P N C L V A D M F F P W A T E S A R K F N V P R I V F H G T G F L S L C 91 Q L S N T I I Q W F Q S H T S P P V A I V S D F F L G W T N S L A S Q L G I P R L V F W P S G V Q R S S A K E V E R L Y R P F K N . . - V S S D D E V V V L P R L P H E V K L T R T Q V S . . E E E W S 188 L V D Y I W Q N D Q L S D S D H Q I Q D N S V I S F P D V P N S P A Y P K W Q A C G L S T Q Y K 190
Dianthus_caryophyllus-chalcononaringenin_2'-GT NmGT3	189 D D D N E F N K R S A R I K E S E V E S Y G V I V N S F Y E L E P E F A D F F R N E L G R . R A W N V G P V 191 K G D P S W E F F K N G V L A N . T Q S W G A I Y N S F R D L E G V Y I D Y I K K K M G H G R V W A V G P L S L C N R K T E D K A R R G K Q A N V N E Q E C L I W L D S K K . . C A S V V Y V C F G S T 285 L P A N . . . - D A S K R G G S C V M P I D D V M T W L D T K T N S D N S V V Y V C F G S R 285
Dianthus_caryophyllus-chalcononaringenin_2'-GT NmGT3	286 A H Y A P A Q L H E I A N A L E A S G H N F V W A V G N V D K K G S D G E E L L P Q G F E Q R T E 286 V E L T T E Q L D S L A A A L E I S G V H F I L C V K L H Q E I S K E Y E D R V A G R G L I I R G W A P Q V L I L E H E A V G A F M T H C G W N S T L E G I S A G V P M V T W P V F A E Q 385 G R G L I I R G W A P Q V A I L R H R A V G A F L T H C G W N S I L E G I A A G V M L T W P M G A D Q 378
Dianthus_caryophyllus-chalcononaringenin_2'-GT NmGT3	386 F Y N E K L V T Q I L K I R V E V G A K K W S R T A M I E H K I S G D A I E K A L K E I M E G E 379 F T N A N L L V D E L K V A M K A C E G G D S N V P N P A M L A N V L A E S I N G G K A E E M R N K A R Q L K E M A W K A V E E G . G S S Y N D L T A L I S E L R N Y K A . . . 475 R A E R E R . - V T E L C D A A L K A V Q S G N G S S A K D L D S L T N Q L N G L K V K I N 464

