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

Trámite : 011
Evento : 378
Actuación : 444

Referencia : Expediente No. **09.070.159 A**
Asunto : Solicitud de patente para: **NOVEDOSOS GENES**
MARCADORES SELECCIONABLES
Solicitante : **DOW AGROSCIENCES LLC**
Fecha de solicitud : 7 de julio 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. **5056** notificado por fijación en lista el 19 de marzo de 2013.
2. Mediante memorial del 20 de junio de 2013 se solicitó oportunamente plazo para responder la acción oficial.
3. Mediante oficio No. **5056** 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 se refieren a la no patentabilidad de las reivindicaciones 3 y 6, a que las reivindicaciones 2, 31 y 32 son reivindicaciones de uso y por consiguiente no son patentables, a la claridad del pliego reivindicatorio, y al nivel inventivo de las reivindicaciones 1-32 en vista de los documentos D1: US 5,561,236 y D2: Bedford D., et al. Gene, vol 104 (1): 39-45.

"EXCELENCIA LEGAL EN UN MUNDO SIN FRONTERAS"

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4. 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:

4.1 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 27 reivindicaciones.

Se han introducido las siguientes modificaciones al pliego reivindicatorio:

- Se eliminaron las reivindicaciones originales 3, 5, 6, 8, y 24.
- Se modificaron las reivindicaciones originales 1, 7, 22 y 31.
- Se reenumeraron las reivindicaciones restantes.

La objeción de excepción de patentabilidad de las reivindicaciones 3 y 6 fue superada al eliminar dichas reivindicaciones.

Las modificaciones realizadas a la reivindicación 1 logran que la invención quede claramente definida para el examinador, al especificar los grupos de los cuales se selecciona el promotor y la secuencia que comprende el polinucleótido.

Con respecto a la objeción a las reivindicaciones 2, 31 y 32 por ser relativas a un uso, estas reivindicaciones fueron modificadas para superar dicha objeción. Además, a partir de la modificación de la reivindicación 2 es claro que lo que se divulga es un método caracterizado por pasos específicos y que por ende no indica un uso. Por favor noten que también se ha corregido la dependencia de esta reivindicación 2; en consecuencia, ahora depende de la reivindicación 1 y no de la reivindicación 4 como erróneamente se encontraba en el capítulo estudiado por Su Despacho.

El Examinador establece que el capítulo reivindicatorio presenta múltiples reivindicaciones independientes, específicamente se refiere a las reivindicaciones 3, 5, 6, 8 y 14. En relación a lo anterior, se han eliminado las reivindicaciones 3, 5, 6 y 8.

Con respecto a la objeción de la reivindicación original 20, en donde el Examinador establece que el método debe definir en cada etapa la estructura del elemento que interviene indicando la secuencia del mismo, queremos llamar la atención del Examinador que su objeción no es clara ya que ésta reivindicación establece: "El método de la reivindicación 14 en donde dicho método es practicado en un invernadero, en una cámara de crecimiento, o en el campo", lo cual claramente es una característica adicional de la reivindicación 14 que indica los lugares en los cuales se puede llevar a cabo dicho método. Por ende, se le solicita al Examinador si es necesario, aclarar dicha objeción.

El Examinador establece que las reivindicaciones 17 y 21 carecen de sustento en la descripción. Sin embargo, estamos en desacuerdo con el Examinador ya que a lo largo de la descripción si se discute e incluso se menciona en varios ejemplos, la inclusión de características de tolerancia a otros herbicidas y adicionalmente esta característica es bien conocida en el arte.

Con respecto a la objeción de las reivindicaciones 22, 23 y 25 éstas se modificaron para hacer más claro el método y se especificaron características adicionales, sin embargo no se detallaron las etapas del método más a fondo ya que se consideran que son procesos plenamente conocidos en el arte previo y para una persona versada en la materia, como lo es una PCR o un Inmunoblot por ejemplo.

Por último la objeción a la reivindicación 7 establece que dicho método no se considera dentro del objeto de la solicitud que es proporcionar un gen para resistencia a herbicidas, sin embargo se deja constancia que en la descripción de la presente solicitud (página 3, párrafo 7) se contempló entre el alcance de la invención los métodos para controlar malezas usando herbicidas.

De esta manera, el alcance del pliego reivindicatorio ha sido limitado teniendo en cuenta las sugerencias del Examinador, y por lo tanto, las demás objeciones de claridad se ven superadas por las modificaciones realizadas y la eliminación de las respectivas reivindicaciones. En consecuencia, se solicita atentamente tener en cuenta este nuevo pliego reivindicatorio para el nuevo análisis de patentabilidad.

