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(54) Titre : PROCEDE DE PRODUCTION D'UNE ROSE AVEC DES PETALES DE COULEUR MODIFIEE
(54) Title: METHOD FOR PRODUCING ROSE WITH ALTERED PETAL COLORS

(57) **Abrégé/Abstract:**

The present application provides a method for producing a rose. The method broadly comprises the steps of: (a) suppressing the rose flavonoid synthesis pathway by (i) suppressing the expression of a rose endogenous dihydroflavonol reductase or (ii) suppressing the expression of a rose endogenous flavonoid 3'-hydroxylase; and (b) expressing a pansy gene coding for a flavonoid 3',5'-hydroxylase and having the nucleotide sequence of SEQ ID NO:1 or 3. Also provided are related rose, rose cell, rose seed and uses thereof.



ABSTRACT

The present application provides a method for producing a rose. The method broadly comprises the steps of: (a) suppressing the rose flavonoid synthesis pathway by (i) suppressing the expression of a rose endogenous dihydroflavonol reductase or (ii) suppressing the expression of a rose endogenous flavonoid 3'-hydroxylase; and (b) expressing a pansy gene coding for a flavonoid 3',5'-hydroxylase and having the nucleotide sequence of SEQ ID NO:1 or 3. Also provided are related rose, rose cell, rose seed and uses thereof.

DESCRIPTION

METHOD FOR PRODUCING ROSE WITH ALTERED PETAL COLORS5 Technical Field

 The present invention relates to a new method for producing a rose with altered petal colors. More specifically, it relates to a method for producing a rose by artificially inhibiting the endogenous metabolic pathway of rose, and expressing the gene coding for pansy flavonoid 3',5'-hydroxylase and the gene coding for dihydroflavonol reductase which reduces dihydromyricetin.

15 Background Art

 Flower petals perform the role of attracting pollinators such as insects and birds, which transport plant pollen, and therefore flower colors, shapes, patterns and odors have evolved in tandem with pollinators (Honda, T. et al., Gendai Kagaku, May, 25-32(1998)). Probably as a result of this, it is rare for a single species of flower to exhibit several different colors, and for example, rose or carnation varieties exhibiting violet to blue colors do not exist, while iris or gentian varieties exhibiting bright red colors do not exist. Because flower color is the most important aspect of petals for purposes of appreciation as well, flowers of different colors have traditionally been bred by crossbreeding. The rose, known as the "queen of flowers" and having high commercial value, has also been crossbred throughout the world.

 For example, the current yellow rose cultivar was created by crossbreeding of *Rosa foetida*, originating from western Asia, with a non-yellow rose variety. However, because flower color is determined by the genetic capacity of the plant, there has been a limit to the flower colors that can currently be produced in cross-bred strains whose available genetic sources are

restricted (Tanaka et al. Plant Cell Physiol. 39, 1119-1126, 1998; Mol et al. Curr. Opinion Biotechnol. 10, 198-201 1999). Among these, the cultivation of blue roses has been thought impossible and has been considered the
5 "holy grail" of colors (Oba, H., "Bara no Tanjo", 1997, Chukoshinsho; Suzuki, M., "Shokubutsu Bio no Mahou: Aoi Bara mo Yume dewanakunatta", 1990, Kodansha Bluebacks; Saisho, H., "Aoi Bara", 2001, Shogakkan).

Although "blue rose" varieties currently exist,
10 these are actually pale violet roses. The first improved variety of "blue rose" by crossbreeding is said to have been the light-violet shaded grey-colored "Grey Pearl" created in 1945. The light-violet pink-colored "Staring Silver" was later created in 1957, and these varieties
15 were crossed to produce several pale violet roses such as "Blue Moon" (1964) and "Madam Violet" (1981). These pale violet roses and other roses were then utilized in further breeding to create light-grey-colored roses such as "Seiryu" (1992) and "Blue Heaven" (2002), which were
20 hailed as new types of "blue roses".

However, these flower colors are not actually blue but merely greyish-dull pink, and despite many years of breeding efforts, there is still no example of a truly "blue" rose. In horticultural industry, the group of
25 colors from violet to blue is generally considered "blue" according to the RHSCC (The Royal Horticultural Society Colour Chart). It is an aim of the present invention to create rose plants having flower colors falling within the "violet group", "violet-blue" group and "blue group"
30 according to the Royal Horticultural Society Colour Chart.

Flower colors derive mainly from the three compound groups of anthocyanins, carotenoids and betalains, but it is the anthocyanins, having the widest absorption
35 wavelength range (from orange to blue), that are responsible for blue color. Anthocyanins belong to the flavonoid family and are biosynthesized by the metabolic

pathway shown in Fig. 1. Anthocyanins are normally localized in the vacuoles of epithelial cells. The color shade of anthocyanins (i.e. flower color) depends largely on the structure of the anthocyanins, with more numerous hydroxyl groups on the B ring resulting in a bluer color. Hydroxylation of the B ring is catalyzed by flavonoid 3'-hydroxylase (F3'H) and flavonoid 3',5'-hydroxylase (F3'5'H). Absence of F3'H and F3'5'H activity leads to synthesis of pelargonidin (orange to red colors), presence of F3'H activity leads to synthesis of cyanidin (red to rouge colors) and presence of F3'5'H activity leads to synthesis of delphinidin (violet color).

These anthocyanidins are modified with sugars and acyl groups to produce an assortment of anthocyanins. Generally speaking, a larger number of modifying aromatic acyl groups correlates to bluer anthocyanins. Anthocyanins also produce quite different colors depending on the vacuole pH and the copresent flavonols and flavones or metal ions (Saito, N., Tanpakushitsu Kakusan Kouso, 47 202-209, 2002; Broullard and Dangles, In the flavonoids: Advances in Research since 1986 (Ed. by Harborne) Capmann and Hall, London pp.565-588; Tanaka et al. Plant Cell Physiol. 39 1119-1126, 1998; Mol et al., Trends in Plant Science 3, 212-217, 1998; Mol et al., Curr. Opinion Biotechnol. 10, 198-201 1999).

Rose flower petal anthocyanins are derivatives of pelargonidin, cyanidin and peonidin, whereas no delphinidin derivatives are known (Biolley and May, J. Experimental Botany, 44, 1725-1734 1993; Mikanagi Y., Saito N., Yokoi M. and Tatsuzawa F. (2000) Biochem. Systematics Ecol. 28:887-902). This is considered to be the main reason for the lack of blue roses. Existing roses have been created by crossbreeding of crossable related rose species (*R. multiflora*, *R. chinensis*, *R. gigantea*, *R. moschata*, *R. gallica*, *R. wichuraiana*, *R. foetida*, etc.).

The fact that no blue rose has been achieved in

spite of repeated efforts at crossbreeding is attributed to the lack of delphinidin production ability by rose-related varieties. Production of delphinidin in rose petals would require expression of F3'5'H in the petals as mentioned above, but F3'5'H is believed to be non-expressed in the petals of rose and rose-related varieties. Thus, it is likely impossible to obtain a blue rose by accumulating delphinidin in the petals through crossbreeding. It is known that trace amounts of the blue pigment rosacyanin are found in rose petals and its chemical structure has been determined (Japanese Unexamined Patent Publication No. 2002-201372), but no reports are known regarding augmentation of rosacyanin to create a blue rose, and no findings have been published on the rosacyanin biosynthesis pathway or the relevant enzymes or genes.

Examples of blue or violet colors produced by biological organisms also include indigo plant-produced indigo (for example, Appl. Microbiol. Biotechnol. Feb. 2003, 60(6):720-5) and microbially-produced violacein (J. Mol. Microbiol. Biotechnol. Oct. 2000 2(4):513-9; Org. Lett., Vol.3, No.13, 2001, 1981-1984), and their derivation from tryptophan and their biosynthetic pathways have been studied.

Blue pigments based on gardenia fruit-derived iridoid compounds (S. Fujikawa, Y. Fukui, K. Koga, T. Iwashita, H. Komura, K. Nomoto, (1987) Structure of genipocyanin G1, a spontaneous reaction product between genipin and glycine. Tetrahedron Lett. 28 (40), 4699-700; S. Fujikawa, Y. Fukui, K. Koga, J. Kumada, (1987), Brilliant skyblue pigment formation from gardenia fruits, J. Ferment. Technol. 65 (4), 419-24) and lichen-derived azulenes (Wako Pure Chemical Industries Co., Ltd.) are also known, but no reports are known of expressing these in plant flower petals to produce blue-colored flowers.

It has been expected that a blue rose could be created by transferring the F3'5'H gene expressed by

other plants into rose and expressing it in rose petals (Saisho, H., "Aoi Bara", 2001, Shogakkan). The F3'5'H gene has been obtained from several plants including petunia, gentian and *Eustoma russellianum* (Holton et al. Nature 366, 276-279, 1993; Tanaka et al. Plan Cell Physiol. 37, 711-716 1996; WO93/18155). There are also reports of transformed varieties of rose (for example, Firoozababy et al. Bio/Technology 12:609-613 (1994); US 5480789; US 5792927; EP 536,327 A1; US 20010007157 A1).

Actual transfer of the petunia F3'5'H gene into rose has also been reported (WO93/18155, WO94/28140).

However, it has not been possible to obtain a blue rose, and it is believed that obtaining a blue rose will require a modification which alters the metabolism of flower pigments suited for rose.

On the other hand, it has been confirmed that transfer of the F3'5'H gene into red carnation, which produces pelargonidin instead of delphinidin, leads to accumulation of both pelargonidin and delphinidin, but that the flower color is only altered to a slightly purplish red (WO94/28140). This result suggests that it is not possible to obtain a "blue" carnation simply by expression of F3'5'H, and that it is necessary to inhibit the metabolic pathway to endogenous synthesis of pelargonidin by carnation.

In order to avoid competition with the carnation endogenous metabolic pathway (reduction of dihydrokaempferol (DHK) by dihydroflavonol reductase (DFR)), a variety lacking DFR was selected from among white carnations. The F3'5'H gene and petunia DFR (which is known to efficiently reduce dihydromyricetin (DHM) without reducing DHK) gene were transferred into carnation. This resulted in one case of successfully obtaining a recombinant carnation with a delphinidin content of about 100% and a blue-violet flower color previously not found in carnation (Tanpakushitsu Kakusan Kouso, Vol.47, No.3, p225, 2002). Thus, further

modification was necessary to realize a blue carnation flower, in addition to accumulating delphinidin by expression of the F3'5'H gene.

DFR has already been cloned from several plants
5 (petunia, tobacco, rose, *Torenia*, snapdragon, transvaal
daisy, orchid, barley, corn, etc.) (Meyer et al., Nature
330, 677-678, 1987; Helariutta et al., Plant Mol. Biol.
22, 183-193 1993; Tanaka et al., Plant Cell Physiol. 36,
1023-1031; Johnson et al., Plant J. 19, 81-85, 1999).
10 Substrate specificity of the DFR gene differs depending
on the plant variety, and it is known that the petunia,
tobacco and orchid DFR genes cannot reduce DHK, whereas
the petunia DFR gene most efficiently reduces DHM among
the dihydroflavonols (Forkmann et al., Z. Naturforsch.
15 42c, 1146-1148, 1987; Johnson et al. Plant J. 19, 81-85,
1999). Nevertheless, no cases have been reported for
expression of these DFR genes in rose.

As a means of avoiding competition with the
endogenous metabolic pathway or between the enzyme and
20 the exogenous gene-derived enzyme such as F3'5'H, as
mentioned above, the gene may be transferred into a
variety lacking the gene. Also, it is known that
expression of the target gene can be artificially
inhibited by deletion methods involving homologous
25 recombination of the target gene, but because of the low
frequency of homologous recombination and the limited
number of suitable plant varieties, this has not been
implemented in practice (for example, Nat. Biotechnol.
2002, 20:1030-4).

30 Inhibition methods on the transcription level
include the antisense method using antisense RNA
transcripts for mRNA of the target gene (van der Krol et
al., Nature 333, 866-869, 1988), the sense
(cosuppression) method using transcripts of RNA
35 equivalent to mRNA of the target gene (Napoli et al.,
Plant Cell 2, 279-289, 1990) and a method of using duplex
RNA transcripts corresponding to mRNA of the target gene

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(RNAi method; Waterhouse et al., Pro. Natl. Acad. Sci. USA 95, 13959-13964, 1998).

Numerous successful examples of these three methods have been published. For rose, cosuppression of chalcone synthase (CHS) gene which is necessary for synthesis of anthocyanins was reported to successfully alter flower color from red to pink (Gutterson HortScience 30:964-966 1995), but this CHS suppression is incomplete and therefore it has not been possible to totally suppress anthocyanin synthesis to obtain a white flower stock.

Patent document 1: Japanese Unexamined Patent Publication No. 2002-201372

Patent document 2: WO93/18155

Patent document 3: USP 5480789

Patent document 4: USP 5792927

Patent document 5: EP 536 327 A1

Patent document 6: US 20010007157 A1

Patent document 7: WO94/28140

Non-patent document 1: Honda T. et al. Gendai Kagaku, May, 25-32(1998)

Non-patent document 2: Tanaka et al. Plant Cell Physiol. 39, 1119-1126, 1998

Non-patent document 3: Mol et al. Curr. Opinion Biotechnol. 10, 198-201 1999

Non-patent document 4: Oba, H., "Bara no Tanjo", 1997, Chukoshinsho

Non-patent document 5: Suzuki, M., "Shokubutsu Bio no Mahou: Aoi Bara mo Yume dewanakunatta", 1990, Kodansha Bluebacks

Non-patent document 6: Saisho, H., "Aoi Bara", 2001, Shogakkan

Non-patent document 7: Saito, N., Tanpakushitsu Kakusan Kousho, 47 202-209, 2002

Non-patent document 8: Brouillard et al. In the flavonoids: Advances in Research since 1986 (Ed by Harborne) Capmann and Hall, London pp565-588

Non-patent document 9: Tanaka et al. Plant Cell

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Physiol. 39 1119-1126, 1998

Non-patent document 10: Mol et al, Trends in Plant Science 3, 212-217 1998

5 Non-patent document 11: Mol et al. Curr. Opinion Biotechnol. 10, 198-201 1999

Non-patent document 12: Biolley and May, J. Experimental Botany, 44, 1725-1734 1993

Non-patent document 13: Mikanagi Y, et al. (2000) Biochem Systematics Ecol. 28:887-902

10 Non-patent document 14: Appl. Microbiol. Biotechnol. 2003 Feb;60(6):720-5

Non-patent document 15: J. Mol. Microbiol. Biotechnol. 2000 Oct; 2 (4): 513-9

15 Non-patent document 16: Org. Lett., Vol. 3, No. 13, 2001, 1981-1984

Non-patent document 17: S. Fujikawa, et al. (1987) Tetrahedron Lett. 28 (40), 4699-700

Non-patent document 18: S. Fujikawa, et al. (1987) J. Ferment. Technol. 65 (4), 419-24

20 Non-patent document 19: Holton et al. Nature 366, 276-279, 1993

Non-patent document 20: Tanaka et al. Plant Cell Physiol. 37, 711-716 1996

25 Non-patent document 21: Firoozababy et al. Bio/Technology 12:609-613 (1994)

Non-patent document 22: Tanpakushitsu Kakusan Kouso, Vol.47, No.3, p225, 2002

Non-patent document 23: Meyer et al. Nature 330, 677-678, 1987

30 Non-patent document 24: Helariutta et al. Plant Mol. Biol. 22 183-193 1993

Non-patent document 25: Tanaka et al. Plant Cell Physiol. 36, 1023-1031

35 Non-patent document 26: Johnson et al. Plant J. 19, 81-85, 1999

Non-patent document 27: Forkmann et al. Z. Naturforsch. 42c, 1146-1148, 1987

Non-patent document 28: Nat Biotechnol 2002,
20:1030-4

Non-patent document 29: van der Krol et al. Nature
333, 866-869, 1988

5 Non-patent document 30: Napoli et al. Plant Cell 2,
279-289, 1990

Non-patent document 31: Waterhouse et al. Pro. Natl.
Acad. Sci. USA 95, 13959-13964 1998

10 Non-patent document 32: Gutterson HortScience
30:964-966 1995

Non-patent document 33: Suzuki, S., "Bara,
Hanazufu", Shogakkann, p.256-260, 1990

Disclosure of the Invention

15 As mentioned above, rose flower colors have been
successfully altered by transferring the F3'5'H gene into
rose and expressing it in the petals. In carnation, the
F3'5'H gene and petunia DFR gene have been expressed in
DFR-deficient varieties to create blue-violet carnations.
20 However, a "blue rose" has not yet been created. It is
therefore an object of the present invention to provide a
rose which blossoms with a blue flower.

The invention thus provides (1) a method for
producing a rose characterized by artificially
25 suppressing the rose endogenous metabolic pathway and
expressing the pansy gene coding for flavonoid 3',5'-
hydroxylase.

The invention further provides (2) a method for
producing a rose characterized by artificially
30 suppressing the rose endogenous metabolic pathway, and
expressing the pansy gene coding for flavonoid 3',5'-
hydroxylase and the gene coding for dihydroflavonol
reductase.

35 The invention still further provides (3) a method
for producing a rose characterized by artificially
suppressing expression of rose endogenous dihydroflavonol
reductase, and expressing the pansy gene coding for

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flavonoid 3',5'-hydroxylase and the gene coding for dihydroflavonol reductase derived from a plant other than rose.

5 The invention still further provides (4) a method for producing a rose characterized by artificially suppressing expression of rose endogenous flavonoid 3'-hydroxylase and expressing the pansy gene coding for flavonoid 3',5'-hydroxylase.

10 The aforementioned pansy gene coding for flavonoid 3',5'-hydroxylase is, for example, the gene listed as SEQ ID NO: 1 or SEQ ID NO: 3. The gene coding for dihydroflavonol reductase is preferably derived from iris, *Nierembergia*, petunia, orchid, gentian or *Eustoma russellianum*.

15 The invention still further provides (5) a rose obtained by the production method according to any one of (1) to (4) above, or a progeny or tissue thereof having the same properties as the rose.

20 The invention still further provides (6) a rose obtained by the production method according to any one of (1) to (4) above, or a progeny or tissue thereof, wherein the petal color of the rose is violet, blue-violet or blue.

25 The invention further provides (7) a rose according to (6) above, or a progeny or tissue thereof, wherein the petal color of the rose belongs to the "Violet group", "Violet-Blue" group or "Blue group" according to the Royal Horticultural Society Colour Chart (RHSCC).

30 The invention further provides (8) a rose according to (7) above, or a progeny or tissue thereof, wherein the petal color of the rose belongs to "Violet group" 85a or 85b according to the Royal Horticultural Society Colour Chart (RHSCC).

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Statement of Invention

According to one aspect of the present invention,
there is provided a method for producing a rose
5 comprising the steps of:

- (a) suppressing the rose flavonoid synthesis
pathway by (i) suppressing the expression of a
rose endogenous dihydroflavonol reductase or
(ii) suppressing the expression of a rose
10 endogenous flavonoid 3'-hydroxylase; and
expressing a pansy gene coding for a flavonoid
3',5'-hydroxylase and having the nucleotide sequence of
SEQ ID NO:1 or 3.

According to another aspect of the present
15 invention, there is provided the use of the rose cell
obtained by the method described herein for providing a
violet color to a petal.

According to still another aspect of the present
invention, there is provided the use described herein,
20 wherein the violet color belongs to the "Violet group"
according to the Royal Horticultural Society Colour
Chart (RHSCC).

According to yet another aspect of the present
invention, there is provided the use described herein,
25 wherein the violet color belongs to "Violet group" 85a or
85b according to the Royal Horticultural Society Colour
Chart (RHSCC).

According to a further aspect of the present
invention, there is provided the use of the rose seed
30 cell obtained by the method described herein for
generating a rose having a petal of a violet color.

According to yet a further aspect of the present
invention, there is provided the use described herein,
wherein the violet color belongs to the "Violet group"
35 according to the Royal Horticultural Society Colour
Chart (RHSCC).

According to still a further aspect of the present

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invention, there is provided the use described herein, wherein the violet color belongs to "Violet group" 85a or 85b according to the Royal Horticultural Society Colour Chart (RHSCC).

5

Brief Description of the Drawings

Fig. 1 shows the flavonoid biosynthesis pathway.

CHS: Chalcone synthase, CHI: Chalcone isomerase

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FNS: Flavone synthase, F3H: Flavanone 3-hydroxylase

F3'H: Flavonoid 3'-hydroxylase

F3'5'H: Flavonoid 3'5'-hydroxylase, FLS: Flavonol
synthase

5 DFR: Dihydroflavonol 4-reductase

ANS: Anthocyanidin synthase, AS: Aurone synthase

C2'GT: Chalcone 2'-glucosyl transferase

Fig. 2 shows the structure of plasmid pBERD1.

Fig. 3 shows the structure of plasmid pBPDBP2.

10 Fig. 4 shows the structure of plasmid pBPDBP8.

Fig. 5 shows the structure of plasmid pSPB461.

Fig. 6 shows the structure of plasmid pSPB472.

Fig. 7 shows the structure of plasmid pSPB130.

Fig. 8 shows the structure of plasmid pSPB919.

15 Fig. 9 shows the structure of plasmid pSPB920.

Fig. 10 shows the structure of plasmid pSPB1106.

Best Mode for Carrying Out the Invention

Several reasons may be postulated for a lack of blue
20 color in rose even with production of delphinidin. The
stability, solubility and color of anthocyanins varies
depending on modification with acyl groups and sugars.
Specifically, it is known that an increased number of
aromatic acyl groups results in greater blueness. Also,
25 formation of complexes between flavonol and flavone
copigments and anthocyanins produce a blue color and
shift the maximum absorption wavelength toward the longer
wavelength end while also increasing the absorbance.
Anthocyanin color is also dependent on pH. Since a lower
30 pH tends toward redness and a more neutral pH produces
blueness, the flower color depends on the pH of the
vacuoles in which the anthocyanins are localized. In
addition, formation of metal chelates in the copresence
of metal ions such as Al^{3+} and Mg^{2+} can significantly
35 affect flower color as well. Trial and error and
assiduous research led to the proposal for a modification
whereby the proportion of delphinidin in flower petals is

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increased.

First, it was attempted to create a blue rose by the same method used to create a blue-violet carnation. Specifically, it was attempted to analyze white rose variety 112 and identify a DFR-deficient line, but unlike carnation, no completely DFR-deficient line could be obtained. This is presumably due to the fact that carnation is diploid while ordinarily cultivated rose is tetraploid, such that it is difficult to find a line deficient in a single gene.

Next, the pansy F3'5'H gene and petunia DFR gene were transferred into the white flower variety Tineke and accumulation of delphinidin was detected, but the amount was minimal and a blue rose was not obtained.

According to the present invention, the DFR gene, an enzyme participating in the rose endogenous flavonoid synthesis pathway, is artificially suppressed by a gene engineering technique, and the pansy F3'5'H gene is expressed while a dihydromyricetin-reducing DFR gene is also expressed, in order to increase the delphinidin content to roughly 80-100% of the total anthocyanidins in the flower petals, thereby allowing realization of a blue rose.

The dihydromyricetin-reducing DFR genes used in this case were derived from iris (*Iridaceae*), *Nierembergia* (*Solanaceae*) and petunia (*Solanaceae*), but as other dihydromyricetin-reducing DFR gene sources there may be mentioned non-pelargonidin-accumulating plants such as tobacco (*Solanaceae*), cyclamen (*Primulaceae*), delphinium (*Ranunculaceae*), orchid (*Orchidaceae*), gentian (*Gentianaceae*), *Eustoma russellianum* (*Gentianaceae*) and the like (Forkmann 1991, Plant Breeding 106, 1-26; Johnson et al., Plant J. 1999, 19, 81-85). The DFR genes used for the present invention are genes that preferentially reduce dihydromyricetin.

According to the invention, the flavonoid 3'-hydroxylase (F3'H) gene, an enzyme participating in the

rose endogenous flavonoid synthesis pathway, is
artificially suppressed by a gene engineering technique,
and the pansy F3'5'H gene is expressed, in order to
increase the delphinidin content to roughly 80-100% of
5 the total anthocyanidins in the flower petals, thereby
allowing realization of a blue rose.

The roses obtained according to the invention have
hitherto non-existent flower colors, and the invention
can provide roses with flower colors belonging not only
10 to the red-purple group, purple group and purple-violet
group but also to the violet group, violet-blue group and
blue group, according to the Royal Horticultural Society
Colour Chart.

15 Examples

The present invention will now be explained in
greater detail by the following examples. Unless
otherwise specified, the molecular biological protocols
used were based on Molecular Cloning (Sambrook and
20 Russell, 2001, Cold Spring Harbor Laboratory Press, Cold
Spring Harbor, New York).

Example 1. Flower color measuring method

The flower petal color shade was evaluated by
measurement using a CM2022 spectrophotometric colorimeter
25 (Minolta Japan) with a 10° visual field and a D65 light
source, and analysis using SpectraMagic color control
software (Minolta Japan). The Royal Horticultural
Society Colour Chart (RHSCC) number is the nearest color
as compared against Color Classification System Version
30 2.1.1 (The Japan Research Institute Co., Ltd.; Japanese
Unexamined Patent Publication No. 2002-016935), based on
the color value (CIE L*a*b* color system) obtained by
visual discrimination and measurement with the device
mentioned above. This system may be used for objective
35 selection of the nearest RHSCC number.

Upon measuring the color shades of flower petals of
cultivars conventionally referred to as "blue roses" and

determining the nearest colors according to the RHSCC by this method, it was determined that Blue Moon and Madam Violet were 186d (Greyed-Purple group), Lavande was 186c (Greyed-Purple group), Seiryu was 189d (Greyed-Green group) and Blue Heaven was 198d (Greyed-Green group). These cultivars are called blue roses but are classified in "Grey" groups according to RHSCC number and therefore do not exhibit the blue color which is the object of the present invention.