Scutellaria_barbata-flavonoid-7GT NmGT4	1	-	M	G	Q	L	H	I	V	L	V	P	M	I	A	H	G	H	M	I	P	M	L	D	M	A	K	L	F	S	S	R	G	V	K	T	T	I	I	A	T	-	-	-	P	A	F	A	E	P	I	R	
	1	M	A	A	Q	L	H	V	V	F	F	P	F	M	A	Q	G	H	L	I	P	T	L	E	M	V	K	L	F	S	S	R	G	L	K	T	T	I	V	T	T	K	F	Y	A	P	A	V	T	K	S	I	E
		K	A	R	E	S	G	H	D	I	G	L	T	T	T	K	F	P	P	K	G	S	S	L	P	D	N	I	R	S	L	D	Q	V	T	D	-	D	L	L	P	H	F	F	R	A	L	E	L	94			
		K	T	K	H	T	G	N	Q	I	N	I	I	I	I	K	F	P	S	A	E	V	G	L	P	E	G	S	E	S	L	D	K	L	K	S	P	D	M	F	K	F	K	A	L	S	L	100					
Scutellaria_barbata-flavonoid-7GT NmGT4	95	L	Q	E	P	V	E	I	M	E	D	L	K	P	D	C	L	V	S	D	M	F	L	P	W	T	T	D	S	A	A	K	F	G	I	P	R	L	L	F	H	G	T	S	L	F	A	R	C	F	A		
	101	L	Q	E	P	F	E	Q	I	L	Q	E	L	S	P	D	C	I	V	S	D	M	F	F	P	W	T	T	A	S	A	A	K	F	D	I	P	R	F	V	F	H	G	L	S	L	F	A	L	C	V	S	
		E	Q	M	S	I	Q	K	P	Y	K	N	V	S	S	-	-	D	S	E	P	F	V	L	R	G	L	P	H	E	V	S	F	V	R	T	Q	I	P	D	Y	E	L	Q	E	G	G	D	D	191			
		E	N	M	R	F	Y	K	P	F	K	N	L	G	S	E	S	L	D	S	E	P	V	M	L	P	D	F	P	N	Q	I	E	F	S	K	V	Q	V	P	E	F	E	V	G	E	S	-	K	N	199		
Scutellaria_barbata-flavonoid-7GT NmGT4	192	A	F	S	K	M	A	K	Q	M	R	D	A	D	K	K	S	Y	G	D	V	I	N	S	F	E	E	L	E	S	E	Y	A	D	Y	N	K	N	V	F	G	K	K	A	W	H	I	G	P	L	K	L	
	200	E	I	M	E	L	N	Q	V	K	E	S	E	V	K	S	Y	G	I	I	I	N	S	F	N	E	L	E	K	D	Y	V	D	Y	Y	R	N	V	F	G	R	R	A	W	L	L	G	P	L	S	L		
		F	N	N	R	A	E	Q	K	S	S	Q	R	G	K	E	S	A	I	D	D	H	E	C	L	A	W	L	N	S	K	K	P	N	S	V	V	Y	M	C	F	G	S	M	A	T	F	T	P	A	291		
		S	N	R	D	D	E	V	K	D	Q	T	-	-	-	-	-	-	D	E	H	G	S	L	K	W	L	D	S	K	K	P	D	S	V	I	Y	V	C	F	G	S	V	A	P	L	S	S	S	292			
Scutellaria_barbata-flavonoid-7GT NmGT4	292	Q	L	H	E	T	A	V	G	L	E	S	S	G	Q	D	F	I	W	V	V	R	N	G	G	E	N	E	D	W	L	P	Q	G	F	E	E	R	I	K	G	K	G	L	M	I	R	G	W	A	P	Q	V
	293	Q	L	H	E	I	A	L	G	L	E	S	S	G	Q	Q	F	I	W	V	V	K	E	R	E	D	G	E	K	W	L	P	E	G	F	E	E	R	I	K	D	K	G	L	I	I	R	G	W	A	P	Q	V
		M	I	L	D	H	P	S	T	G	A	F	V																																								