4.2 Argumentos a favor de una solicitud divisional

Mediante el Artículo 36 de la Decisión 486 en el cual se establece la posibilidad de dividir la solicitud en dos o más fraccionarias, se observa que los requerimientos exigidos por el artículo mencionado, son que no implique la ampliación de la materia contenida en la solicitud inicial. Por esta razón, se le aclara al Examinador que la presente solicitud divisional no solo no amplía la materia de la solicitud inicial sino que además está limitando la invención, por lo que se solicita continuar con el trámite de la presente solicitud.

4.3 Argumentos a favor del nivel inventivo de la presente solicitud

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.

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. Por favor tengase presente que la jurisprudencia vignete señala que el Examinador es quién está a cargo de esta evaluación y 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.

La presente solicitud sí tiene nivel inventivo

4.3.1 El documento D2 muestra que el K_m de la enzima DSM-2 (denominado como “producto del gen bar de *S. coelicolor*” en D2) es aproximadamente 20 veces mayor que la del producto del gen de *S. hydroscopicus*, es decir el documento D2 enseña que la actividad de DSM-2 es mucho menor que la del producto de *S. hydroscopicus*. En general, el Examinador no aprecia lo suficiente el hecho de que una persona versada en la materia estaría fuertemente desalentada por el muy bajo K_m de la enzima DSM-2 para intentar emplear el gen relacionado para proporcionar resistencia transgénica de glufosinato en plantas. Tal y como lo enseña el documento D2, el K_m del gen DSM-2, el cual era aproximadamente 1mM, era cerca de 20 veces el del producto del gen de *S. hydroscopicus*. Una persona versada en la materia no hubiera esperado que una enzima con un nivel tan alto de K_m pudiera proporcionar una resistencia a herbicida efectiva, ni en una bacteria ni en plantas transgénicas.

4.3.2 En el documento de Castle *et al.*, (2004), anexado junto a esta respuesta, se observa una experiencia análoga, en donde los autores trabajaron con genes “GAT” que tenían como sustrato el herbicida glifosato. Los genes nativos que tenían un K_m de aproximadamente 1.2mM, no proporcionaban alguna resistencia a glifosato en *E. coli* ni en plantas transgénicas. El artículo cita en la página 1152: “La producción de las proteínas GAT en *E. coli* no permitieron que las células crecieran en medio suplementado con concentraciones inhibitorias de glifosato. Similarmente, la expresión de *gat* en tabaco transgénico y *Arabidopsis* no confirió tolerancia a herbicida.”. De hecho estos resultados

con los genes nativos llevaron a que Castle *et al.* realizaran evolución dirigida de las enzimas para mejorar K_m y k_{cat} para lograr una tolerancia efectiva del herbicida en las plantas y encontraron que las variantes que tenían K_m para glifosato comparable con el de la enzima para glufosinato de *S. hydroscopicus* proporcionaban resistencia a glifosato en plantas transgénicas (Castle *et al.* pg. 1154). Sin embargo, los productos que emergieron de este esfuerzo en última instancia, no han salido al mercado; un vocero para Pioneer Hi-Bred, que continuó con la investigación y el desarrollo de la tecnología GAT, admitió en el 2009 que la tecnología "no estaba cumpliendo con los estándares de Pioneer para la tolerancia de glifosato" (ver Anexo Noticia Bloomberg by Kaskey, 2009).

4.3.3 Una persona versada en la materia, tomando el documento D2, habría notado el número K_m , que era comparable con el de la enzima nativa GAT en Castle, y que mostraba similarmente una no resistencia en una copia cromosomal (D2: Sección resultados, pg. 43, columna de la derecha), específicamente el documento D2 establece que una copia cromosomal del gen bar de *S. coelicolor* "no confiere resistencia apreciable a [bialphos]". Basado en el K_m reportado y la falta de tolerancia al glufosinato, y en vista de los resultados reportados con GAT y la resistencia a glifosato, una persona versada en la materia no hubiera usado el gen DSM-2 bacteriano en un intento de crear plantas transgénicas resistentes a glufosinato.

