

Señor Director

DIRECCION DE NUEVAS CREACIONES

Superintendencia de Industria y Comercio

E. S. D.

SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO



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Referencia : Expediente **No. 09.106.063**

Asunto : Solicitud de patente para: **MÉTODO PARA PRODUCIR UNA PLANTA QUIMÉRICA CON UNA CAPA SUPERFICIAL TRANSGÉNICA**

Solicitante : **SUNTORY HOLDINGS LIMITED**

Fecha de Solicitud : 28 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. **17287** notificado por fijación en lista del 02 de octubre de 2012.

2. Mediante oficio No. **17287** 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:

"EXCELENCIA LEGAL EN UN MUNDO SIN FRONTERAS"

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2.1 Excepción a la patentabilidad

El objeto de las reivindicaciones 1 a 28 es una planta floricultural que no se considera patentable de acuerdo con el artículo 20 literal c).

2.2 De la solicitud de patente

2.2.1 Descripción

La descripción no presenta información suficiente para reproducir la invención porque:

- No revela elementos promotores para obtener la expresión en la capa L1.
- No es claro como se obtiene la transformación específica en los tejidos de la capa L1 y que no se expresan en la capa L2 (células germinales).

2.2.2 Reivindicaciones

De manera resumida el Examinador ha expuesto lo siguiente:

- El término planta floricultural no es claro.
- Los métodos reclamados en las reivindicaciones 29 y 30 son caracterizados a partir de los resultados a alcanzar.

2.3 Análisis de patentabilidad

2.3.1 Novedad

Para la evaluación de este requerimiento se emplean los documentos D1: WO2004020637, y D2: WO2005017147. El Examinador afirma que la reivindicación 29 carece de novedad a la luz de las enseñanzas de los mencionados documentos.

2.3.2 Nivel Inventivo

Para la evaluación de este requerimiento se emplean los documentos D1: WO2004020637 y D3: T. Schmülling, J. Schell. Plant Molecular Biology 21: 705-708. El Examinador afirma que la reivindicación 30 carece de nivel inventivo teniendo en cuenta la combinación de enseñanzas de D1 y D3 o de los documentos D1 y D2.

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 un nuevo pliego reivindicatorio conformado por 6 reivindicaciones.

Específicamente, las modificaciones realizadas al capítulo reivindicatorio son:

- Se han eliminado las antiguas reivindicaciones 1 a 28 y 30.
- Se ha reemplazado el término “planta floricultural” por el término “rosal” en la nueva reivindicación 1.
- La nueva reivindicación 1 (Antigua reivindicación 29) se ha modificado de acuerdo a las observaciones hechas por el Examinador.

Respecto a la excepción de patentabilidad del capítulo reivindicatorio, se supera la objeción con la eliminación de las reivindicaciones 1 a 28.

Respecto a la objeción hecha a la descripción, el solicitante considera que la figura 3 muestra la estructura del vector binario pSPB130 el cual contiene una variante del promotor 35S del virus del mosaico de la coliflor, es decir, E2-358 en el cual dos regiones potenciadoras se ligan. Adicionalmente, una persona normalmente versada en la materia logra entender qué promotor puede ser usado refiriéndose al ejemplo 2 de la descripción de la presente solicitud. Por otro lado, tal y como se establece en la argumentación de novedad, el método instantáneo para producir una planta de rosa que contiene un gen extraño en las células en la capa L1, pero no en las células de polen ni en las células ovulares, está caracterizado porque comprende la etapa de selección de una planta de rosa que contiene el gen extraño en las células de la capa L1, pero no en las células de polen ni en las células ovulares.

Así entonces, consideramos que las modificaciones efectuadas son suficientes para aclarar la materia reivindicada de manera fácilmente comprensible y las mismas deben tenerse por sustentadas en la descripción, de acuerdo con el conocimiento de una persona medianamente versada en la materia.

Por lo anterior, es posible establecer que el pliego reivindicatorio define claramente el método para la producción de una planta de rosa modificada.

En consecuencia, se han atendido las recomendaciones del Examinador con respecto a las objeciones de claridad, excepción y descripción de la solicitud. Por lo tanto, se solicita atentamente tener en cuenta este nuevo pliego reivindicatorio para el análisis de patentabilidad de la solicitud.

3.2 Argumentos a favor de la patentabilidad de la presente solicitud

3.2.1 Novedad

Siguiendo los lineamientos establecidos para el análisis de novedad, es posible establecer que una anterioridad afecta la novedad de una solicitud cuando revela todas y cada una de las características técnicas del producto.

El Examinador considera que el documento citado D1, es una anterioridad que afecta la novedad de la presente invención. Respetuosamente manifestamos nuestro desacuerdo por las razones mencionadas a continuación:

La nueva reivindicación 1 ahora incluye la característica técnica esencial *“la etapa de selección de una planta de rosa que contiene el gen extraño en las células que comprenden la capa L1, pero no en las células de polen ni en las células ovulares”*. A pesar de que D1 y D2 describen que el gen puede ser exclusivamente expresado en partes de la planta tales como las flores, tallos, hojas, ninguno de los documentos divulga, enseña o sugiere que es necesario un paso de selección. De esta manera, el solicitante presenta que la novedad de la invención reclamada en la nueva reivindicación 1 no debe ser negada con base en los documentos D1 o D2.

De acuerdo con los argumentos anteriormente explicados, la nueva reivindicación independiente 1 es novedosa sobre el estado de la técnica. Como consecuencia de lo anterior, las reivindicaciones dependientes 2-6 son también novedosas en virtud de su dependencia.