Fig.17

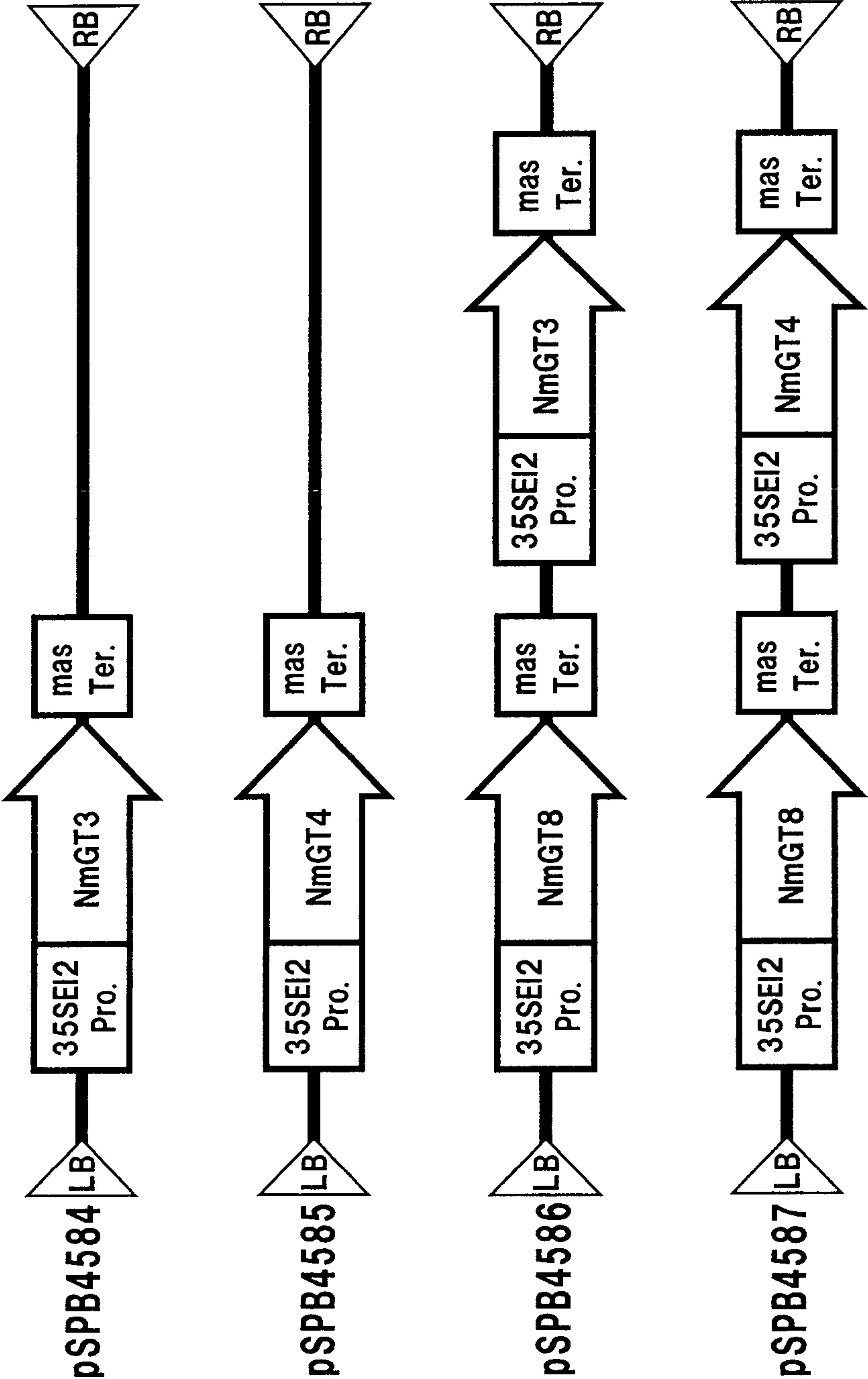
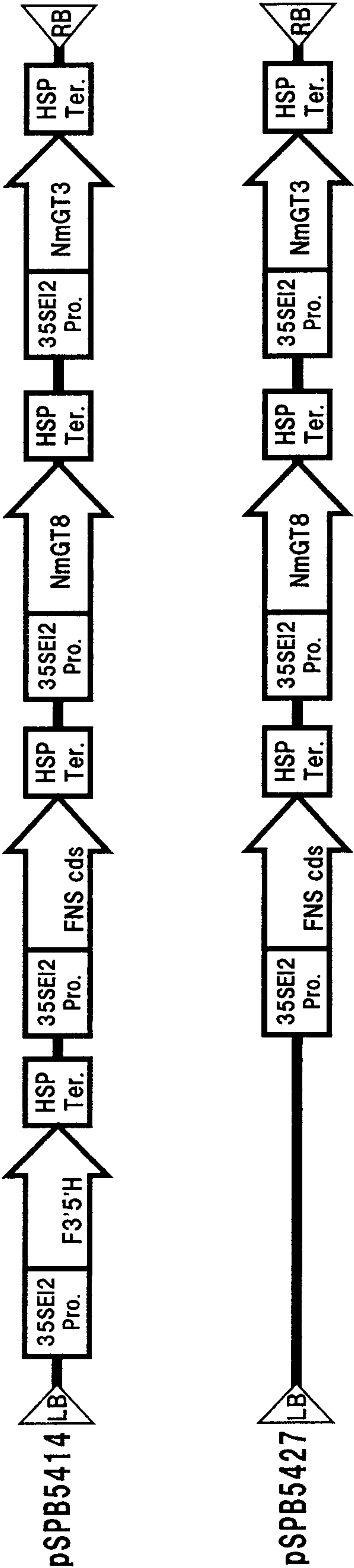