4.3.4 En la medida que el Examinador está sugiriendo poner múltiples copias del transgén a la planta (análogo al plásmido de "altas copias" que se enseña en el documento D2 que proporciona resistencia), una persona versada en la materia en el momento de la aplicación de esta solicitud hubiera sabido que esto hubiera sido improbable que produjera un nivel de resistencia efectivo. Era bien conocido que las células que tuvieran múltiples copias de transgén eran asociadas frecuentemente con silenciamiento post- transcripcional de genes (ver anexo Gelvin, pg. 24, columna derecha, segundo párrafo completo). De esta manera, simplemente poner copias adicionales del transgén dentro del genoma de la planta para superar las deficiencias de K_m de la enzima y lograr la resistencia bialphos que los autores del documento D2 encontraron en una bacteria con un plásmido de alto número de copias, no hubiera sido una estrategia que una persona versada en la materia hubiera empleado.

4.3.5 La sugerencia del Examinador que una persona versada en la materia hubiera compensado el pobre K_m de la enzima al simplemente ligarla a un promotor poderoso para obtener una mayor expresión, es también una suposición incorrecta, como la anterior. En el momento que se hizo la presentación de la solicitud era bien conocido que obteniendo incluso una copia única de un transgén usando un poderoso promotor puede llevar al silenciamiento de genes y bajar los niveles de expresión a menos de lo que se esperaba, ya fuera por silenciamiento post-transcripcional (ver Elmayan & Vaucheret., pg. 787, en donde establece que el silenciamiento post-transcripcional “puede afectar copias únicas del transgén” cuando el gen se expresa demasiado) o por metilación (ver Day *et al.* (2000), encontraron el silenciamiento asociado a la metilación de transgenes de copia única recientemente integrados, cuando se empleaba el promotor poderoso 35S ARN del virus del mosaico de la coliflor). De esta manera, incluso si una persona versada en la materia hubiera considerado ligar el gen DSM-2 a un promotor poderoso en el intento de lograr la resistencia de bialphos que reportaron en el documento D2 que se producía por un plásmido de alto número de copias, la persona versada hubiera considerado los posibles problemas conocidos del silenciamiento de genes asociados con el uso de promotores poderosos, haciendo esta opción incluso menos deseada que lo que indicaba el alto valor de K_m , como se describió anteriormente.

4.3.6 Por último, consideramos que el Examinador debe tener en cuenta lo impredecible de expresar genes bacterianos para tolerancia de herbicidas en plantas, como se observa en el Ejemplo 1 y 17 de la patente US Patent No. 7,838,733. En el Ejemplo 1 se establece que el gen AAD-1 fue seleccionado de más de 100 “coincidencias” en GENBANK usando como referencia un gen activo conocido (*tfdA*). El Ejemplo 17 muestra que otro gen, AAD-2, que era significativamente más cercano a *tfdA* por identidad de la secuencia, era esencialmente inactivo en plantas, mientras que AAD-1 (menos relacionado con *tfdA* por secuencia) era sorprendentemente muy activo en plantas. De esta manera, esto también demuestra que no se puede predecir que un dado gen bacteriano de tolerancia a herbicidas se exprese bien en plantas.

Todos estos argumentos que se establecieron con evidencia demuestran que una persona versada en la materia no se vería motivada a aplicar las enseñanzas de D1 al gen divulgado en D2, y por lo tanto los citados documentos no afectan el nivel inventivo de la presente solicitud.

4.4 Conclusiones

De acuerdo a todos los argumentos presentados, la presente solicitud cumple a cabalidad con los requerimientos exigidos en la Decisión 486 y las objeciones formuladas por el Examinador se ven superadas. Con respecto al nivel inventivo, consideramos que el Examinador está excluyendo el hecho de que el inventor en el momento de solucionar el problema técnico de la solicitud tuvo que emplear habilidad inventiva, pues la presente invención no resulta de la mera extrapolación de las enseñanzas del arte previo, como se afirma. Se está desconociendo el mérito investigativo empleado y que está aportando una ventaja al arte previo.

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. 5056, 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.

6. Anexos

- Nuevo pliego reivindicatorio conformado por 27 reivindicaciones
- Anexo artículo Science, Castle *et al.* 2004
- Anexo artículo Genes, Day *et al.* 2000
- Anexo artículo Plant Journal, Elmayan & Vaucheret, 1996
- Anexo noticia Bloomberg, Kaskey, 2009.