Example 2. Flavonoid analysis

1) Extraction of flower petal color

A 0.5 g portion of freeze-dried rose petals was subjected to extraction in 4 ml of 50% acetonitrile (CH_3CN) containing 0.1% TFA for 20 minutes under ultrasonic vibration and then filtered with a 0.45 μm filter. High-performance liquid chromatography (HPLC) of the anthocyanins in the extract was conducted under the following conditions. Isocratic elution was carried out using an RSpak DE-413L (4.6 mm ϕ x 25 cm, Shoko Co., Ltd.) column with a flow rate of 0.6 ml/min, and a mobile phase at a linear concentration gradient of 10% $\rightarrow$ 50% $\text{CH}_3\text{CN}/\text{H}_2\text{O}$ containing 0.5% trifluoroacetic acid (TFA) for 15 minutes followed by 50% $\text{CH}_3\text{CN}/\text{H}_2\text{O}$ containing 0.5% TFA for 10 minutes. Detection was performed using an SPD-M10A photodiode array detector (Shimadzu Laboratories), with detection in the wavelength range of 600-250 nm and calculation of the abundance ratio of each anthocyanin based on the 520 nm absorbance area.

2) Anthocyanidin analysis

A 0.2 ml portion of the filtrate was dried completely under reduced pressure in a glass test tube and dissolved in 0.2 ml of 6N hydrochloric acid (HCl), and subjected to hydrolysis at 100°C for 20 minutes. The hydrolyzed anthocyanidins were extracted with 0.2 ml of 1-pentanol, and the organic layer was analyzed by HPLC

under the following conditions. The column used was an ODS-A312 (6 mm ϕ x 15 cm, YMC Co., Ltd.), and elution was performed at a flow rate of 1 ml/min using a CH₃COOH:CH₃OH:H₂O = 15:20:65 solution as the mobile phase.

5 Detection was performed by spectral measurement at 600-400 nm using an SPD-M10A photodiode array detector (Shimadzu Laboratories), identification based on absorption maximum ($\lambda_{\max}$) and retention time (RT), and quantitation based on 520 nm absorbance area. The
10 retention time and $\lambda_{\max}$ of delphinidin and cyanidin under these HPLC conditions were 4.0 min, 5.2 min and 534 nm, 525 nm, respectively. Delphinidin hydrochloride and cyanidin hydrochloride purchased from Funakoshi Co., Ltd. were used as samples for identification and quantitation.

15 3) Flavonol analysis

 A 0.2 ml portion of the flower petal-extracted filtrate was dried to hardness under reduced pressure in a 1.5 ml Eppendorf tube and dissolved in 0.2 ml of 0.1 M potassium phosphate buffer (KPB) at pH 4.5, and then 6
20 units of β -glucosidase (Shinnihon Kagaku Co., Ltd.) and 1 unit of naringenase (Sigma Chemical Co., MO, USA) were added and the mixture was kept at 30°C for 16 hours. After the reaction, 0.2 ml of 90% CH₃CN was added to the enzyme reaction solution to terminate the reaction. The
25 solution was filtered with a 0.45 μ m filter and subjected to HPLC under the following conditions.

 Isocratic elution was carried out using a Develosil C30-UG-5 (4.6 mm ϕ x 15 cm, Nomura Chemical Co., Ltd.) column with a flow rate of 0.6 ml/min, and a mobile phase
30 at a linear concentration gradient of 18% $\rightarrow$ 63% CH₃CN/H₂O containing 0.1% TFA for 10 minutes followed by 63% CH₃CN/H₂O containing 0.1% TFA for 10 minutes. Detection was performed using an SPD-M10A photodiode array detector, with detection in the wavelength range of 400-
35 250 nm. The R.T. and $\lambda_{\max}$ of kaempferol and quercetin under these conditions were 11.6 min, 365 nm and 10.3

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min, 370 nm, respectively. Kaempferol and quercetin purchased from Funakoshi Co., Ltd. were used as samples for quantitation based on the A330 nm area.

Example 3. pH measurement method

5 Approximately 2 g of rose petals frozen at -80°C for 1 hour or longer was pressed with a homogenizer to obtain the petal juice. The pH was measured by connecting a 6069-10C microelectrode (Horiba Laboratories) to a pH meter (F-22, Horiba Laboratories).

10 Example 4. Transformation of rose

 Several methods have been reported for transformation of roses (for example, Firoozababy et al. Bio/Technology 12:609-613 (1994); US 5480789; US 5792927; EP 536,327 A1; US 20010007157 A1), and transformation may
15 be carried out by any of these techniques. Specifically, rose calli taken from aseptic seedling leaves were immersed for 5 minutes in a bacterial suspension of *Agrobacterium tumefaciens* Ag10 (Lazo et al., Bio/Technology 9:963-967, 1991), the excess bacterial
20 suspension was wiped off with sterile filter paper, and the calli were transferred to subculturing medium and cocultivated for 2 days in a dark room.

 After subsequently rinsing with MS liquid medium containing 400 mg/L carbenicillin, the calli were
25 transferred to selection/elimination medium prepared by adding 50 mg/L kanamycin and 200 mg/L carbenicillin to subculturing medium. Upon repeating transfer and cultivation of the portions which grew normally in selection medium without growth inhibition, the
30 kanamycin-resistant calli were selected out. The kanamycin-resistant transformed calli were cultivated in redifferentiation medium containing 50 mg/L kanamycin and 200 mg/L carbenicillin to obtain kanamycin-resistant
35 shoots. The obtained shoots were rooted in 1/2MS medium and then habituated. The habituated plants were potted and then cultivated in a closed greenhouse until blooming.

Example 5. Obtaining rose flavonoid gene

A cDNA library derived from Kardinal rose variety flower petals was screened using the petunia DFR gene (described in WO96/36716) as the probe, to obtain rose DFR cDNA which was designated as pCGP645. The details have already been reported (Tanaka et al., Plant Cell Physiol. 36, 1023-1031 1995).

Likewise, the same library was screened with the petunia chalcone synthase-A (CHS-A) gene (Koes et al., Gene (1989) 81, 245-257) and the anthocyanidin synthase (ANS) gene (Martin et al., Plant J., (1991) 1, 37-49) according to a publicly known procedure (Tanaka et al., Plant Cell Physiol. 36, 1023-1031 1995), to obtain rose chalcone synthase (CHS) and anthocyanidin synthase (ANS) homologs which were designated as pCGP634 and pCGP1375, respectively. The nucleotide sequence for rose CHS is listed as SEQ ID NO: 5, and the nucleotide sequence for rose ANS is listed as SEQ ID NO: 6.

Example 6. Screening for white rose

For creation of a blue cultivar by gene recombination, cultivars lacking only the DFR gene may be selected, in order to avoid competition between the endogenous anthocyanin synthesis pathway and the introduced genes (particularly the F3'5'H gene), and the petunia DFR gene and F3'5H gene transferred into those cultivars (WO96/36716).

A screening was conducted among the numerous existing white rose varieties, for those lacking only the DFR gene and normally expressing other anthocyanin biosynthesis enzyme genes. The cause of flower color whitening is believed to be occasional mutation or deletion of structural genes involved in anthocyanin biosynthesis, and occasional loss of transcription regulating factors which control transcription of structural genes involved in anthocyanin biosynthesis. Roses lacking DFR gene mRNA were examined according to the method described in WO96/36716.

First, 112 primarily white rose lines were analyzed for flavonoid composition of the flower petals by the method described in Example 1, and lines with high accumulation of flavonols were selected. The pH of each petal juice was then measured and 80 cultivars with relatively high pH values were chosen as primary candidates.

RNA was then extracted from petals of these cultivars. The RNA extraction was accomplished by a publicly known method (Tanaka et al., Plant Cell Physiol. 36, 1023-1031, 1995). The obtained RNA was used to examine the presence or absence of mRNA corresponding to the rose DFR gene (Tanaka et al., Plant Cell Physiol. 36, 1023-1031, 1995) and the rose anthocyanidin synthase (ANS) gene. RT-PCR was performed and eight cultivars (WKS-11, 13, 22, 36, 43, White Killarney, Tsuru No.2, Tineke) having low endogenous expression of DFR mRNA and normal ANS mRNA levels were selected.

RT-PCR was carried out with a Script First-strand Synthesis System for RT-PCR (Invitrogen) using RNA obtained from petals of each cultivar. The DFR mRNA was detected using DFR-2F (5'-CAAGCAATGGCATCGGAATC-3') (SEQ ID NO: 13) and DFR-2B (5'-TTTCCAGTGAGTGGCGAAAGTC-3') (SEQ ID NO: 14) primers, and the ANS mRNA was detected using ANS-2F (5'-TGGACTCGAAGAACTCGTCC-3') (SEQ ID NO: 15) and ANS-2B (5'-CCTCACCTTCTCCCTTGTT-3') (SEQ ID NO: 16) primers.

These eight cultivars showed lower levels of DFR mRNA and normal levels of ANS mRNA in Northern blotting (Table 1), and their cultivating properties were excellent. Two of the transformable cultivars (Tineke, WKS36) were decided on for actual transfer of the delphinidin-producing construct.

Table 1

Cultivar name	Flavonols (mg/g petal)			pH	RT-PCR		
	Q	K	Total		DFR	CHS	ANS
WKS-36	0.082	8.095	8.177	4.81	-	+	+
White Killarney	1.343	6.113	7.456	4.7	+	+	+
Tsuru No.2	0.715	5.188	5.903	4.7	+	+	+
WKS-11	2.028	0.475	2.503	4.51	+	+	+
Tineke	0.097	4.337	4.434	4.45	-	+	+
WKS-13	0.320	3.993	4.313	4.45	-	+	+
WKS-22	0.145	10.469	10.614	4.41	-	+	+
WKS-43	0.045	2.104	2.149	4.07	-	+	+

+: mRNA detected at same level as colored rose (Rote Rose cultivar)

5 -: mRNA detected at lower level than colored rose (Rote Rose cultivar)

Q: Quercetin, K: kaempferol

10 Example 7. Transfer of rose DFR gene into Tineke

Plasmid pE2113 (Mitsuhara et al., Plant Cell Physiol. 37, 49-59, 1996) comprises the enhancer sequence repeat-containing cauliflower mosaic virus 35S (E1235S) promoter and the nopaline synthase terminator. This
15 plasmid was digested with SacI and the ends were blunted using a Blunting Kit (Takara). The DNA fragment was ligated with an 8 bp SalI linker (Takara) and the obtained plasmid was designated as pUE5.

Plasmid pUE5 was digested with HindIII and EcoRI to
20 obtain an approximately 3 kb DNA fragment, which was introduced into pBin19 (Bevan M., Binary Agrobacterium Vector for plant transformation. Nucl. Acid Res. 12. 8711-21, 1984) previously digested with HindIII and EcoRI, to obtain plasmid pBE5. Next, pCGP645 was
25 digested with BamHI and XhoI to obtain a DNA fragment containing full-length rose DFR cDNA. This was ligated with pBE5 digested with BamHI and XhoI to construct pBERD1 (Fig. 2). The plasmid was transferred into

Agrobacterium tumefaciens Ag10.

Plasmid pBERD1 (Fig. 2) was transferred into the white rose cultivar "Tineke", and 18 transformants were obtained. Flower color was altered in six of the
 5 obtained transformants. Pigment analysis of two plants in which a clear color change from white to pink was observed confirmed accumulation of cyanidin and pelargonidin in both (Table 2). These results suggested that the Tineke cultivar is a cultivar lacking the DFR
 10 gene.

Table 2

Plant No.	Cya (mg/g)	Pel (mg/g)
1	0.014	0.005
2	0.014	0.006

Cya: Cyanidin, Pel: Pelargonidin

15 Example 8. Transfer of pansy F3'5'H gene (#18) and
 petunia DFR gene into Tineke

RNA was extracted from young budding pansy (Black Pansy variety) petals by the method of Turpen and Griffith (BioTechniques 4:11-15, 1986), and Oligotex-dT
 20 (Qiagen) was used for purification of polyA⁺RNA. This polyA⁺RNA and a λZAPII/GigapackII Cloning Kit (Stratagene) were used to construct a cDNA library from the young budding pansy petals. After transferring approximately 100,000 pfu of phage plaques grown on an NZY plate onto a
 25 Colony/PlaqueScreen (DuPont), treatment was conducted by the manufacturer's recommended protocol. The plaques were ³²P-labeled and screened using petunia Hf1cDNA (pCGP602, Holton et al., Nature, 366, p276-279, 1993) as the probe.

30 The membrane was subjected to pre-hybridization for 1 hour at 42°C in hybridization buffer (10% (v/v) formamide, 1 M NaCl, 10% (w/v) dextran sulfate, 1% SDS), and then the ³²P-labeled probe was added to 1 x 10⁶ cpm/ml

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and hybridization was performed for 16 hours at 42°C. The membrane was then rinsed for 1 hour in 2xSSC, 1% SDS at 42°C, fresh rinsing solution was exchanged, and rinsing was again performed for 1 hour. The rinsed membrane was exposed on a Kodak XAR film together with an intensifying screen, and the hybridization signal was detected.

The results of cDNA analysis demonstrated that the two obtained cDNA had high identity with petunia Hf1. The two cDNA types were designated as pansy F3'5'H cDNA, BP#18 (pCGP1959) and BP#40 (pCGP1961). The nucleotide sequence for #18 is listed as SEQ ID NO: 1, and its corresponding amino acid sequence is listed as SEQ ID NO: 2, the nucleotide sequence for #40 is listed as SEQ ID No. 3, and its corresponding amino acid sequence is listed as SEQ ID NO: 4. BP#18 and BP#40 have 82% identity on the DNA level. Also, BP#18 and BP#40 both exhibit 60% identity with petunia Hf1 and 62% identity with petunia Hf2 (Holton et al., Nature, 366, p276-279, 1993), on the DNA level.

Separately, plasmid pUE5 was digested with EcoRI and the ends were blunted using a Blunting Kit (Takara), and the obtained DNA fragment was ligated with an 8 bp HindIII linker (Takara), producing a plasmid which was designated as pUE5H. There was recovered an approximately 1.8 kb DNA fragment obtained by subjecting plasmid pCGP1959 containing pansy F3'5'H #18 cDNA to complete digestion with BamHI and partial digestion with XhoI. The plasmid obtained by ligation of this with pUE5H digested with BamHI and XhoI was designated as pUEBP18.

Separately, a DNA fragment containing petunia DFR cDNA was recovered by digestion of pCGP1403 (W096/36716) with BamHI and XhoI, and this DNA fragment was ligated with pBE5 that had been digested with BamHI and XhoI, to prepare pBEPD2. Next, pUEBP18 was partially digested with HindIII and an approximately 2.8 kb DNA fragment was recovered containing the El235S promoter, pansy F3'5'H

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#18 cDNA and the nos terminator. This fragment was ligated with a DNA fragment obtained by partial digestion of pBEPD2 with HindIII to obtain a binary vector plasmid pBPDBP2 (Fig. 3). This plasmid was introduced into *Agrobacterium tumefaciens* Ag10.

Plasmid pBPDBP2 (Fig. 3) was transferred into the white rose cultivar "Tineke", and 40 transformants were obtained. Flower color was altered in 23 of the obtained transformants, and pigment analysis confirmed accumulation of delphinidin in 16 of the 19 analyzed transformants (Table 3). The delphinidin content was 100% at maximum (average: 87%), but the maximum amount of pigment was very low at 0.035 mg per gram of petals and the flower color was only altered from RHS Color Chart 158d (Yellow-White group) to 56a (Red group) or 65b (Red-Purple group), while no color of the Violet group, Violet-Blue group or Blue group according to the RHSCC was achieved and the target blue rose could not be obtained.

20

Table 3

Plant No.	Del content (%)	Del (mg/g)	Cya (mg/g)	M (mg/g)	Q (mg/g)	K (mg/g)
1	87	0.002	0.000	0.000	0.058	0.354
2	100	0.004	0.000	0.338	0.059	1.921
3	82	0.002	0.001	0.203	0.039	1.382
4	100	0.003	0.000	0.245	0.050	1.840
5	76	0.005	0.001	0.000	0.280	3.288
6	0	0.000	0.000	0.000	0.098	0.409
7	0	0.000	0.001	0.000	0.101	0.358
8	0	0.000	0.001	0.000	0.030	2.277
9	83	0.013	0.003	0.000	0.117	0.841
10	85	0.011	0.002	0.000	0.104	3.300
11	84	0.020	0.004	0.000	0.168	3.137
12	91	0.025	0.002	0.294	0.119	1.252
13	90	0.028	0.003	0.000	0.075	1.912
14	91	0.014	0.001	0.000	0.152	2.667
15	90	0.035	0.004	0.000	0.086	1.616
16	83	0.023	0.005	0.000	0.117	2.267
17	91	0.014	0.001	0.000	0.113	0.825
18	76	0.003	0.001	0.000	0.085	2.351
19	82	0.005	0.001	0.000	0.054	1.616

Del: delphinidin, M: Myricetin

5 Example 9. Transfer of pansy F3'5'H gene (#40) and
petunia DFR gene into Tineke

Plasmid pE2113 (Mitsuhara et al., Plant Cell
 Physiol. 37, 49-59, 1996) was digested with HindIII and
 XbaI to obtain an approximately 800 bp DNA fragment,
 10 which was ligated with pBin19 (Bevan M., Binary
 Agrobacterium Vector for plant transformation. Nucl. Acid
 Res. 12. 8711-21, 1984) previously digested with HindIII
 and XbaI. The obtained plasmid was designated as
 pCGP1391. Another plasmid, pCGP669 (WO94/21840),
 15 contains the petunia chalcone synthase A (CHS-A) gene
 promoter. This plasmid was digested with EcoRI, blunted
 and then digested with HindIII.

The approximately 700 bp DNA fragment was ligated
 with pCGP1391 that had been digested with HindIII and
 20 SnaBI, and the obtained plasmid was designated as

pCGP1707. Also, there was recovered an approximately 1.8 kb DNA fragment obtained by subjecting plasmid pCGP1961 containing pansy F3'5'H #40 cDNA to complete digestion with BamHI and partial digestion with XhoI. The plasmid
5 obtained by ligation of this with pUE5H digested with BamHI and XhoI was designated as pUEBP40. Plasmid pUEBP40 was digested with EcoRV and XbaI and an approximately 5.5 kb DNA fragment was recovered.

This fragment was ligated with an approximately 700
10 bp fragment obtained by digesting plasmid pCGP1707 with HindIII, blunting the ends and further digesting with XbaI, to obtain plasmid pUFBP40. Next, pUFBP40 was partially digested with HindIII and an approximately 3.4 kb DNA fragment was recovered containing the cauliflower
15 35S promoter enhancer, CHS-A promoter, pansy F3'5'H #40 cDNA and the nos terminator. This fragment was ligated with a DNA fragment obtained by partial digestion of pBEPD2 with HindIII to obtain a binary vector plasmid pBPDBP8 (Fig. 4). This plasmid was introduced into
20 *Agrobacterium tumefaciens* Ag10.

Plasmid pBPDBP8 (Fig. 4) was transferred into the white rose cultivar "Tineke", and 53 transformants were obtained. Flower color was altered in 17 of the obtained transformants, and pigment analysis confirmed
25 accumulation of delphinidin in 8 of the 9 analyzed transformants (Table 4). The delphinidin content was 93% at maximum (average: 79%), but the maximum amount of pigment was very low at 0.014 mg per gram of petals and the flower color was only altered from RHS Color Chart
30 158d (Yellow-White group) to 56a (Red group) or 65b (Red-Purple group), while no color of the Violet group, Violet-Blue group or Blue group according to the RHSCC was achieved and the target blue rose could not be
35 a variety lacking only the DFR gene.

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Table 4

Plant No.	Del content (%)	Del (mg/g)	Cya (mg/g)	M (mg/g)	Q (mg/g)	K (mg/g)
1	0	0.000	0.001	0.000	0.018	2.023
2	9	0.001	0.006	na	na	na
3	93	0.011	0.001	0.000	0.036	2.724
4	86	0.007	0.001	0.000	0.076	2.957
5	71	0.013	0.006	0.000	0.073	2.503
6	87	0.014	0.002	0.000	0.058	3.390
7	78	0.005	0.002	0.000	0.049	1.241
8	47	0.004	0.004	0.000	0.070	1.800
9	78	0.004	0.001	0.000	0.029	2.326

na: no analysis/measurement

5 Example 10. Transfer of pansy F3'5'H gene (#18) and
petunia DFR gene into WKS36

Plasmid pBPDBP2 (Fig. 3) was transferred into the white rose "WKS36", and 138 transformants were obtained. Flower color was altered in 10 of the obtained transformants, and accumulation of delphinidin was confirmed in all of the plants (Table 5). The delphinidin content was 91% at maximum (average: 60%), but the maximum amount of pigment was very low at 0.033 mg per gram of petals and the flower color was only altered to very light pink, while no color of the Violet group, Violet-Blue group or Blue group according to the RHSCC was achieved and the target blue rose could not be obtained. This suggested that the WKS36 variety is not a variety lacking only the DFR gene.

20

Table 5

Plant No.	Del content (%)	Del (mg/g)	Cya (mg/g)	M (mg/g)	Q (mg/g)	K (mg/g)
1	60	0.008	0.005	0.381	0.169	2.291
2	40	0.006	0.009	0.633	0.486	2.911
3	54	0.005	0.005	0.654	0.336	3.460
4	43	0.016	0.021	0.000	0.656	2.469
5	53	0.009	0.008	0.404	0.325	2.397
6	53	0.004	0.003	0.498	0.251	2.768
7	45	0.013	0.016	0.000	0.381	1.537
8	83	0.004	0.001	0.000	0.156	1.632
9	80	0.033	0.008	0.000	0.557	3.766
10	91	0.013	0.000	0.000	0.184	2.610

5 Example 11. Transfer of pansy F3'5'H gene (#18) and
petunia DFR gene into WKS36

10 A plasmid obtained by replacing the AscI site of
plasmid pUCAP (van Engelen et al., Transgenic Research 4,
288-290, 1995) with PacI linker was designated as pUCPP.
Separately, an expression cassette prepared by linking
the rose chalcone synthase promoter, pansy F3'5'H #18
cDNA and nos terminator was obtained in the following
manner.

15 Chromosomal DNA was extracted from young leaves of
the Kardinal rose cultivar (Tanaka et al., Plant Cell
Physiol. 36, 1023-1031, 1995). An approximately 100 µg
portion of DNA was partially digested with Sau3AI, and
approximately 20-kb DNA fragments were recovered by
sucrose density gradient.

20 These were ligated with lambda phage EMBL3 (for
example, Stratagene) that had been digested with BamHI,
and a chromosomal DNA library was prepared by the
manufacturer's recommended protocol. The library was
screened by a publicly known method (Tanaka et al., Plant
25 Cell Physiol. 36, 1023-1031, 1995) using rose chalcone
synthase cDNA (DNA database: GenBank Accession No.
AB038246) as the probe. Among the obtained chalcone

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synthase chromosome clones, there existed lambda CHS20 which included an approximately 6.4 kb DNA sequence upstream from the start codon of chalcone synthase. The approximately 2.9 kb DNA fragment obtained by digestion of lambda CHS20 with HindIII and EcoRV includes the chalcone synthase promoter region.

This fragment was ligated with a fragment obtained by digestion of pUC19 (Yanisch-Perron C et al., Gene 33:103-119, 1985) with HindIII and SmaI. This was designated as pCGP1116. The sequence of the chalcone synthase promoter region included therein is listed as SEQ ID NO: 21. An approximately 2.9 kb DNA fragment obtained by digestion of pCGP1116 with HindIII and KpnI was ligated with a DNA fragment obtained by digestion of pJB1 (Bodeau, Molecular and genetic regulation of Bronze-2 and other maize anthocyanin genes. Dissertation, Stanford University, USA, 1994) with HindIII and KpnI to obtain pCGP197.

Separately, an approximately 300 bp DNA fragment containing the nopaline synthase terminator, obtained by digestion of pUE5 with SacI and KpnI, was blunted and linked with pBluescriptSK- which had been digested with EcoRV and BamHI and blunted. A plasmid of those obtained in which the 5' end of the terminator was close to the SalI site of pBluescriptSK- was designated as pCGP1986. A DNA fragment obtained by digesting pCGP1986 with XhoI, blunting the ends and further digesting with SalI was linked with a DNA fragment obtained by digesting pCGP197 with HindIII, blunting the ends and further digesting with SalI, to obtain pCGP2201.

Next, a DNA fragment obtained by digesting pCGP2201 with SalI and blunting the ends was linked with an approximately 1.7 kb DNA fragment (containing the pansy flavonoid 3',5'-hydroxylase gene) obtained by digesting pCGP1959 with BamHI and KpnI and blunting the ends. A plasmid of those obtained in which the rose chalcone synthase promoter had been inserted in a direction

allowing transcription of the pansy flavonoid 3',5'-hydroxylase gene in the forward direction was designated as pCGP2203. Plasmid pCGP2203 was recovered by digestion with HindIII and SacI. The DNA fragment was cloned at the HindIII and SacI sites of pUCPP, and the resulting plasmid was designated as pSPB459. Next, plasmid pE2113 was digested with SnaBI and a BamHI linker (Takara) was inserted to obtain a plasmid designated as pUE6.

An approximately 700 bp DNA fragment obtained by digestion of pUE6 with HindIII and BamHI was linked with an approximately 2.2 kb DNA fragment obtained by digestion of pCGP1405 (WO96/36716) with BamHI and BglII and with the binary vector pBinplus (van Engelen et al., Transgenic Research 4, 288-290, 1995) digested with HindIII and BamHI, to obtain pSPB460. An approximately 5 kb DNA fragment obtained by digestion of pSPB459 with PacI was introduced into the PacI site of pSPB460 to obtain pSPB461 (Fig. 5) having the petunia DFR and pansy F3'5'H #18 genes linked in the forward direction on the binary vector. This plasmid is modified for constitutive expression of the petunia DFR gene in plants and specific transcription of the pansy F3'5'H #18 gene in flower petals. The plasmid was transferred into *Agrobacterium tumefaciens* Ag10.