Fig.18



17/22

Fig. 19

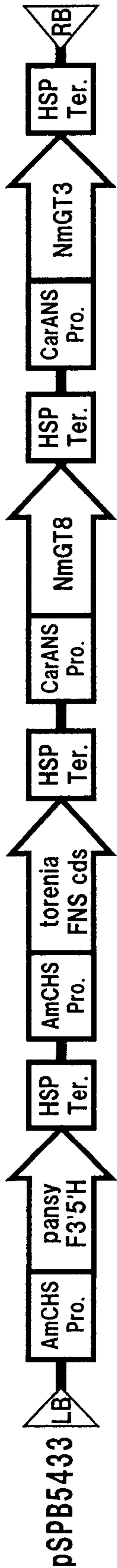
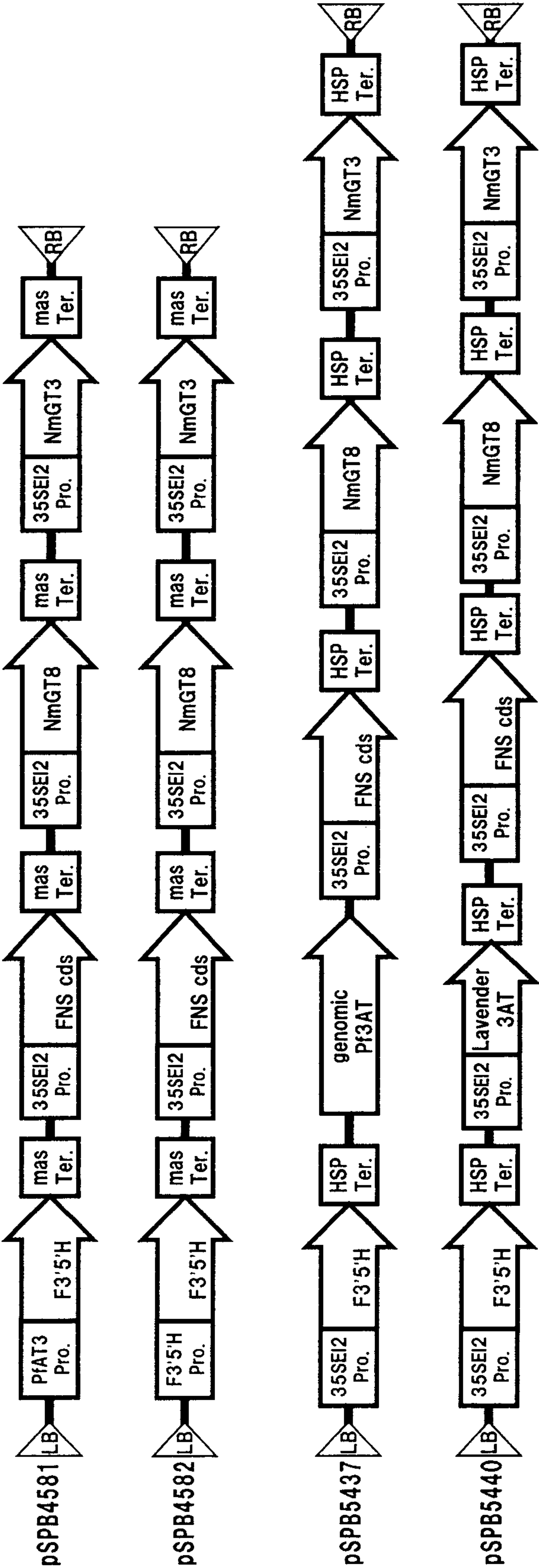
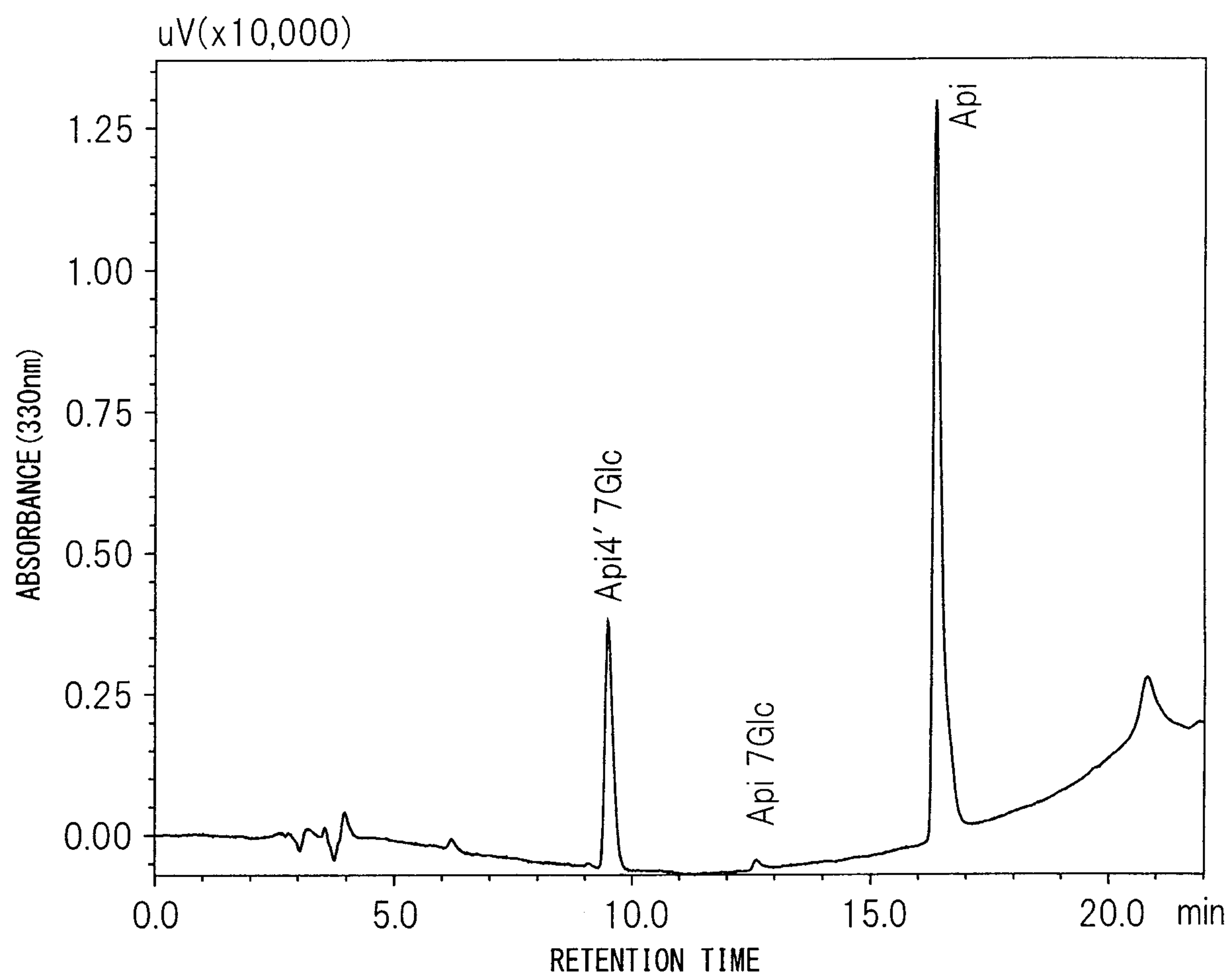