3.2.2 Nivel inventivo

Con el fin de demostrar que la presente solicitud cumple con el requerimiento de nivel inventivo, se pone de presente lo siguiente:

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 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.

Respecto a la objeción del nivel inventivo de la reivindicación 30, ésta es superada con la eliminación de dicha reivindicación. Por lo tanto, los argumentos presentados a continuación se refieren al nivel inventivo de la nueva reivindicación 1.

En primer lugar, Schmuelling (D3) divulga la transformación quimérica mediada por *Agrobacterium* en una planta transgénica periclinal de tabaco.

Es conocido que la probabilidad de generar quimeras es generalmente baja y es esperado que el efecto de introducir un gen en una cierta planta, en las características que ésta expresa, puede variar dependiendo de los tipos de genes y plantas que se utilicen. Por estas razones, se requiere que el método reclamado en la nueva reivindicación 1 de la presente solicitud incluya, como característica esencial de la invención, *"la etapa de selección de una planta de rosa que contiene el gen extraño en las células que comprenden la capa L1, pero no en las células de polen ni en las células ovulares"*.

Para demostrar lo anterior se anexa el documento Oono et al., *Plant Cell Physiol*, 34 (5) : 745-752 (1993), el cual fue citado como el documento 8 en el reporte de búsqueda internacional, que describe:

“Aún no hemos establecido condiciones estándar para la producción de quimeras transgénicas periclinales. Las condiciones, tales como la concentración y la duración de la aplicación de antibióticos, para la selección de plantas transgénicas, deben ser estandarizadas. La producción de quimeras transgénicas abrirá un nuevo camino para el progreso de la biología desarrolladora de plantas” (Página 751, columna derecha, líneas 28-34).

A partir de esta descripción, es claro que considerando cada una de las especies de plantas usadas y cada una de las variedades de especies, el estudio detallado y el ensayo y error requeriría no sólo un método específico para la transformación sino también un método para la producción de quimeras. Dado que una rosa difiere de las otras especies, y la ocurrencia de una quimera periclinal en una rosa requeriría un delicado proceso biológico, una persona medianamente versada en la materia no tendría una expectativa razonable de éxito al producir una rosa que contiene un gen extraño específico en las células de la capa L1, pero no en las células de polen ni en las células ovulares.

Tal y como se indicó anteriormente, Schmülling (D3) revela la producción de una quimera periclinal de planta de tabaco usando *Agrobacterium*, y los métodos para investigar quimeras periclinales para capas transformadas y no transformadas. D3 también describe que la capa L2 dan lugar a gametos, que únicamente la capa L3 fue transformada y por lo tanto, un gen extraño está presente en la capa L3 pero no en la capa L2, y que la invasión celular de células transgénicas L3 a la capa no transformada L2 puede haber ocurrido (Página 707, párrafo que culmina la columna izquierda y columna derecha). Así, la persona versada en la materia, buscando una planta donde únicamente una parte de las células, y otra parte no (especialmente las células de la capa L2), contenga un gen extraño con el fin de evitar la dispersión del gen extraño al medio ambiente, al contrario, se habría abstenido de aplicar el método divulgado en D3, ya que

puede ocurrir una invasión no deseada de células transformadas a la capa L2 con este método.

De igual forma, tal y como se mencionó anteriormente, la probabilidad de generar quimeras es generalmente baja, y es esperado que el efecto de introducir un gen en una cierta planta en las características que ésta expresa puede variar dependiendo de los tipos de genes y plantas que se utilicen. En consecuencia, incluso si D1 y D2 describen que el gen puede ser expresado exclusivamente en partes de la planta tales como flores, tallos, hojas, una persona versada en el arte no podría fácilmente haber concebido la idea de aplicar las enseñanzas de D1 y D2 a las enseñanzas de D3 para llegar a la invención reivindicada en la presente solicitud, la cual comprende , **la etapa de selección de una planta de rosa que contiene el gen extraño en las células que comprenden la capa L1, pero no en las células de polen ni en las células ovulares**, y donde el gen extraño es seleccionado del grupo formado por el gen flavonoide 3' ,5' -hidroxilasa y el gen transferasa del grupo acilo aromático. Cabe resaltar, que debido a la presencia de esta etapa, el método reclamado en la presente solicitud **inevitablemente** lleva al éxito, es decir, que éste contiene todos los pasos relevantes necesarios para llegar directamente a la invención reivindicada (sin producir plantas que lleven el transgen en su línea germinal).

4. Conclusión

Con las modificaciones introducidas al pliego reivindicatorio y los argumentos de novedad y nivel inventivo acá presentados, se superan las objeciones formuladas por el Examinador en el oficio No. **17287**.

5. 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. **17287**, toda vez que al no haber sido trasladadas a mí representada, dicha decisión violaría tanto el tenor literal del artículo 45 de la Decisión 486 como el derecho constitucional al debido proceso. De la misma manera, en el evento que como consecuencia del nuevo análisis de

patentabilidad sobre el pliego reivindicatorio modificado, el Examinador llegue a la conclusión que solamente algunas de las reivindicaciones son patentables, respetuosamente solicitamos que se proceda al otorgamiento parcial del privilegio.

6. Anexos

- Nuevo pliego reivindicatorio conformado por 6 reivindicaciones.
- Formulario de modificaciones en solicitud pendiente.
- Documento: Oono et al., Plant Cell Physiol, 34 (5) : 745-752 (1993).