Plasmid pSPB461 (Fig. 5) was transferred into the white rose "WKS36", and 229 transformants were obtained. Flower color was altered in 16 of the obtained transformants, and accumulation of delphinidin was confirmed in all 12 of the pigment-analyzed plants (Table 6). The delphinidin content was 79% at maximum (average: 58%), but the amount of pigment was very low at 0.031 mg per gram of petals and the flower color was only altered to very light pink, while no color of the Violet group, Violet-Blue group or Blue group according to the RHSCC was achieved and the target blue rose could not be obtained. This suggested that the WKS36 variety is not a variety lacking only the DFR gene.

Table 6

Plant No.	Del content (%)	Del (mg/g)	Cya (mg/g)	M (mg/g)	Q (mg/g)	K (mg/g)
1	39	0.002	0.004	0.000	0.414	3.744
2	52	0.006	0.005	0.000	0.465	3.363
3	27	0.002	0.005	0.000	0.342	3.703
4	58	0.014	0.010	0.000	0.430	2.780
5	62	0.008	0.005	0.498	0.281	2.189
6	72	0.002	0.001	0.000	0.193	2.391
7	71	0.010	0.004	0.000	0.152	4.021
8	79	0.031	0.008	0.403	0.215	2.660
9	26	0.004	0.011	0.000	0.249	2.331
10	54	0.007	0.006	0.000	0.299	2.085
11	74	0.017	0.006	0.145	0.248	3.505
12	74	0.013	0.005	0.000	0.229	2.005

5

Example 12. Transfer of pansy F3'5'H gene (#18),
petunia DFR gene and perilla anthocyanin 3-glucoside
acyltransferase gene into WKS36

10 A gene comprising a start codon added to the perilla
hydroxycinnamoyl CoA: anthocyanin 3-glucoside
acyltransferase (3AT) gene was designated as pSAT208F
(Yonekura-Sakakibara et al., Plant Cell Physiol. 41, 495-
502, 2000). An approximately 3.9 kb DNA fragment
15 obtained by digestion of pSPB580 (PCT/AU03/00079) with
BamHI and XhoI was linked with an approximately 1.8 kb
DNA fragment obtained by digestion of pSAT208F with BamHI
and XhoI.

20 The obtained plasmid was digested with AscI, and a
DNA fragment was recovered containing the El235S
promoter, the perilla 3AT gene and the petunia
phospholipid transfer protein terminator. The DNA
fragment was inserted into the AscI site of pSPB461 to
obtain plasmid pSPB472 (Fig. 6) having the perilla 3AT,
petunia DFR and pansy F3'5'H #18 gene transcription
25 directions in the forward direction. This plasmid is
modified for constitutive expression of the perilla 3AT

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gene and the petunia DFR gene in plants and specific transcription of the pansy F3'5'H #18 gene in flower petals. The plasmid was transferred into *Agrobacterium tumefaciens* Ag10.

5 Plasmid pSPB472 (Fig. 6) was transferred into the white rose "WKS36", and 75 transformants were obtained. Flower color was altered in four of the obtained transformants, and accumulation of delphinidin was confirmed in all three of the pigment-analyzed plants
10 (Table 7). The delphinidin content was 67% at maximum (average: 49%), but the amount of pigment was very low at 0.011 mg per gram of petals and the flower color was only altered to very light pink, while no color of the Violet group, Violet-Blue group or Blue group according to the
15 RHSCC was achieved and the target blue rose could not be obtained. This suggested that the WKS36 variety is not a variety lacking only the DFR gene.

Table 7

20

Plant No.	Del content (%)	Del (mg/g)	Cya (mg/g)	M (mg/g)	Q (mg/g)	K (mg/g)
1	32	0.003	0.006	0.219	0.236	1.972
2	67	0.011	0.005	0.520	0.329	3.234
3	46	0.006	0.007	0.000	0.579	3.874

Thus, despite screening of several white roses, it was not possible to obtain a cultivar lacking only the
25 DFR gene. In other words, it was not possible to obtain a blue rose by the method for creation of blue carnation (WO94/28140).

Example 13. Inhibition of rose DFR gene by cosuppression

30 Plasmid pBERD1 was transferred into the pale violet rose "Lavande", and 26 transformants were obtained. However, none of the plants exhibited altered flower color, suggesting that it is difficult to inhibit the

rose endogenous DFR gene by cosuppression.

Example 14. Screening for colored roses

5 Cultivars for creation of blue roses were then selected from among colored roses. After visually selecting 136 lines from colored rose cultivars with relatively blue shades, 89 of the lines were subjected to pigment analysis. The values obtained for the examined colored roses are shown in Tables 8 to 10.

10

Table 8

Name	Cya (mg/g)	Pel (mg/g)	Peo (mg/g)	Q (mg/g)	K (mg/g)
Lavande	0.078	0.000	0.000	0.451	0.078
Madam Violet	0.055	0.000	0.000	1.780	0.189
Vol de Nuit	0.317	0.003	0.000	2.661	0.316
Blue Moon	0.049	0.000	0.000	1.341	0.119
Seiryu	0.015	0.000	0.000	3.030	1.300
WKS077	1.875	0.008	0.000	1.430	0.247
WKS078	0.211	0.000	0.000	1.286	0.133
WKS079	2.864	0.003	0.000	1.030	0.106
WKS080	0.040	0.000	0.000	0.362	0.047
WKS081	0.032	0.000	0.000	4.480	1.563
WKS082	0.074	0.000	0.000	2.400	0.196
WKS083	0.018	0.405	0.000	0.146	0.962
WKS084	0.055	0.000	0.000	1.269	0.159
WKS087	0.032	0.000	0.000	0.797	0.134
WKS089	0.030	0.000	0.000	1.484	0.317
WKS090	1.571	0.007	0.000	1.346	0.339
WKS091	0.045	0.169	0.000	0.186	0.899
WKS092	0.038	0.002	0.000	1.358	0.135
WKS095	0.015	0.000	0.000	2.945	0.255
WKS096	0.024	0.000	0.000	2.032	0.349
WKS097	0.991	0.002	0.000	1.659	0.185
WKS100	0.051	0.000	0.000	1.410	0.615
WKS101	0.424	0.000	0.000	2.194	0.482
WKS104	0.066	0.000	0.000	2.347	0.424
WKS107	1.202	0.004	0.000	3.134	0.460
WKS114	0.429	0.000	0.000	3.509	0.541
WKS116	0.026	0.000	0.000	3.440	0.868
WKS117	0.027	0.000	0.000	0.227	0.149
WKS121	0.669	0.006	0.000	1.336	0.453
WKS123	0.487	0.003	0.000	3.663	0.826

Peo: Peonidin

Table 9

Name	Cya (mg/g)	Pel (mg/g)	Peo (mg/g)	Q (mg/g)	K (mg/g)
WKS124	0.022	0.045	0.000	0.192	2.012
WKS125	0.187	0.002	0.000	0.349	0.089
WKS126	0.544	0.002	0.000	2.226	0.895
WKS127	1.609	0.008	0.006	2.278	0.528
WKS128	1.844	0.003	0.007	2.576	0.409
WKS129	1.645	0.002	0.006	0.450	0.160
WKS130	1.332	0.008	0.005	1.599	0.525
WKS131	0.582	0.002	0.001	2.460	0.567
WKS132	1.101	0.006	0.000	0.298	0.208
WKS133	2.773	0.003	0.000	1.263	0.230
WKS133	3.487	0.011	0.023	0.414	0.108
WKS134	1.084	0.001	0.002	2.777	0.413
WKS135	0.241	0.007	0.001	0.803	0.113
WKS136	0.637	0.000	0.003	1.451	0.062
WKS137	1.208	0.014	0.002	1.034	1.027
WKS138	1.955	0.006	0.000	3.857	0.855
WKS139	0.285	0.003	0.000	1.363	0.538
WKS140	0.075	0.000	0.000	0.291	0.097
WKS141	0.197	0.000	0.000	0.358	0.045
WKS142	1.906	0.029	0.106	1.890	1.860
WKS143	1.125	0.027	0.020	1.596	1.129
WKS144	2.685	0.484	0.000	0.160	0.184
WKS145	0.948	0.006	0.000	3.086	1.222
WKS146	3.108	0.047	0.000	0.228	0.398
WKS147	0.593	0.003	0.004	3.619	0.924
WKS148	0.059	0.000	0.000	3.113	0.466
WKS149	1.101	0.013	0.000	1.481	1.866
WKS150	0.498	0.562	0.000	0.061	0.156
WKS151	0.947	1.073	0.000	0.038	0.227
WKS152	0.303	1.599	0.000	0.015	0.464

Peo: Peonidin

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Table 10

Name	Cya (mg/g)	Pel (mg/g)	Peo (mg/g)	Q (mg/g)	K (mg/g)
WKS153	1.178	0.796	0.000	0.020	0.179
WKS154	0.219	0.659	0.000	0.007	0.265
WKS155	0.547	0.006	0.000	1.274	0.073
WKS156	0.851	0.005	0.000	1.139	0.238
WKS157	0.955	0.555	0.000	0.133	1.315
WKS158	0.634	0.005	0.000	0.526	0.219
WKS159	0.106	0.320	0.000	0.034	0.959
WKS160	0.750	0.005	0.000	2.283	0.768
WKS161	0.262	0.419	0.000	0.197	1.115
WKS162	0.039	0.564	0.000	0.041	0.447
WKS163	0.184	0.002	0.000	0.756	0.105
WKS164	0.918	0.012	0.000	1.954	2.832
WKS165	0.097	0.604	0.000	0.026	0.197
WKS166	0.116	0.015	0.000	0.488	0.566
WKS167	0.647	0.002	0.000	2.507	0.499
WKS168	1.109	0.029	0.000	1.797	2.328
WKS169	0.070	0.003	0.000	0.208	1.369
Baby Faurax	2.247	0.022	0.058	4.518	0.580
Indigo	0.891	0.006	0.000	5.781	3.820
Intermezzo	0.040	0.000	0.000	1.075	0.443
James Veitch	1.281	0.004	0.002	2.087	0.923
Lagoon	0.053	0.000	0.000	2.887	0.315
Magenta	0.126	0.000	0.000	1.062	0.191
MRS COLVILLE	1.666	0.012	0.000	3.500	2.940
Mme. Isaac Pereire	0.629	0.003	0.000	1.021	0.105
Mme. de La Roche-Lambert	0.869	0.005	0.000	4.994	2.794
Roseraie de L'hay	0.364	0.005	1.256	0.156	0.077
Rose de Rescht	1.348	0.004	0.000	4.027	0.842
Rose du Roi a Fleurs Pourpres	2.556	0.017	0.000	0.968	0.411

Peo: Peonidin

5 Example 15. Transfer of pansy F3'5'H gene (#40) and
Torenia anthocyanin 5-acyltransferase gene into Lavande

10 Modification of anthocyanins with aromatic acyl
groups can stabilize the anthocyanins and produce a bluer
color (for example, W096/25500). The following
experiment was conducted with the goal of producing
acylated delphinidin-type anthocyanins.

RNA was obtained from *Torenia* Summer Wave flower petals, and polyA⁺RNA was prepared therefrom. A cDNA library was prepared from the polyA⁺RNA with λ ZAPII (Stratagene) as the vector, using a directional cDNA library preparation kit (Stratagene) according to the manufacturer's recommended protocol. The major anthocyanin of *Torenia* is modified with an aromatic acyl group at the 5-position glucose (Suzuki et al., Molecular Breeding 2000 6, 239-246), and therefore anthocyanin acyltransferase is expressed in *Torenia* petals.

Anthocyanin acyltransferase includes the conserved amino acid sequence Asp-Phe-Gly-Trp-Gly-Lys, and corresponding synthetic DNA can be used as primer to obtain the anthocyanin acyltransferase gene (W096/25500). Specifically, 10 ng of single-stranded cDNA synthesized for construction of the *Torenia* cDNA library was used as template, and 100 ng of ATC primer (5'-GA(TC)TT(TC)GGITGGGGIAA-3', I: inosine) (SEQ ID NO: 17) and 100 ng of oligo dT primer (5'-TTTTTTTTTTTTTTTTTCTCGAG-3') (SEQ ID NO: 18) were used as primers for PCR with Taq polymerase (Takara, Japan), under the manufacturer's recommended conditions.

The PCR was carried out in 25 cycles of reaction with one cycle consisting of 1 minute at 95°C, 1 minute at 55°C and 1 minute at 72°C. The approximately 400 bp DNA fragment that was obtained was recovered with Gene Clean II (BIO,101. Inc.) according to the manufacturer's recommended protocol, and was subcloned in pCR-TOPO. Determination of the nucleotide sequence revealed a sequence homologous to the gentian acyltransferase gene (Fujiwara et al., 1998, Plant J. 16 421-431). The nucleotide sequence was determined by the Dye Primer method (Applied Biosystems), using Sequencer 310 or 377 (both by Applied Biosystems).

The DNA fragment was labeled with DIG using a DIG-labeling detection kit (Japan Roche), and used for

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screening of a *Torenia* cDNA library by plaque hybridization according to the manufacturer's recommended protocol. Twelve of the obtained positive signal clones were randomly selected, the plasmids were recovered, and
5 their nucleotide sequences were determined. These exhibited high homology with anthocyanin acyltransferase. The total nucleotide sequence of the cDNA in the clone designated as pTAT7 was determined. The nucleotide sequence is listed as SEQ ID NO: 7, and the corresponding
10 amino acid sequence is listed as SEQ ID NO: 8.

After digesting pBE2113-GUS (Mitsuhara et al., Plant Cell Physiol. 37, 49-59, 1996) with SacI, the ends were blunted and an 8 bp XhoI linker (Takara) was inserted. An approximately 1.7 kb DNA fragment obtained by
15 digesting pTAT7 with BamHI and XhoI was inserted at the BamHI and XhoI sites of this plasmid, to obtain pSPB120. After digesting pSPB120 with SnaBI and BamHI, the ends were blunted and ligation was performed to obtain pSPB120'. Separately, plasmid pCGP1961 containing pansy
20 F3'5'H #40 cDNA was completely digested with BamHI and then partially digested with XhoI to obtain an approximately 1.8 kb DNA fragment which was recovered and ligated with pUE5H previously digested with BamHI and XhoI, to obtain a plasmid which was designated as
25 pUEBP40.

After digesting pUEBP40 with SnaBI and BamHI, the ends were blunted and ligation was performed to obtain pUEBP40'. This plasmid pUEBP40' was partially digested with HindIII to obtain an approximately 2.7 kb DNA
30 fragment which was recovered and linked with a DNA fragment obtained by partial digestion of pSPB120' with HindIII. Of the obtained plasmids, a binary vector having the neomycin phosphotransferase gene, pansy F3'5'H #40 gene and *Torenia* 5AT gene linked in that order in the
35 same direction from the right border sequence on the binary vector, was designated as pSPB130 (Fig. 7). This plasmid is modified for constitutive expression of the

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pansy F3'5'H #40 gene and the *Torenia* 5AT gene in plants and specific transcription of the genes in the flower petals. The plasmid was transferred into *Agrobacterium tumefaciens* Ag10.

5 Plasmid pSPB130 (Fig. 7) was transferred into the pale violet rose variety "Lavande", and 41 transformants were obtained. Accumulation of delphinidin was confirmed in 20 of the 32 pigment-analyzed plants (Tables 11 and 12). The delphinidin content was 71% at maximum
10 (average: 36%). The flower color was altered from RHS Color Chart 186c (Greyed-Purple group) to 79d (Purple group). The proportion of acylated anthocyanins was only about 30% of the total anthocyanins. Upon spectral
15 measurement of the acylated anthocyanins, the maximum absorption wavelength had shifted toward longer wavelength by 4 nm from delphinidin 3,5-diglucoside, but because of the low proportion among the total
20 anthocyanins, no clear effect was achieved for the flower color.

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Table 11

Plant No.	Acylation (%)	Del content (%)	Del (mg/g)	Cya (mg/g)	M (mg/g)	Q (mg/g)	K (mg/g)
1	0	9	0.005	0.050	na	na	na
2	0	11	0.009	0.069	na	na	na
3	0	10	0.010	0.087	na	na	na
4	0	22	0.028	0.102	na	na	na
5	5	51	0.073	0.069	na	na	na
6	4	57	0.093	0.069	na	na	na
7	5	48	0.039	0.042	na	na	na
8	13	0	0.000	0.065	na	na	na
9	17	9	0.006	0.062	na	na	na
10	26	0	0.000	0.104	na	na	na
11	17	67	0.074	0.036	na	na	na
12	0	0	0.000	0.131	na	na	na
13	0	0	0.000	0.083	na	na	na
14	6	48	0.084	0.092	na	na	na
15	0	20	0.020	0.081	na	na	na
16	42	13	0.020	0.131	0.000	0.637	0.020
17	32	36	0.032	0.058	na	na	na
18	7	0	0.000	0.146	na	na	na
19	0	0	0.000	0.069	na	na	na
20	0	0	0.000	0.142	na	na	na
21	0	0	0.000	0.080	na	na	na

na: no analysis/measurement

5

Table 12

Plant No.	Acylation (%)	Del content (%)	Del (mg/g)	Cya (mg/g)	M (mg/g)	Q (mg/g)	K (mg/g)
22	0	0	0.000	0.069	na	na	na
23	0	0	0.000	0.057	na	na	na
24	18	4	0.006	0.149	na	na	na
25	17	4	0.008	0.208	na	na	na
26	0	0	0.000	0.188	na	na	na
27	0	0	0.000	0.078	na	na	na
28	17	67	0.090	0.044	na	na	na
29	17	71	0.057	0.024	na	na	na
30	16	40	0.040	0.059	na	na	na
31	21	70	0.082	0.036	0.305	0.062	0.008
32	18	62	0.066	0.040	na	na	na

na: no analysis/measurement

Example 16. Transfer of pansy F3'5'H gene (#40) and

Torenia anthocyanin 5-acyltransferase gene into WKS100

Plasmid pSPB130 (Fig. 7) was transferred into the pale violet rose variety "WKS100", and 146 transformants were obtained. Accumulation of delphinidin was confirmed in 56 of the 63 pigment-analyzed plants (Tables 13-15). The delphinidin content was 95% at maximum (average: 44%). The flower color was altered from RHS Color Chart 56d (Red group) to 186d (Greyed-Purple group). However, no color of the Violet group, Violet-Blue group or Blue group according to the RHSCC was achieved and the target blue rose could not be obtained.

Table 13

Plant No.	Acylation (%)	Del content (%)	Del (mg/g)	Cya (mg/g)	Pel (mg/g)	M (mg/g)	Q (mg/g)	K (mg/g)
1	20	75	0.036	0.012	0.000	2.944	0.974	0.322
2	16	51	0.027	0.027	0.000	1.685	1.734	0.512
3	13	50	0.024	0.024	0.000	0.000	1.382	1.912
4	23	50	0.037	0.037	0.000	na	na	na
5	9	25	0.013	0.033	0.005	na	na	na
6	10	26	0.034	0.097	0.000	na	na	na
7	13	65	0.053	0.028	0.000	1.936	1.184	0.760
8	13	65	0.044	0.024	0.000	1.622	1.065	0.562
9	14	62	0.033	0.021	0.000	2.096	1.444	0.710
10	14	95	0.137	0.008	0.000	0.000	0.156	1.097
11	10	62	0.036	0.022	0.000	2.025	1.194	0.799
12	5	59	0.054	0.038	0.000	2.194	1.289	0.783
13	9	43	0.033	0.044	0.000	2.542	1.803	0.734
14	9	50	0.030	0.031	0.000	0.020	1.971	0.741
15	1	70	0.066	0.028	0.000	1.652	1.659	0.867
16	0	20	0.008	0.023	0.008	0.308	2.632	1.463
17	1	63	0.068	0.040	0.000	2.037	2.128	1.554
18	21	51	0.037	0.035	0.000	2.659	1.936	1.002
19	0	0	0.000	0.095	0.000	na	na	na
20	0	0	0.000	0.037	0.000	na	na	na
21	0	23	0.026	0.086	0.003	0.182	4.554	3.083
22	4	71	0.110	0.044	0.000	3.265	1.643	1.341
23	12	65	0.051	0.025	0.002	1.356	0.888	0.387
24	6	58	0.038	0.027	0.000	2.374	2.016	0.809
25	5	52	0.044	0.040	0.000	2.651	2.546	1.108

na: no analysis/measurement

Table 14

Plant No.	Acylation (%)	Del content (%)	Del (mg/g)	Cya (mg/g)	Pel (mg/g)	M (mg/g)	Q (mg/g)	K (mg/g)
26	6	64	0.033	0.019	0.000	2.707	1.546	0.605
27	16	0	0.000	0.041	0.000	na	na	na
28	16	13	0.007	0.050	0.000	0.249	3.359	1.459
29	12	7	0.007	0.095	0.000	na	na	na
30	15	9	0.007	0.069	0.000	na	na	na
31	15	8	0.007	0.081	0.000	na	na	na
32	7	7	0.007	0.094	0.000	na	na	na
33	13	10	0.006	0.055	0.000	na	na	na
34	14	46	0.078	0.090	0.002	na	na	na
35	7	8	0.007	0.078	0.000	na	na	na
36	3	48	0.045	0.039	0.010	3.050	2.304	1.326
37	2	39	0.029	0.046	0.000	na	na	na
38	1	55	0.073	0.059	0.000	1.608	2.138	1.015
39	1	33	0.030	0.063	0.000	na	na	na
40	2	59	0.050	0.035	0.000	3.651	2.727	1.076
41	17	15	0.011	0.061	0.000	na	na	na
42	0	0	0.000	0.048	0.002	na	na	na
43	3	17	0.009	0.046	0.000	na	na	na
44	40	32	0.027	0.058	0.000	na	na	na
45	2	0	0.000	0.031	0.000	na	na	na
46	2	0	0.000	0.038	0.000	na	na	na
47	1	8	0.004	0.048	0.000	na	na	na
48	19	57	0.046	0.034	0.000	2.626	2.165	0.900
49	10	59	0.047	0.032	0.000	1.737	1.901	1.054
50	2	70	0.057	0.024	0.000	1.545	0.880	0.694

na: no analysis/measurement

Table 15

Plant No.	Acylation (%)	Del content (%)	Del (mg/g)	Cya (mg/g)	Pel (mg/g)	M (mg/g)	Q (mg/g)	K (mg/g)
51	4	10	0.006	0.056	0.000	na	na	na
52	16	12	0.006	0.039	0.002	na	na	na
53	34	84	0.156	0.030	0.000	5.100	1.056	0.511
54	32	89	0.131	0.017	0.000	3.907	0.803	0.431
55	29	89	0.098	0.013	0.000	3.687	0.453	0.226
56	21	83	0.083	0.017	0.000	2.679	0.817	0.431
57	14	8	0.007	0.082	0.000	na	na	na
58	9	44	0.034	0.041	0.002	2.258	2.054	0.672
59	7	51	0.040	0.038	0.000	2.246	2.151	0.765
60	0	7	0.008	0.111	0.000	na	na	na
61	1	48	0.069	0.073	0.000	1.558	1.730	0.565
62	13	0	0.000	0.036	0.000	na	na	na
63	16	14	0.005	0.029	0.000	na	na	na

na: no analysis/measurement

5 Example 17. Transfer of pansy F3'5'H gene (#40) and
Torenia anthocyanin 5-acyltransferase gene into WKS116

Plasmid pSPB130 (Fig. 7) was transferred into the pale violet rose variety "WKS116", and 282 transformants were obtained. Accumulation of delphinidin was confirmed
10 in 33 of the 36 pigment-analyzed plants (Tables 16 and 17). The delphinidin content was 80% at maximum (average: 73%). The flower color was altered from RHS Color Chart 196d (Greyed-Green group) to 186d (Greyed-Purple group). However, no color of the Violet group,
15 Violet-Blue group or Blue group according to the RHSCC was achieved and the target blue rose could not be obtained.

Table 16

Plant No.	Acylation (%)	Del content (%)	Del (mg/g)	Cya (mg/g)	M (mg/g)	Q (mg/g)	K (mg/g)
1	1.8	78	0.015	0.004	0.746	0.753	0.507
2	12.7	78	0.097	0.028	1.826	2.352	1.572
3	5.9	78	0.030	0.009	1.000	1.452	0.934
4	0.0	76	0.030	0.010	0.813	0.990	0.480
5	2.6	72	0.038	0.015	1.279	1.835	0.832
6	0.0	72	0.019	0.007	0.839	0.983	0.642
7	3.1	75	0.033	0.011	1.131	1.476	0.877
8	1.9	75	0.028	0.009	0.761	0.977	0.466
9	2.6	76	0.034	0.011	na	na	na
10	2.7	73	0.031	0.011	na	na	na
11	4.4	77	0.033	0.010	1.001	1.003	0.618
12	7.0	74	0.035	0.012	0.849	0.945	0.577
13	9.3	74	0.025	0.009	na	na	na
14	3.2	80	0.044	0.011	1.045	0.959	0.545
15	4.5	75	0.031	0.010	1.115	1.256	0.729
16	10.5	71	0.028	0.012	1.055	1.155	0.670
17	1.7	51	0.016	0.016	0.330	1.537	1.052
18	10.5	77	0.112	0.033	2.008	2.976	2.216
19	0.0	0	0.000	0.010	na	na	na
20	0.0	30	0.007	0.015	na	na	na
21	na	56	0.013	0.010	0.197	1.960	1.463
22	4.4	47	0.006	0.007	na	na	na
23	3.6	77	0.026	0.008	na	na	na

na: no analysis/measurement

Table 17

Plant No.	Acylation (%)	Del content (%)	Del (mg/g)	Cya (mg/g)	M (mg/g)	Q (mg/g)	K (mg/g)
24	7.2	82	0.028	0.006	1.295	1.272	0.805
25	3.5	83	0.035	0.007	na	na	na
26	17.4	26	0.009	0.025	na	na	na
27	39.3	91	0.101	0.010	3.499	0.563	0.178
28	28.2	85	0.047	0.005	na	na	na
29	0.0	0	0.000	0.025	na	na	na
30	10.4	89	0.092	0.012	na	na	na
31	1.9	0	0.000	0.036	na	na	na
32	5.8	76	0.027	0.009	na	na	na
33	16.8	88	0.066	0.009	na	na	na
34	10.5	87	0.103	0.015	na	na	na
35	13.7	38	0.021	0.034	na	na	na
36	18.3	95	0.051	0.003	na	na	na

na: no analysis/measurement

5 Example 18. Transfer of pansy F3'5'H gene (#40) and
Torenia anthocyanin 5-acyltransferase gene into WKS124

Plasmid pSPB130 (Fig. 7) was transferred into the pale orange rose variety "WKS124", and 50 transformants were obtained. Accumulation of delphinidin was confirmed in 13 of the 15 pigment-analyzed plants (Table 18). The delphinidin content was 95% at maximum (average: 82%). The flower color was altered from RHS Color Chart 52d (Red group) to 71c (Red-Purple group). However, no color of the Violet group, Violet-Blue group or Blue group according to the RHSCC was achieved and the target blue rose could not be obtained.