Fig.20



19/22

Fig.21



20/22

Fig.22

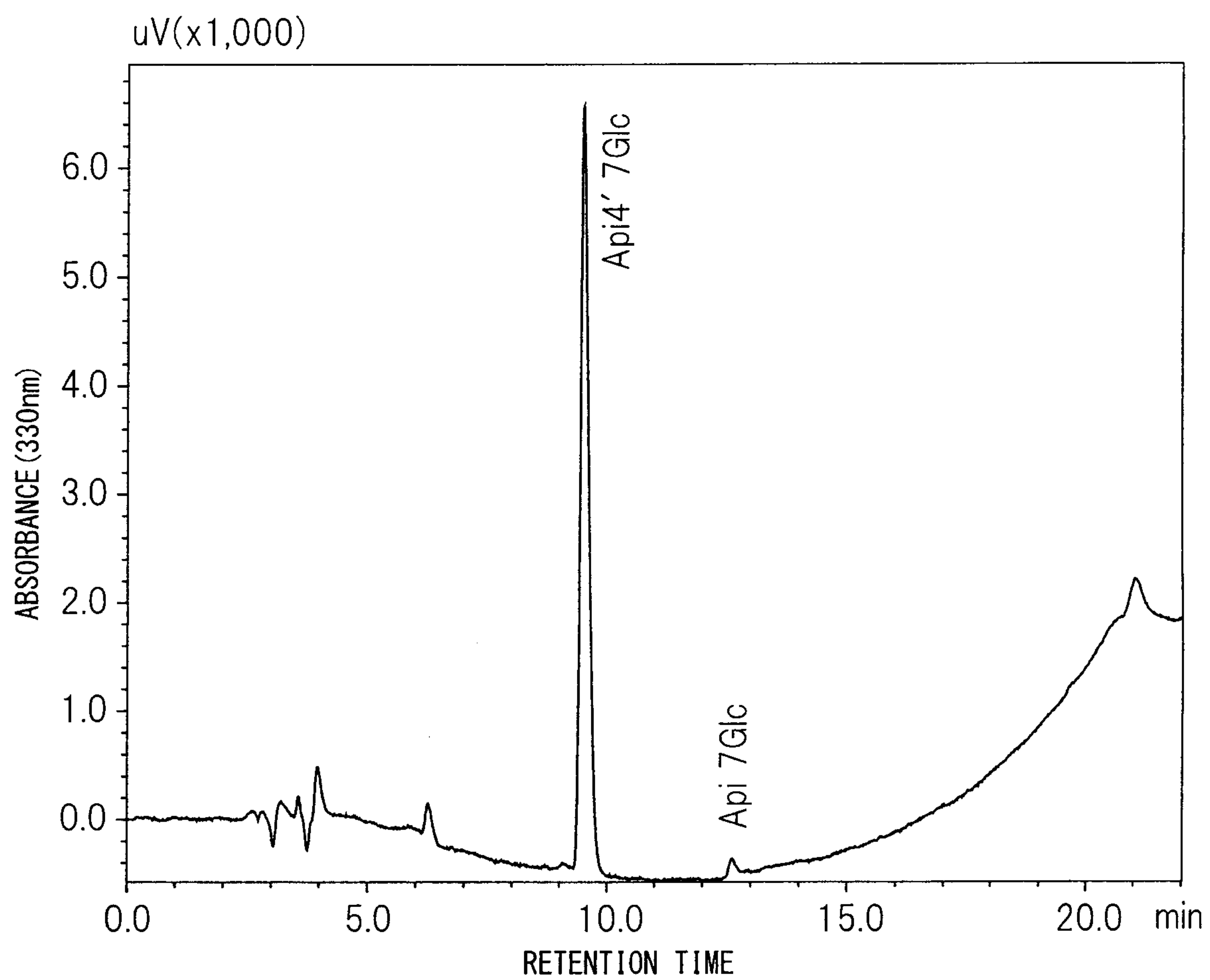


Fig.23

SuGTAK5 NmGT3	1	-	-	-	-	M	K	Q	L	H	I	A	L	F	P	S	M	A	H	G	H	M	I	P	M	F	E	L	A	K	L	F	T	S	R	G	L	K	A	T	I	I	A	T	P	A	F	A	A	P	I			
	1	M	P	S	I	L	S	N	S	A	H	I	L	L	F	P	F	P	T	S	G	H	I	I	P	I	L	D	L	A	N	Q	L	L	A	R	G	L	T	I	L	I	T	P	A	N	L	T	L	L				
		T	K	A	Q	Q	S	G	L	D	V	G	-	-	-	L	A	I	T	P	F	P	P	K	G	S	S	L	P	Q	N	I	I	A	F	D	Q	M	T	N	P	D	L	T	T	S	F	L	R	A	92			
		S	T	Q	L	I	E	L	D	R	L	G	S	L	H	T	L	V	L	P	F	P	-	N	P	P	N	P	S	E	T	S	L	A	A	R	V	H	A	S	S	Q	L	S	N	T	I	I	Q	W	99			
SuGTAK5 NmGT3	93	M	E	L	L	Q	E	P	V	E	A	I	L	Q	E	L	H	P	D	C	L	V	S	D	M	F	L	P	W	T	A	D	S	A	A	K	F	Q	I	P	R	L	A	F	Y	G	T	S	Y	F	S	R		
	100	F	Q	S	H	T	S	P	-	-	-	-	-	-	-	-	P	V	A	I	V	S	D	F	F	L	G	W	T	N	S	L	A	S	Q	L	G	I	P	R	L	V	F	W	P	S	G	V	Q	R	S			
		C	V	S	E	Q	V	G	D	K	P	-	-	-	-	-	F	N	N	V	T	S	D	S	E	P	V	L	V	P	G	L	P	Q	Q	I	K	F	V	R	S	Q	F	S	P	V	L	L	E	E	T	188		
		S	L	V	D	Y	I	W	Q	N	D	Q	L	S	D	S	D	H	Q	I	Q	D	N	S	V	I	S	F	P	D	V	P	N	S	P	A	Y	P	K	W	Q	A	C	G	L	S	T	Q	Y	K	190			
SuGTAK5 NmGT3	189	Q	N	D	-	F	A	K	L	F	K	Q	M	T	E	A	W	K	K	T	Y	G	E	V	V	N	S	F	N	E	L	E	S	D	Y	A	N	H	Y	K	N	V	I	G	R	-	K	A	W	E	I			