Señor Director,


LUZ CLEMENCIA DE PÁEZ

C.C. 35.456.344 de Usaquén

T.P.A. No. 23.555

COLO-17511-0843-003-2
COLO-15218-0841-004-6
DGA\DGA\ID-233354\WPC17511
No. M-2012-233354\DGA

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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
Japón	1-40 Dojimahama 2-chome, Kita-Ku, Osaka 530-8203,	Osaka-shi
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
---	--	--------------------------------------

Dirección y domicilio	Ciudad	
Carrera 4ª No. 72 –35	Bogotá	
Dirección electrónica	No. Fax	Número telefónico
cavelier@cavelier.com	2 11 86 50	3 47 36 11

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

4. Datos del trámite**Corrección:****Expediente No 09.106.063****Aparece****Debe ser****Modificación:****Modificación al capítulo reivindicatorio.**

Presento nuevo capítulo reivindicatorio compuesto por 6 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 LUZ CLEMENCIA DE PAEZ	Firma 
C.C. 35.456.344 DE Usaquén	Tarjeta Profesional 23.555

an DGAINCA\WPC17511\ID-235370

REIVINDICACIONES

1. Un método para producir una planta de rosa que contiene un gen extraño en las células que comprenden la capa L1, pero no en las células de polen o células ovulares, que comprende las etapas de:

Introducción de un gen extraño dentro de una planta de rosa mediado por *Agrobacterium tumefaciens*; y

Selección de una planta de rosa que contenga el gen extraño en las células que comprenden la capa L1, pero no en las células de polen o células ovulares,

donde el gen extraño es seleccionado del grupo formado por el gen flavonoide 3',5' –hidroxilasa y el gen transferasa del grupo acilo aromático.

2. El método de acuerdo con la reivindicación 1, donde el gen extraño es seleccionado del grupo formado por el gen flavonoide 3',5' –hidroxilasa, derivado de una planta que pertenece a la familia de las violetas, y el gen transferasa del grupo acilo aromático, derivado de una planta que pertenece a la familia de la boca de dragón.

3. El método de acuerdo con la reivindicación 2, donde la planta que pertenece a la familia de las violetas es un pensamiento.

4. El método de acuerdo con la reivindicación 2, donde la planta que pertenece a la familia de la boca de dragón es una torenia.

5. El método de acuerdo con cualquiera de las reivindicaciones 1 a 4, donde la rosa es Rosa híbrida.

6. El método de acuerdo con la reivindicación 5, donde la rosa es rosa híbrida de té, rosa floribunda o miniatura.

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Plant Cell Physiol. 34(5): 745-752 (1993)
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論文
番号 0012

Effects of the Over-Expression of the *rolC* Gene on Leaf Development in Transgenic Periclinal Chimeric Plants

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and Hirofumi Uchimiya³

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Using *Agrobacterium tumefaciens* harboring a vector that carried the *rolC* gene under the control of the 35S RNA promoter of cauliflower mosaic virus, we produced several transgenic plants. Two of them were periclinal chimeras with altered leaves that had wrinkled dark green margins and inner pale green regions. One chimeric plant had shortened internodes, reduced apical dominance, small flowers and exhibited male sterility, but the other had a normal phenotype. Analysis of proteins, RNA and DNA indicated that the inner pale green tissues consisted of transformed cells while the outer dark green tissues were composed of non-transformed cells. Histological analysis indicated that mesophyll cells were distorted and larger intercellular spaces were present in the transformed pale green regions. Furthermore, in young leaves, transformed mesophyll cells were larger than non-transformed cells. However, the normal parts had larger numbers of cells per unit area than the transformed parts. These observations suggest that the expression of 35S-*rolC* in leaves caused inhibition of cell division in developing leaves and that the undulating margins, composed of non-transformed cells, were a consequence of the requirement for accommodating more cells in less space within the region of *rolC*-transformed cells.

Key words: *Agrobacterium rhizogenes* — Cell lineage — Periclinal chimeras — *rolC* — Tobacco — Transgenic plants.

Hairy root disease is a pathogenic condition caused by *Agrobacterium rhizogenes*, and it is the result of the expression of T-DNA genes of a Ri plasmid that have become stably integrated into the host plant genome (Chilton et al. 1982). The adventitious roots induced by *A. rhizogenes* can proliferate in hormone-free medium, and that regenerate into morphologically and physiologically altered plants (David et al. 1984, Tepfer 1984). These regenerated plants have unusual phenotypes, exhibiting dwarfism, reduced apical dominance in both roots and stems, reduced fertility, wrinkled leaves, altered flower morphology, plagiotropic

root growth (altered geotropism), and reduced production of pollen and seeds. The combination of these phenotypes, designated hairy root syndrome, is stably inherited to the progeny (Tepfer 1984). The best-defined Ri plasmid, the agropine-type plasmid, has two T-DNAs (TL- and TR-DNA), while the cucumopine and mannopine type each contains a single T-DNA that is homologous to TL-DNA. These observations, together with the experimental results obtained from infection experiments, and the analysis of DNA from plants regenerated from hairy roots, demonstrate that the TL-DNA is sufficient for induction of hairy roots and the hairy root syndrome (Vilaine et al. 1987, Jouanin et al. 1987). Four loci, designated as *rolA*, *rolB*, *rolC*, and *rolD*, are involved in hairy root formation and have been identified in the TL-DNA of the agropine-type Ri plasmid pRiA4 by transposon mutagenesis (White et al. 1985). DNA sequence analysis of TL-DNA has revealed the presence of 18 open reading frames (ORFs) and the *rolA*, *rolB*, *rolC*, and *rolD* loci correspond to ORFs 10, 11, 12, and 15, respectively (Slightom et al. 1986).

