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SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO



No. 09-103864- -00004-0000

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Señor Director
DIRECCION DE NUEVAS CREACIONES
Superintendencia de Industria y Comercio
E. S. D.

Trámite : 011
Evento : 378
Actuación : 444

Referencia : Expediente No. **09103864**
Asunto : Solicitud de patente para: **MÉTODO PARA LA DETERMINACIÓN DE PRESENCIA DE CRUCES CON ROSA CULTIVADA CON ROSA SILVESTRE**
Solicitante : SUNTORY HOLDINGS LIMITED
Fecha : 23 de septiembre de 2009

1. Yo, Luz Clemencia DE PÁEZ, en mi condición de apoderada de la sociedad solicitante de la patente de la referencia, me refiero al oficio No. **15120** notificado por fijación en lista del 28 de agosto de 2012.

2. Mediante oficio No. **15120** se puso en conocimiento del solicitante el informe que contiene las observaciones hechas por su Despacho sobre la presente solicitud de patente. Las observaciones realizadas en el oficio son las siguientes:

2.1 No es una invención

El examinador ha expuesto:

- El objeto de la reivindicación 15 no se considera una invención, ya que las secuencias mencionadas son aislamientos de *Rosa* sp.

2.2 Reivindicaciones:

De manera resumida, el Examinador ha expuesto lo siguiente:

- Reivindicación 1: no es clara porque no define el método por una serie de pasos sucesivos para llevarlo a cabo, sino por un resultado a alcanzar. La misma observación aplica para las reivindicaciones 2 a 4.
- Reivindicaciones 12 y 14: pretenden caracterizar el método por ser utilizado sobre ciertas especies de rosas.

2.3 Análisis de patentabilidad

2.3.1 Novedad

El examinador considera que las características esenciales del producto de la reivindicación 1 habían sido previamente divulgadas en D1: JP2006149202, el cual da a conocer un método que comprende la identificación de la presencia de un gen KSN que contiene un trasnposon indicador, que se utiliza en el documento de la presente invención obteniendo de esta manera el mismo producto. Además, D1 divulga las SEQ ID NO 11 y 15 que son idénticas a las SEQ ID NO 3 y 4 que se utilizan en el documento de la presente invención, obteniendo de esa manera el mismo producto.

3. Respuesta a las objeciones

De este modo y con el objeto de atender las objeciones arriba mencionadas, se desea presentar las siguientes modificaciones, aclaraciones y argumentos:

3.1 Modificación de una solicitud en trámite

De acuerdo con el artículo 34 de la Decisión 486 de la Comisión de la Comunidad Andina, donde se establece que la modificación de una solicitud es posible en cualquier momento del trámite mientras no implique una ampliación de la solicitud inicial, me permito presentar nuevo pliego reivindicatorio conformado por 8 reivindicaciones.

Siguiendo las amables sugerencias del Examinador, se han introducido las siguientes modificaciones al pliego reivindicatorio:

- Las reivindicaciones originales 1 a 5, 9 y 10 han sido unificadas para formar la nueva reivindicación 1.
- Las reivindicaciones originales 6 y 7 han sido unificadas para formar la nueva reivindicación 2.

- Las reivindicaciones originales 8, 11, 12, 13 y 14 ahora corresponden a las reivindicaciones 3, 4, 5, 6 y 7, respectivamente.
- Las reivindicaciones 15 y 16 han sido eliminadas.
- La nueva reivindicación 8 corresponde a la reivindicación 15 original antes de ser modificada, pero ahora está dirigida a un método.

Por lo anterior, las objeciones formuladas por el Examinador en cuanto a la claridad y concisión del pliego reivindicatorio se ven superadas. En consecuencia, se solicita atentamente tener en cuenta este nuevo pliego reivindicatorio para el análisis de patentabilidad.

3.2 Argumentos a favor de la novedad de la presente solicitud

El artículo 16 de la Decisión 486 señala lo siguiente:

“Artículo 16.- Una invención se considerará nueva cuando no está comprendida en el estado de la técnica.

El estado de la técnica comprenderá todo lo que haya sido accesible al público por una descripción escrita u oral, utilización, comercialización o cualquier otro medio antes de la fecha de presentación de la solicitud de patente o, en su caso, de la prioridad reconocida.

Sólo para el efecto de la determinación de la novedad, también se considerará dentro del estado de la técnica, el contenido de una solicitud de patente en trámite ante la oficina nacional competente, cuya fecha de presentación o de prioridad fuese anterior a la fecha de presentación o de prioridad de la solicitud de patente que se estuviese examinando, siempre que dicho contenido esté incluido en la solicitud de fecha anterior cuando ella se publique o hubiese transcurrido el plazo previsto en el artículo 40”.

Siguiendo entonces los lineamientos de la Jurisprudencia aplicable al análisis de novedad de una solicitud de patente, se considera que cuando las características técnicas esenciales son idénticas a las características de la materia divulgada en cada documento del estado de la técnica, puede afectar dicho requerimiento de patentabilidad, en cuyo caso se considera que no tiene novedad. Adicionalmente se considerará nueva la invención si se encuentra alguna diferencia o un sólo aspecto que no hubiese sido revelado en el arte previo.

Con base en lo anterior, respetuosamente manifestamos nuestro desacuerdo con el análisis llevado a cabo por el Examinador y con las conclusiones del mismo y solicitamos atentamente la reconsideración de las razones por las cuales se objeta la novedad, con base en la siguiente información:

Como puede verse a partir de la traducción en Inglés de la solicitud de patente No. JP2006149202, citada por el examinador como estado de la técnica más cercano a la presente solicitud, es evidente que D1 no describe en absoluto **un método para determinar si o no una rosa silvestre de interés se cruza con una rosa cultivada**, aunque D1 si describe un método de impartir una propiedad perpetua de floración a una angiosperma mediante la regulación de la expresión del gen específico que utiliza el ADN específico o transposón. Esto se encuentra soportado en el pliego reivindicatorio de D1, específicamente en las reivindicaciones 1 y 8. Además en la sección "Una breve descripción de la invención" se encuentra establecido el objeto de la invención del documento D1 [párrafo 0008], donde se especifica el método descrito anteriormente. El párrafo [0008] establece lo siguiente:

"Los presentes inventores han tenido éxito en identificar una proteína y un gen que participan en la floración perpetua de angiospermas, y un ADN que regula la expresión de dicho gen, y han encontrado que cuando la expresión de este gen es regulada, la formación de brotes de flores en el ápice del brote se promueve de este modo haciendo que dicha planta tenga floración perpetua. La presente invención se basa en tal investigación."

Por esta razón, se puede concluir que las características técnicas del método revelado en la nueva reivindicación 1 (pasos y condiciones) no aparecen reveladas en D1 y por ende, la novedad de la invención no se encuentra afectada, y por lo tanto, la objeción se debe tener por superada.

3.3 Argumentos a favor del nivel inventivo de la presente solicitud

Inicialmente, ponemos de presente que si bien el Despacho no hizo objeción alguna por nivel inventivo, con el fin de agilizar el trámite de este asunto, procederemos a demostrar que la invención además cumple con este requisito. Sin embargo, respetuosamente ponemos de presente que en el evento que su Despacho considere pertinente hacer una objeción a este respecto, resultará necesario que se expida una acción oficial adicional, por cuanto la misma no nos ha sido trasladada.