	191	K	G	D	P	S	W	E	F	F	K	N	G	V	L	A	N	T	Q	S	W	G	A	I	Y	N	S	F	R	D	L	E	G	V	Y	I	D	Y	I	K	K	M	G	H	G	R	V	W	A	V				
		G	P	L	L	L	C	S	S	K	G	G	E	K	N	Q	Q	R	G	K	E	S	A	I	D	E	H	E	C	L	A	W	L	D	S	K	N	-	-	P	N	S	V	V	Y	V	C	A	G	S	284			
		G	P	L	L	P	A	N	D	A	S	-	-	-	-	-	K	R	G	G	S	C	V	M	P	I	D	D	V	M	T	W	L	D	T	K	T	N	S	D	N	S	V	Y	V	C	F	G	S	284				
SuGTAK5 NmGT3	285	V	A	S	F	S	Q	A	Q	L	R	E	T	A	M	G	L	E	A	S	G	Q	N	F	V	W	V	V	R	K	N	K	E	D	D	D	D	W	L	P	V	G	F	E	E	R	V	G	N					
	285	R	V	E	L	T	T	E	Q	L	Q	L	D	S	L	A	A	L	E	I	S	G	V	H	F	I	L	C	V	K	L	H	Q	E	I	S	-	-	-	-	K	E	Y	E	D	R	V	A	G					
		R	G	L	I	I	R	G	W	A	P	Q	V	M	I	L	N	H	A	A	V	G	A	F	V	T	H	C	G	W	N	S	T	L	E	G	V	C	A	G	L	P	M	V	T	W	P	V	F	A	E	Q	F	384
		R	G	L	I	I	R	G	W	A	P	Q	V	A	I	L	R	H	R	A	V	G	A	F	L	T	H	C	G	W	N	S	I	L	E	G	I	A	A	G	V	V	M	L	T	W	P	M	G	A	D	Q	F	379
SuGTAK5 NmGT3	385	F	N	E	K	L	V	T	E	V	L	G	T	G	V	S	V	G	N	K	R	W	M	L	R	E	S	E	G	V	E	R	D	A	V	A	R	A	V	E	E	I	M	V	G	G	A	E	E					
	380	T	N	A	N	L	L	V	D	E	L	K	V	A	M	K	A	C	E	G	-	-	-	G	D	S	N	V	P	N	P	A	M	L	A	N	V	L	A	E	S	I	N	G	G	R	A	E	R					
		M	R	S	R	A	E	N	Y	K	E	M	A	K	K	A	V	E	E	G	-	G	S	S	Y	N	N	L	N	A	L	I	E	E	L	S	N	Y	V	A	P	T	I	Q	D	K	N	478						
	E	-	-	R	V	T	E	L	C	D	A	A	L	K	A	V	Q	S	G	N	G	S	S	A	K	D	L	D	S	L	T	N	Q	L	N	G	L	K	V	K	I	N	-	-	-	-	-	464						