Table 18

Plant No.	Acylation (%)	Del content (%)	Del (mg/g)	Cya (mg/g)	Pel (mg/g)	M (mg/g)	Q (mg/g)	K (mg/g)
1	0.6	0	0.000	0.013	0.069	na	na	na
2	35.5	75	0.256	0.051	0.034	0.066	0.093	1.190
3	43.0	78	0.385	0.068	0.041	0.039	0.046	1.197
4	44.2	85	0.811	0.120	0.028	0.106	0.094	1.021
5	na	86	0.907	0.123	0.024	0.219	0.066	0.852
6	4.6	0	0.000	0.023	0.075	na	na	na
7	7.9	90	1.498	0.169	0.008	0.905	0.143	0.679
8	8.4	90	1.403	0.146	0.008	0.971	0.145	0.827
9	26.7	88	0.521	0.066	0.003	0.623	0.108	0.853
10	21.9	89	0.504	0.058	0.003	0.636	0.098	0.727
11	26.0	85	0.928	0.145	0.019	0.424	0.152	0.455
12	3.8	95	1.017	0.058	0.000	1.161	0.140	0.262
13	11.6	84	0.939	0.156	0.025	0.748	0.128	0.262
14	38.5	69	0.166	0.071	0.007	0.000	0.059	0.776
15	27.1	55	0.137	0.040	0.074	0.000	0.021	2.330

na: no analysis/measurement

5 Example 19. Transfer of pansy F3'5'H gene (#40) and
Torenia anthocyanin 5-acyltransferase gene into WKS132

Plasmid pSPB130 (Fig. 7) was transferred into the bright red rose variety "WKS132", and 24 transformants were obtained. Accumulation of delphinidin was confirmed in 6 of the 7 pigment-analyzed plants (Table 19). The delphinidin content was 43% at maximum (average: 12%). The flower color was altered from RHS Color Chart 57a (Red-Purple group) to 66a (Red-Purple group). However, no color of the Violet group, Violet-Blue group or Blue group according to the RHSCC was achieved and the target blue rose could not be obtained.

Table 19

Plant No.	Acylation (%)	Del content (%)	Del (mg/g)	Cya (mg/g)	Pel (mg/g)
1	1.8	0.4	0.008	1.872	0.009
2	1.0	0.0	0.000	1.409	0.010
3	21.3	11.4	0.237	1.841	0.007
4	6.8	42.5	0.461	0.619	0.006
5	7.6	9.5	0.204	1.936	0.011
6	na	1.3	0.016	1.227	0.007
7	23.7	5.4	0.081	1.407	0.005

5 Example 20. Transfer of pansy F3'5'H gene (#40) and
Torenia anthocyanin 5-acyltransferase gene into WKS133

Plasmid pSPB130 (Fig. 7) was transferred into the dark red-violet rose variety "WKS133", and 16 transformants were obtained. Accumulation of delphinidin was confirmed in all eight of the pigment-analyzed plants (Table 20). The delphinidin content was 34% at maximum (average: 11%). The flower color was altered from RHS Color Chart 53a (Red group) to 61a (Red-Purple group). However, no color of the Violet group, Violet-Blue group or Blue group according to the RHSCC was achieved and the target blue rose could not be obtained.

Table 20

Plant No.	Acylation (%)	Del content (%)	Del (mg/g)	Cya (mg/g)	Pel (mg/g)	Peo (mg/g)	M (mg/g)	Q (mg/g)	K (mg/g)
1	10.3	23.7	1.322	4.253	0.009	0.004	0.691	0.792	0.133
2	11.8	33.8	1.192	2.324	0.005	0.003	0.621	0.422	0.093
3	6.1	12.9	0.009	0.060	0.000	0.000	0.102	0.500	0.048
4	3.8	9.1	0.363	3.627	0.005	0.008	na	na	na
5	15.8	2.0	0.078	3.774	0.009	0.000	0.045	0.939	0.472
6	11.5	2.7	0.135	4.771	0.011	0.005	0.046	0.576	0.034
7	13.3	3.0	0.180	5.800	0.009	0.009	0.100	0.937	0.179
8	12.2	3.5	0.161	4.470	0.009	0.009	0.068	0.738	0.148

na: no analysis/measurement

Example 21. Transfer of pansy F3'5'H gene (#40) and
Torenia anthocyanin 5-acyltransferase gene into WKS137

5 Plasmid pSPB130 (Fig. 7) was transferred into the
dark red-violet rose variety "WKS137", and 20
transformants were obtained. Accumulation of delphinidin
was confirmed in all 17 of the pigment-analyzed plants
(Table 21). The delphinidin content was 1.3% at maximum
(average: 0.4%). No alteration in flower color was
10 observed from RHS Color Chart 61b (Red-Purple group).

Table 21

Plant No.	Acylation (%)	Del content (%)	Del (mg/g)	Cya (mg/g)	Pel (mg/g)	Peo (mg/g)	M (mg/g)	Q (mg/g)	K (mg/g)
1	0.5	0.3	0.008	2.821	0.037	0.000	na	na	na
2	0.8	0.3	0.010	3.384	0.051	0.000	na	na	na
3	0.4	0.3	0.005	1.982	0.014	0.000	na	na	na
4	0.6	0.2	0.008	3.344	0.057	0.000	na	na	na
5	0.7	0.4	0.011	3.145	0.035	0.000	na	na	na
6	0.7	1.3	0.025	2.919	0.040	0.003	na	na	na
7	0.4	0.3	0.008	2.820	0.045	0.000	na	na	na
8	0.5	0.4	0.010	2.467	0.042	0.000	na	na	na
9	0.7	0.2	0.010	3.836	0.024	0.000	na	na	na
10	0.1	0.5	0.008	1.743	0.016	0.000	na	na	na
11	0.7	0.4	0.011	2.593	0.027	0.003	na	na	na
12	0.6	0.3	0.007	2.393	0.022	0.000	0.048	3.026	2.812
13	1.4	0.2	0.009	3.756	0.065	0.000	na	na	na
14	0.7	0.4	0.008	2.149	0.024	0.001	na	na	na
15	0.8	0.5	0.007	2.281	0.041	0.000	na	na	na
16	0.5	0.5	0.007	1.314	0.014	0.000	na	na	na
17	1.0	0.2	0.007	2.892	0.051	0.000	na	na	na

na: no analysis/measurement

Example 22. Transfer of pansy F3'5'H gene (#40) and
Torenia anthocyanin 5-acyltransferase gene into WKS140

5 Plasmid pSPB130 (Fig. 7) was transferred into the
pale violet rose variety "WKS140", and 197 transformants
were obtained. Accumulation of delphinidin was confirmed
in 37 of the 45 pigment-analyzed plants (Tables 22 and
23). The delphinidin content was 94% at maximum
(average: 47%). The flower color was altered from RHS
10 Color Chart 186d (Greyed-Purple group) to 79d (Purple
group). However, no color of the Violet group, Violet-
Blue group or Blue group according to the RHSCC was
achieved and the target blue rose could not be obtained.

Table 22

Plant No.	Acylation (%)	Del content (%)	Del (mg/g)	Cya (mg/g)	M (mg/g)	Q (mg/g)	K (mg/g)
1	3.5	0.0	0.000	0.090	na	na	na
2	2.5	0.0	0.000	0.093	0.096	2.429	0.246
3	5.5	63.5	0.061	0.035	0.688	1.090	0.106
4	13.2	17.7	0.013	0.059	na	na	na
5	5.4	11.6	0.017	0.129	na	na	na
6	3.6	12.3	0.011	0.078	na	na	na
7	13.6	11.7	0.009	0.069	na	na	na
8	4.1	22.3	0.012	0.041	0.057	1.950	0.492
9	3.3	0.0	0.000	0.071	na	na	na
10	2.6	18.6	0.017	0.076	na	na	na
11	4.2	18.6	0.012	0.052	0.130	3.101	1.172
12	6.5	25.0	0.026	0.079	0.251	2.300	0.592
13	1.3	0.0	0.000	0.062	0.000	2.200	0.552
14	22.7	85.4	0.261	0.045	1.649	0.943	0.126
15	20.9	57.4	0.093	0.069	0.481	1.418	0.182
16	16.4	39.9	0.052	0.078	na	na	na
17	15.2	50.8	0.074	0.072	na	na	na
18	6.1	22.6	0.036	0.111	0.148	2.152	0.279
19	2.7	0.0	0.000	0.033	na	na	na
20	9.1	52.6	0.041	0.037	na	na	na
21	4.4	46.2	0.075	0.087	na	na	na
22	8.5	34.7	0.040	0.075	0.195	1.847	0.394
23	11.0	30.9	0.018	0.040	0.155	1.106	0.142
24	13.4	46.8	0.056	0.063	na	na	na
25	2.8	5.1	0.006	0.107	na	na	na

na: no analysis/measurement

Table 23

Plant No.	Acylation (%)	Del content (%)	Del (mg/g)	Cya (mg/g)	M (mg/g)	Q (mg/g)	K (mg/g)
26	4.1	6.8	0.007	0.098	na	na	na
27	31.4	93.4	0.252	0.018	1.434	0.361	0.052
28	13.4	86.7	0.101	0.016	1.237	1.740	0.499
29	32.3	94.2	0.200	0.012	0.862	0.131	0.029
30	13.0	89.7	0.176	0.020	0.553	0.289	0.026
31	12.3	87.1	0.150	0.022	1.007	0.674	0.135
32	6.7	9.9	0.009	0.086	na	na	na
33	11.5	67.4	0.108	0.052	na	na	na
34	5.0	11.2	0.014	0.110	0.074	2.588	0.659
35	12.5	79.7	0.088	0.022	1.192	1.185	0.574
36	15.0	83.4	0.065	0.013	1.478	1.147	0.570
37	1.8	0.0	0.000	0.068	na	na	na
38	1.3	44.3	0.105	0.132	0.582	3.259	1.232
39	2.5	73.6	0.114	0.041	na	na	na
40	14.0	85.3	0.165	0.028	1.881	1.035	0.180
41	0.5	4.3	0.006	0.144	na	na	na
42	9.9	53.3	0.040	0.035	0.373	1.038	0.164
43	33.5	87.4	0.275	0.040	1.851	0.701	0.148
44	1.3	0.0	0.000	0.073	na	na	na
45	1.5	0.0	0.000	0.062	na	na	na

na: no analysis/measurement

5 Example 23. Transfer of pansy F3'5'H gene (#40) and
Torenia anthocyanin 5-acyltransferase gene into WKS77

Plasmid pSPB130 (Fig. 7) was transferred into the
dark red-purple rose variety "WKS77", and 35
transformants were obtained. Accumulation of delphinidin
10 was confirmed in all 17 of the pigment-analyzed plants
(Table 24). The delphinidin content was 57% at maximum
(average: 33%). The flower color was altered from RHS
Color Chart 57a (Red-Purple group) to 71a (Red-Purple
group). However, no color of the Violet group, Violet-
15 Blue group or Blue group according to the RHSCC was
achieved and the target blue rose could not be obtained.

Table 24

Plant No.	Acylation (%)	Del content (%)	Del (mg/g)	Cya (mg/g)	Pel (mg/g)	M (mg/g)	Q (mg/g)	K (mg/g)
1	6.2	42.5	1.153	1.552	0.008	0.484	0.679	0.196
2	7.6	38.6	0.618	0.979	0.005	0.267	0.465	0.094
3	3.9	40.4	0.706	1.030	0.011	1.266	1.768	0.722
4	2.0	46.9	0.372	0.417	0.004	0.363	0.608	0.276
5	5.4	40.6	0.540	0.784	0.005	1.077	1.809	0.645
6	2.0	44.7	1.078	1.325	0.009	0.516	1.034	0.382
7	2.1	46.5	0.398	0.453	0.005	0.353	0.792	0.569
8	5.8	39.7	0.647	0.980	0.005	0.425	0.706	0.183
9	4.7	40.0	0.844	1.268	0.000	0.310	0.764	0.199
10	7.6	39.7	1.345	2.033	0.009	0.350	0.635	0.119
11	14.1	2.9	0.068	2.274	0.013	na	na	na
12	12.8	6.9	0.126	1.688	0.009	na	na	na
13	12.7	4.2	0.109	2.468	0.012	0.060	1.541	0.366
14	13.0	20.9	0.704	2.669	0.000	0.407	2.502	0.694
15	19.3	43.5	1.011	1.308	0.007	0.357	0.843	0.276
16	19.6	6.1	0.092	1.414	0.010	0.120	1.740	0.477
17	22.8	56.6	1.068	0.814	0.004	0.604	0.503	0.126

na: no analysis/measurement

5 Example 24. Transfer of pansy F3'5'H gene (#40) and
Torenia anthocyanin 5-acyltransferase gene into WKS82

Plasmid pSPB130 (Fig. 7) was transferred into the pale violet rose variety "WKS82", and 89 transformants were obtained. Accumulation of delphinidin was confirmed in all 44 of the pigment-analyzed plants (Tables 25 and 26). The delphinidin content was 91% at maximum (average: 49%). The flower color was altered from RHS Color Chart 186d (Greyed-Purple group) to 80c (Purple-Violet group). However, no color of the Violet group, Violet-Blue group or Blue group according to the RHSCC was achieved and the target blue rose could not be obtained.

Table 25

Plant No.	Acylation (%)	Del content (%)	Del (mg/g)	Cya (mg/g)	Pel (mg/g)	M (mg/g)	Q (mg/g)	K (mg/g)
1	10.5	52.3	0.055	0.050	0.000	0.430	0.883	0.083
2	15.9	62.5	0.091	0.054	0.000	0.570	0.549	0.030
3	15.9	36.6	0.044	0.076	0.000	0.622	2.221	0.102
4	6.8	40.0	0.023	0.034	0.000	0.247	0.986	0.172
5	15.0	82.9	0.087	0.018	0.000	5.451	0.403	0.042
6	na	89.7	0.072	0.008	0.000	0.853	0.163	0.062
7	9.5	89.5	0.101	0.012	0.000	0.719	0.144	0.019
8	14.7	11.4	0.012	0.090	0.000	na	na	na
9	11.6	29.3	0.024	0.059	0.000	na	na	na
10	8.7	15.2	0.010	0.053	0.000	na	na	na
11	7.9	59.0	0.046	0.032	0.000	0.580	0.619	0.022
12	8.5	55.6	0.060	0.048	0.000	1.318	1.615	0.165
13	13.9	42.3	0.026	0.035	0.000	0.603	1.094	0.052
14	10.1	10.3	0.008	0.073	0.000	na	na	na
15	10.6	18.8	0.018	0.079	0.000	na	na	na
16	9.3	11.7	0.009	0.066	0.000	na	na	na
17	14.3	76.2	0.112	0.035	0.000	3.741	1.587	0.377
18	12.7	76.7	0.101	0.031	0.000	1.608	0.656	0.075
19	9.8	71.7	0.057	0.022	0.000	1.403	0.455	0.041
20	5.3	14.1	0.011	0.068	0.000	0.132	2.999	0.720
21	3.5	18.5	0.008	0.035	0.000	na	na	na
22	7.7	23.1	0.017	0.055	0.000	0.141	0.929	0.034
23	5.4	19.0	0.015	0.065	0.000	0.297	4.128	1.350

na: no analysis/measurement

Table 26

Plant No.	Acylation (%)	Del content (%)	Del (mg/g)	Cya (mg/g)	Pel (mg/g)	M (mg/g)	Q (mg/g)	K (mg/g)
24	1.1	42.1	0.036	0.050	0.000	0.609	2.929	0.679
25	22.7	91.0	0.079	0.008	0.000	0.964	0.218	0.018
26	6.1	61.3	0.048	0.030	0.000	0.490	0.468	0.029
27	8.7	91.3	0.097	0.009	0.000	2.053	0.339	0.123
28	9.4	59.9	0.060	0.040	0.000	1.537	1.631	0.422
29	5.5	51.2	0.040	0.038	0.000	0.688	0.723	0.038
30	5.1	61.4	0.056	0.032	0.003	0.637	0.537	0.087
31	7.0	53.3	0.037	0.032	0.000	0.706	1.032	0.051
32	5.7	58.1	0.071	0.051	0.000	1.592	1.478	0.220
33	4.3	64.6	0.092	0.050	0.000	0.849	0.753	0.035
34	6.4	61.7	0.042	0.026	0.000	0.477	0.468	0.023
35	8.9	58.8	0.048	0.034	0.000	0.646	0.928	0.063
36	6.2	11.6	0.007	0.057	0.000	0.094	1.132	0.066
37	7.1	51.2	0.038	0.036	0.000	0.911	1.135	0.079
38	5.8	50.8	0.029	0.028	0.000	0.868	1.105	0.096
39	5.5	47.0	0.027	0.023	0.007	1.366	1.632	0.105
40	4.9	67.0	0.044	0.022	0.000	0.795	0.586	0.051
41	na	61.1	0.053	0.033	0.000	1.310	1.466	0.259
42	9.6	71.0	0.074	0.030	0.000	0.460	0.337	0.023
43	1.2	27.6	0.009	0.024	0.000	na	na	na
44	5.2	13.8	0.013	0.078	0.000	na	na	na

na: no analysis/measurement

Example 25. Transfer of pansy F3'5'H gene (#40) and
Torenia anthocyanin 5-acyltransferase gene into WKS91

Plasmid pSPB130 (Fig. 7) was transferred into the
light orange rose variety "WKS91", and 10 transformants
were obtained. Accumulation of delphinidin was confirmed
in only one of the two pigment-analyzed plants (Table
27). The delphinidin content was 2% at maximum. No
alteration in flower color was observed from RHS Color
Chart 43c (Red group).

Table 27

Plant No.	Acylation (%)	Del content (%)	Del (mg/g)	Cya (mg/g)	Pel (mg/g)
1	0.7	0.0	0.000	0.090	0.307
2	0.0	1.8	0.006	0.040	0.295

Example 26. Expression of pansy F3'5'H gene (#40)
and iris DFR gene and suppression of rose endogenous DFR
gene in Lavande

RNA was obtained from blue iris petals of cut
flowers, and polyA⁺RNA was prepared therefrom. A cDNA
library was prepared from the polyA⁺RNA with λ ZAPII
(Stratagene) as the vector, using a cDNA library
preparation kit (Stratagene) according to the
manufacturer's recommended protocol. An iris DFR gene
fragment was prepared by the same method as reported for
obtaining gentian DFR gene fragment (Tanaka et al. Plant
Cell Physiol. 37, 711-716 1996).

The approximately 400 bp DNA fragment obtained was
recovered with Gene Clean according to the manufacturer's
recommended protocol, and was subcloned in pCR-TOPO.
Determination of the nucleotide sequence revealed a
sequence homologous to the rose DFR gene. The DNA
fragment was used for screening of the iris cDNA library,

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and iris DFR cDNA including the full-length amino acid sequence was obtained. The total nucleotide sequence of the cDNA in the clone designated as pSPB906 was determined. The nucleotide sequence is listed as SEQ ID NO: 9, and the corresponding amino acid sequence is listed as SEQ ID NO: 10.

Next, an approximately 3.9 kb DNA fragment obtained by digestion of pSPB580 with BamHI and XhoI was linked with an approximately 1.5 kb DNA fragment obtained by digestion of pSPB906 with BamHI and XhoI, and the obtained plasmid was designated as pSPB909.

A vector for transcription of double-stranded RNA for the rose DFR cDNA in plants was prepared in the following manner. An approximately 3.5 kb DNA fragment (including MacI promoter, rose DFR cDNA and mas terminator) obtained by partial digestion of pCGP1364 (Tanaka et al., Plant Cell Physiol. (1995) 36, 1023-1031) with PstI was inserted at the PstI site of pUC19 (Yanisch-Perron C et al., Gene 33:103-119, 1985) to obtain plasmids, among which a plasmid having the HindIII site of pUC19 near the MacI promoter was designated as pCGP1394.

Next, an approximately 1.4 kb DNA fragment obtained by digestion of pCGP1394 with HindIII and SacII was ligated with an approximately 1.9 kb DNA fragment obtained by digestion of pCGP1394 with PstI, blunting of the ends and further digestion with SacII, and with a binary vector fragment obtained by digestion of pBinPLUS with SacI, blunting of the ends and further digestion with HindIII, to obtain pSPB185. Plasmid pSPB185 was digested with XbaI, blunted and ligated with a SalI linker to obtain pSPB521. An approximately 700 bp DNA fragment obtained by digestion of pUE6 with HindIII and BamHI was ligated with a binary vector DNA fragment obtained by digestion of pSPB521 with HindIII and SacI and with a GUS gene fragment obtained by digestion of pE2113 with BamHI and SacI, to obtain pSPB528.

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Plasmid pSPB528 is a binary vector having a structural gene inserted between the enhancer-containing cauliflower mosaic virus 35S promoter and the manopine synthase terminator, which is expressible in plants.

5 Also, in order to shorten the 5'-end non-translated sequence of rose DFR cDNA in pCGP645, plasmid pCGP645 was digested with SmaI and PvuI, blunted and re-ligated to obtain pCGP645s.

10 The 5'-end sequence of rose DFR cDNA was obtained by PCR amplification using pCGP645s as the template and a reverse primer and the synthetic primer RDF310 (5'-CCCTCGAGCCCTTGATGGCCTCGTCG-3') (SEQ ID NO: 19) as the primers, and was cloned in pCRTOP0. The DNA nucleotide sequence was determined and absence of errors by PCR was confirmed. This plasmid was designated as pSPB569.

15 Also, a rose DFR cDNA 5'-end sequence with a different length was obtained by amplification using pCGP645s as the template and a reverse primer and the synthetic primer RDF830 (5'-GGGTCGACGCGGCCCTCTGCTTTCGG-3') (SEQ ID NO: 20) as the primers, and was cloned in pCRTOP0. The DNA nucleotide sequence was determined and absence of errors by PCR was confirmed.

20 This plasmid was designated as pSPB570. A binary vector DNA fragment obtained by digestion of pSPB528 with BamHI and SacI, and an approximately 0.3 kb DNA fragment obtained by digestion of pSPB569 with SacI and XhoI, were ligated with a DNA fragment obtained by digestion of pSPB570 with BamHI and SalI, to obtain pSPB572. This vector is designed for transcription of double-stranded

25 RNA for rose DFR cDNA in plants.

30 Plasmid pUE6 was digested with SacI and blunted, and a SalI linker was inserted to obtain pUE8. A DNA fragment obtained by digesting pUE8 with HindIII and EcoRI was introduced at the HindIII and EcoRI sites of pBinPLUS to obtain plasmid pSPB189. An approximately 3.7 kb DNA fragment obtained by digestion of pSPB189 with BamHI and SalI was ligated with an approximately 1.8 kb

35

DNA fragment obtained by complete digestion of pCGP1961 with BamHI followed by partial digestion with XhoI, to obtain plasmid pSPB567. After PacI digestion and dephosphorylation treatment of pSPB572, it was linked with an approximately 2.8 kb DNA fragment obtained by digestion of pSPB567 with PacI, and a plasmid with transcription of the nptII gene and pansy F3'5'H #40 in the same direction was selected and designated as pSPB905.

After AscI digestion and dephosphorylation treatment of pSPB905, it was linked with an approximately 2.5 kb DNA fragment obtained by digestion of pSPB909 with AscI, and a plasmid with transcription of the iris DFR gene in the same direction as the nptII gene was obtained and designated as pSPB919 (Fig. 8). This plasmid is expected to allow transcription of the iris DFR gene and pansy F3'5'H #40 gene in rose, while suppressing expression of the rose DFR gene due to transcription of double-stranded RNA. The plasmid was transferred into *Agrobacterium tumefaciens* Ag10.

Plasmid pSPB919 (Fig. 8) was transferred into the pale violet rose variety "Lavande", and 87 transformants were obtained. Accumulation of delphinidin was confirmed in 31 of the 38 pigment-analyzed plants (Tables 28 and 29). The delphinidin content was 100% at maximum (average: 76%). The flower color was altered from RHS Color Chart 186c (Greyed-Purple group) to 85a,b (Violet group).

RNA was extracted from rose petals in the same manner as explained above, and after separating the RNA by agarose gel electrophoresis, it was transferred onto Hybond N (Amersham) (for example, Tanaka et al., 1995). The mRNA was detected using a DIG Northern Starter Kit (Roche) by the manufacturer's recommended protocol. The rose DFR mRNA was detected using pCGP645 (Tanaka et al., Plant Cell Physiol. 36, 1023-1031, 1995) as template and a T7 primer transcript as the probe.