El artículo 18 de la Decisión 486 señala lo siguiente:

Artículo 18.- Se considerará que una invención tiene nivel inventivo, si para una persona del oficio normalmente versada en la materia técnica correspondiente, esa invención no hubiese resultado obvia ni se hubiese derivado de manera evidente del estado de la técnica.

Consecuentemente, una invención se considerará inventiva si la misma no hubiese resultado obvia a partir del arte previo, para una persona del oficio normalmente versada en la materia. Esto a su vez implica que el Examinador a cargo de esta evaluación debe colocarse en el momento exacto en el que el inventor buscaba resolver el problema técnico contemplado en la solicitud y para el cual poseía la literatura y el conocimiento disponibles en dicho momento.

Siguiendo entonces los lineamientos de la Jurisprudencia aplicable al análisis del nivel inventivo de una solicitud de patente, se considera que una anterioridad o una combinación de dos anterioridades puede afectar dicho requerimiento de patentabilidad, si una persona medianamente versada en la materia encuentra enseñanzas o sugerencias claras que, a partir de un conocimiento medio en dicho campo, le permitan llegar de manera obvia a la invención reivindicada. Adicionalmente se considera que una mejora sustancial, un efecto inesperado o una ventaja con respecto al arte previo es un indicio de nivel inventivo, por cuanto aporta un salto tecnológico en la regla técnica.

Respecto a lo anterior, el Tribunal de Justicia de la Comunidad Andina ha expuesto en la Interpretación Prejudicial del proceso 13-IP-2010:

"En este orden de ideas, se puede concluir que una invención goza de nivel inventivo cuando a los ojos de un experto medio en el asunto de que se trate, se necesita algo más que la simple aplicación de los conocimientos técnicos en la materia para llegar a ella, es decir, que de conformidad con el estado de la técnica el invento no es consecuencia clara y directa de dicho estado".

[señalado fuera del texto]

Por lo anterior, se entiende que para que una persona medianamente versada en la materia pudiera derivar de manera obvia una invención, requeriría simplemente aplicar sus conocimientos técnicos. En contraste, todo aquello que va más allá de la simple aplicación de

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los conocimientos técnicos y que no ha sido revelado en el estado de la técnica, debe ser considerado inventivo.

Inicialmente, se debe tener en cuenta que las rosas cultivadas son de floración perpetua (floración) mientras que la mayoría de las rosas silvestres son de una temporada de floración. D1 divulga el hallazgo que la causa de la floración perpetua es la presencia de un gene KSN que contiene un transposón, y basado en este hallazgo, proporciona un método de conferir una propiedad de floración perpetua para angiospermas.

Por el contrario, el objetivo (propósito) de la presente invención es proporcionar un método que permita una fácil determinación de si o no una rosa silvestre de interés es cruzada con el polen de una rosa transformante, es decir, proporcionar un método de determinación si o no un gen indicador de una rosa cultivada (que es tetraploide (4x) y de floración perpetua) ha sido transferido a una rosa silvestre (que generalmente es diploide (2x) y de una temporada de floración). Con el fin de alcanzar este objetivo, es necesario que **la rosa cultivada contenga tal gen indicador en la configuración homóloga**. La presente invención utiliza este concepto técnico con el fin de alcanzar el objetivo. Sin embargo, D1 no enseña o sugiere tal concepto.

Como se describe en la columna 3, línea 49 a la columna 4, línea 3 de US8206928 B2 (correspondiente a la descripción de esta solicitud), el gen indicador debe satisfacer todos los siguientes 3 requerimientos:

1. el gen indicador siempre está presente en las rosas cultivadas y no en las rosas silvestres nativas;
2. las rosas cultivadas son tetraploides (4x) y un gen indicador para el cruce debe estar presente en por lo menos tres de los cuatro cromosomas homólogos, y preferiblemente puede estar presente en todos los cuatro cromosomas homólogos. Las rosas cultivadas son tetraploides (4x), mientras que las rosas silvestres nativas son diploides (2x). En consecuencia, los gametos producidos de rosas cultivadas pueden ser usualmente 2x, las rosas silvestres pueden ser x, y los híbridos producidos del cruce entre ellos pueden llegar a ser tripoides (3x). Basado en esto, es imperativo que el gen indicador para el cruce esté presente en por lo menos tres de los genes homólogos de las rosas cultivadas. Teniendo en cuenta la posibilidad que un gameto haploide (x) puede ser producido mediante meiosis heterogénea de rosas cultivadas, sin embargo, se prefiere, para una determinación exacta, que el gen indicador esté presente en todos los cuatro cromosomas homólogos; y

3. las mismas secuencias de la secuencia parcial de la secuencia base del gen indicador no está presente en las otras regiones del cromosoma.

Antes de la fecha de prioridad de esta solicitud, no se conocían genes indicadores que satisfacen los tres requisitos anteriores que habían sido conocidos por una persona del oficio medianamente versada en la materia. Los inventores de esta solicitud llevaron a cabo una investigación extensa en un número de genes y encontraron inesperadamente un gen KSN que tiene un transposón insertado en el mismo, que calificó como tal un gen indicador. Amablemente solicitamos tener en cuenta que se ha encontrado hasta la fecha que hay genes distintos del gen KSN, que satisfacen los tres requisitos anteriores. A este respecto, un gen KSN que tiene un transposón insertado en el mismo es de gran valor.

Por estas razones, respetuosamente consideramos que la invención revelada en la nueva reivindicación 1 cumple el requisito de nivel inventivo frente al documento D1.

3.4 Conclusión

Por todo lo anterior, el documento D1 no resulta una anterioridad válida capaz de afectar la novedad del producto de la presente solicitud. De la misma manera, el documento D1 no resulta anterioridad válida capaz de afectar el nivel inventivo del producto de la presente solicitud.

Por lo tanto, consideramos que el Examinador está pasando por alto que una sola diferencia con respecto al estado de la técnica, permite concluir que el objeto reivindicado es nuevo. Además, se está desconociendo el mérito investigativo empleado y que está aportando una ventaja al arte previo.

Dicho mérito investigativo ha sido reconocido por otras oficinas de patentes del mundo, las cuales han otorgado patentes para invenciones equivalentes. Tal es el caso de Estados Unidos, patente No. US8206928 (B2).

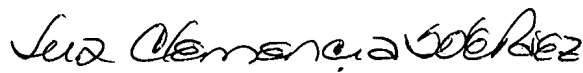
Si bien es cierto que cada país es autónomo en conceder o negar el privilegio de patente para una solicitud, también es cierto que los requisitos de concesión de dichos privilegios son universales, esto es, novedad, nivel inventivo y aplicación industrial, como también la forma de evaluarlos. Así las cosas, el hecho de que la misma solicitud cumpla con los mencionados requisitos en diferentes países, como se mencionó anteriormente, es un claro indicio del merecimiento de la patente en Colombia.

4. Puesto que se ha dado respuesta a todas las objeciones formuladas por el Examinador, se solicita atentamente proceder al otorgamiento de la solicitud de patente. En este sentido, respetuosamente recordamos al Examinador que la patente no podrá ser negada con base en objeciones diferentes a las expuestas en la acción oficial No. 15120 toda vez que al no haber sido trasladadas a mi representada, dicha decisión violaría tanto el tenor literal del artículo 45 de la Decisión 486 como el derecho constitucional al debido proceso. En este evento, y con base en el inciso 2 del artículo 45, respetuosamente solicitamos al Examinador expedir una nueva acción oficial que permita a mi representada contra argumentar las mismas o modificar la solicitud con el fin de cumplir los requisitos de patentabilidad.