Abbreviations: CaMV 35S promoter, promoter that directs the production of the 35S RNA transcript of cauliflower mosaic virus; DAPI, 4',6-diamidino-2-phenylindole; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; 2-ME, 2-mercaptoethanol; NPT II, neomycin phosphotransferase II; PCR, polymerase chain reaction; SDS-PAGE, SDS-polyacrylamide gel electrophoresis; T-DNA, DNA transferred from a Ti or Ri plasmid to a plant cell.

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Although there are many reports about *rol* genes, the relationship between the functions of the products of *rol* genes and the physiological and morphological changes associated with hairy root syndrome is still unclear (for review, see Oono et al. 1993). Transgenic tobacco plants containing only the *rolC* gene have altered phenotypes, such as dwarfism, reduced apical dominance, altered leaf and flower morphology, partial male sterility, early flowering and autonomous proliferation of roots in vitro (Oono et al. 1987, 1990, Schmülling et al. 1988). The expression of the *rolC* gene under the regulation of its own promoter is restricted mainly to phloem tissues of dicot and monocot plants (Sugaya et al. 1989, Sugaya and Uchimiya 1992, Schmülling et al. 1989, Matsuki et al. 1989). When the promoter of the *rolC* gene is replaced by the CaMV 35S promoter, new morphological characteristics, such as pale green and lanceolate leaves, are observed (Schmülling et al. 1988).

In this communication, we report the production of periclinal chimeric tobacco plants that consist of non-transformed and transformed cells possessing the *rolC* gene under the control of the CaMV 35S promoter.

Materials and Methods

Construction of plasmids.—The DNA fragment containing the coding sequence of *rolC* and the 3'-flanking sequence of pR1A4 (Slightom et al. 1986) was digested with *Hpa*I and *Eco*R I and subcloned into the *Sma*I/*Eco*R I site of pUC12. Then the *Hind*III/*Bam*H I fragment of the CaMV 35S promoter from pBI121 (Jefferson et al. 1987) was inserted at the *Hind*III/*Bam*H I site of this construct. The resulting plasmid pUYO9 was digested with *Hind*III/*Eco*R I for isolation of the CaMV 35S-*rolC* fragment, which was introduced into the *Hind*III and *Eco*R I sites of the binary vector Bin19 (Bevan 1984). The resulting plasmid pBYO9 was transferred from *Escherichia coli* to *A. tumefaciens* LBA4404 by triparental mating (Bevan 1984).

Establishment of transformants.—Leaves of tobacco (*Nicotiana tabacum* cv. Petite Havana SR1) were cut into square of about 1 cm² and infected with the *A. tumefaciens* LBA4404 that harbored the binary plasmid containing pBYO9. The infected leaf explants were incubated in Murashige and Skoog's (1962) medium supplemented with 1 mg liter⁻¹ benzyladenine and 0.1 mg liter⁻¹ NAA (MSNB medium). After two days, explants were transferred to MSNB medium that contained 300 mg liter⁻¹ Claforan (Hoechst, Germany). After one week, explants were transferred to the MSNB medium that contained 100 mg liter⁻¹ kanamycin sulfate (Meiji Seika, Japan). After several weeks, the green shoots regenerated from leaf explants were excised and placed onto MS medium without plant hormones. Plants with roots were then transferred to soil

and allowed to grow to maturity in a greenhouse.

Seeds were plated on 0.8% agar medium that contained MS salts and kanamycin sulfate (300 mg liter⁻¹) for selection of kanamycin-resistant seedlings.

Extraction of proteins and immunoblot analysis.—Leaves of the tobacco plants were homogenized in extraction buffer (50 mM Tris-HCl (pH 7.5), 0.4 M sucrose, and 0.7% 2-mercaptoethanol) and centrifuged at 3,000 × g for 15 min. The supernatants were recentrifuged at 200,000 × g for 30 min to obtain the soluble fraction. All extraction procedures were performed at 4°C. Amounts of protein were determined by the method of Bradford (1976). Proteins were denatured in electrophoresis sample buffer, separated by SDS-PAGE on a 15% polyacrylamide gel, and transferred to an Immobilon-P membrane (MILLIPORE, Japan). Immunodetection of RolC protein in plant extracts was performed as reported previously (Oono et al. 1991) with the exception of the addition of 0.1 mg ml⁻¹ protein extracted from a leaf of normal plants to the affinity-purified antiserum against RolC when membranes were treated with antiserum.

Dot-blot hybridization.—Total RNA was extracted from leaf tissues by the method of Logemann et al. (1987). RNA (1.7 µg) was blotted onto a HybondTM-N+ membrane (Amersham, Japan) with 0.05 M NaOH and allowed to hybridize with the radioactive probe. A purified DNA fragment corresponding to the *rolC*-coding region (Cla I-*Bgl*II fragment) was labeled with [α -³²P]dATP using the Klenow large fragment of DNA polymerase I and a random primer. Hybridization and washing were carried out according to the protocols provided by Amersham.

Detection of NPT II.—Leaf tissues were homogenized with extraction buffer [10% glycerol, 40 mM EDTA-Na₂, 150 mM NaCl, 100 mM NH₄Cl, 10 mM Tris-HCl (pH 7.5) and 3.0 mg ml⁻¹ DTT]. After centrifugation at 3,000 × g for 10 min, 10 µl of each supernatant fraction were analyzed by electrophoresis on a non-denaturing polyacrylamide gel for detection of NPT II as described by Scott et al. (1988).