Detection of pansy F3'5'H #40 mRNA was accomplished using pCGP1961 as template and a T7 primer transcript as the probe. Detection of iris DFR mRNA was accomplished using pSPB906 as template and a T7 primer transcript as the probe. Pansy F3'5'H #40 and iris DFR gene mRNA were detected in the altered-color roses. On the other hand, rose DFR mRNA was significantly reduced compared to the host and a band was detected at the low molecular weight position, indicating decomposition of the rose DFR mRNA.

10

Table 28

Plant No.	Del content (%)	Del (mg/g)	Cya (mg/g)	M (mg/g)	Q (mg/g)	K (mg/g)
1	0.0	0.000	0.105	0.036	0.856	0.038
2	0.0	0.000	0.125	na	na	na
3	0.0	0.000	0.091	0.023	0.851	0.101
4	0.0	0.000	0.116	0.000	1.336	0.087
5	0.0	0.000	0.048	na	na	na
6	88.5	0.086	0.011	1.626	1.187	0.411
7	90.8	0.089	0.009	0.797	1.548	0.087
8	84.0	0.046	0.009	0.163	0.699	0.016
9	87.8	0.062	0.009	0.193	0.760	0.022
10	89.3	0.072	0.009	0.210	0.575	0.033
11	91.5	0.049	0.005	0.398	0.805	0.050
12	91.5	0.032	0.003	0.100	0.811	0.014
13	85.7	0.040	0.007	0.092	0.497	0.012
14	64.9	0.040	0.021	0.263	0.327	0.015
15	88.3	0.041	0.005	na	na	na
16	66.4	0.011	0.006	0.036	1.221	0.030
17	79.7	0.008	0.002	0.030	0.765	0.009
18	100.0	0.010	0.000	0.048	1.343	0.067
19	95.9	0.040	0.002	0.159	0.136	0.004
20	65.4	0.016	0.008	0.090	1.244	0.048
21	18.8	0.011	0.049	0.048	0.855	0.020
22	0.0	0.000	0.110	0.000	1.274	0.079
23	0.0	0.000	0.140	0.000	1.952	0.200

na: no analysis/measurement

Table 29

Plant No.	Del content (%)	Del (mg/g)	Cya (mg/g)	M (mg/g)	Q (mg/g)	K (mg/g)
24	41.4	0.102	0.144	0.265	0.417	0.015
25	34.3	0.042	0.081	0.167	0.429	0.024
26	34.6	0.023	0.043	na	na	na
27	41.4	0.082	0.116	0.232	0.385	0.019
28	37.7	0.046	0.076	0.254	0.429	0.018
29	36.1	0.032	0.057	0.151	0.235	0.042
30	97.2	0.052	0.002	0.208	0.088	0.004
31	93.0	0.038	0.003	0.347	0.137	0.007
32	98.2	0.101	0.002	0.339	0.258	0.029
33	91.3	0.039	0.004	na	na	na
34	91.9	0.041	0.004	0.332	0.120	0.007
35	96.8	0.052	0.002	na	na	na
36	96.7	0.084	0.003	0.342	0.168	0.010
37	88.0	0.014	0.002	0.076	1.000	0.029
38	84.5	0.016	0.003	0.074	1.121	0.025

na: no analysis/measurement

5 Example 27. Expression of pansy F3'5'H gene (#40)
and *Nierembergia* DFR gene, and suppression of rose
endogenous DFR gene in Lavande

10 RNA was obtained from petals of the *Nierembergia*
hybrida cultivar Fairy Bell Patio Light Blue (Suntory
Flowers Co., Ltd.), and polyA⁺RNA was prepared therefrom.
A cDNA library was prepared from the polyA⁺RNA with λ ZAPII
(Stratagene) as the vector, using a cDNA library
synthesis kit (Stratagene) according to the
manufacturer's recommended protocol. The cDNA library
15 was screened using DIG-labeled petunia DFR cDNA (from
pCGP1405).

20 The screening conditions were according to the
plaque hybridization method using a DIG-labeling system,
according to the manufacturer's recommended protocol.
However, the formaldehyde concentration was 30% for the
pre-hybridization and hybridization buffers, and
hybridization was carried out overnight at 37°C. The
membrane was rinsed at 55°C in 5xSSC containing 1% SDS.

Plasmids were recovered from 20 plaques among the numerous positive signals, and their nucleotide sequences were determined using Reverse Primer (Takara). These exhibited high homology with the DFR genes of other plants including petunia. The total nucleotide sequence of the cDNA in the clone designated as pSPB709 was determined. The nucleotide sequence is listed as SEQ ID NO: 11, and the corresponding amino acid sequence is listed as SEQ ID NO: 12

An approximately 3.9 kb DNA fragment obtained by digestion of pSPB580 with BamHI and XhoI was linked with an approximately 1.5 kb DNA fragment obtained by digestion of pSPB709 with BamHI and XhoI, to obtain plasmid pSPB910. After AscI digestion and dephosphorylation treatment of pSPB910, it was linked with an approximately 2.5 kb DNA fragment obtained by digestion of pSPB910 with AscI, and a plasmid with transcription of the *Nierembergia* DFR gene in the same direction as the *nptII* gene was obtained and designated as pSPB920 (Fig. 9). This plasmid is expected to allow transcription of the *Nierembergia* DFR gene and pansy F3'5'H #40 gene in rose, while suppressing expression of the rose DFR gene due to transcription of double-stranded RNA. The plasmid was transferred into *Agrobacterium tumefaciens* Ag10.

Plasmid pSPB920 (Fig. 9) was transferred into the pale violet rose variety "Lavande", and 56 transformants were obtained. Accumulation of delphinidin was confirmed in 23 of the 24 pigment-analyzed plants (Table 30). The delphinidin content was 100% at maximum (average: 43%). The flower color was altered from RHS Color Chart 186c (Greyed-Purple group) to 85b (Violet group).

Table 30

Plant No.	Del content (%)	Del (mg/g)	Cya (mg/g)	M (mg/g)	Q (mg/g)	K (mg/g)
1	69.5	0.025	0.002	0.081	2.265	0.066
2	85.4	0.024	0.004	0.114	1.355	0.032
3	71.8	0.006	0.002	0.043	0.781	0.027
4	100.0	0.012	0.000	0.414	0.283	0.030
5	88.2	0.015	0.002	0.506	0.126	0.030
6	100.0	0.013	0.000	0.430	0.123	0.008
7	33.3	0.019	0.038	na	na	na
8	37.3	0.012	0.020	na	na	na
9	48.2	0.012	0.013	na	na	na
10	18.9	0.011	0.049	0.053	1.023	0.022
11	39.7	0.037	0.056	0.120	1.157	0.035
12	9.4	0.010	0.095	na	na	na
13	11.0	0.008	0.062	na	na	na
14	24.4	0.017	0.054	0.128	1.852	0.181
15	12.4	0.015	0.102	na	na	na
16	89.7	0.089	0.010	0.530	1.424	0.165
17	15.4	0.006	0.035	na	na	na
18	22.3	0.006	0.019	0.018	1.286	0.038
19	10.4	0.007	0.058	0.039	1.673	0.045
20	28.3	0.006	0.015	0.028	0.932	0.025
21	35.2	0.015	0.028	0.105	0.743	0.028
22	16.0	0.010	0.052	na	na	na
23	0.0	0.000	0.018	0.013	1.764	0.027
24	13.7	0.007	0.042	0.033	1.469	0.041

na: no analysis/measurement

5

Example 28. Inheritance of traits to progeny

Cross-breeding was carried out using a transformant (LA/919-2-13) obtained by transfer of pSPB919 (Fig. 8) into the pale violet rose variety "Lavande" as the pollen parent and non-recombinant WKS77 or WKS133 as the maternal parent (Suzuki, S., "Bara, Hanazufu", Shogakkann, p.256-260, 1990). Fruit was collected on the 100th day after pollination. Seed production was accomplished by first peeling the fruit, harvesting the achene, peeling the achene, and then removing the germ and embedding it on moistened filter paper in a dish. The water used for seed production was sterilized water

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containing 1 ml/l PPM™ (Plant Preservative Mixture, Plant Cell Technology, Inc.) and 50 mg/l kanamycin, and seedlings were raised by potting only the normally budded plants.

- 5 Accumulation of delphinidin was confirmed in all 40 of the pigment-analyzed transformant progeny (Tables 31 and 32). The delphinidin content was 99% at maximum (average: 46%).

10

Table 31

Plant No.	Del content (%)	Del (mg/g)	Cya (mg/g)	Pel (mg/g)	Peo (mg/g)
1	89.8	0.494	0.056	0.000	0.000
2	96.1	3.900	0.153	0.005	0.000
3	55.9	0.836	0.660	0.000	0.000
4	24.6	0.041	0.127	0.000	0.000
5	23.5	1.108	3.605	0.009	0.002
6	25.9	0.191	0.545	0.003	0.000
7	0.5	0.013	2.552	0.012	0.002
8	75.8	0.283	0.090	0.000	0.000
9	95.9	1.420	0.061	0.000	0.000
10	30.8	0.862	1.841	0.007	0.105
11	13.3	0.068	0.441	0.004	0.000
12	23.9	0.529	1.667	0.023	0.000
13	43.7	0.280	0.362	0.000	0.000
14	19.3	0.035	0.145	0.000	0.000
15	0.6	0.008	1.418	0.021	0.000
16	20.8	0.048	0.183	0.000	0.000
17	92.5	2.257	0.177	0.007	0.000
18	66.4	2.496	1.247	0.015	0.000
19	42.4	0.369	0.497	0.004	0.000
20	75.6	0.597	0.183	0.010	0.000
21	19.6	0.271	1.103	0.008	0.000
22	71.0	0.107	0.044	0.000	0.000
23	0.6	0.006	0.850	0.004	0.000

Table 32

Plant No.	Del content (%)	Del (mg/g)	Cya (mg/g)	Pel (mg/g)	Peo (mg/g)
24	16.7	0.053	0.263	0.000	0.000
25	71.8	0.211	0.083	0.000	0.000
26	18.6	0.177	0.769	0.003	0.000
27	1.3	0.009	0.652	0.004	0.000
28	59.7	0.183	0.124	0.000	0.000
29	39.6	0.124	0.187	0.003	0.000
30	21.4	0.187	0.684	0.003	0.000
31	0.6	0.005	0.763	0.004	0.000
32	38.8	0.226	0.353	0.003	0.000
33	50.5	0.154	0.151	0.000	0.000
34	28.0	0.267	0.682	0.003	0.000
35	83.9	0.204	0.039	0.000	0.000
36	64.9	0.380	0.205	0.000	0.000
37	78.8	0.239	0.064	0.000	0.000
38	97.4	0.614	0.016	0.000	0.000
39	98.7	0.805	0.011	0.000	0.000
40	54.9	0.083	0.068	0.000	0.000

5 Example 29. Expression of pansy F3'5'H #40 gene and
iris DFR gene and suppression of rose endogenous DFR gene
in WKS140

10 Plasmid pSPB919 was transferred into the pale violet
rose variety "WKS140", and 89 transformants were
obtained. Accumulation of delphinidin was confirmed in
74 of the 79 pigment-analyzed plants. The delphinidin
content was 100% at maximum (average: 68%). The flower
color was altered from RHS Color Chart 186d (Greyed-
Purple group) to primarily 84c (Violet group).

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Table 33

Plant No.	Del (%)	Del (mg/g)	Cya (mg/g)	Pel (mg/g)
1	0.0%	0.0000	0.0423	0.0000
2	89.9%	0.0242	0.0027	na
3	90.0%	0.0245	0.0027	na
4	88.6%	0.0093	0.0012	na
5	43.5%	0.0042	0.0054	na
6	91.2%	0.0118	0.0011	na
7	81.2%	0.0027	0.0006	na
8	81.0%	0.0173	0.0041	na
9	73.9%	0.0733	0.0259	na
10	62.9%	0.0321	0.0190	na
11	91.9%	0.0962	0.0084	na
12	99.1%	0.1606	0.0015	na
13	94.7%	0.0588	0.0033	na
14	100.0%	0.0839	0.0000	na
15	0.0%	0.0000	0.0005	na
16	98.4%	0.0296	0.0005	na
17	80.4%	0.1748	0.0451	na
18	94.6%	0.0190	0.0000	na
19	0.0%	0.0000	0.0714	na
20	34.3%	0.0099	0.0191	na
21	30.9%	0.0126	0.0282	na
22	65.6%	0.0294	0.0154	na
23	24.1%	0.0205	0.0646	na

na: no analysis/measurement

5 Example 30. Expression of pansy F3'5'H #40 gene and iris DFR gene and suppression of rose endogenous DFR gene in WKS77

10 Plasmid pSPB919 was transferred into the dark red-purple rose variety "WKS77", and 50 transformants were obtained. Accumulation of delphinidin was confirmed in 21 of the 23 pigment-analyzed plants. The delphinidin content was 81% at maximum (average: 19%). The flower color was altered from RHS Color Chart 57a (Red-Purple group) to 77b (Purple group).

Table 34

Plant No.	Del (%)	Del (mg/g)	Cya (mg/g)	Pel (mg/g)
1	26.0%	1.2028	3.4033	0.0117
2	41.5%	0.6473	0.9093	0.0048
3	80.8%	0.2210	0.0526	na
4	68.0%	0.1865	0.0878	na
5	68.5%	0.2090	0.0951	0.0010
6	1.5%	0.0119	0.7731	0.0051
7	1.5%	0.0114	0.7304	0.0041
8	0.2%	0.0069	2.9266	0.0063
9	0.2%	0.0017	1.0791	0.0062
10	0.0%	0.0000	0.5013	0.0043
11	0.1%	0.0028	2.3418	0.0110
12	0.4%	0.0091	2.4603	0.0126
13	0.2%	0.0040	1.7766	0.0096
14	0.3%	0.0026	0.9046	0.0052
15	0.0%	0.0000	1.6063	0.0100
16	22.2%	0.3279	1.1392	0.0049
17	24.0%	0.2638	0.8288	0.0052
18	1.4%	0.0240	1.6777	0.0118
19	1.1%	0.0186	1.6352	0.0101
20	26.7%	0.2645	0.7230	0.0037
21	22.7%	0.2200	0.7460	0.0046
22	40.1%	0.8929	1.3374	0.0071

na: no analysis/measurement

5 Example 31. Expression of pansy F3'5'H #40 gene and
Nierembergia DFR gene and suppression of rose endogenous
DFR gene in WKS77

10 Plasmid pSPB920 was transferred into the dark red-
purple rose variety "WKS77", and 30 transformants were
obtained. Accumulation of delphinidin was confirmed in
26 of the 27 pigment-analyzed plants. The delphinidin
content was 98% at maximum (average: 60%). The flower
color was altered from RHS Color Chart 57a (Red-Purple
group) to 77b (Purple group).

Table 35

Plant No.	Del (%)	Del (mg/g)	Cya (mg/g)	Pel (mg/g)
1	93.9%	0.1679	0.0110	0.0000
2	97.6%	0.2311	0.0058	na
3	96.3%	0.1684	0.0065	na
4	97.1%	0.1012	0.0017	na
5	9.6%	0.0946	0.7810	0.1104
6	21.9%	0.1462	0.5166	0.0034
7	12.7%	0.1097	0.7495	0.0049
8	97.9%	0.1942	0.0042	na
9	98.1%	0.1228	0.0024	na
10	3.2%	0.0360	1.0689	0.0035
11	3.1%	0.0267	0.9587	0.0032
12	4.8%	0.1138	2.2562	0.0049
13	6.2%	0.1066	1.5999	0.0080
14	96.5%	0.3541	0.0132	na
15	2.1%	0.0173	0.7852	0.0068
16	94.7%	0.2898	0.0160	0.0000
17	96.7%	0.0819	0.0020	0.0000
18	95.8%	0.6969	0.0309	na
19	96.4%	0.4868	0.0181	na
20	64.3%	0.3092	0.1724	na
21	26.9%	0.2740	0.7431	0.0025
22	19.9%	0.3760	1.5028	0.0071
23	88.2%	0.0316	0.0042	na
24	94.2%	0.0259	0.0016	na
25	90.4%	0.0481	0.0051	na

na: no analysis/measurement

5 Example 32. Expression of pansy F3'5'H #40 gene and
petunia DFR gene and suppression of rose endogenous DFR
gene in WKS77

10 Plasmid pSPB921 was transferred into the dark red-
purple rose variety "WKS77", and 15 transformants were
obtained. Accumulation of delphinidin was confirmed in
12 of the 13 pigment-analyzed plants. The delphinidin
content was 98% at maximum (average: 60%). The flower
color was altered from RHS Color Chart 57a (Red-Purple
group) to 72b (Red-Purple group).

Table 36

Plant No.	Del (%)	Del (mg/g)	Cya (mg/g)	Pel (mg/g)
1	90.0%	0.0549	0.0061	na
2	38.4%	0.3397	0.5402	0.0041
3	56.9%	0.7834	0.5824	0.0099
4	58.5%	0.0196	0.0139	na
5	90.3%	0.1336	0.0144	na
6	90.9%	0.1251	0.0126	na
7	86.7%	0.1771	0.0274	na
8	91.6%	0.0113	0.0010	na
9	97.5%	0.0864	0.0022	na
10	9.5%	0.2687	2.6591	0.0000
11	8.8%	0.1421	1.4598	0.0071
12	0.4%	0.0060	1.3554	0.0053

na: no analysis/measurement

5 Example 33. Inheritance of traits to progeny

10 Cross-breeding was carried out in the same manner as Example 28, using a transformant (LA/919-4-10) obtained by transfer of pSPB919 into the pale violet rose variety "Lavande" as the pollen parent and the non-recombinant rose variety "Black Baccara" as the maternal parent. Fruit was collected on the 100th day after pollination. Seed production was accomplished by first peeling the fruit, harvesting the achene, peeling the achene, and then removing the germ and embedding it on moistened

15 filter paper in a dish. The water used for seed production was sterilized water containing 1 ml/l PPMTM (Plant Preservative Mixture, Plant Cell Technology, Inc.) and 50 mg/l kanamycin, and seedlings were raised by potting only the normally budded plants.

20 Accumulation of delphinidin was confirmed in all 18 of the pigment-analyzed transformant progeny. The delphinidin content was 99.8% at maximum (average: 98.7%).

Table 37

Plant No.	Del (%)	Del (mg/g)	Cya (mg/g)	Pel (mg/g)
1	97.8%	0.6633	0.0142	0.0009
2	99.0%	0.9002	0.0096	na
3	98.5%	0.5385	0.0080	na
4	99.5%	2.0561	0.0087	0.0016
5	99.8%	1.6556	0.0034	na
6	96.6%	0.5601	0.0200	na
7	99.0%	0.6148	0.0063	na
8	98.9%	1.6867	0.0193	na
9	95.0%	0.5740	0.0304	na
10	96.9%	0.1152	0.0036	na
11	99.3%	0.0683	0.0005	na
12	99.6%	0.1248	0.0005	na
13	99.5%	0.3574	0.0010	0.0000
14	99.6%	0.5500	0.0021	na
15	99.6%	1.2322	0.0049	na
16	99.7%	1.4384	0.0042	na
17	99.8%	0.5117	0.0010	na
18	98.3%	0.8073	0.0140	na

na: no analysis/measurement

5 Example 34. Expression of pansy F3'5'H #40 gene and suppression of rose endogenous F3'H gene in WKS77

Plasmid pSPB1106 (Fig. 10) was transferred into the dark red-purple rose variety "WKS77", and 40 transformants were obtained. Accumulation of delphinidin was confirmed in all 26 of the pigment-analyzed plants. The delphinidin content was 80.0% at maximum (average: 30.5%). The flower color underwent a major alteration from RHS Color Chart 57a (Red-Purple group) to 83d (Violet group).

Table 38

Plant No.	Del (%)	Del (mg/g)	Cya (mg/g)	Pel (mg/g)	M (mg/g)	Q (mg/g)	K (mg/g)
1	68.7%	0.5497	0.2275	0.0241	na	na	na
2	78.8%	0.3449	0.0830	0.0096	na	na	na
3	80.0%	0.6949	0.1604	0.0144	na	na	na
4	71.2%	0.4377	0.1563	0.0214	na	na	na
5	72.7%	0.5260	0.1715	0.0266	0.3812	0.2275	1.7669
6	70.7%	0.3829	0.1449	0.0146	na	na	na
7	10.3%	0.0358	0.3031	0.0071	na	na	na
8	15.6%	0.1847	0.9530	0.0444	na	na	na
9	4.8%	0.0739	1.4586	0.0149	na	na	na
10	1.1%	0.0114	1.0411	0.0144	na	na	na
11	54.0%	1.3206	1.1166	0.0092	na	na	na
12	57.8%	0.8842	0.6410	0.0056	na	na	na
13	0.9%	0.0242	2.5500	0.0168	na	na	na
14	23.0%	0.2087	0.6909	0.0062	na	na	na
15	12.7%	0.1645	1.1271	0.0058	na	na	na
16	26.4%	0.5275	1.4645	0.0132	na	na	na
17	18.7%	0.3555	1.5310	0.0109	na	na	na
18	24.2%	0.4388	1.3687	0.0072	na	na	na
19	64.7%	0.4029	0.1945	0.0249	0.6368	0.3949	2.0567
20	0.1%	0.0021	1.8646	0.0077	na	na	na
21	0.0%	0.0000	0.9708	0.0062	na	na	na
22	0.1%	0.0022	2.6049	0.0127	na	na	na
23	0.4%	0.0066	1.8002	0.0066	na	na	na
24	0.5%	0.0079	1.4670	0.0056	0.0000	1.3096	0.2414
25	17.3%	0.1000	0.4671	0.0099	na	na	na
26	18.3%	0.1232	0.5418	0.0052	na	na	na

na: no analysis/measurement

5 Example 35. Expression of pansy F3'5'H #40 gene and suppression of rose endogenous F3'H gene in Lavande

10 Plasmid pSPB1106 was transferred into the pale violet rose variety "Lavande", and 40 transformants were obtained. Accumulation of delphinidin was confirmed in 23 of the 25 pigment-analyzed plants. The delphinidin content was 98.3% at maximum (average: 46.9%).

Table 39

Plant No.	Del (%)	Del (mg/g)	Cya (mg/g)	Pel (mg/g)	M (mg/g)	Q (mg/g)	K (mg/g)
1	76.8%	0.0732	0.0188	0.0032	0.5705	0.1595	0.3073
2	80.1%	0.1441	0.0296	0.0061	0.5298	0.1881	4.3294
3	3.7%	0.0086	0.2174	0.0027	na	na	na
4	4.4%	0.0079	0.1691	0.0034	na	na	na
5	8.8%	0.0158	0.1557	0.0070	na	na	na
6	39.0%	0.0212	0.0128	0.0204	0.0000	0.0363	1.3107
7	44.4%	0.0089	0.0027	0.0084	0.0756	0.0573	1.3689
8	40.4%	0.0165	0.0071	0.0172	0.0365	0.0592	2.5211
9	42.0%	0.0087	0.0036	0.0084	0.0752	0.0596	1.2661
10	13.5%	0.0153	0.0939	0.0040	0.1288	1.0594	0.5440
11	81.6%	0.2252	0.0447	0.0061	0.3947	0.1401	0.3947
12	78.8%	0.1022	0.0239	0.0036	0.6700	0.2137	0.5847
13	81.7%	0.2125	0.0438	0.0036	1.3616	0.4621	0.7478
14	80.9%	0.1829	0.0388	0.0044	0.4100	0.2405	0.0567
15	70.9%	0.0664	0.0204	0.0069	0.4230	0.1221	0.1788
16	0.0%	0.0000	0.0844	0.0000	na	na	na
17	98.0%	0.2363	0.0048	0.0000	0.0000	1.0613	0.2698
18	98.3%	0.1398	0.0025	0.0000	0.0479	0.7060	0.1299
19	4.2%	0.0078	0.1724	0.0040	0.0000	0.8627	0.2075
20	0.0%	0.0000	0.1696	0.0043	na	na	na
21	60.0%	0.0333	0.0115	0.0107	0.0000	0.0740	1.8678
22	14.3%	0.0091	0.0454	0.0088	0.1096	0.5305	0.6453
23	15.1%	0.0082	0.0408	0.0053	na	na	na
24	17.6%	0.0082	0.0324	0.0059	na	na	na
25	24.4%	0.0147	0.0375	0.0080	0.0000	0.2147	0.9765

na: no analysis/measurement

5 These results demonstrate that the transferred
exogenous gene was inherited and expressed by the
progeny, and that the trait of delphinidin production
which is not found in ordinary rose petals was
successfully inherited by the rose progeny. Thus, this
10 gene can be used for cross-breeding cultivation of roses
with altered colors to create roses with new colors
including blue and purple.

Industrial Applicability

15 By artificially suppressing function of the
endogenous metabolic pathway such as, for example,
expression of dihydroflavonol reductase, in rose, and

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expressing the gene coding for pansy flavonoid 3',5'-hydroxylase and a gene coding for dihydroflavonol reductase from species other than rose, it is possible to create blue to violet roses. These genes are inherited
5 by subsequent generations, and the blue rose trait can be utilized for cross-breeding.