5. Anexos

- Formulario de modificaciones en solicitud pendiente
- Nuevo pliego reivindicatorio conformado por 8 reivindicaciones
- Primera hoja de la solicitud de patente de Estados Unidos
- Traducción exacta al Inglés del documento D1: JP2006149202.

Señor Director,


LUZ CLEMENCIA DE PÁEZ

C.C. 35.456.344 de Usaquén

T.P.A. No. 23.555

 COLO-15218-0841-003-8
COLO-17511-0843-002-4
SGD\SGD\ID-230550\WPC17511
No. M-2012-230550\SGD

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DIRECCIÓN DE NUEVAS CREACIONES
MODIFICACIONES Y/O CORRECCIONES

1. Identificación del Trámite

☐ Error material

☒ Modificación a una Solicitud en trámite

☒ Patente de Invención

☐ Patente de Modelo de Utilidad

☐ Registro de Diseño Industrial

☐ Esquema de Trazado de Circuitos Integrados

2. Datos del solicitante

Persona Natural☐

Persona Jurídica☒

SUNTORY HOLDINGS LIMITED

Nombre o denominación / Nombre o razón social completa

Nombre representante legal

Tipo de empresa Micro☐ Pequeña☐ Mediana☐ Otra☐

Documento de identificación: ☐ C.C. ☐ C.E. ☐ NIT ☐ Otro Número_____

Nacionalidad/País de constitución	Dirección y domicilio del titular	Ciudad
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Dirección electrónica	No. Fax	Número telefónico
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☐ Autorizo expresamente a la Superintendencia de Industria y Comercio para que todas las comunicaciones sean enviadas a través de la dirección de correo electrónico

3. Datos del apoderado:

Apellidos y Nombre
De Páez Luz Clemencia

Documento de identificación
C.C. 35.456.344

Tarjeta profesional
T.P.A. 23.555

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En caso de haber presentado poder general para asuntos que se adelanten ante la Delegatura de Propiedad Industrial, sírvase indicar el número de su radicación o protocolo de poder general

4. Datos del trámite**Corrección:****Expediente No 09.103.864****Aparece****Debe ser****Modificación:****Modificación al capítulo reivindicatorio.**

Presento nuevo capítulo reivindicatorio compuesto por 8 reivindicaciones y pido respetuosamente que el examen de patentabilidad sea realizado sobre estas nuevas reivindicaciones presentadas.

Respetuosamente manifiesto que a pesar de eliminar materia del pliego reivindicatorio originalmente presentado, mi representada no renuncia a la protección que pueda obtener sobre dicha materia y se reserva el derecho de presentar una solicitud divisional que la contenga durante el trámite de la presente solicitud, beneficiándose de la fecha de prioridad inicialmente reivindicada.

**Modificación al capítulo descriptivo.****Modificación al resumen.****Modificación a los dibujos.****Modificación al solicitante.****5. Anexos****Poder, si fuere el caso.****Documento que contiene las modificaciones o correcciones.****Copia de la solicitud y sus anexos en formato magnético.**

6. Firma	
Nombre y apellidos	Firma
LUZ CLEMENCIA DE PAEZ	<i>LUZ CLEMENCIA DE PAEZ</i>
C.C. 35.456.344 DE Usaquén	Tarjeta Profesional 23.555

SGD\NCA\WPC17511\ID-230656

REIVINDICACIONES

1. Un método para determinar si o no una rosa silvestre de interés está cruzada con una rosa cultivada, que comprende
detectar si una semilla de la rosa silvestre comprende un gen KSN que contiene un transposón indicador insertado llevando a cabo una amplificación específica mediante de la reacción en cadena de la polimerasa (PCR) vía de un iniciador delantero:
CATATTATGGCATAGGGTGTGGC (SEQ ID NO: 3) y un iniciador inverso:
TGTAATCTGTAGGAGATCCCATGC (SEQ ID NO: 4); y
determinar si o no la rosa silvestre está cruzada con una rosa cultivada, en donde la presencia del gen KSN que contiene un transposón indicador insertado en la semilla de la rosa silvestre indica un cruce entre la rosa salvaje y la rosa cultivada, en donde la ausencia del gen KSN que contiene el transposón indicador insertado en la semilla de la rosa silvestre no indica cruce entre la rosa silvestre y la rosa cultivada.
2. El método de acuerdo a la reivindicación 1, en donde la rosa cultivada comprende un gen relacionado con el color de la flor, en donde el gen relacionado con el color de la flor es el gen de la enzima flavonoide 3', 5'-hidroxilasa derivado de pensamiento de la familia violácea.
3. El método de acuerdo a la reivindicación 1, en donde la hibridación se lleva a cabo para detectar si la semilla de la rosa silvestre comprende el gen KSN que contiene el transposón indicador insertado.

4. El método de acuerdo a la reivindicación 1, en donde la rosa silvestre tiene, en la configuración homóloga, un gen KSN que no contiene un transposón indicador insertado.
5. El método de acuerdo a la reivindicación 1, en donde la rosa silvestre se selecciona del grupo que consiste de NOIBARA (*R. multiflora* Thunb. ex Murray), TERIHANOIBARA (*R. wichuraiana* Crep.), HAMANASU (*R. rugosa* Thunb. ex Murray), OOTAKANEBARA (*R. acicularis* Lindl.), KARAFUTOIBARA (*R. marretii* Lev.), OOFUJIIBARA, AZUMAIBARA, YAMATERIHANOIBARA (*R. luciae* Franch. et Rochebr.), YAMAIBARA (*R. sambucina* Koidz.), KAKAYANBARA, YAEYAMANOIBARA (*R. bracteata* Wendl.), NANIWAIBARA (*R. laevigata* Michx.), SANSHOUBARA (*R. roxburghii* Tratt. var. *hirtula* (Regel) Rehd. et Wils.), TAKANEBARA (*R. acicularis* var. *nipponensis* (Crép.) Koehne.), TSUKUSHIIBARA (*R. multiflora* var. *adenochaeta* (Koidz.) Makino), MORIIBARA (*R. luciae* var. *hakonensis* Franch. et Sav.), FUJIIBARA (*R. luciae* var. *fujisanensis* Makino), YABUIBARA, NIOIIBARA (*R. luciae* var. *onoei* (Makino) Momiyama), y MIYAKOIBARA (*R. luciae* var. *paniculgera* (Makino) Momiyama).
6. El método de acuerdo a la reivindicación 1, en donde la rosa cultivada tiene, en la configuración homóloga, un gen KSN que tiene un transposón indicador insertado en ésta.
7. El método de acuerdo a la reivindicación 1, en donde la rosa cultivada es un té híbrido, una floribunda o una miniatura.

8. El método de acuerdo a la reivindicación 1, en donde la PCR se lleva a cabo usando un primer par iniciador capaz de hibridizar con una región de la secuencia base establecida en la SEQ ID NO: 1, y un segundo iniciador capaz de hibridizar con una región de la secuencia base establecida en la SEQ ID NO: 2.

SGD\SGD\ID-230524\WPC17511



US008206928B2

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(12) **United States Patent**
Nakamura et al.