PCR analysis.—Leaf extracts were prepared by the method of Edwards et al. (1991). The 2.5 µl aliquots of extracts ($A_{260}=1.0$) were added to 47.5 µl of the reaction mixture for PCR (400 µM each dNTP, 0.2 µM each primer, 2 mM MgCl₂, 10 mM Tris-HCl (pH 8.3), 50 mM KCl and 0.5 U µl⁻¹ of *AmpliTag* DNA polymerase (Perkin Elmer Cetus, U.S.A.); final concentrations). The synthesized primers corresponded to the 5' and 3' terminal of *rolC* (5'-ATCGCTGAAGACGACCTGTGT-3' and 5'-TTAGCCGATTGCAAACTTGAC-3'; Slightom et al. 1986) or *psaD*, which encodes PSI-D1 protein (5'-TCTAGAATTCTATGCAACAAAAATAGCTT-3' and 5'-TCTAGAATTCTGTTAAAGACAATTACA-3'; Yamamoto et al. in preparation). The temperature cycle for amplification of *rolC* was 94°C for 2 min, 60°C for 2 min, and 72°C for 3

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min, followed by 39 cycles of 94°C for 1 min, 60°C for 2 min, and 72°C for 3 min. The final extension was carried out at 72°C for 15 min. For *psaD*b, the aforementioned reactions at 60°C were conducted at 37°.

Histological observations.—Leaf pieces were fixed in FAA [a mixture of formalin, acetic acid, and 70% ethanol, 90 : 5 : 5 (v/v)]. After dehydration and clearing with an ethanol/n-butanol series, tissues were embedded in paraffin. Sections (10 µm) were stained with hematoxylin.

Observation of the density of distribution of leaf cells and preparation of protoplasts.—The lower epidermis was removed from pieces of leaves that included both pale green and dark green regions. Then these pieces were incubated in enzyme solution [1.4% Cellulase Onozuka R-10 (Yakult, Japan), 0.4% Macerozyme R-10 (Yakult) and 0.4 M sucrose] for 2 h (for observations of the density of distribution of leaf cells) or for 5 h (for preparation of protoplasts).

Staining with 4,6-diamidino-2-phenylindole (DAPI).—Pieces of leaves from which the lower epidermis had been peeled off were fixed in ethanol and acetic acid (3 : 1 (v/v)) for 2 h. After washing in distilled water, the leaf pieces were stained in NS buffer (Kuroiwa et al. 1981) that contained 0.5 µg ml⁻¹ DAPI for 30 min. The samples were mounted on glass slides and observed under a Nikon fluorescence microscope (EFD2; Nikon, Japan) in the ultra violet excitation mode (HBO 100W/2 mercury lamp from OSRAM (Germany) as the light source; 330–380 nm excitation filter (Nikon), DM400 dichroic mirror (Nikon) and BA420 barrier filter (Nikon)).

Results

Production of chimeric transformants.—We attempted to obtain transformed tobacco plants that expressed the *rolC* gene under the control of the CaMV 35S promoter. *Agrobacterium*-mediated transformation yielded 21 tobacco regenerants. In 13 regenerants, we noted specific morphological changes that are characteristic of CaMV 35S-*rolC* plants: pale green and lanceolate leaves; dramatically reduced apical dominance and internodal length; and very small and male-sterile flowers (Schmülling et al. 1988). Six regenerants had wild-type characteristics. The mature leaves of two remaining plants had unexpected morphology: the periphery of the leaves was severely wrinkled with and was darker than the central region (Fig. 1a). Irregularly delineated areas of the darker tissue invaded the central regions of leaves in many cases (Fig. 1a, indicated by asterisk). In younger leaves, this periclinal chimerism was not visible (Fig. 1a).

Differences between the two unusual regenerants were also apparent: one showed reduced apical dominance and internodal length, as well as very small and male-sterile flowers, characteristic of 35S-*rolC* transformants (designat-

ed chimera-2; Fig. 1c and 2), whereas the other transformant did not exhibit such morphological alterations in its stem and flowers (designated chimera-1; Fig. 1a, 1b, and 2).

Detection of the transgene.—From the morphological appearance of the transformants, we assumed that the chimeric plants consisted of tissues that expressed *rolC* as judged by the pale green and lanceolate leaves (Schmülling et al. 1989), and tissues that did not. To investigate the expression of the *rolC* in pale green and dark green regions independently, immunoblot analysis and RNA dot-blot hybridization analysis were performed. Figure 3a demonstrates the presence of RolC protein in the pale green region of chimera-2. Furthermore, *rolC* RNA was detected only in pale green regions but not in dark green regions (Fig. 3b). The level of expression of *rolC* in pale green regions of chimera-2 was higher than in chimera-1. To examine the expression of the *npt II* gene, which was co-transformed with the 35S-*rolC* gene, the activity of the NPT II enzyme was analyzed. Figure 3c shows the NPT II activity in extracts of pale green tissue and 35S-transformed leaves. The results of immunoblot analysis, dot-blot hybridization and assay of the NPT II activity suggested that both the 35S-*rolC* and *npt II* genes were inactivated in or absent from the dark green tissues. To confirm the presence or absence of *rolC* gene in these chimeric tissues, PCR analysis was performed using primers that corresponded to the 5' start region and the 3' stop region of the coding sequence of *rolC*. Agarose gel electrophoresis clearly revealed a band of approximately 540 bp that corresponded to a fragment of *rolC* in the reaction mixture prepared with DNA from pale green tissues or 35S-*rolC* transformed leaves as template (Fig. 4a). The absence of a signal was not due to specific inhibition of the PCR reaction by the extract of dark green regions, because products of equivalent reactions in each lane could be seen when primers specific for *psaD*b, a nucleus-encoded gene, were used as controls (Fig. 4b).