71a

SEQUENCE LISTING

<110> International Flower Developments Proprietary Limited

<120> Method for producing rose with altered petal colors

<130> 5837-195CA

<140> CA 2,537,065

<141> 2004-08-13

<150> JP 2003-293121

<151> 2003-08-13

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Ala Val Ile Leu Phe Leu Ile Thr Arg Phe Leu Val Arg Ser Leu Phe	
15 20 25	
aag aaa cca acc gga ccg ctc ccg ccg ggt cct tca ggc tgg ccc ttg	147
Lys Lys Pro Thr Gly Pro Leu Pro Pro Gly Pro Ser Gly Trp Pro Leu	
30 35 40	
gtg ggc gcg ctc cct ctc cta ggc gcc atg cct cac gtc aca cta gcc	195
Val Gly Ala Leu Pro Leu Leu Gly Ala Met Pro His Val Thr Leu Ala	
45 50 55	
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Asn Leu Ala Lys Lys Tyr Gly Pro Ile Met Tyr Leu Lys Met Gly Thr	
60 65 70	

71b

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Cys Asp Met Val Val Ala Ser Thr Pro Asp Ser Ala Arg Ala Phe Leu	
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aaa acc cta gac ctc aac ttc tcc gac cgc ccg ccc aac gcc ggc gcc	339
Lys Thr Leu Asp Leu Asn Phe Ser Asp Arg Pro Pro Asn Ala Gly Ala	
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acc cat ttg gcg tac ggc gcg cag gac ttg gtc ttc gcg aag tac ggt	387
Thr His Leu Ala Tyr Gly Ala Gln Asp Leu Val Phe Ala Lys Tyr Gly	
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cca agg tgg aag acc cta aga aaa ttg agc aac ctc cac atg cta ggc	435
Pro Arg Trp Lys Thr Leu Arg Lys Leu Ser Asn Leu His Met Leu Gly	
125 130 135	
ggg aag gcg ctg gac gat tgg gct cac gtg agg gct aac gag cta ggc	483
Gly Lys Ala Leu Asp Asp Trp Ala His Val Arg Ala Asn Glu Leu Gly	
140 145 150	
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His Met Leu Asn Ala Met Cys Glu Ala Ser Arg Cys Gly Glu Pro Val	
155 160 165 170	
gtg ctg gcc gag atg ctc acg tac gcc atg gcc aac atg atc ggt caa	579
Val Leu Ala Glu Met Leu Thr Tyr Ala Met Ala Asn Met Ile Gly Gln	
175 180 185	
gtg ata ctg agt cgg cgc gtg ttc gtc acc aaa ggg aca gag tcg aac	627
Val Ile Leu Ser Arg Arg Val Phe Val Thr Lys Gly Thr Glu Ser Asn	
190 195 200	
gag ttc aaa gat atg gtg gtc gag ttg atg act tcc gcg ggg tat ttc	675
Glu Phe Lys Asp Met Val Val Glu Leu Met Thr Ser Ala Gly Tyr Phe	
205 210 215	
aac att ggt gac ttc ata ccg tcg att gct tgg atg gat ttg caa ggg	723
Asn Ile Gly Asp Phe Ile Pro Ser Ile Ala Trp Met Asp Leu Gln Gly	
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Ile Glu Arg Gly Met Lys Lys Leu His Thr Lys Phe Asp Val Leu Leu	
235 240 245 250	
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Thr Lys Met Met Lys Glu His Arg Ala Thr Ser His Glu Arg Glu Gly	
255 260 265	
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Gly Glu Lys Leu Asn Val Thr Asn Val Lys Ala Val Leu Leu Asn Leu	
285 290 295	

71c

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tcg aaa ctc ccg tat tta caa gcc ata gcg aaa gaa aca tat cgt aaa	1107
Ser Lys Leu Pro Tyr Leu Gln Ala Ile Ala Lys Glu Thr Tyr Arg Lys	
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cac cca tcg aca cct cta aac ctg ccg agg att gcg atc caa gca tgt	1155
His Pro Ser Thr Pro Leu Asn Leu Pro Arg Ile Ala Ile Gln Ala Cys	
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ttc tcg cct gaa aga ttc ttg tct gag gag aat ggg aag atc agt cca	1299
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Gly Gly Asn Asp Phe Glu Leu Ile Pro Phe Gly Ala Gly Arg Arg Ile	
430 435 440	
tgt gct ggg aca agg atg gga atg gtc ctt gta agt tat att ttg ggc	1395
Cys Ala Gly Thr Arg Met Gly Met Val Leu Val Ser Tyr Ile Leu Gly	
445 450 455	
act ttg gtc cat tct ttt gat tgg aaa tta cca aat ggg gtc agt gag	1443
Thr Leu Val His Ser Phe Asp Trp Lys Leu Pro Asn Gly Val Ser Glu	
460 465 470	
att aac atg gat gag agt ttt ggg ctt gcg ttg caa aag gcc gtg cct	1491
Ile Asn Met Asp Glu Ser Phe Gly Leu Ala Leu Gln Lys Ala Val Pro	
475 480 485 490	
ctc tcg gct acg gtc agt cca cga ttg gcc cca agc gcg tac gtt ata	1539
Leu Ser Ala Thr Val Ser Pro Arg Leu Ala Pro Ser Ala Tyr Val Ile	
495 500 505	
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71d

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 35 40 45
 Leu Gly Ala Met Pro His Val Thr Leu Ala Asn Leu Ala Lys Lys Tyr
 50 55 60
 Gly Pro Ile Met Tyr Leu Lys Met Gly Thr Cys Asp Met Val Val Ala
 65 70 75 80
 Ser Thr Pro Asp Ser Ala Arg Ala Phe Leu Lys Thr Leu Asp Leu Asn
 85 90 95
 Phe Ser Asp Arg Pro Pro Asn Ala Gly Ala Thr His Leu Ala Tyr Gly
 100 105 110
 Ala Gln Asp Leu Val Phe Ala Lys Tyr Gly Pro Arg Trp Lys Thr Leu
 115 120 125
 Arg Lys Leu Ser Asn Leu His Met Leu Gly Gly Lys Ala Leu Asp Asp
 130 135 140
 Trp Ala His Val Arg Ala Asn Glu Leu Gly His Met Leu Asn Ala Met
 145 150 155 160
 Cys Glu Ala Ser Arg Cys Gly Glu Pro Val Val Leu Ala Glu Met Leu
 165 170 175
 Thr Tyr Ala Met Ala Asn Met Ile Gly Gln Val Ile Leu Ser Arg Arg
 180 185 190
 Val Phe Val Thr Lys Gly Thr Glu Ser Asn Glu Phe Lys Asp Met Val
 195 200 205
 Val Glu Leu Met Thr Ser Ala Gly Tyr Phe Asn Ile Gly Asp Phe Ile
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 Pro Ser Ile Ala Trp Met Asp Leu Gln Gly Ile Glu Arg Gly Met Lys
 225 230 235 240

71e

Lys	Leu	His	Thr	Lys	Phe	Asp	Val	Leu	Leu	Thr	Lys	Met	Met	Lys	Glu	245	250	255
His	Arg	Ala	Thr	Ser	His	Glu	Arg	Glu	Gly	Lys	Ser	Asp	Phe	Leu	Asp	260	265	270
Val	Leu	Leu	Glu	Glu	Cys	Glu	Asn	Thr	Asn	Gly	Glu	Lys	Leu	Asn	Val	275	280	285
Thr	Asn	Val	Lys	Ala	Val	Leu	Leu	Asn	Leu	Phe	Thr	Ala	Gly	Thr	Asp	290	295	300
Thr	Ser	Ser	Ser	Ile	Ile	Glu	Trp	Ala	Leu	Thr	Glu	Met	Met	Lys	Asn	305	310	315
Pro	Thr	Ile	Leu	Lys	Lys	Thr	Gln	Glu	Glu	Met	Asp	Arg	Val	Ile	Gly	325	330	335
Arg	Asp	Arg	Arg	Leu	Leu	Glu	Ser	Asp	Val	Ser	Lys	Leu	Pro	Tyr	Leu	340	345	350
Gln	Ala	Ile	Ala	Lys	Glu	Thr	Tyr	Arg	Lys	His	Pro	Ser	Thr	Pro	Leu	355	360	365
Asn	Leu	Pro	Arg	Ile	Ala	Ile	Gln	Ala	Cys	Glu	Val	Asp	Gly	Tyr	Tyr	370	375	380
Ile	Pro	Lys	Asp	Thr	Arg	Leu	Ser	Val	Asn	Ile	Trp	Ala	Ile	Gly	Arg	385	390	395
Asp	Pro	Ser	Val	Trp	Glu	Asn	Pro	Ser	Glu	Phe	Ser	Pro	Glu	Arg	Phe	405	410	415
Leu	Ser	Glu	Glu	Asn	Gly	Lys	Ile	Ser	Pro	Gly	Gly	Asn	Asp	Phe	Glu	420	425	430
Leu	Ile	Pro	Phe	Gly	Ala	Gly	Arg	Arg	Ile	Cys	Ala	Gly	Thr	Arg	Met	435	440	445
Gly	Met	Val	Leu	Val	Ser	Tyr	Ile	Leu	Gly	Thr	Leu	Val	His	Ser	Phe	450	455	460
Asp	Trp	Lys	Leu	Pro	Asn	Gly	Val	Ser	Glu	Ile	Asn	Met	Asp	Glu	Ser	465	470	475
Phe	Gly	Leu	Ala	Leu	Gln	Lys	Ala	Val	Pro	Leu	Ser	Ala	Thr	Val	Ser	485	490	495
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Ala Ile Ile Phe Leu Ile Thr Arg Phe Leu Val Arg Ser Leu Phe Lys																
15 20 25																
aaa cca acc cga ccg ctc ccc ccg ggt cct ctc ggt tgg ccc ttg gtg																149
Lys Pro Thr Arg Pro Leu Pro Pro Gly Pro Leu Gly Trp Pro Leu Val																
30 35 40																
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Gly Ala Leu Pro Leu Leu Gly Ala Met Pro His Val Ala Leu Ala Lys																
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Leu Ala Lys Lys Tyr Gly Pro Ile Met His Leu Lys Met Gly Thr Cys																
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His Leu Ala Tyr Gly Ala Gln Asp Leu Val Phe Ala Lys Tyr Gly Pro																
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Arg Trp Lys Thr Leu Arg Lys Leu Ser Asn Leu His Met Leu Gly Gly																
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140 145 150 155																

71g

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Leu Ala Glu Met Leu Thr Tyr Ala Met Ala Asn Met Ile Gly Gln Val	
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365 370 375	

71h

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Trp Ala Ile Gly Arg Asp Pro Asn Val Trp Glu Asn Pro Leu Glu Phe
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ttg ccg gaa aga ttc ttg tct gaa gag aat ggg aag atc aat ccc ggt 1301
Leu Pro Glu Arg Phe Leu Ser Glu Glu Asn Gly Lys Ile Asn Pro Gly
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ggg aat gat ttt aag ctg att ccg ttt gga gcc ggg agg aga att tgt 1349
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gcg ggg aca agg atg gga atg gtc ctt gta agt tat att ttg ggc act 1397
Ala Gly Thr Arg Met Gly Met Val Leu Val Ser Tyr Ile Leu Gly Thr
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460                               465                               470                               475

aat atg gat gaa agt ttt ggg ctt gca ttg caa aag gcc gtg ccg ctc 1493
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Ser Ala Leu Val Ser Pro Arg Leu Ala Ser Asn Pro Tyr Ala Thr
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atgactttat tgtataatta tttatttttc ctttctgggt attttatcca tttaattttt 1718

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<213> Artificial Sequence

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Leu	Pro	Pro	Gly	Pro	Leu	Gly	Trp	Pro	Leu	Val	Gly	Ala	Leu	Pro	Leu
		35					40					45			
Leu	Gly	Ala	Met	Pro	His	Val	Ala	Leu	Ala	Lys	Leu	Ala	Lys	Lys	Tyr
	50					55					60				
Gly	Pro	Ile	Met	His	Leu	Lys	Met	Gly	Thr	Cys	Asp	Met	Val	Val	Ala
65					70					75					80
Ser	Thr	Pro	Glu	Ser	Ala	Arg	Ala	Phe	Leu	Lys	Thr	Leu	Asp	Leu	Asn
				85					90					95	
Phe	Ser	Asn	Arg	Pro	Pro	Asn	Ala	Gly	Ala	Ser	His	Leu	Ala	Tyr	Gly
			100					105					110		
Ala	Gln	Asp	Leu	Val	Phe	Ala	Lys	Tyr	Gly	Pro	Arg	Trp	Lys	Thr	Leu
		115					120					125			
Arg	Lys	Leu	Ser	Asn	Leu	His	Met	Leu	Gly	Gly	Lys	Ala	Leu	Asp	Asp
	130					135					140				
Trp	Ala	Asn	Val	Arg	Val	Thr	Glu	Leu	Gly	His	Met	Leu	Lys	Ala	Met
145					150					155					160
Cys	Glu	Ala	Ser	Arg	Cys	Gly	Glu	Pro	Val	Val	Leu	Ala	Glu	Met	Leu
				165					170					175	
Thr	Tyr	Ala	Met	Ala	Asn	Met	Ile	Gly	Gln	Val	Ile	Leu	Ser	Arg	Arg
			180					185					190		
Val	Phe	Val	Thr	Lys	Gly	Thr	Glu	Ser	Asn	Glu	Phe	Lys	Asp	Met	Val
		195					200					205			
Val	Glu	Leu	Met	Thr	Ser	Ala	Gly	Tyr	Phe	Asn	Ile	Gly	Asp	Phe	Ile
	210					215					220				
Pro	Ser	Ile	Ala	Trp	Met	Asp	Leu	Gln	Gly	Ile	Glu	Arg	Gly	Met	Lys
225					230					235					240
Lys	Leu	His	Thr	Lys	Phe	Asp	Val	Leu	Leu	Thr	Lys	Met	Val	Lys	Glu
				245					250					255	
His	Arg	Ala	Thr	Ser	His	Glu	Arg	Lys	Gly	Lys	Ala	Asp	Phe	Leu	Asp
			260					265					270		
Val	Leu	Leu	Glu	Glu	Cys	Asp	Asn	Thr	Asn	Gly	Glu	Lys	Leu	Ser	Ile
		275					280					285			
Thr	Asn	Ile	Lys	Ala	Val	Leu	Leu	Asn	Leu	Phe	Thr	Ala	Gly	Thr	Asp
	290					295					300				
Thr	Ser	Ser	Ser	Ile	Ile	Glu	Trp	Ala	Leu	Thr	Glu	Met	Ile	Lys	Asn
305					310						315				320

71j

Pro Thr Ile Leu Lys Lys Ala Gln Glu Glu Met Asp Arg Val Ile Gly
 325 330 335
 Arg Asp Arg Arg Leu Leu Glu Ser Asp Ile Ser Ser Leu Pro Tyr Leu
 340 345 350
 Gln Ala Ile Ala Lys Glu Thr Tyr Arg Lys His Pro Ser Thr Pro Leu
 355 360 365
 Asn Leu Pro Arg Ile Ala Ile Gln Ala Cys Glu Val Asp Gly Tyr Tyr
 370 375 380
 Ile Pro Lys Asp Ala Arg Leu Ser Val Asn Ile Trp Ala Ile Gly Arg
 385 390 395 400
 Asp Pro Asn Val Trp Glu Asn Pro Leu Glu Phe Leu Pro Glu Arg Phe
 405 410 415
 Leu Ser Glu Glu Asn Gly Lys Ile Asn Pro Gly Gly Asn Asp Phe Lys
 420 425 430
 Leu Ile Pro Phe Gly Ala Gly Arg Arg Ile Cys Ala Gly Thr Arg Met
 435 440 445
 Gly Met Val Leu Val Ser Tyr Ile Leu Gly Thr Leu Val His Ser Phe
 450 455 460
 Asp Trp Lys Leu Pro Asn Gly Val Ala Glu Leu Asn Met Asp Glu Ser
 465 470 475 480
 Phe Gly Leu Ala Leu Gln Lys Ala Val Pro Leu Ser Ala Leu Val Ser
 485 490 495
 Pro Arg Leu Ala Ser Asn Pro Tyr Ala Thr
 500 505

<210> 5
 <211> 1474
 <212> DNA
 <213> Rose

<220>
 <223> Nucleotide sequence encoding rose chalcone synthase

<400> 5
 ggagatatca aaatggtgac cgtcgaggaa gtccgcaagg ctcaacgcgc tgagggtccg 60
 gctaccgtcc tggccatcgg gacagcaact cctcccaact gtattgacca gagcacatac 120
 cccgactact acttccgtat cactaagagc gagcacaagg ctgagctcaa ggagaaattc 180
 cagcgcgatgt gtgacaaatc tatgatcaag aagcgctaca tgtacttgac cgaagaaatt 240
 cttaaggaga atcctagtat gtgtgagtac atggcccctt cacttgatgc aagacaagat 300

71k

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atggtggttg ttgaaattcc aaagcttgga aaagaggctg ccactaaggc tattaaggaa      360
tggggtcagc ccaagtccaa aatcacccac ttggtctttt gtaccactag tggcgtcgac      420
atgcccgggg ccgattacca gctcactaag ctcttaggcc tccgcccgtc cgtgaagcgt      480
ctcatgatgt accaacaagg gtgtttcgcc ggaggcacgg tgctccggtt ggctaaggac      540
ttggccgaga acaacaaggg tgcacgtggt cttgttggtt gctcagagat cactgccgtg      600
actttccgtg ggcttagcga caccatctc gatagtcttg tgggccaagc cttgttcggt      660
gatggtgctg cggccattat tggtggggcc gaccattgc cggaggttga gaagccttcg      720
ttcgagttgg tctcggcagc ccaaactatc ctctctgaca gtgacggagc catcgacggg      780
catcttcgtg aagttgggct cacatttcac ctctcaaag atgttcccgg gctgatttca      840
aagaacatcg agaagagcct caacgaggcc ttcaaacctt tgaacatcac agactggaac      900
tcacttttct ggattgcaca cccgggtggc cctgcaattt tagaccaagt agaggctaaa      960
ttgggcctga agcccgaaaa gttagaagcc acaaggcata tattatccga gtacggcaat     1020
atgtctagtg cttgtgtggt gtttattttg gacgagggtg ggagaaagtc tgcagctaat     1080
gggcacaaga ccactggaga aggctggag tggggtgtcc tatttggttt tgggccaggg     1140
ctcacgctcg agaccgtcgt gcttcacagt gtggctgctt aaacttgaag gcatctgggt     1200
tcacttgagt gatctgctcc tggatttggt cttatatatg tctgtttcc actctacttt     1260
ccttggtaga tttccttttt tggatttatt tttctggtga atttagcaat atatgtaatg     1320
atgaataata ttattccaca aatttcatac gagcaaaagt tcctgcaata atttagttag     1380
aagttgactt tccggaagat ttagagcggg gaatatatct ccactagct gaaagattat     1440
ccggggatag agtacgttca aaaaaaaaaa aaaa                                1474

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```

<210> 6
<211> 420
<212> DNA
<213> Rose

```

```

<220>
<223> Nucleotide sequence encoding part of rose anthocyanidin synthase

```

```

<400> 6
gaagaaggga ggctggagaa ggaggtcggt ggactcgaag aactcgtcct gcaaatgaaa      60
atcaactact acccaaaatg ccctcagccg gaacttgccc tcggcgtgga agcccacacc     120
gacataagtg cactcacctt catcctccac aacatgggtc ccggcctgca gctcttctac     180

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711

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ggcgggcaaat ggggtgacagc gaaatgcgtg cccaactcca tcgtcatgca catcggcgac      240
aacttgagaga ttctgagcaa cggcaagtac aagagcattt ttcacagggg ggattgtcaa      300
caagggagaaa ggtgagggttc tcgttggcgg ttttcttgta gccacccagg aggaggtcat      360
tctcaagccg ttgcgacgac tgtctcgagg aggaaccgcg tcttccaccc gacttttcgg      420

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```

<210> 7
<211> 1808
<212> DNA
<213> Torenia

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<220>
<223> Nucleotide sequence encoding Torenia anthocyanin acyl transferase

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```

<400> 7
cttcaaagcc aaaaagaaac aattaatca atg gct gtt gaa gcc ccc aaa aca      53
                               Met Ala Val Glu Ala Pro Lys Thr
                               1                               5

ata tgt gca gtc ctc gaa aac tct ctt att aca cca caa agt acc gat      101
Ile Cys Ala Val Leu Glu Asn Ser Leu Ile Thr Pro Gln Ser Thr Asp
   10                               15                               20

aca gaa caa act ctt tca ctc aca ttc ttt gac atc aaa tgg gtt cat      149
Thr Glu Gln Thr Leu Ser Leu Thr Phe Phe Asp Ile Lys Trp Val His
   25                               30                               35                               40

ttt cat cca atg caa tgc ctt gtg ttg tac aac ttc cca tgt tct aag      197
Phe His Pro Met Gln Cys Leu Val Leu Tyr Asn Phe Pro Cys Ser Lys
                               45                               50                               55

tca cat ttt ctc gaa gcc aca gtt ccg agc ttc aaa tca tca ctc tcc      245
Ser His Phe Leu Glu Ala Thr Val Pro Ser Phe Lys Ser Ser Leu Ser
                               60                               65                               70

aaa act ctc aga cac tat ctt cca tta tca gga aac tta tac tat cca      293
Lys Thr Leu Arg His Tyr Leu Pro Leu Ser Gly Asn Leu Tyr Tyr Pro
   75                               80                               85

aac ccg acc cat gac atg gat gat gat gaa tcg aac atg ccc gag atc      341
Asn Pro Thr His Asp Met Asp Asp Asp Glu Ser Asn Met Pro Glu Ile
   90                               95                               100

cgt tat aaa cct ggc gac tcg gtt tct cta acc gtt gca gag tac ttc      389
Arg Tyr Lys Pro Gly Asp Ser Val Ser Leu Thr Val Ala Glu Tyr Phe
  105                               110                               115                               120

tcc ggt cat gaa gac aat acg act act gaa gaa tac ttc aat tac ctc      437
Ser Gly His Glu Asp Asn Thr Thr Thr Glu Glu Tyr Phe Asn Tyr Leu
                               125                               130                               135

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71m

act gga aat ttc cag aga gat tgc gat caa ttc tat gat ctc tta ccc	485
Thr Gly Asn Phe Gln Arg Asp Cys Asp Gln Phe Tyr Asp Leu Leu Pro	
140 145 150	
gat ttt cga gac ccg gaa acc gaa tcc aat tgc aca gta atc cca ctt	533
Asp Phe Arg Asp Pro Glu Thr Glu Ser Asn Cys Thr Val Ile Pro Leu	
155 160 165	
ata gca gtt caa atc aca ctc ttt cca ggt gct ggg ata tgt ctg ggg	581
Ile Ala Val Gln Ile Thr Leu Phe Pro Gly Ala Gly Ile Cys Leu Gly	
170 175 180	
gtc atc aac agt cac gta gtt ggc gat gcg agt tcc ata gtg gga ttc	629
Val Ile Asn Ser His Val Val Gly Asp Ala Ser Ser Ile Val Gly Phe	
185 190 195 200	
atc aaa gct tgg agt aaa gtt gca atg tat gaa gac gat gaa gag att	677
Ile Lys Ala Trp Ser Lys Val Ala Met Tyr Glu Asp Asp Glu Glu Ile	
205 210 215	
cta gct aac aac aat ttg att cca tct tat gac aga tca gtc gtg aaa	725
Leu Ala Asn Asn Asn Leu Ile Pro Ser Tyr Asp Arg Ser Val Val Lys	
220 225 230	
gat cca aaa ggg atc aaa tct ttg ctc tgg aac aag atg aag aac gtg	773
Asp Pro Lys Gly Ile Lys Ser Leu Leu Trp Asn Lys Met Lys Asn Val	
235 240 245	
aaa tat caa ccc caa ccc gca aaa cat ctc cca aca aac aag gtc cga	821
Lys Tyr Gln Pro Gln Pro Ala Lys His Leu Pro Thr Asn Lys Val Arg	
250 255 260	
gcc aca tac acc ttg aga aag aac gat atc gag agg ctg aaa acc cga	869
Ala Thr Tyr Thr Leu Arg Lys Asn Asp Ile Glu Arg Leu Lys Thr Arg	
265 270 275 280	
atc cga tcc aag aaa cca ggc aca acc tgc tta tca tct ttc aca atc	917
Ile Arg Ser Lys Lys Pro Gly Thr Thr Cys Leu Ser Ser Phe Thr Ile	
285 290 295	
gca aca gcc tat gct tgg aca tgc ctt gca aaa tct gca gca gaa gct	965
Ala Thr Ala Tyr Ala Trp Thr Cys Leu Ala Lys Ser Ala Ala Glu Ala	
300 305 310	
gaa gaa caa gta gtc caa gac agt gac gag cac ttg ctc atg ccc	1013
Glu Glu Gln Val Val Gln Asp Ser Asp Asp Glu His Leu Leu Met Pro	
315 320 325	
gtt gat ttg aga cca aga ata gat cct cca tta cca cct tct tac ttt	1061
Val Asp Leu Arg Pro Arg Ile Asp Pro Pro Leu Pro Pro Ser Tyr Phe	
330 335 340	
gga aac tgc gtt ctt cca tct ttt gcg aaa acg acg cat ggg ctt ttg	1109
Gly Asn Cys Val Leu Pro Ser Phe Ala Lys Thr Thr His Gly Leu Leu	
345 350 355 360	

71n

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aaa gga gag tta ggg ctt ttt aat gca gtg gaa gtg att agt gat gtc      1157
Lys Gly Glu Leu Gly Leu Phe Asn Ala Val Glu Val Ile Ser Asp Val
                               365                               370                               375

att acc ggt atc gtt agc aag aaa tat gac ttg ttc aaa gac tta gac      1205
Ile Thr Gly Ile Val Ser Lys Lys Tyr Asp Leu Phe Lys Asp Leu Asp
                               380                               385                               390

aga caa ggt gag att ttt cgt gcc ttg ttc gga aaa cga gtg ttg gcg      1253
Arg Gln Gly Glu Ile Phe Arg Ala Leu Phe Gly Lys Arg Val Leu Ala
                               395                               400                               405

atc atg ggt tcg cct aag ttc gat ctc tac gaa gtt gat ttc ggg tgg      1301
Ile Met Gly Ser Pro Lys Phe Asp Leu Tyr Glu Val Asp Phe Gly Trp
                               410                               415                               420

ggt aag ccg aag aag att gaa cct gtg tcc att gat aga gag agg acg      1349
Gly Lys Pro Lys Lys Ile Glu Pro Val Ser Ile Asp Arg Glu Arg Thr
425                               430                               435                               440

act atg tgg att agc aag tct ggc gag ttt gag ggt gga ttg gag att      1397
Thr Met Trp Ile Ser Lys Ser Gly Glu Phe Glu Gly Gly Leu Glu Ile
                               445                               450                               455

ggt ttt tct ttc aat aag aag aaa atg gat gct ttt ggc gag tgt ttt      1445
Gly Phe Ser Phe Asn Lys Lys Lys Met Asp Ala Phe Gly Glu Cys Phe
                               460                               465                               470

aac agc ggt ttg aag gat att taatttaaaa aattgttttag ctttgatgca      1496
Asn Ser Gly Leu Lys Asp Ile
                               475

tgcgttttat atatgttgtg aaataatgtg gtgtgcaata actagagtaa ctttaggtta      1556

ataaattcgg tttttctgtt aaatctggat gattcgtgca agcaaactgt cgatgcgttg      1616

gatggatgtc ggggtggtgtg gagattgttg aagaaggaaa tggatgcttt ttttatggtg      1676

gtttgaagga tttgaatgtg tagattattg gtttattgag gttgtttata tttgtgtatg      1736

ttgtttatgc atgaaaaata tttagatccc aacattttat gtatgacgtg gtttaaatatt      1796

tcgatttcga tc                                                         1808

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<210> 8
 <211> 479
 <212> PRT
 <213> Torenia