(10) **Patent No.:** **US 8,206,928 B2**
(45) **Date of Patent:** **Jun. 26, 2012**

(54) **METHOD FOR DETERMINATION OF
PRESENCE OF CROSSING WITH
CULTIVATED ROSE IN WILD ROSE**

(75) Inventors: **Noriko Nakamura**, Mishima-gun (JP);
Masako Mizutani, Mishima-gun (JP);
Yoshikazu Tanaka, Mishima-gun (JP)

(73) Assignee: **Suntory Holdings Limited**, Osaka-shi,
Osaka (JP)

(*) Notice: Subject to any disclaimer, the term of this
patent is extended or adjusted under 35
U.S.C. 154(b) by 295 days.

(21) Appl. No.: **12/532,246**

(22) PCT Filed: **Mar. 21, 2008**

(86) PCT No.: **PCT/JP2008/056000**
§ 371 (c)(1),
(2), (4) Date: **Sep. 21, 2009**

(87) PCT Pub. No.: **WO2008/117860**
PCT Pub. Date: **Oct. 2, 2008**

(65) **Prior Publication Data**
US 2010/0143914 A1 Jun. 10, 2010

(30) **Foreign Application Priority Data**
Mar. 23, 2007 (JP) 2007-077882

(51) Int. Cl.
C12Q 1/68 (2006.01)
C12P 19/34 (2006.01)

(52) U.S. Cl. **435/6.12; 435/91.2**
(58) Field of Classification Search **435/6.12,**
435/91.2; 536/24.33
See application file for complete search history.

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(57) **ABSTRACT**

Disclosed is a method for determining whether or not a wild rose of interest is crossed with a cultivated rose. The method comprises the steps of: examining whether or not a KSN gene containing a transposon (an indicator) is contained in the rose of interest; and determining that the rose of interest is crossed with a cultivated rose when the individual has the transposon-containing KSN gene.

10 Claims, 1 Drawing Sheet

Accurate Trans. of JP 2006-149202 A 2006.6.15

(54) [Title of the Invention] Protein And Gene Participating In Perpetual Blooming Of Angiosperm

[Scope of Claims]

[Claim 1] A protein described in the following (a) or (b):

(a) a protein containing the amino acid sequence represented by SEQ ID NO: 2, and

(b) a protein containing an amino acid sequence derived from the amino acid sequence represented by SEQ ID NO: 2 by substitution, addition or insertion of one or more amino acids and being capable of regulating flower bud formation at the shoot apex of an angiosperm.

[Claim 2] The protein according to claim 1 wherein said angiosperm belongs to the genus Rosa.

[Claim 3] A gene encoding the protein according to claim 1 or 2.

[Claim 4] The protein according to claim 3 comprising the nucleotide sequence represented by SEQ ID NO: 1.

[Claim 5] A single stranded nucleic acid molecule comprising all or part of a nucleotide sequence identical to the sense strand of the gene according to claim 3 or 4.

[Claim 6] A single stranded nucleic acid molecule comprising all or part of a nucleotide sequence identical to the antisense strand of the gene according to claim 3 or 4.

[Claim 7] A double stranded nucleic acid molecule comprising all or part of a nucleotide sequence identical to the gene according to claim 3 or 4.

[Claim 8] A method of imparting a perpetual blooming property to an angiosperm, which comprises regulating the expression of the gene according to claim 3 or 4 by using the single stranded nucleic acid molecule as defined in claim 5 or 6 or the double stranded nucleic acid molecule as defined in claim 7.

[Claim 9] The method according to claim 8 wherein said angiosperm belongs to the genus Rosa.

[Claim 10] A DNA described in the following (a) or (b):

(a) a DNA containing the nucleotide sequence represented by SEQ ID NO: 3, and

(b) a DNA containing a nucleotide sequence derived from the nucleotide sequence represented by SEQ ID NO: 3 by deletion, substitution, addition or insertion of one or more nucleotides and being capable of functioning as a transposon of an angiosperm.

[Claim 11] The DNA according to claim 10 wherein said angiosperm belongs to the genus Rosa.

[Claim 12] A transposon containing the DNA according to claim 10 or 11.

[Claim 13] A method of imparting a perpetual blooming property to an angiosperm, which comprises regulating the expression of the gene of claim 3 or 4 using the DNA according to claim 10 or 11 or the transposon according to claim 12.

[Claim 14] The method according to claim 13 wherein said angiosperm belongs to the genus Rosa.

[Detailed Description of the Invention]

[0001]

[Background of the Invention]

Field of the Invention

The present invention relates to a gene and a DNA fragment participating in perpetual blooming of angiosperms.

[0002]

Background Art

In the flower industry today, top three sales flowers are varieties that can be bloomed throughout the year. Thus, year-round shipment is one of the most important factors in flower production. Chrysanthemums are produced throughout the year by devising appropriate cultivation methods. For roses and carnations, varieties having the genetic (perpetual blooming) property of flowering throughout the year have been produced. In home and other gardens as well, perpetual blooming is a very important advantage since flowers, once planted, can be enjoyed all year round, and thus perpetual blooming varieties are also important in the seed industry.

[0003]

Many of rose cultivars are perpetual blooming, and all of them were obtained by cross breeding. Today's perpetual blooming roses such as hybrid tea and floribunda roses were established over 100 years starting at the 18th century by a series of cross breeding between Chinese perpetual blooming roses and then-existing European roses.

[0004]

Perpetual blooming varieties created by cross breeding are products obtained by accident. In particular, since many of rose cultivars are polyploids of four or higher orders, the mode of trait transmission by crossing is specifically complicated. Thus, it is difficult to guarantee the imparting of a perpetual blooming property to specific varieties or the introduction of a specific trait into a perpetual blooming variety. Therefore, even varieties of ornamental value may not be marketed since they are not perpetual blooming. Also, in many other flowers, there exist very few perpetual blooming varieties due to the lack of suitable breeding materials.

[0005]

Research is underway on the relationship between the centroradialis (cen') gene of Antirrhinum and the terminal flower 1 (TFL1) gene of Arabidopsis and flowering (Patent Document 1; Kohyo (National Publication of Translated Version) No. 11-512289). For example, it is known that the effect of the TFL1 gene of Arabidopsis and its ortholog is to regulate flower bud formation (anthogenesis) at the shoot apex. At the shoot apex of the wild type, no flower bud formation occurs and its growth continues under an appropriate cultivation condition such as temperature, water and fertilizers. On the other hand, in artificially induced

mutants that do not express the TFL1 gene or its ortholog, flower bud formation occurs early, shoot elongation ceases, and the plant height becomes small.

[0006]

However, no research has been made on the perpetual blooming property of angiosperms, specifically roses.

[0007]

[Patent Document 1]

Kohyo (National Publication of Translated Version) No. 11-512289

[0008]

[Summary of the Invention]

The present inventors have succeeded in identifying a protein and gene participating in perpetual blooming of angiosperms, and a DNA that regulates the expression of said gene, and have found that when the expression of this gene is regulated, flower bud formation at the shoot apex is promoted thereby making said plant perpetual blooming. The present invention is based on such a finding.

[0009]

Thus, it is an object of the present invention to provide a protein and gene participating in perpetual blooming, and a DNA that can regulate the expression of said gene.

[0010]

Accordingly, the protein according to the present invention is described in the following (a) or (b):

(a) a protein containing the amino acid sequence represented by SEQ ID NO: 2, and

(b) a protein containing an amino acid sequence derived from the amino acid sequence represented by SEQ ID NO: 2 by substitution, addition or insertion of one or more amino acids and being capable of regulating flower bud formation at the shoot apex of an angiosperm.