Histological analysis.—The hematoxylin-stained cross section of a mature leaf with both pale green and dark green sectors is shown in Figure 5a. The pale green region has a smaller number of mesophyll cells with palisade cells in disarray, as compared with the dark green region. Plastids in pale green regions were weakly stained and enlarged. In young leaves, the difference between normal cells at the leaf edge and the enlarged and disorganized mesophyll cells in the inner region were even clearer (Fig. 5b), although the chimeric phenotype was not apparent in the case of young leaves (Fig. 1a).

Comparison of diameters of mesophyll protoplasts obtained from dark green and pale green tissues.—The lower epidermis was peeled off from 3 pieces of chimera-1 leaves, and 6 pieces of chimera-2 leaves that contained dark and pale green regions. Then the pale green region and the dark

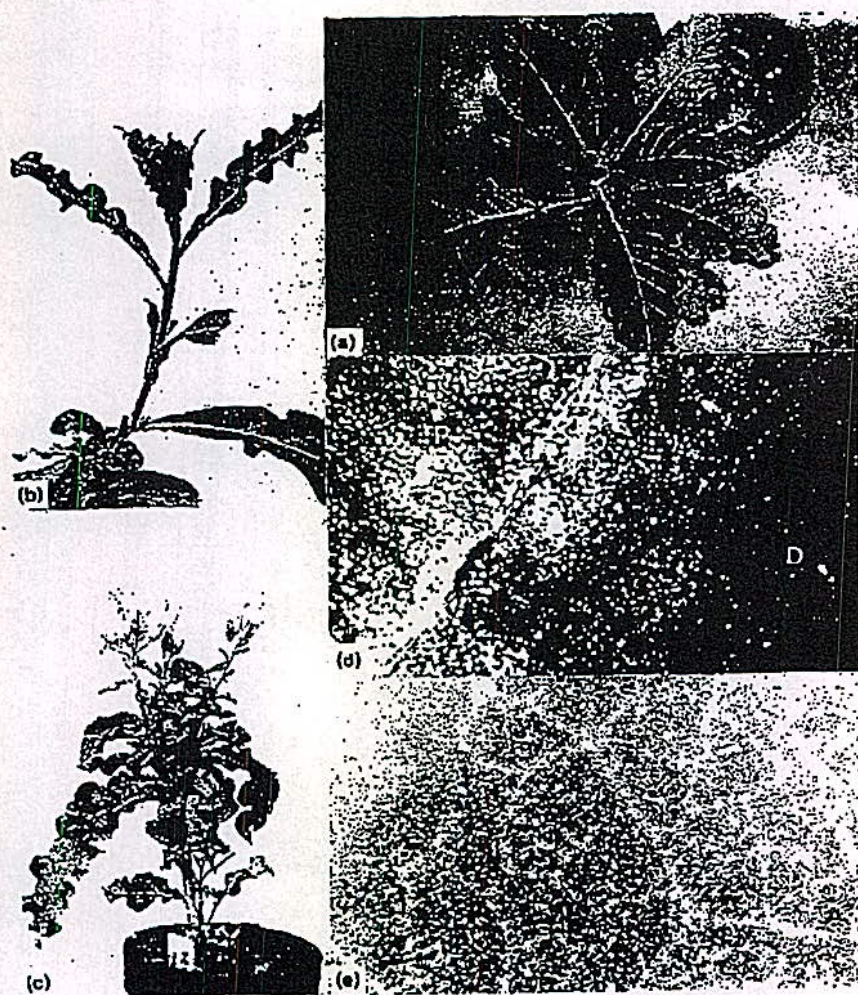


Fig. 1 Morphology of chimeric plants and photomicrographs of the boundary region between pale (P) and a dark green (D) region. (a): Chimera-1. The young leaves did not show chimerism (indicated by an arrow). The invasion by dark green tissues of central regions of leaves was often observed (indicated by an asterisk; the excised region of this leaf was used for PCR analysis). (b): Chimera-1 did not have shortened internodes and reduced apical dominance. (c): Chimera-2 had shortened internodes or reduced apical dominance. (d): Boundary region between a dark and a pale green region of a piece of a leaf from chimera-1 that had been treated with enzyme solution. (e): A similar boundary region after staining with DAPI.

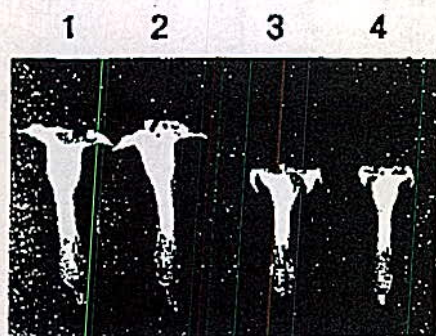


Fig. 2 Morphology of flowers. Flowers from a non-transformant (1), chimera-1 (2), chimera-2 (3), and a 35S-roIC transformant (4) are shown.