Señor Director,


LUZ CLEMENCIA DE PÁEZ

C.C. 35.456.344 de Usaquén

T.P.A. No. 23.555

COLO-10975-0841-142-4
FBJ\FBJ\ID-247838\WPC10975
No. M-2013-247838\FBJ

REIVINDICACIONES

1. Un vector que comprende un promotor seleccionado del grupo que consiste de AtUb10, CsVMV y ZmUbi1, que puede operar en una célula de planta, y un polinucleótido que comprende la secuencia SEQ ID NO: 3, enlazado operativamente a dicho promotor, en donde dicho polinucleótido codifica una proteína que tiene actividad de fosfinotricin acetiltransferasa, y en donde dicho polinucleótido produce híbridos bajo condiciones de 6XSSC a 65°C con el pleno complemento de una sonda de ácido nucleico que codifica la secuencia identificada con SEQ ID NO:2.

2. Un método para identificar células resistentes a la fosfinotricina por medio de un gen de resistencia a la fosfinotricina por medio de un vector de acuerdo con la reivindicación 1, **CARACTERIZADO PORQUE** dicho método comprende los pasos de:

- a. someter una pluralidad de células de planta a transformación con un vector de la reivindicación 1
- b. cultivar las células en un medio
- c. exponer las células a la fosfinotricina, y
- d. determinar si las células son resistentes a la fosfinotricina.

3. Un proceso para la producción de una planta resistente a la fosfinotricina, en donde dicho método comprende incorporar al genoma de la planta el vector de la reivindicación 1, y seleccionar por resistencia a la fosfinotricina.

4. Un proceso para proteger un grupo de plantas cultivadas en un campo destruyendo las malezas por medio del vector de la reivindicación 1, **CARACTERIZADO PORQUE** dicho proceso comprende los pasos de:

- a. Incorporar en el genoma de las células de dichas plantas un vector de la reivindicación 1,
- b. Destruir dichas malezas luego de la aplicación de un herbicida que comprende un inhibidor de glutamin sintetasa como ingrediente activo.

5. Un método para producir una proteína sintética que tiene actividad de fosfinotricin acetiltransferasa, en donde dicho método comprende modificar una proteína que codifica la secuencia con número de identificación 2 (SEC ID NO:2) para producir una proteína variante, y analizar dicha proteína variante para determinar dicha actividad.

6. El método de la reivindicación 5, en donde dicho método comprende modificar un polinucleótido seleccionado del grupo integrado por la secuencia con número de identificación 1 (SEC ID NO:1) y la secuencia con número de identificación 3 (SEC ID NO:3) para crear una pluralidad de polinucleótidos modificados, y utilizar dichos polinucleótidos modificados en un procedimiento de entremezclado de genes.

7. El método de la reivindicación 5, en donde dicho método comprende modificar un polinucleótido seleccionado del grupo integrado por la secuencia con número de

identificación 1 (SEC ID NO:1) y la secuencia con número de identificación 3 (SEC ID NO:3) mediante PCR con propensión a error.

8. El método de la reivindicación 5, en donde dicho método comprende modificar al menos un polinucleótido que codifica por lo menos una de dichas secuencias de aminoácidos.

9. Una célula bacteriana diferente de *Streptomyces* en donde dicha célula comprende un vector de la reivindicación 1.

10. Un método para seleccionar una célula de planta que comprende un vector de acuerdo con la reivindicación 1, en donde dicho método comprende

- a. proveer dicho vector a una pluralidad de células ; y
- b. cultivar dicha pluralidad de células en una concentración de un herbicida que permita que las células que expresan dicho polinucleótido crezcan mientras destruyen o inhiben el desarrollo de las células que carecen de dicho vector, en donde dicho herbicida comprende fosfinotricina.

11. El método de la reivindicación 10, en donde dicho método se utiliza en un sistema de expresión seleccionado del grupo integrado por un sistema de expresión bacteriano, un sistema de expresión de cultivo de células de planta, un sistema de expresión de algas, un sistema de expresión de células animales, un sistema de expresión viral, un sistema de expresión de hongos y un sistema de expresión de levaduras.

12. El método de la reivindicación 11, en donde dicho sistema de expresión es un sistema de expresión de *Pseudomonas fluorescens*.

13. La célula de planta transgénica de la reivindicación 1, en donde dicha célula comprende además un gen con resistencia a herbicidas seleccionado del grupo integrado por AAD-1 y AAD-12.

14. Un método para seleccionar o distinguir una planta individual o un grupo de plantas de cultivo que contienen un gen DSM-2 entre una población de plantas de la misma especie que no contienen el gen.