[0011]

Furthermore, the gene according to the present invention encodes the protein according to the present invention.

[0012]

Furthermore, the DNA according to the present invention is described in the following (a) or (b):

(a) a DNA containing the nucleotide sequence represented by SEQ ID NO: 3, and

(b) a DNA containing a nucleotide sequence derived from the nucleotide sequence represented by SEQ ID NO: 3 by deletion, substitution, addition or insertion of one or more nucleotides and being capable of functioning as a transposon of an angiosperm.

[0013]

The present invention can make an angiosperm perpetual blooming and provides a target protein, a target gene, a nucleic acid molecule, and a DNA regulating the expression of said gene that are useful in developing perpetual blooming angiosperms.

[0014]

[Specific description of the Invention]

As used herein the term "perpetual blooming" refers to the property of a plant that permits perpetual flowering throughout all the seasons.

[0015]

As used herein the term "angiosperm" refers to plants in

which ovules are contained in the ovary, and most of the plants for ornamental purpose belong to the angiosperm. The angiosperm includes a variety of plants such as, but not limited to, plants belonging to the order Rosales, more specifically the family Rosaceae, and most preferably Rosa.

[0016]

As used herein the term "nucleic acid molecule" is used in the sense that it includes DNA, RNA and PNA (peptide nucleic acid).

[0017]

As used herein the term "specific" when used in reference to a nucleic acid molecule means that the nucleotide sequence of the nucleic acid molecule is only located in the subject gene and not in other genes of angiosperms.

[0018]

Numerical values for homology as used herein may be those calculated using a homology search program known to those skilled in the art unless otherwise specified, but preferably they are numerical values calculated using default (initial setting) parameters (for example, Protein-Based BLAST Search (Filler: ON, Scoring Matrix: BLOSUM62, Word Size: 3, E value: 10, Gap costs: 11, 1, Alignments: 50) in BLAST [J. Mol. Biol., 215, 403 (1990)]).

[0019]

The protein according to the present invention contains the amino acid sequence represented by SEQ ID NO: 2, and said protein can regulate flower bud formation at the shoot apex of an angiosperm. Also, the protein according to the present invention is not limited to those described above, but may be a mutant containing an amino acid sequence derived from the amino acid sequence represented by SEQ ID NO: 2 by substitution, addition or insertion of one or more amino acids, as long as it can regulate flower bud formation at the shoot apex of an angiosperm. In said mutant, the number of amino acids that are substituted, deleted, added or inserted may preferably be, but not limited to, 1 to 9, more preferably 1 to 5, and most preferably 1 to 3. The amino acid sequence of said mutant may preferably have a homology of at least 83%, more preferably at least 90%, and most preferably at least 95% to the amino acid sequence represented by SEQ ID NO: 2.

[0020]

The gene according to the present invention may encode the protein according to the present invention. The nucleotide sequence is not specifically limited, but may preferably be the nucleotide sequence represented by SEQ ID NO: 1. The nucleotide sequence represented by SEQ ID NO: 1 may be a sequence from the translation initiation codon through the translation stop codon of cDNA relative to mRNA, and may encode the amino acid sequence represented by SEQ ID NO: 2. The gene according to the present invention may contain not only the sequence of the cDNA but that of the genomic DNA. The gene containing such a sequence of the genomic DNA may contain the sequence of an intron other than the sequence of the exon encoding the protein according to the present invention, and may preferably be a gene having inserted a sequence corresponding to an intron in the nucleotide sequence represented by SEQ ID NO: 1, for example a gene containing the nucleotide sequence (SEQ ID NO: 19)

shown in Fig. 4. Furthermore, the gene according to the present invention may contain a nucleotide sequence derived from the nucleotide sequence represented by SEQ ID NO: 1 by substitution, addition or insertion of one or more nucleotides. As used herein, the number of nucleotides that are substituted, deleted, added or inserted may preferably be, but not limited to, 1 to 9, more preferably 1 to 5, and most preferably 1 to 3. Such a nucleotide sequence may preferably have a homology of at least 81%, more preferably at least 85%, and most preferably at least 90% to the nucleotide sequence represented by SEQ ID NO: 1.

[0021]

The gene and protein according to the present invention can be obtained according to a method known to those skilled in the art. As a method for obtaining the gene according to the present invention, there can be mentioned, for example, a chemical synthetic method such as the phosphoramidite method, and a nucleic acid amplification method that employs a primer designed based on the nucleotide sequence of the gene of interest with a nucleic acid material from an angiosperm such as Rosa chinensis spontanea as a template. As a method for obtaining the protein according to the present invention, there can be mentioned, for example, a method of purifying it as a natural product from the shoot apex of an angiosperm, and a method of integrating the gene according to the present invention into an expression system with *Escherichia coli* or yeast as the host to produce the protein.

[0022]

The protein according to the present invention regulates flower bud formation at the shoot apex of an angiosperm and the gene according to the present invention encodes said protein. Accordingly, if the function of the protein or gene according to the present invention is inhibited in an angiosperm, the regulation of flower bud formation is released, and thus flower bud formation is promoted, thereby making the angiosperm perpetual blooming. Therefore, the present invention provides a method of imparting a perpetual blooming property to an angiosperm, said method comprising inhibiting the function of the protein or gene according to the present invention. Said method may be performed according to a method known to those skilled in the art, but preferably it may comprise regulating the expression of the gene according to the present invention. Examples of such methods include, for example, an antisense RNA/DNA technology [Bioscience & Industry 50, 322 (1992); Kagaku (Chemistry) 46, 681 (1992); Biotechnology 9, 358 (1992); Trends in Biotechnology 10, 87 (1992); Trends in Biotechnology 10, 152 (1992); Saibo Kogaku (Cell Engineering) 16, 1463 (1997)], a triple helix technology [Trends in Biotechnology 10, 132 (1992)], and RNA interference (RNAi) [Chuang CF & Meyerowitz EM: Proc. Natl. Acad. Sci. U.S.A. 97, 4985 (2000); Elbashir, S.M. et al., Nature 411, 494-498 (2001)].

[0023]

In accordance with a preferred embodiment of the present invention, the regulation of the gene according to the present invention may be performed using a single stranded nucleic acid molecule comprising all or part of a nucleotide sequence identical to the antisense of the gene according to the present invention. Such a method is also known as the antisense method. In an antisense method, the expression of

the target gene is regulated by expressing, at a high level, RNA having a sequence complementary to the gene of which expression is desired to be regulated. In such a method, a single stranded RNA comprising all of the nucleotide sequence identical to the antisense strand of the gene according to the present invention may be used. Also, in the above method, a single stranded RNA comprising part of the nucleotide sequence identical to the antisense strand of the gene according to the present invention may be used. Though such a partial single stranded RNA may be any RNA as long as it can regulate the expression of the gene according to the present invention and may be designed as appropriate by a person skilled in the art, it may preferably be specific to the gene according to the present invention, and the chain length may preferably be 5 to 100 nucleotides, more preferably 5 to 50 nucleotides, and most preferably 10 to 20 nucleotides.