<220>
 <223> Amino acid sequence of Torenia anthocyanin acyl transferase

710

<400> 8

Met	Ala	Val	Glu	Ala	Pro	Lys	Thr	Ile	Cys	Ala	Val	Leu	Glu	Asn	Ser	1	5	10	15
Leu	Ile	Thr	Pro	Gln	Ser	Thr	Asp	Thr	Glu	Gln	Thr	Leu	Ser	Leu	Thr	20	25	30	
Phe	Phe	Asp	Ile	Lys	Trp	Val	His	Phe	His	Pro	Met	Gln	Cys	Leu	Val	35	40	45	
Leu	Tyr	Asn	Phe	Pro	Cys	Ser	Lys	Ser	His	Phe	Leu	Glu	Ala	Thr	Val	50	55	60	
Pro	Ser	Phe	Lys	Ser	Ser	Leu	Ser	Lys	Thr	Leu	Arg	His	Tyr	Leu	Pro	65	70	75	80
Leu	Ser	Gly	Asn	Leu	Tyr	Tyr	Pro	Asn	Pro	Thr	His	Asp	Met	Asp	Asp	85	90	95	
Asp	Glu	Ser	Asn	Met	Pro	Glu	Ile	Arg	Tyr	Lys	Pro	Gly	Asp	Ser	Val	100	105	110	
Ser	Leu	Thr	Val	Ala	Glu	Tyr	Phe	Ser	Gly	His	Glu	Asp	Asn	Thr	Thr	115	120	125	
Thr	Glu	Glu	Tyr	Phe	Asn	Tyr	Leu	Thr	Gly	Asn	Phe	Gln	Arg	Asp	Cys	130	135	140	
Asp	Gln	Phe	Tyr	Asp	Leu	Leu	Pro	Asp	Phe	Arg	Asp	Pro	Glu	Thr	Glu	145	150	155	160
Ser	Asn	Cys	Thr	Val	Ile	Pro	Leu	Ile	Ala	Val	Gln	Ile	Thr	Leu	Phe	165	170	175	
Pro	Gly	Ala	Gly	Ile	Cys	Leu	Gly	Val	Ile	Asn	Ser	His	Val	Val	Gly	180	185	190	
Asp	Ala	Ser	Ser	Ile	Val	Gly	Phe	Ile	Lys	Ala	Trp	Ser	Lys	Val	Ala	195	200	205	
Met	Tyr	Glu	Asp	Asp	Glu	Glu	Ile	Leu	Ala	Asn	Asn	Asn	Leu	Ile	Pro	210	215	220	
Ser	Tyr	Asp	Arg	Ser	Val	Val	Lys	Asp	Pro	Lys	Gly	Ile	Lys	Ser	Leu	225	230	235	240
Leu	Trp	Asn	Lys	Met	Lys	Asn	Val	Lys	Tyr	Gln	Pro	Gln	Pro	Ala	Lys	245	250	255	
His	Leu	Pro	Thr	Asn	Lys	Val	Arg	Ala	Thr	Tyr	Thr	Leu	Arg	Lys	Asn	260	265	270	
Asp	Ile	Glu	Arg	Leu	Lys	Thr	Arg	Ile	Arg	Ser	Lys	Lys	Pro	Gly	Thr	275	280	285	
Thr	Cys	Leu	Ser	Ser	Phe	Thr	Ile	Ala	Thr	Ala	Tyr	Ala	Trp	Thr	Cys	290	295	300	

71p

Leu Ala Lys Ser Ala Ala Glu Ala Glu Glu Gln Val Val Gln Asp Ser
305 310 315 320

Asp Asp Glu His Leu Leu Met Pro Val Asp Leu Arg Pro Arg Ile Asp
325 330 335

Pro Pro Leu Pro Pro Ser Tyr Phe Gly Asn Cys Val Leu Pro Ser Phe
340 345 350

Ala Lys Thr Thr His Gly Leu Leu Lys Gly Glu Leu Gly Leu Phe Asn
355 360 365

Ala Val Glu Val Ile Ser Asp Val Ile Thr Gly Ile Val Ser Lys Lys
370 375 380

Tyr Asp Leu Phe Lys Asp Leu Asp Arg Gln Gly Glu Ile Phe Arg Ala
385 390 395 400

Leu Phe Gly Lys Arg Val Leu Ala Ile Met Gly Ser Pro Lys Phe Asp
405 410 415

Leu Tyr Glu Val Asp Phe Gly Trp Gly Lys Pro Lys Lys Ile Glu Pro
420 425 430

Val Ser Ile Asp Arg Glu Arg Thr Thr Met Trp Ile Ser Lys Ser Gly
435 440 445

Glu Phe Glu Gly Gly Leu Glu Ile Gly Phe Ser Phe Asn Lys Lys Lys
450 455 460

Met Asp Ala Phe Gly Glu Cys Phe Asn Ser Gly Leu Lys Asp Ile
465 470 475

<210> 9
<211> 1252
<212> DNA
<213> Iris

<220>
<223> Nucleotide sequence encoding Iris dihydrofravonol reductase

<400> 9
aaacaatata tcgag atg atg agc ccc gtt gtc gtg acc gga gcg agc ggc 51
Met Met Ser Pro Val Val Val Thr Gly Ala Ser Gly
1 5 10

tac gtc ggt tca tgg ctt gtt atg aag ctc ctt cgc gac ggc tac gcc 99
Tyr Val Gly Ser Trp Leu Val Met Lys Leu Leu Arg Asp Gly Tyr Ala
15 20 25

gtt cga gcc act gtc aga gac cca acc aat gtg gag aag acg aag ccg 147
Val Arg Ala Thr Val Arg Asp Pro Thr Asn Val Glu Lys Thr Lys Pro
30 35 40

71q

ctg ttg gac ctc ccc gga gct gac gcg ctg ctc acc atc tgg aag gca	195
Leu Leu Asp Leu Pro Gly Ala Asp Ala Leu Leu Thr Ile Trp Lys Ala	
45 50 55 60	
gac ctc ggc cag gac gga agc ttc gac aag gcg gtc gca gga tgc acc	243
Asp Leu Gly Gln Asp Gly Ser Phe Asp Lys Ala Val Ala Gly Cys Thr	
65 70 75	
gcg gtc ttc cac gtc gcc acg ccc atg gat ttc gag tcc aag gac cca	291
Ala Val Phe His Val Ala Thr Pro Met Asp Phe Glu Ser Lys Asp Pro	
80 85 90	
gaa aac gag gtg atc aag ccg acc ata aat ggc gtt tta agt atc atg	339
Glu Asn Glu Val Ile Lys Pro Thr Ile Asn Gly Val Leu Ser Ile Met	
95 100 105	
agg tcc tgt aag aag gcc gga acg gtc aaa cgc gtc gtc ttc act tca	387
Arg Ser Cys Lys Lys Ala Gly Thr Val Lys Arg Val Val Phe Thr Ser	
110 115 120	
tcc gcc ggg acg gtg gac gtg aaa gaa cat cag cag acg gag tac gac	435
Ser Ala Gly Thr Val Asp Val Lys Glu His Gln Gln Thr Glu Tyr Asp	
125 130 135 140	
gag agc tcg tgg agc gac gtc gac ttc tgc aga cgt gtc aag atg aca	483
Glu Ser Ser Trp Ser Asp Val Asp Phe Cys Arg Arg Val Lys Met Thr	
145 150 155	
ggc tgg atg tat ttt gtg tcg aag act ctg gcc gag aga gca gcc tgg	531
Gly Trp Met Tyr Phe Val Ser Lys Thr Leu Ala Glu Arg Ala Ala Trp	
160 165 170	
gaa ttt gca aga gag aat ggc ata gac ttc ata agc atc atc ccc acg	579
Glu Phe Ala Arg Glu Asn Gly Ile Asp Phe Ile Ser Ile Ile Pro Thr	
175 180 185	
cta gtc gtc ggt cct ttc atc acc aca act atg cca ccc agc atg gtg	627
Leu Val Val Gly Pro Phe Ile Thr Thr Thr Met Pro Pro Ser Met Val	
190 195 200	
act gcg cta tca ttc atg aca gga aac gaa gca cac tat cac ata atc	675
Thr Ala Leu Ser Phe Met Thr Gly Asn Glu Ala His Tyr His Ile Ile	
205 210 215 220	
aag cac gcg cag ctc gtc cac ctt gac gac ctg tgc gct gcc cac att	723
Lys His Ala Gln Leu Val His Leu Asp Asp Leu Cys Ala Ala His Ile	
225 230 235	
tac ctc ctg aat cgc ccc gaa gcg aac ggg agg tac ata tgc tca tcg	771
Tyr Leu Leu Asn Arg Pro Glu Ala Asn Gly Arg Tyr Ile Cys Ser Ser	
240 245 250	
cac gaa gcc acc atc cac gac ctg gcg agg atg gtc agg gag agg cac	819
His Glu Ala Thr Ile His Asp Leu Ala Arg Met Val Arg Glu Arg His	
255 260 265	

<210>	10
<211>	361
<212>	PRT
<213>	Iris

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<400> 10
Met Met Ser Pro Val Val Val Thr Gly Ala Ser Gly Tyr Val Gly Ser
  1             5             10             15

Trp Leu Val Met Lys Leu Leu Arg Asp Gly Tyr Ala Val Arg Ala Thr
      20             25             30

Val Arg Asp Pro Thr Asn Val Glu Lys Thr Lys Pro Leu Leu Asp Leu
      35             40             45

Pro Gly Ala Asp Ala Leu Leu Thr Ile Trp Lys Ala Asp Leu Gly Gln
      50             55             60

Asp Gly Ser Phe Asp Lys Ala Val Ala Gly Cys Thr Ala Val Phe His
      65             70             75             80

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71s

Val	Ala	Thr	Pro	Met	Asp	Phe	Glu	Ser	Lys	Asp	Pro	Glu	Asn	Glu	Val	85	90	95	
Ile	Lys	Pro	Thr	Ile	Asn	Gly	Val	Leu	Ser	Ile	Met	Arg	Ser	Cys	Lys	100	105	110	
Lys	Ala	Gly	Thr	Val	Lys	Arg	Val	Val	Phe	Thr	Ser	Ser	Ala	Gly	Thr	115	120	125	
Val	Asp	Val	Lys	Glu	His	Gln	Gln	Thr	Glu	Tyr	Asp	Glu	Ser	Ser	Trp	130	135	140	
Ser	Asp	Val	Asp	Phe	Cys	Arg	Arg	Val	Lys	Met	Thr	Gly	Trp	Met	Tyr	145	150	155	160
Phe	Val	Ser	Lys	Thr	Leu	Ala	Glu	Arg	Ala	Ala	Trp	Glu	Phe	Ala	Arg	165	170	175	
Glu	Asn	Gly	Ile	Asp	Phe	Ile	Ser	Ile	Ile	Pro	Thr	Leu	Val	Val	Gly	180	185	190	
Pro	Phe	Ile	Thr	Thr	Thr	Met	Pro	Pro	Ser	Met	Val	Thr	Ala	Leu	Ser	195	200	205	
Phe	Met	Thr	Gly	Asn	Glu	Ala	His	Tyr	His	Ile	Ile	Lys	His	Ala	Gln	210	215	220	
Leu	Val	His	Leu	Asp	Asp	Leu	Cys	Ala	Ala	His	Ile	Tyr	Leu	Leu	Asn	225	230	235	240
Arg	Pro	Glu	Ala	Asn	Gly	Arg	Tyr	Ile	Cys	Ser	Ser	His	Glu	Ala	Thr	245	250	255	
Ile	His	Asp	Leu	Ala	Arg	Met	Val	Arg	Glu	Arg	His	Pro	Trp	Cys	Gly	260	265	270	
Ser	Ile	Pro	Glu	Lys	Phe	Asp	Gly	Ile	Glu	Lys	Asp	Val	Arg	Thr	Val	275	280	285	
His	Phe	Ser	Ser	Lys	Arg	Leu	Leu	Asp	Leu	Gly	Phe	Glu	Phe	Lys	Tyr	290	295	300	
Thr	Val	Glu	Glu	Met	Phe	Asp	Glu	Ala	Ile	Arg	Ser	Cys	Val	Glu	Lys	305	310	315	320
Lys	Leu	Ile	Pro	Leu	Pro	Glu	Asn	Gly	Asn	Val	Asp	Ala	Ala	Ala	Gly	325	330	335	
Ala	Lys	Asp	Met	Val	His	Gly	Ala	Glu	Glu	His	Ala	Arg	Ile	Ala	Met	340	345	350	
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<223> Nucleotide sequence encoding Nierembergia hybrida dihydrofravonol reductase

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gtt cat gct act gtt cgt gat cct gag aac aag aag aag gtg aaa cat      199
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gac ttg gcg gta gaa gga agc ttt gat gaa gcc att aaa ggc tgt caa      295
Asp Leu Ala Val Glu Gly Ser Phe Asp Glu Ala Ile Lys Gly Cys Gln
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71u

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Gly Lys Glu Asn Ala Pro Val Ala Asn His Lys Val Lys Leu Thr Ser	
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 50 55 60
 Trp Lys Ala Asp Leu Ala Val Glu Gly Ser Phe Asp Glu Ala Ile Lys
 65 70 75 80
 Gly Cys Gln Gly Val Phe His Val Ala Thr Pro Met Asp Phe Glu Ser
 85 90 95
 Lys Asp Pro Glu Asn Glu Val Ile Lys Pro Thr Val Gln Gly Met Leu
 100 105 110
 Ser Ile Ile Glu Ser Cys Val Lys Ala Asn Thr Val Lys Arg Leu Val
 115 120 125
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 130 135 140
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 Lys Met Thr Gly Trp Met Tyr Phe Val Ser Lys Ile Leu Ala Glu Lys
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 210 215 220

71w

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Cys Ser Ser His His Ala Ile Ile Tyr Asp Val Ala Lys Met Ile Arg
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Lys Asp Leu Pro Val Val Ala Phe Ser Ser Lys Lys Leu Thr Asp Met
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Gly Phe Gln Phe Lys Tyr Thr Leu Glu Asp Met Tyr Lys Gly Ala Ile
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71aa

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What is claimed is:

1. A method for producing a rose comprising the steps of:
 - (a) suppressing the rose flavonoid synthesis pathway by (i) suppressing the expression of a rose endogenous dihydroflavonol reductase or (ii) suppressing the expression of a rose endogenous flavonoid 3'-hydroxylase; and
 - (b) expressing a pansy gene coding for a flavonoid 3',5'-hydroxylase and having the nucleotide sequence of SEQ ID NO:1 or 3.
2. The method for producing a rose according to claim 1, further comprising expressing the gene coding for dihydroflavonol reductase from a plant other than the rose when the expression of the rose dihydroflavonol is suppressed.
3. The method for producing a rose according to claim 2, wherein the plant is iris, Nierembergia or petunia.
4. A rose cell obtained by the method according to any one of claims 1 to 3.
5. The rose cell according to claim 4 for providing a violet color to a petal.
6. The rose cell according to claim 5, wherein the violet color belongs to the "Violet group" according to the Royal Horticultural Society Colour Chart (RHSCC).

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7. The rose cell according to claim 6, wherein the violet color belongs to "Violet group" 85a or 85b according to the Royal Horticultural Society Colour Chart (RHSCC).
8. A rose seed cell obtained by the method according to any one of claims 1 to 3.
9. The rose seed cell according to claim 8 for generating a rose having a petal of a violet color.
10. The rose seed cell according to claim 9, wherein the violet color belongs to the "Violet group" according to the Royal Horticultural Society Colour Chart (RHSCC).
11. The rose seed cell according to claim 10, wherein the violet color belongs to "Violet group" 85a or 85b according to the Royal Horticultural Society Colour Chart (RHSCC).
12. The use of the rose cell obtained by the method according to any one of claims 1 to 3 for providing a violet color to a petal.
13. The use according to claim 12, wherein the violet color belongs to the "Violet group" according to the Royal Horticultural Society Colour Chart (RHSCC).
14. The use according to claim 13, wherein the violet color belongs to "Violet group" 85a or 85b according to the Royal Horticultural Society Colour Chart (RHSCC).

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15. The use of the rose seed cell obtained by the method according to any one of claims 1 to 3 for generating a rose having a petal of a violet color.
16. The use according to claim 15, wherein the violet color belongs to the "Violet group" according to the Royal Horticultural Society Colour Chart (RHSCC).
17. The use according to claim 16, wherein the violet color belongs to "Violet group" 85a or 85b according to the Royal Horticultural Society Colour Chart (RHSCC).