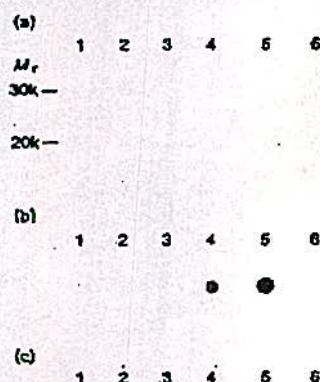


Fig. 3 Expression of the transgene in chimeric plants. (a): Immunoblot (the position of RoIC protein is indicated by an arrowhead), (b): Dot hybridization with a roIC probe, (c): Assay of NPT II activity. RNA or protein was extracted from dark green region of a leaf of chimera-1 (lane 1), a pale green region of chimera-1 (lane 2), a dark green region of chimera-2 (lane 3), a pale green region of chimera-2 (lane 4), a 35S-roIC transformed leaf (lane 5), and a non-transformed leaf (lane 6).

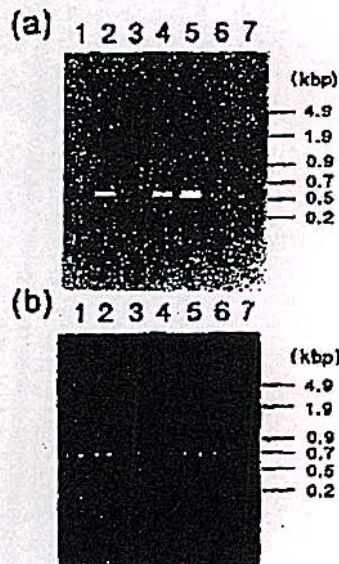


Fig. 4 PCR analysis of chimeric plants. PCR was performed with (a) roIC primers and (b) pzeD6 primers. DNA was extracted from the dark green regions of chimera-1 (lane 1), pale green regions of chimera-1 (lane 2), dark green regions of chimera-2 (lane 3), pale green regions of chimera-2 (lane 4), a 35S-roIC transformant (lane 5), and a non-transformed leaf (lane 6). There were no signals when PCR was performed without extracted DNA (lane 7).

green region were separated. After treatment with enzymes, the diameters of 20 protoplasts from each region were estimated. As shown in Table 1, diameters of protoplasts from the pale green region were significantly larger than those from the dark green region in 5 of 9 cases. The average diameter of protoplasts from 9 samples of pale green regions was $34.9 \pm 5.4 \mu\text{m}$ ($n=180$), compared with $30.2 \pm 5.8 \mu\text{m}$ ($n=180$) on average for 9 samples of dark green region.

Observation of a boundary region along the horizontal plane—Figures 1d and 1e show the boundary region between the two phenotypes along the horizontal plane of a leaf. The pale green region has apparently a smaller number of cells in a given area than the dark green region (Fig. 1d). This difference in the number of cells in a given area was confirmed by nuclei staining with DAPI (Fig. 1e).

Phenotype of progeny—The seeds of chimera-1

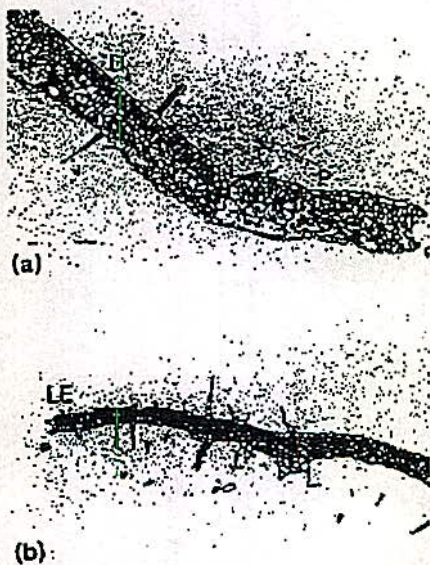


Fig. 5 Cross sections of leaves of chimera-1. (a) A mature leaf and (b) a young leaf were cut into 10- μ m sections. D, dark green region; P, pale green region; LE, edge of leaf. Bar in (a) = 100 μ m. The two micrographs are shown at the same magnification.

were obtained by self-fertilization, whereas the seeds of chimera-2 were obtained by cross-fertilization with wild-

Table 1 Comparison of the average diameter (μ m) of protoplasts obtained from dark and pale green tissues

	Block	Dark green	Pale green
Chimera-1	A	29.3 ^{abc}	34.6 ^{ac}
	B	31.6 ^{ab}	36.9 ^{ab}
	C	27.3 ^c	30.8 ^{cd}
Chimera-2	A	31.4 ^{abc}	38.6 ^a
	B	38.9 ^a	37.9 ^{ab}
	C	27.9 ^{cd}	37.1 ^{ab}
	D	28.5 ^{cd}	31.6 ^{cd}
	E	30.1 ^{cd}	30.8 ^{cd}
	F	26.9 ^e	35.9 ^{ab}

Values with the same superscripts are not significantly different at the 5% level.

type pollen because the flowers of chimera-2 were male-sterile like those of 35S-*rolC* transgenic plants. Among the progeny of both chimeric lines, no plants had the altered phenotypes characteristic of 35S-*rolC* transgenic or chimeric plants. Furthermore, no plants were resistant to kanamycin. In 35S-*rolC* transgenic plants, the observed altered phenotypes and resistance to kanamycin were transmitted to the progeny by cross-fertilization with wild-type pollen (data not shown).