[0024]

In accordance with another preferred embodiment of the present invention, the regulation of the gene according to the present invention may be performed using a single stranded nucleic acid molecule comprising all or part of a nucleotide sequence identical to the sense of the sense strand of the according to the present invention. Thus, this sense single stranded nucleic acid, like the antisense single stranded nucleic acid described above, may be used to regulate the expression of the gene according to the present invention. In such a method, a single stranded RNA comprising all of the nucleotide sequence identical to the sense strand of the gene according to the present invention may be used. Also, in the above method, a single stranded RNA comprising part of the nucleotide sequence identical to the sense strand of the gene according to the present invention may be used. Though such a partial single stranded RNA may be any RNA as long as it can regulate the expression of the gene according to the present invention and may be designed as appropriate by a person skilled in the art, it may preferably be specific to the gene according to the present invention, and the chain length may preferably be 5 to 100 nucleotides, more preferably 5 to 50 nucleotides, and most preferably 10 to 20 nucleotides.

[0025]

In accordance with another preferred embodiment of the present invention, the regulation of the gene according to the present invention may be performed using a double stranded nucleic acid molecule comprising all or part of a nucleotide sequence identical to the gene according to the present invention. For example, using this double stranded nucleic acid molecule, an antisense or sense single stranded nucleic acid of the gene according to the present invention may be expressed in an angiosperm. The double stranded nucleic acid molecule according to the present invention may preferably be DNA, in which the chain length and specific nucleotide sequence should correspond to the chain length and nucleotide sequence of the single stranded nucleic acid molecule of interest. For example, when the above antisense single stranded nucleic acid is to be expressed, the double stranded nucleic acid molecule according to the present invention should comprise the antisense strand of the gene according to the present invention as the coding strand. Also, when the above sense single stranded nucleic acid is to be

expressed, the double stranded nucleic acid molecule according to the present invention should comprise the sense strand of the gene according to the present invention as the coding strand.

[0026]

A double stranded nucleic acid molecule according to the present invention may be expressed in an angiosperm using a method known to those skilled in the art. For example, by introducing an expression construct containing a promoter, a double stranded nucleic acid molecule according to the present invention, a transcription terminator etc. into an angiosperm of interest, and cultivating the plant obtained, the double stranded nucleic acid molecule according to the present invention may be expressed. The introduction of the expression construct into the plant may preferably be performed by a method known to those skilled in the art, such as an *Agrobacterium* method, a binary vector method, an electroporation method, a PEG method, a particle gun method and the like. In particular, the transformation of roses for gene introduction may be performed according to a method described in U.S. Pat. No. 5480789 etc.

[0027]

As a method of regulating gene expression using a nucleic acid molecule according to the present invention, there can be mentioned a transformation method with an antisense strand (Proc. Natl. Acad. Sci. U.S.A. 90: 6160-6164, 1993). In this method, an antisense double stranded DNA having the entire nucleotide sequence of the gene according to the present invention may be introduced into a plant of interest. This will permit the expression of an antisense single stranded RNA according to the present invention as RNA complementary to mRNA of the gene according to the present invention, and this complementary RNA will extremely regulate the expression of said gene.

[0028]

As another method of regulating gene expression using a nucleic acid molecule according to the present invention, there can be mentioned a cosuppression method. In this method, a sense double stranded DNA having the entire nucleotide sequence of the gene according to the present invention may be introduced into a plant of interest. This will permit the expression of a sense single stranded RNA according to the present invention, and this RNA will extremely regulate the expression of said gene (Plant Cell 9: 1357-1368, 1997).

[0029]

The DNA according to the present invention comprises the nucleotide sequence represented by SEQ ID NO: 3, and can function as a transposon in an angiosperm. In the nucleotide sequence represented by SEQ ID NO: 3, residues 1 through 856 are LTR, residues 857 through 6105 are ORF, and residues 8069 through 8925 are LTR. Also, the DNA according to the present invention is not specifically limited to those described above, but may comprise a nucleotide sequence derived from the nucleotide sequence represented by SEQ ID NO: 3 by substitution, addition or insertion of one or more nucleotides as long as it can function as a transposon in an angiosperm. As used herein, the number of nucleotides that are substituted, deleted, added or inserted may preferably be, but not limited to, 1 to 9, more preferably 1 to 5, and most preferably 1 to 3. Such a nucleotide sequence may preferably

have a homology of at least 70%, more preferably at least 80%, and most preferably at least 90% to the nucleotide sequence represented by SEQ ID NO: 3.

[0030]

The DNA according to the present invention may be obtained by a method known to those skilled in the art, such as chemical synthesis by the phosphoramidite method etc., and a nucleic acid amplification method that employs primers designed based on the nucleotide sequence of the gene of interest with a nucleic acid material from a perpetual blooming rose such as Old blush as a template.

[0031]

The DNA according to the present invention functions as a transposon in an angiosperm, and regulates the expression of the gene according to the present invention by being inserted into said gene. This will release the regulation of flower bud formation at the shoot apex and thus promote flower bud formation, thereby making the angiosperm perpetual blooming. Thus the present invention provides a transposon containing the DNA according to the present invention, and furthermore a method of imparting a perpetual blooming property to an angiosperm, said method comprising regulating the expression of the gene according to the present invention by using the DNA according to the present invention or a transposon containing it.

[0032]

In this method, the DNA or transposon according to the present invention may be introduced into an angiosperm. This may be performed as appropriate by a person skilled in the art so as to regulate the expression of the gene according to the present invention, wherein the DNA or transposon according to the present invention may preferably be introduced into or in the vicinity of the gene according to the present invention, more preferably into the second intron of the gene according to the present invention, and most preferably into the same position as the perpetual blooming rose.

[0033]

The introduction of DNA into the plant may preferably be performed by a method known to those skilled in the art, such as an Agrobacterium method, a binary vector method, an electroporation method, a PEG method, a particle gun method and the like. In particular, the transformation of roses for gene introduction may be performed according to a method described in U.S. Pat. No. 5480789 etc.

[0034]

[Examples]

Working Example 1: Identification of factors participating in perpetual blooming

In perpetual blooming rose varieties of today, shoots and axillary buds that have extended about 4 to 7 leaves form flower buds at the shoot apex. Immediately after flowering stops, axillary buds thereunder start to grow and form flower buds similarly. Thus, if the cultivation conditions such as temperature, water and fertilizers are suitable, shoots extend one after another, form flower buds on the tips thereof, and thus bloom throughout the year. In contrast, climbing roses, even if they have extended over 30 or 40 leaves, continue to extend without forming flower buds at the shoot apex, and thus become one-time flowering.

[0035]

Based on the finding on the wild type (indefinite inflorescence) and a mutant (definite inflorescence) of Arabidopsis and the fact that mutation to one-time flowering is often seen in perpetual blooming roses of today, the present inventors have set up the following hypothesis and proved it.

[0036]

(Hypothesis 1)

Since the orthologous gene of the TFL1 gene is active in a one-time flowering climbing rose, flower bud formation at the shoot apex is regulated, whereas since the activity of said gene is decreased due to mutation in a perpetual blooming rose, flower bud formation at the shoot apex is not regulated.

[0037]

(Hypothesis 2)

A perpetual blooming rose is created by mutation of transposon insertion into of the orthologous gene of the TFL1 gene, whereas a one-time flowering climbing rose is created by reverse mutation of transposon deletion.

[0038]

(1) Sequencing of the para-orthologous gene of the TFL1 gene and the presence or absence of a transposon in said gene

The nucleotide sequence of the para-ortholog of the TFL1 gene was clarified in the following manner, and it was demonstrated that in the one-time flowering type there is at least one gene in which a transposon has not been inserted.