22/22

Fig.24

SuGTAk5 NmGT4	1	-	M	K	Q	L	H	I	A	L	F	P	S	M	A	H	G	H	M	I	P	M	F	E	L	A	K	L	F	T	S	R	G	L	K	A	T	I	I	A	T	-	-	-	-	P	A	F	A	A	P	I		
	1	M	A	A	Q	L	H	V	V	F	F	P	F	M	A	Q	G	H	L	I	P	T	L	E	M	V	K	L	F	S	S	R	G	L	K	T	T	I	V	T	T	K	F	Y	A	P	A	V	T	K	S	I		
		T	K	A	Q	Q	S	G	L	D	V	G	L	A	I	T	P	F	P	P	K	G	S	S	L	P	Q	N	I	I	A	F	D	Q	M	T	N	P	D	L	T	T	S	F	L	R	A	M	E	L	95			
		E	K	T	K	H	T	G	N	Q	I	N	I	I	I	I	K	F	P	S	A	E	V	G	L	P	E	G	S	E	S	L	D	K	L	K	S	P	D	M	F	M	K	F	F	K	A	L	S	L	100			
SuGTAk5 NmGT4	96	L	Q	E	P	V	E	A	I	L	Q	E	L	H	P	D	C	L	V	S	D	M	F	L	P	W	T	A	D	S	A	A	K	F	Q	I	P	R	L	A	F	Y	G	T	S	Y	F	S	R	C	V	S	E	
	101	L	Q	E	P	F	E	Q	I	L	Q	E	L	S	P	D	C	I	V	S	D	M	F	F	P	W	T	T	A	S	A	A	K	F	D	I	P	R	F	V	F	H	G	L	S	L	F	A	L	C	V	S	E	
		Q	V	G	-	D	K	P	F	N	N	V	T	S	-	-	-	D	S	E	P	V	L	V	P	G	L	P	Q	Q	I	K	F	V	R	S	Q	F	S	P	V	L	L	E	E	T	Q	N	D	191				
		N	M	R	F	Y	K	P	F	N	L	G	S	E	S	L	D	S	E	P	V	M	L	P	D	F	P	N	Q	I	E	F	S	K	V	Q	V	P	E	F	E	V	G	E	S	K	N	E	200					
SuGTAk5 NmGT4	192	F	A	K	L	F	K	Q	M	T	E	A	W	K	K	T	Y	G	E	V	V	N	S	F	N	E	L	E	S	D	Y	A	N	H	Y	K	N	V	I	G	R	K	A	W	E	I	G	P	L	L	C			
	201	I	M	E	L	L	N	Q	V	K	E	S	E	V	K	S	Y	G	I	I	I	N	S	F	N	E	L	E	K	D	Y	V	D	Y	Y	R	N	V	W	G	R	R	A	W	L	L	G	P	L	S	S			
		S	S	S	K	G	G	E	K	N	Q	Q	R	G	K	E	S	A	I	D	E	H	E	C	L	A	W	L	D	S	K	N	P	N	S	V	V	Y	V	C	A	G	S	V	A	S	F	S	Q	A	291			
		-	-	-	-	-	-	-	-	-	N	R	D	D	E	V	K	D	Q	T	D	E	H	G	S	L	K	W	L	D	S	K	K	P	D	S	V	I	Y	V	C	F	G	S	V	A	P	L	S	S	292			
SuGTAk5 NmGT4	292	Q	L	R	E	T	A	M	G	L	E	A	S	G	Q	N	F	V	W	V	V	R	K	N	K	E	D	D	D	D	W	L	P	V	G	F	E	E	R	V	G	N	R	G	L	I	I	R	G	W	A	P	Q	V
	293	Q	L	H	E	I	A	L	G	L	E	S	S	G	Q	Q	F	I	W	V	V	K	E	-	R	E	D	G	E	K	W	L	P	E	G	F	E	E	R	I	K	D	K	G	L	I	I	R	G	W	A	P	Q	V
		M	I	L	N	H	A	A	V	G	A	F	V	T	H	C	G	W	N	S	T	L	E	G	V	C	A	G	L	P	M	V	T	W	P	V	F	A	E	Q	F	F	N	E	K	L	V	T	391					
		S	I	L	E	H	E	S	T	G	G	F	V	T	H	C	G	W	N	S	V	L	E	A	V	S	A	G	V	V	M	A	T	L	P	T	F	A	E	Q	P	F	F	N	E	K	L	L	T	391				
SuGTAk5 NmGT4	392	E	V	L	G	T	G	V	S	V	G	N	K	R	W	M	L	R	E	S	E	G	V	E	R	D	A	V	A	R	A	V	E	E	I	M	V	G	G	A	E	E	M	R	S	R	A	E	N	Y				
	392	K	V	L	K	I	G	I	P	I	G	S	P	-	L	S	N	R	G	K	S	G	V	K	K	E	E	I	A	E	A	M	K	G	I	M	E	G	E	A	L	E	M	R	I	R	A	K	S	L				
		K	E	M	A	K	K	A	V	E	E	G	G	S	S	Y	N	N	L	N	A	L	I	E	E	L	S	N	Y	V	A	P	T	I	Q	D	K	N	478															
		K	E	M	A	W	K	A	V	E	E	G	G	S	S	Y	N	D	L	T	S	L	I	D	G	V	K	A	Y	R	S	Q	S	N	K	I	-	-	475															