Discussion

In this report, we have described that periclinal chimeras can be obtained by *Agrobacterium*-mediated transformation. The chimeric leaves are characterized by a wrinkled, dark green outer margin and a pale green inner region. Christou and McCabe (1992) analyzed chimeric transgenic soybean plants derived from experiments that involved acceleration of particles in an electric field. In our experiments, the dramatic change in the phenotype of leaves that contained 35S-*rolC* made it both possible and easy to detect chimeras. The dot-blot hybridization analysis of mRNA prepared from leaves indicated that *rolC* RNA was expressed only in the inner pale green regions. The expression of *rolC* under control of the CaMV 35S promoter has been shown to cause the production of small, lanceolate, pale green leaves in transgenic plants (Schmülling et al. 1988) and this effect is cell-autonomous (Spena et al. 1989). Thus, in the present study, chimerism was a result of chimeric expression of *rolC*. The assay of NPT II activity and the analysis of DNA after PCR revealed that the enzymatic activity of NPT II and the *rolC* gene were present only in the inner pale green regions, suggesting that only the inner region was transformed. It is, therefore, conceivable that these chimeras consisted of non-transformed tissues (outer regions) and transformed tissues (inner regions). The low level of expression of the *rolC* gene in the pale green regions of chimera-1 prevented detection of *RolC* protein by immunoblot analysis.

According to reviews by Tilney-Bassett (1986) and Pechig (1989), periclinal chimeras usually arise from a spontaneous or induced somatic mutation that becomes incorporated into a lateral shoot. Once a mutant lineage populates one of the three layers of cells (termed L-I, L-II and L-III) in a shoot, the pattern of cell division in the shoot ensures that this condition is perpetuated and inherited by all the lateral shoot produced by the original chimeric meristem. Thus, the chimeras found in the present study could have originated from transformed cells that had been incorporated into the inner layer and from non-transformed cells or cells that had lost the transgene incorporated into the outer layer of the meristem of adventitious shoots during regeneration. The absence of kanamycin-resistant or phenotypically altered progeny suggests that the

germ-line cells were derived from non-transformed cells. Judged from the pattern of dark green and pale green tissues and from the germ-line genotype, tissues derived from the L-II layer may consist of non-transformed cells and tissues derived from the L-III layer may be transformed. The tissues derived from the L-I layer (epidermis) were probably non-transformed because extracts from dark green tissues containing epidermal cells gave no signals after PCR (Fig. 4a, lanes 1, 3). The invasion by dark green tissue of inner regions, as shown in Figure 1a, may have resulted from intrusion of cells derived from L-II into the L-III layer (Stewart 1986).

The difference between chimera-1 and chimera-2 was presumably a consequence of the difference in the level of expression of *rolC* in L-III-derived cells. Recently, Szymkowiak and Sussex (1992) reported that the internal meristem layer (L-III) determines floral morphology in periclinal chimeras of tomato. Results of immunoblot analysis and dot-blot hybridization suggested that the level of expression of *rolC* in pale green regions of chimera-1 was higher than that in pale green regions of chimera-2 (compare lanes 2 and 4 in Figs. 3a and 3b).

The effects of *rolC* on the development of tobacco leaves were determined by histological analysis. We found that the parenchyma tissues were disorganized in inner pale green regions that contained the 35S-*rolC* gene. In young leaves, cells in the inner region were larger and fewer in number per unit area than those in the outer region. In leaf pieces from which the lower epidermis had been peeled off and which had been soaked in enzyme solution, the number of cells per unit area in the inner pale green region was lower than that in the outer dark green region. Staining with DAPI also gave the same result. These observations suggest that the difference of cell number between the pale and the dark regions caused the physical tension in the pale green regions that resulted in disorganized and/or enlarged cells in the pale green regions, and undulating leaf morphology in the dark green regions. The diameter of protoplasts from pale green tissues was apparently larger than that of protoplasts from dark green tissues. However, the inner pale green region contained cells derived from both L-II and L-III layers. Thus, it is possible that the large mesophyll cells in inner regions, observed by histological analysis, were due not only to physical tension exerted by non-transformed cells (L-I and L-II) but also to their own size which was altered by the expression of *rolC*. However, enlargement in pale green regions did not contribute very much to negation of a wrap in leaf.

The results published by Estruch et al. (1991b) on the size and number of cells in *rolC*-chimeric tissues are in conflict with the results presented here. The discrepancy might be due to the fact that in our case only one layer of cells was transformed whereas in their case all three layers expressed the *rolC* gene. These differences might be worthy of

further analysis.

The difference in a numbers of cells between pale and dark regions may have arisen from delayed progression and/or earlier interruption of cell division in *rolC*-transformed cells. Thus, the effect of *rolC* on the division of plant cells also requires further analysis.

The high level of expression of *rolC* under control of the 35S promoter in leaf tissues results in pale green, lanceolate leaves (Schmülling et al. 1988). In leaves of lanceolate mutants of tomato (*La*⁺), Dangler (1984) observed that measurable enlargement of cells was initiated at an earlier stage than in normal plants. Furthermore, he suggested that cell division ceases at an early stage during the expansion of the lamina of leaves. Similar events, such as inhibition of cell division and early enlargement of cells may occur during the development of the cells transformed with the 35S-*rolC*.

With respect to the molecular function of the products of the *rolC*, the *rolC* protein is present in the soluble fraction (Oono et al. 1991, Estruch et al. 1991b) and has cytokinin- β -glucosidase activity (Estruch et al. 1991a). Estruch et al. hypothesized that *rolC* converts an inactive cytokinin-glucoside conjugate to an active form and causes shortened internodes and reduced apical dominance. However, this finding is contradicted by the fact that in 35S-*rolC* plants root growth was promoted and pale green leaves were formed.

We have not yet established standard conditions for the production of transgenic periclinal chimeras. Conditions, such as concentration and duration of antibiotics application for selection of transgenic plants, have to be standardized. Production of transgenic chimeras will open new avenues to the progress in plant developmental biology.

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