[0039]

The TFL1 gene belongs to a gene family called the phosphatidylethanolamine-binding protein (PEBP). In Arabidopsis, 6 types of PEBP genes are known. Based on the nucleotide sequences of known PEBP genes of Arabidopsis, rice, Antirrhinum, petunia, tomato etc., highly conserved regions were selected to design the following primers:

Forward primer (Utf1F1): GATAGACCCAGATGTTCCAGGTCC (SEQ ID NO: 4);

Reverse primer (Utf1R1): CCTGGAATATCTGTCACCATCCT (SEQ ID NO: 5).

[0040]

Using these primers, PCR was performed with the DNA of Rosa chinensis spontanea (Rcs) as the template to obtain a plurality of amplification products. The nucleotide sequences of these amplification products were determined, and four genes estimated to be PEBP family genes were selected from the nucleotide sequences and designated as RPEBP1, RPEBP2, RPEBP3 and KSN. Among these amplification products, the nucleotide sequence of a KSN product of more than 400 bp is as shown in Fig. 1. The nucleotide sequence had a high homology with the nucleotide sequence of a region from exon 2 to 3 of the Arabidopsis TFL1 gene.

[0041]

Based on the partial nucleotide sequence of KSN determined as described above, the nucleotide sequence of the entire KSN gene was determined in the following manner. Thus, it was sequenced by the Inverse PCR method with Rcs DNA as the template.

[0042]

First, a few μ g of Rcs DNA was digested with NEB's

restriction enzyme *Cac 8I* (DNA 17 μ l, enzyme 1 μ l and 10 $\times$ buffer 2 μ l were reacted at 37°C for 4 hours), heated to inactivate the enzyme (at 65°C for 20 minutes), and then circularized using NEB's T4 DNA ligase (enzyme-digested DNA 20 μ l, ligase 1 μ l, 10 $\times$ buffer 12.5 μ l and purified water 91.5 μ l were reacted overnight at 8 °C). With the circular DNA as the template, PCR was performed using the following two primers:

KSN-1R1: CTGTAAGTTATACCAAGTGCAGGTGC (SEQ ID NO: 6).

KSN-1F1: GGAAGTACATATTATGGCATAATC (SEQ ID NO: 7).

The nucleotide sequence of the amplification product obtained is shown in Fig. 2. As can be seen from Fig. 2, the nucleotide sequence of 416 bp upstream to *Utf1-F1* was determined.

[0043]

Furthermore, a few μ g of Rcs DNA was digested with NBE's restriction enzyme *Sau96I* (DNA 17 μ l, enzyme 1 μ l and 10 $\times$ buffer 2 μ l were reacted at 37°C for 4 hours), heated to inactivate the enzyme (at 80°C for 20 minutes), and then circularized using NEB's T4 DNA ligase (enzyme-digested DNA 20 μ l, ligase 1 μ l, 10 $\times$ buffer 12.5 μ l and purified water 91.5 μ l were reacted overnight at 8°C). With the circular DNA as the template, PCR was performed using the above primer KSN-1R1 and the following primer KSN-1F1:

KSN-1F1: AGGAGCTAACATTTTTGCCTTG (SEQ ID NO: 8).

The nucleotide sequence of the amplification product obtained is shown in Fig. 3. As can be seen from Fig. 3, the nucleotide sequence of 401 bp downstream to *Utf1R1* was determined.

[0044]

The nucleotide sequence of KSN that assembled the sequences thus determined is shown in Fig. 4. In Fig. 4, the region estimated to be an exon as compared to the nucleotide sequence of *Arabidopsis TFL1* gene was boxed. Furthermore, the nucleotide sequence of the KSN coding region is shown in SEQ ID NO: 1, and an amino acid sequence encoded thereby is shown in SEQ ID NO: 2.

[0045]

In a similar manner, the nucleotide sequences of RPEBP1, RPEBP2 and RPEBP3 were determined (the sequences are not shown).

[0046]

Based on the nucleotide sequences of RPEBP1, RPEBP2, RPEBP3 and KSN thus as described above, primers for specifically amplifying the almost entire coding regions of these genes were designed. For example, primers for KSN amplification are as follows:

KSNF1: ATTAGGAGTACAATCCTTCCTTCC (SEQ ID NO: 9);

KSNR2: ACCTGCCTCCTGCTAGCTGC (SEQ ID NO: 10).

[0047]

With the DNAs of perpetual blooming Old Blush (OBB) and its mutant, one-season blooming Old BLush Climbing (OBC1), as the template, PCR was performed using the above primers for amplifying the coding region under the condition for obtaining about 1 Kbp amplification products (Promega's PCR Master Mix was used according to the attached protocol). As a result, for the amplification products of RPEBP1, RPEBP2 and RPEBP3, no difference was noted between OBB and OBC1. Since

no amplification products of a predicted size were noted for KSN, PCR was performed again by changing the PCR condition to one for obtaining about 10 Kbp amplification products (TaKaRa's One Shot LAPCR Mix was used according to the attached protocol). As a result, an about 10 Kbp amplification product was obtained for OBB, and an about 2 kbp amplification product was obtained for OBC1.

[0048]

For the amplification products obtained with DNAs of OBB and OBC1 as the template, it was estimated that some DNA has been inserted into KSN. Thus, the nucleotide sequence of the insert was demonstrated in the following manner.

[0049]

First, by designing several primers before and after the estimated insertion point, PCR was performed to obtain amplification products. As a result, the following primers were shown to be located most closely to the inserted DNA:

KSNIF3: CATATTATGGCATAGGGTGTGGC (SEQ ID NO: 11);

KSNR5: CAAATGTAACATCTGTGGTGCCTG (SEQ ID NO: 12).

[0050]

For the short insert (Si) estimated to be about 900 bp, the above two primers were used to demonstrate the nucleotide sequence.

[0051]

Furthermore, for the long insert (Li) estimated to be about 9 Kbp, the nucleotide sequence was demonstrated in the following manner. First, according to the attached protocol, PCR amplification products were ligated to pT7BlueT-Vector (manufactured by Novagen) using the DNA Ligation Kit Ver. 2 (manufactured by TaKaRa) and circularized. Using this plasmid, the competent cell JM109 strain (manufactured by TaKaRa) was transformed according to the attached protocol. The transformed *Escherichia coli* was plated on a L-agar medium (containing 50 µg/ml of ampicillin) which had previously plated with 50 µl of 2% 5-bromo-4-chloro-3-indolyl-β-D-galactoside (Xgal) and 10 µl of 0.1 M isopropyl-β-D-thiogalacto-pyranoside (IPTG). From the resulting colonies, white colonies were grown.

[0052]

From the resulting colonies, clone 6 was estimated to contain a PCR amplification product, and thus the nucleotide sequence was clarified in the following manner. First, it was cleaved with restriction enzymes HindIII or EcoRI that has a breaking point in the cloning site of this vector, and ligated again using the DNA Ligation Kit Ver. 2 (manufactured by TaKaRa), and circularized. Using this plasmid, the competent cell JM109 strain (manufactured by TaKaRa) was transformed according to the attached protocol, and the colonies were obtained (clone 6-12 and clone 6-16, respectively). Clone 6-16 having a large DNA fragment obtained by using EcoRI was further cleaved with SpeI, NdeI, XbaI and PstI at corresponding sites, respectively, and clones were obtained similarly. Among the clones obtained, five clones of clone 6-16-27, clone 6-16-32, clone 6-16-34, clone 6-16-40 and clone 6-16-43 were estimated to have DNA fragments different from one another.