Fig.1

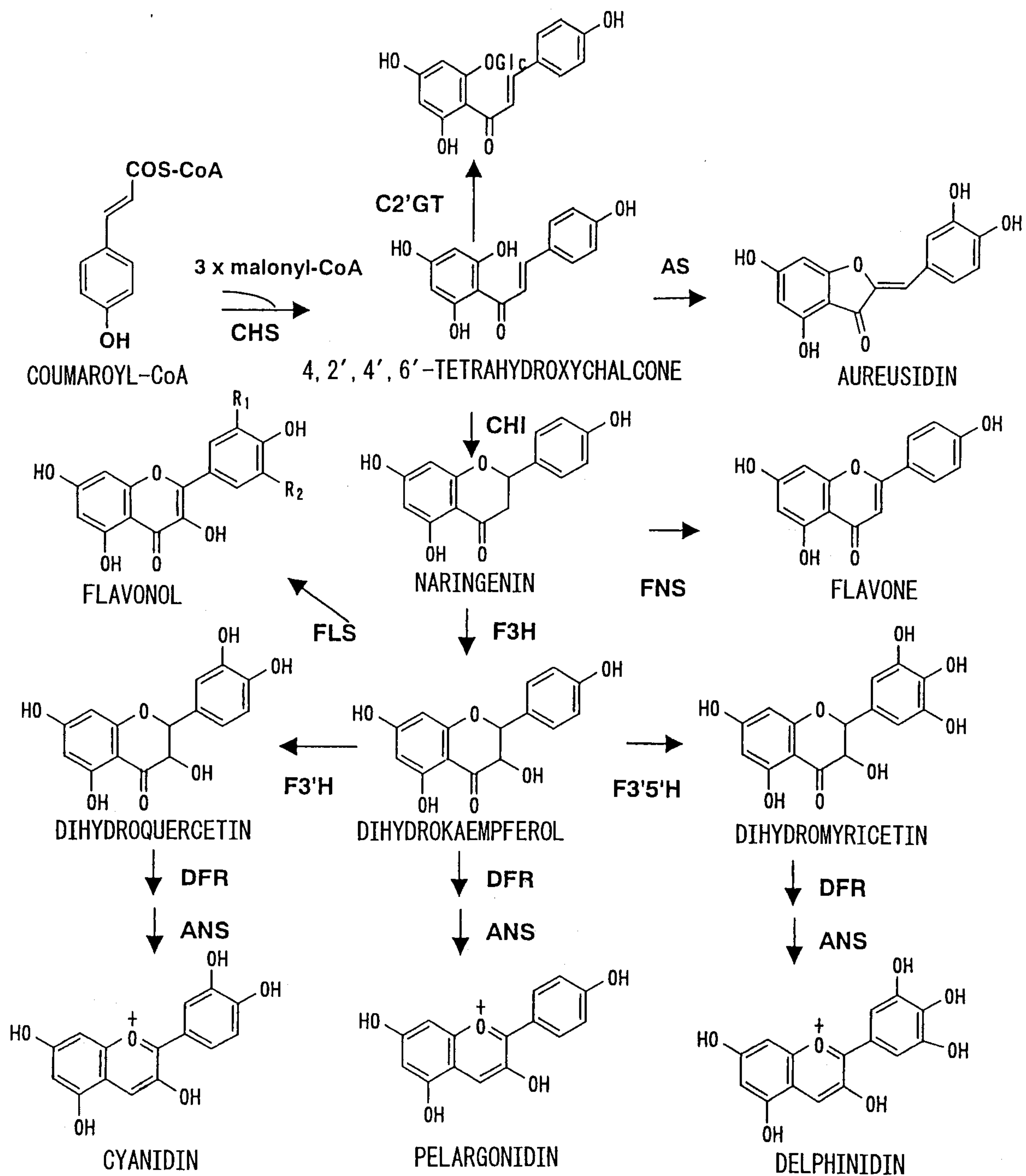


Fig.2

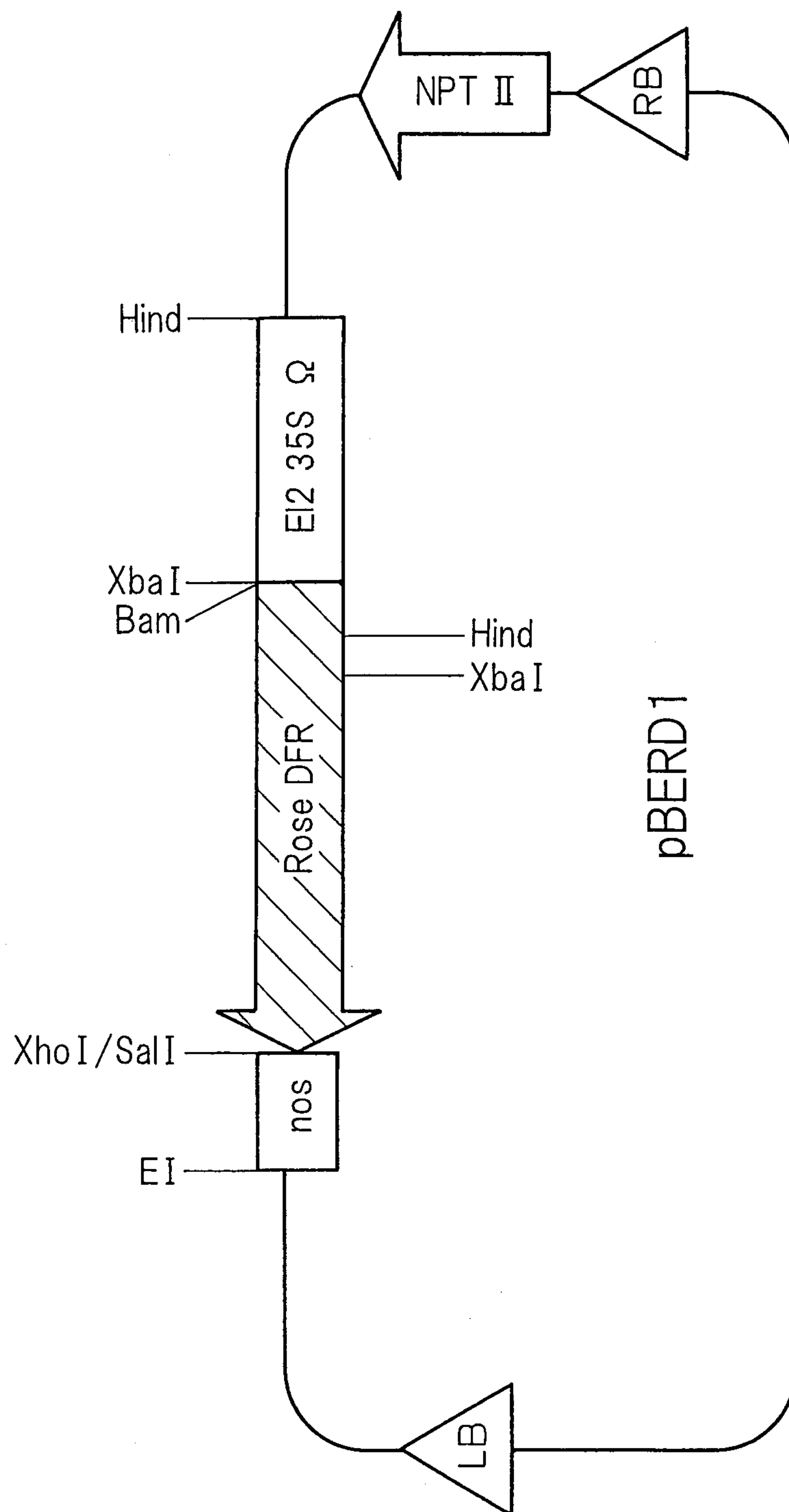


Fig.3

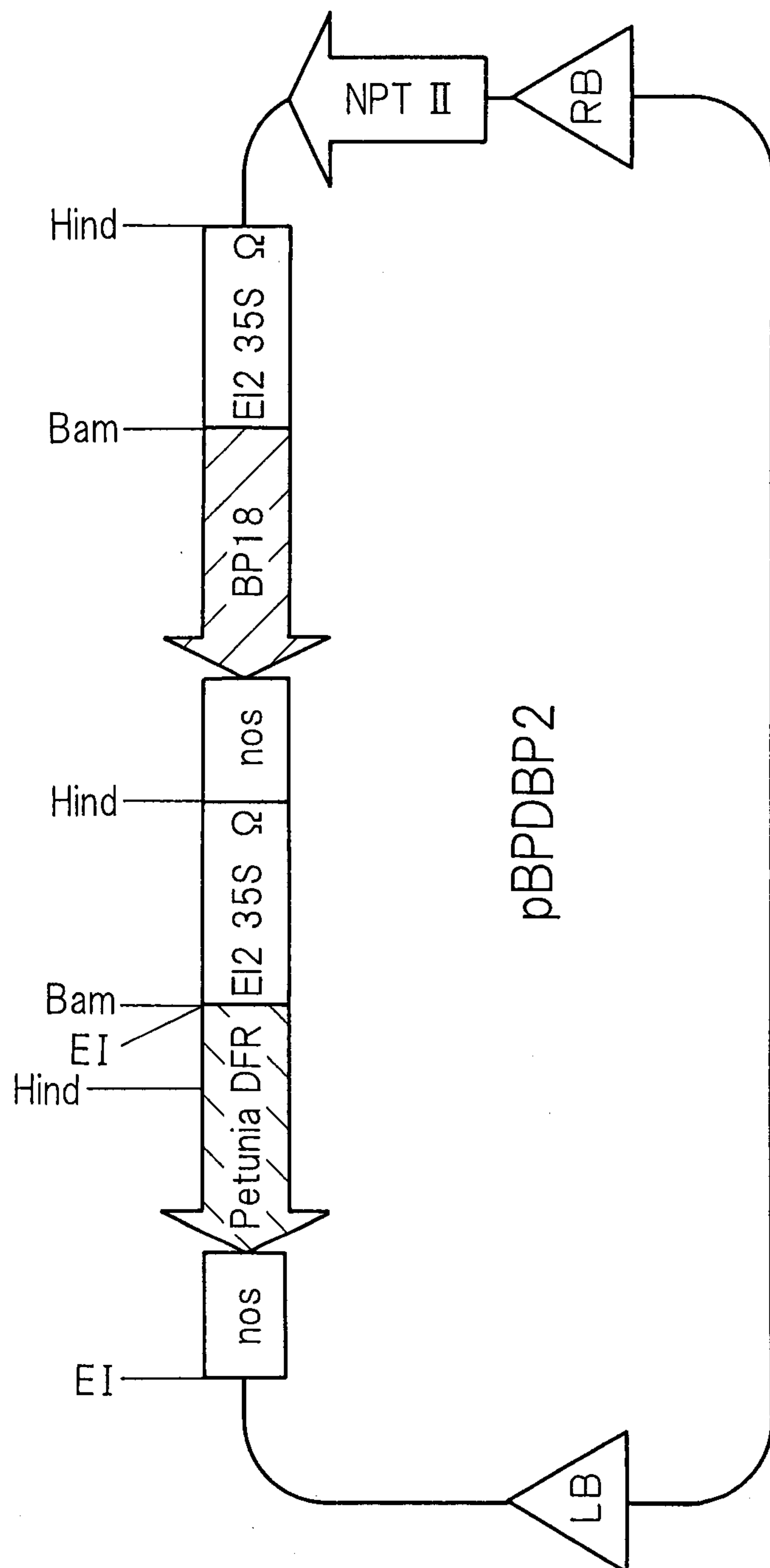


Fig.4

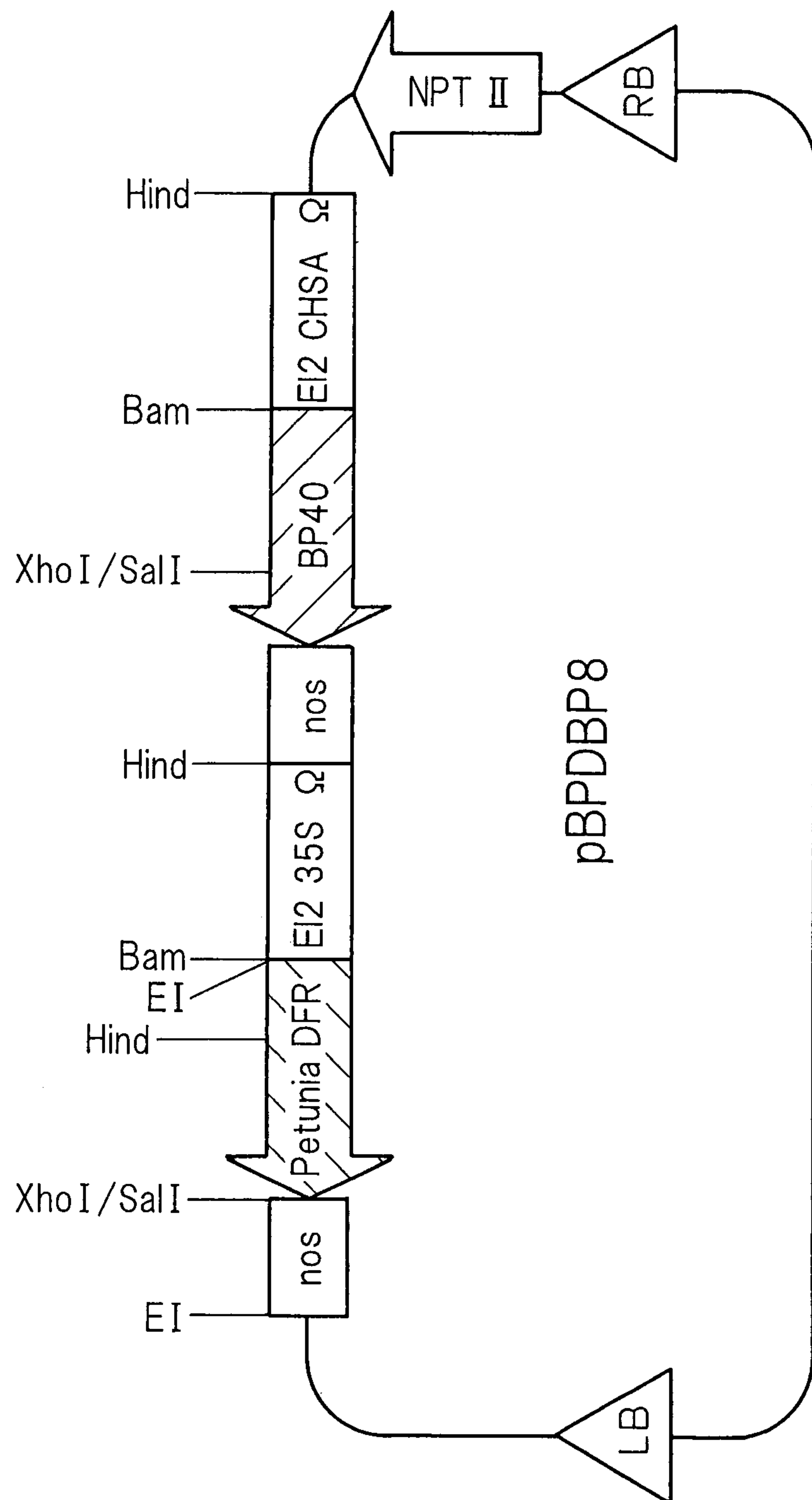
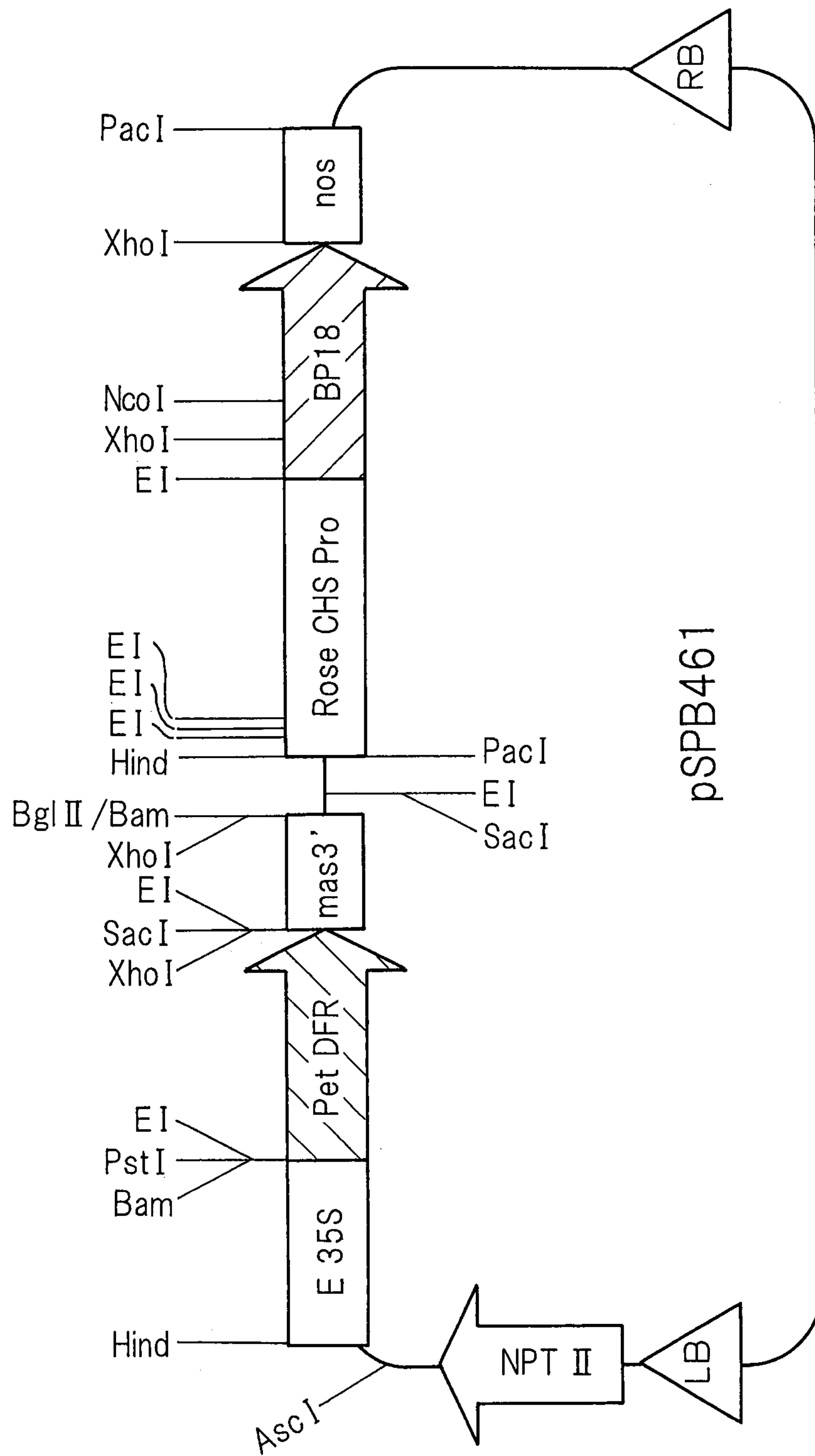


Fig.5



உரித்தே

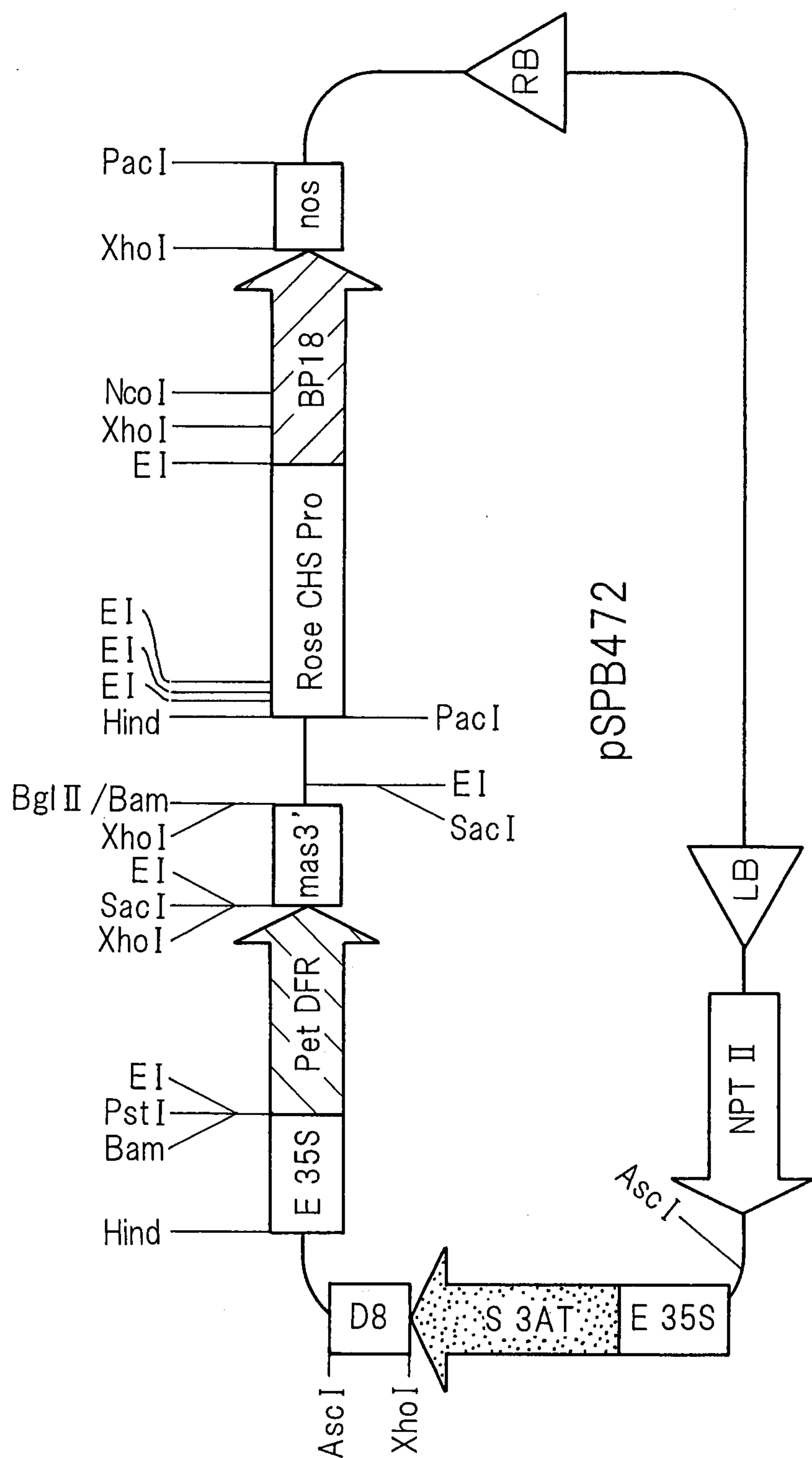


Fig.7

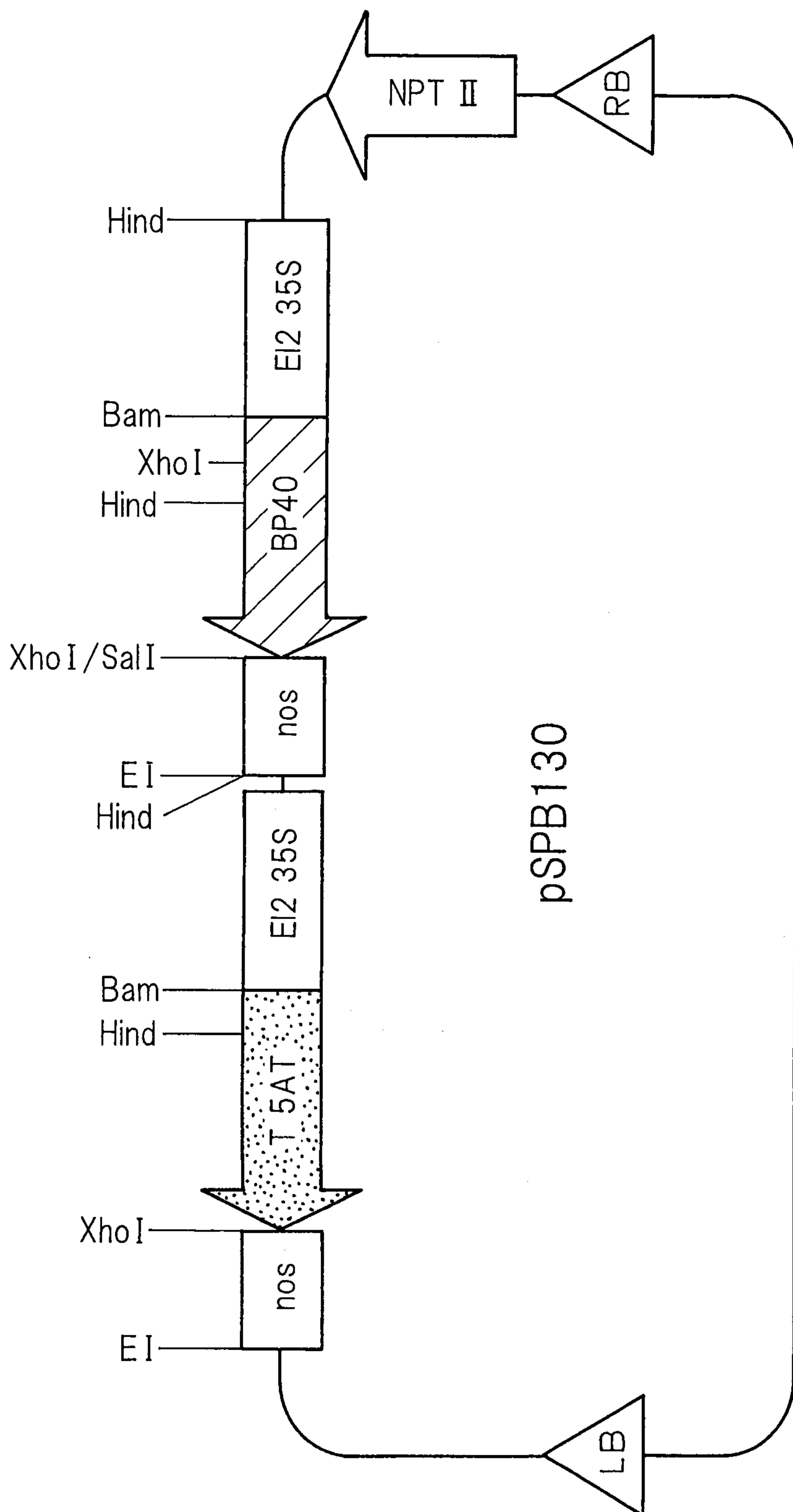


Fig.8

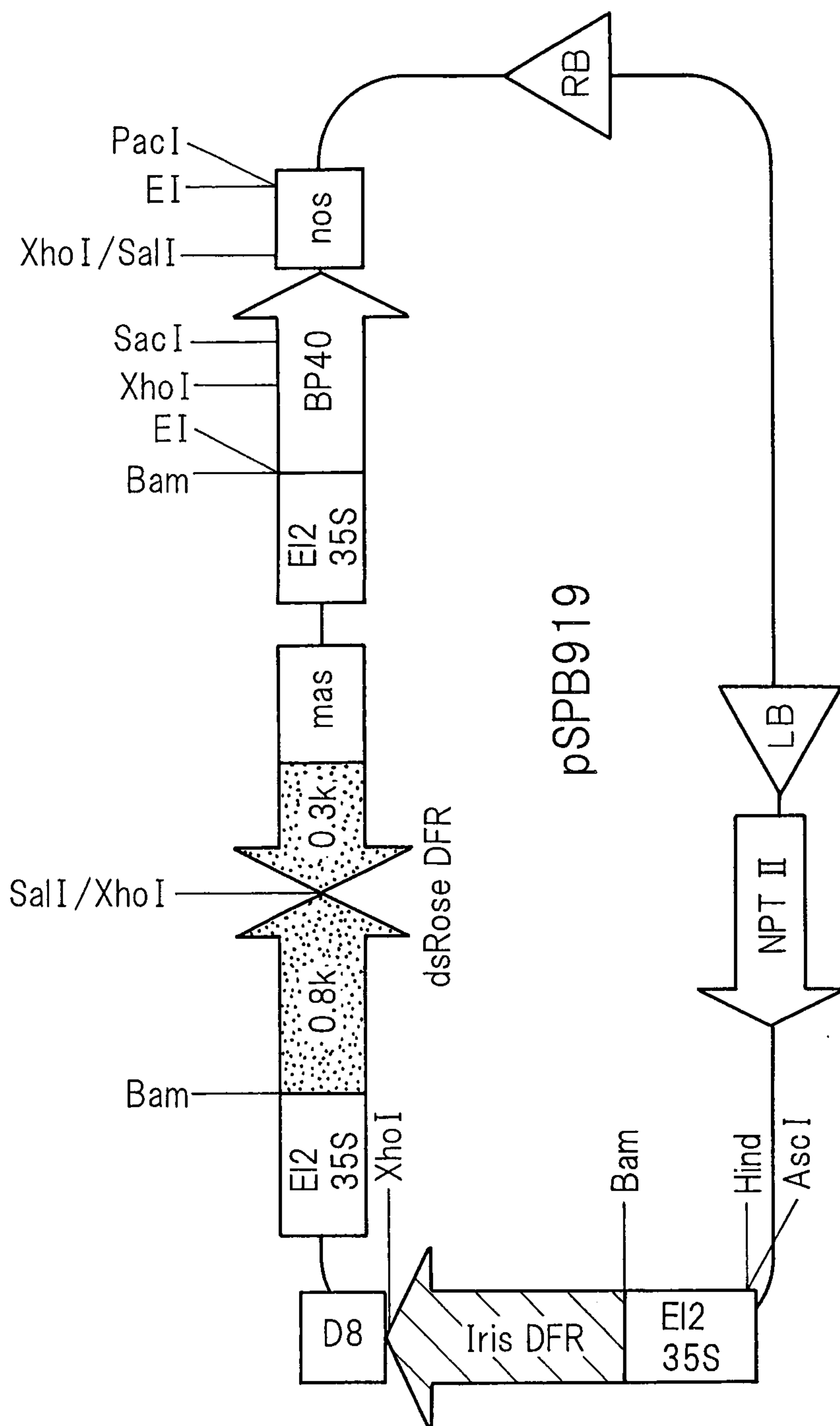


Fig.9

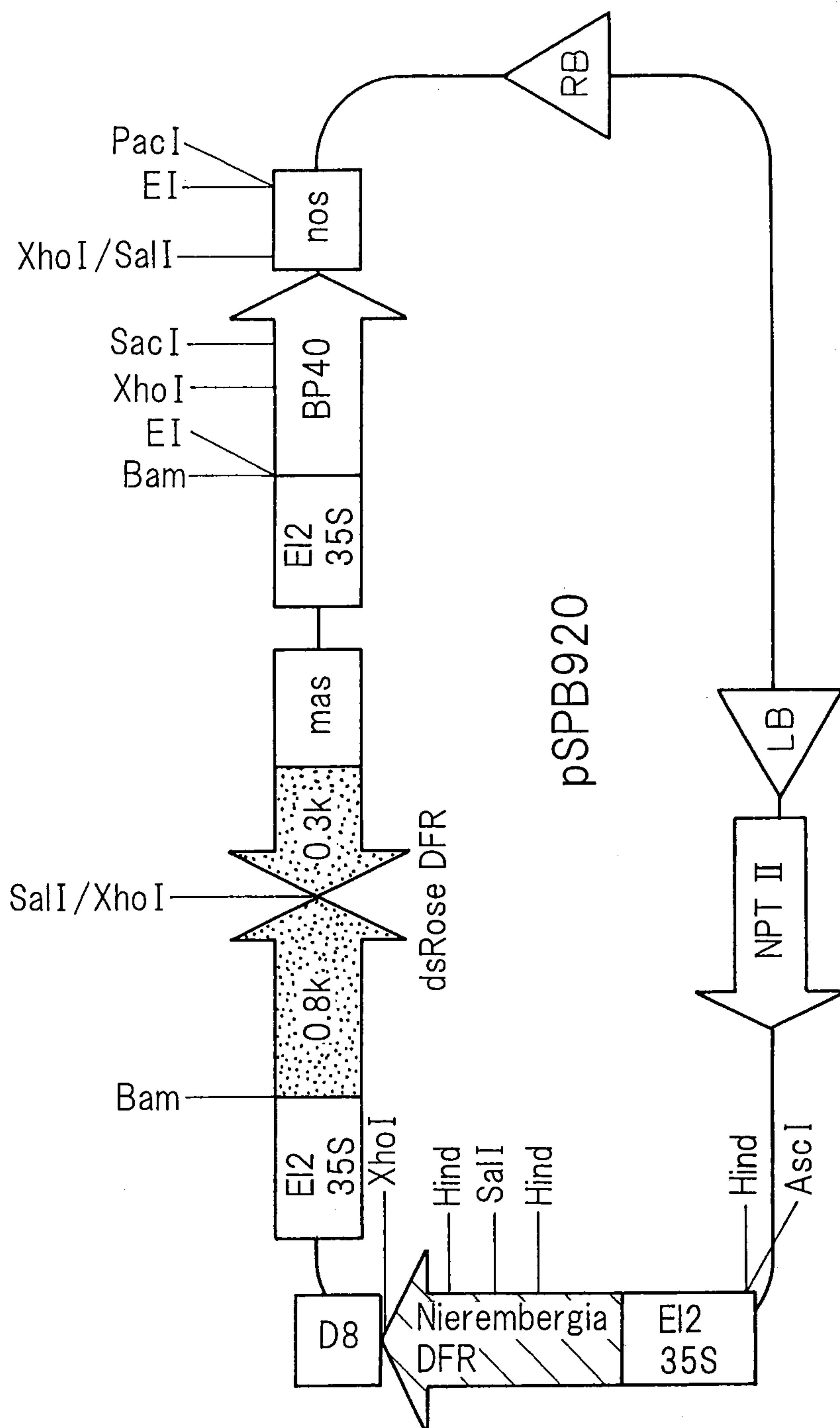


Fig.10