[0053]

The nucleotide sequence of DNAs inserted into the plasmids of the above eight clones were sequenced from the

front and behind using the following primers and then ligated:

M13R18: CAGGAAACAGCTATGACC (SEQ ID NO: 13);

MR13F18: TGTAACGACGGCCAGT (SEQ ID NO: 14).

[0054]

As a result, this DNA fragment (Li) was found to be about 9 Kbp long and, as a result of blast homology search, had a high homology with a copia-type retrotransposon. This transposon (SEQ ID NO: 3) had been inserted into intron 2 of the KSN gene of OBB. The about 900 bp insert of OBC1 was found to be an about 900 bp LTR (long terminal repeat) that had been left behind when this retrotransposon disappeared.

[0055]

As described above, as the KSN of Rcs, OBB and OBC1, three types were noted: the wild type (w type, about 1 Kbp) having no insertions; a type (Li type, about 10 Kbp) having a retrotransposon; a type (Si type) in which a retrotransposon has exited leaving LTR behind.

[0056]

The above result suggests that in the Li type mRNA transcription may be greatly inhibited by the insertion of an about 9000 bp DNA leading to the substantial inhibition of KSN expression, and in the Si type the regulation of KSN expression is slight due to the insertion of a short 900 bp DNA. This coincides with the fact that OBB becomes perpetual blooming while OBC1 blooms sporadically from summer to autumn though it extends well like climbing roses.

[0057]

Subsequently, the structure of the KSN gene was examined for some garden varieties. When the primers designed earlier for the front (KSNIF3) and behind (KSNR5) of the transposon insertion site, the w type and Si type provided amplification products of different sizes of 250 bp and 1150 bp, respectively. Also, a primer KSNInsR3 that, when used with KSNIF3, amplifies part of the retrotransposon was designed further inside of LTR (KSNInsR3: TGTAATCTGTAGGAGATCCCATGC, SEQ ID NO: 15). Using KSNIF3, KSNR5 and KSNInsR3, KSNs of six garden varieties ("Flaming Peace", "Peace", "Wendy Cussons", "Gold Bunny", "Ice Berg", and "Pink Chiffon") for which climbing mutant garden varieties have been created were examined. For all of these varieties, the primer pair of KSNIF3 and KSNR5 gave no amplification products whereas the primer pair of KSNIF3 and KSNInsR3 gave amplification products in perpetual blooming varieties. This indicates that the retrotransposon had been inserted into all KSNs for perpetual blooming varieties. On the other hand, for climbing mutant varieties, a 1150 bp amplification product of the Si type was obtained by the primer pair of KSNIF3 and KSNR5 in addition to a amplification product by the primer pair of KSNIF3 and KSNInsR3, indicating the presence of KSN having a residual LTR in addition to the retrotransposon-containing KSN. Thus, it was demonstrated that in these six garden varieties too, transposon has been inserted in perpetual blooming varieties, and that at least one KSN is present in which the transposon transferred leaving LTR behind in climbing (one-season blooming) varieties.

[0058]

(2) Comparison of the expression level of mRNA of the rose TFL1 gene ortholog between perpetual blooming roses and one-season blooming roses

That the expression level of KSN mRNA of perpetual blooming roses is markedly lower than that of one-season blooming roses due to the insertion of a transposon shown in the above (1) was demonstrated in the following manner.

[0059]

First, from the axillary bud of Rcs having the wild type KSN, RNA was extracted. Three types of Rcs axillary buds were used: 15 cm long, 10 cm long and 5 cm long. Each sample was collected from the position shown in Fig. 5. Similarly, RNA was extracted from the shoot apex of the axillary buds of OBB and the axillary buds containing the buds of OBC1. For RNA extraction, the Rneasy Plant Mini Kit (manufactured by Qiagen) was used.

Subsequently, the amount of KSN mRNA was compared using the Quanti Tect SYBR Green RT-PCR Kit (manufactured by Qiagen) and the Light Cycler System (manufactured by Roche Molecular Biochemicals). In this experiment, mRNA of rose actin was used as the control. Since the amount of KSN mRNA was too small to be determined by the the QuantiTect SYBR Green RT-PCR Kit, it was further determined using the limiting dilution method. The limiting dilution method is a method in which a 3-fold dilution series of a RNA extract is prepared, and the dilution factor at which KSN mRNA can no longer detected is determined by the OneStep RT-PCR Kit (manufactured by Qiagen) to estimate the amount of mRNA. The result for Rcs is shown in the following Table 1. In Table 1, each numerical value represents a relative amount when the measured value of Rcs3p was set as 1.

[0060]

Table 1: The amount of mRNA expressed for each Rcs-derived sample

Measured site	Actin mRNA	KSN mRNA	The amount of KSN mRNA normalized to the amount of actin mRNA
Rcs1p	2.745	0.4720	0.172
Rsc2p	0.998	0.735	0.736
Rsc3p	1.000	1.000	1.000
Rsc1l	0.662	0.082	0.124
Rsc2m	0.886	0.0204	0.023
Rsc3l	0.377	0.0023	0.006

[0061]

Table 1 revealed that the amount expressed of KSN mRNA at the shoot apex increases as the axillary buds grow, and decreases at positions as they are further away from the shoot apex. Furthermore, the amount expressed of KSN mRNA at the OBC1 shoot apex was about 1/1000 that of Rcs3p, and the expression was not detected at the OBB shoot apex.

[0062]

(3) Discussion

The above results demonstrated that a transposon has been inserted into the KSN gene of perpetual blooming roses, which inhibits the expression of said gene thereby promoting flower bud formation at the shoot apex. Therefore, by inhibiting the KSN gene, a perpetual blooming property can be imparted to roses.

[0063]

Since no difference is noted in the phenotypes of TFL1 and ATC of Arabidopsis, CEN of Antirrhinums and LpTFL1 of ryegrasses (grasses of monocotyledons) when they were

expressed in Arabidopsis, it is reported, the difference in their functions is not due to the structural difference of the proteins but to the difference in gene expression (Shepard, K.A. and Purugganon M.D., The genetics of plant morphological evolution., Current Opinion in Plant Biology 5:49-55, 2002). Furthermore, considering the fact that the structure of the KSN gene has been well conserved among the eukaryotic organisms as a whole and that the genes of monocotyledonous plants exhibit its original function in dicotyledonous plants as well, it is believed that the mechanism of perpetual blooming discovered in the genus Rosa also function in other angiosperms.

[0064]

[Sequence Listing]

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[Brief Explanation of the Drawings]

[Fig.1] Fig. 1 is a drawing showing the nucleotide sequence (SEQ ID NO: 16) of a partially sequenced KSN gene.

[Fig.2] Fig. 2 is a drawing showing the nucleotide sequence (SEQ ID NO: 17) of a partially sequenced KSN gene.

[Fig.3] Fig. 3 is a drawing showing the nucleotide sequence (SEQ ID NO: 18) of a partially sequenced KSN gene.

[Fig.4] Fig. 4 is a drawing showing the nucleotide sequence (SEQ ID NO: 19) of the coding region of the KSN gene.

[Fig.5] Fig. 5 is a drawing showing the amount expressed of KSN mRNA at various sites of rose axillary buds.