15. El método de la reivindicación 14 que comprende la aplicación de glufosinato o bialafos a la colección de plantas.

16. El método de la reivindicación 14 en donde dicho método es practicado en un invernadero, en una cámara de crecimiento, o en el campo.

17. Un método para controlar por lo menos una maleza en el campo, comprendiendo dicho método la aplicación a por lo menos una porción de dicho campo de un primer herbicida seleccionado entre un grupo que consta de glufosinato, fosfinotricin y bialafos; y

aplicando dicho por lo menos uno de otros herbicidas a dicha por lo menos una parte de dicho campo, en donde dicho campo comprende por lo menos una planta transgénica que contiene un vector de acuerdo con la reivindicación 1, comprendiendo dicha planta

- a. un polinucleótido heterólogo que codifica una enzima que confiere resistencia a un herbicida inhibidor de la glutamin sintetasa, y
- b. un segundo polinucleótido heterólogo que codifica una enzima que confiere resistencia a por lo menos otro herbicida;

18. Un método para detectar si una planta contiene un polinucleótido de la reivindicación 1 en donde dicho método comprende los pasos de

- a. recolectar una muestra de dicha planta
- b. analizar dicha muestra para probar la presencia de dicho polinucleótido, usando un cebador de PCR o una sonda que hibridiza con el vector o polinucleótido de la reivindicación 1.

19. El método de la reivindicación 18 en donde dicho método comprende los pasos de

- a. Recolectar una muestra de dicha planta
- b. analizar dicha muestra para probar la presencia de la proteína codificada por dicho polinucleótido.

20. El método de la reivindicación 19 en donde dicho método comprende analizar dicha muestra para detectar la presencia de dicha proteína por medio de un anticuerpo.

21. Un método para controlar por lo menos una maleza en el campo, comprendiendo dicho método la aplicación de un herbicida inhibidor de la glutamin sintetasa a una colección de plantas que comprenden el vector de la reivindicación 1, en un campo que tiene malezas.

22. El método de la reivindicación 4 en donde dicha planta es una monocotiledónea.

23. El método de la reivindicación 22 en donde dicha monocotiledónea se selecciona entre un grupo que consta de maíz, arroz, trigo, cebada, centeno, caña de azúcar, pasto césped de estaciones cálidas y frías, avena, sorgo, y las hierbas para pastura.

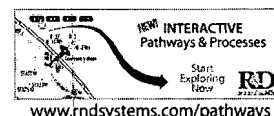
24. El método de la reivindicación 21 en donde dicha planta es una dicotiledónea.

25. El método de la reivindicación 14 en donde dicho gen DSM-2 funciona como un marcador seleccionable y dicho método se practica en invernadero, una cámara de crecimiento o en el campo.

26. El vector de la reivindicación 1 caracterizado porque el polinucleótido comprende un codón que incrementa la expresión de la planta.

27. Un método que comprende una proteína para comunicar a una planta tolerancia a los herbicidas, en donde dicho método comprende integrar dentro de una planta de cultivo un polinucleótido, en donde dicho polinucleótido codifica dicha proteína y dicha proteína tiene la actividad de la fosfinotricin acetiltransferasa, en donde dicho polinucleótido produce híbridos bajo condiciones de 6XSSC a 65°C con el pleno complemento de una sonda de ácido nucleico que codifica la secuencia con número de identificación 2 (SEQ ID NO:2), y en donde la expresión de dicho polinucleótido y la producción de dicha proteína dan a dicha planta tolerancia a un herbicida de fosfinotricina.

SPONSORED BY R&D SYSTEMS INC.



Discovery and Directed Evolution of a Glyphosate Tolerance Gene

Linda A. Castle *et al.*
Science **304**, 1151 (2004);
 DOI: 10.1126/science.1096770

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Michael W. Lassner¹**

The advantage of glyphosate detoxification is the removal of herbicidal residue, which may result in more robust tolerance and allow spraying during reproductive development. *N*-Acetylgllyphosate is not herbicidal (10) and is not an effective inhibitor of EPSPS (11). We sought a soluble enzyme

To find a new enzyme exhibiting activity on glyphosate, we searched within our microbial collection. Because *Bacillus* species produce a wide variety of enzymes involved in secondary metabolism, we focused our search on bacilli isolated from non-extreme environments. We developed a mass spectrometry method (11) that detects $<0.5 \mu\text{M}$ *N*-acetylgllyphosate. Several hundred isolates were grown to stationary phase, permeabilized, and incubated with the substrates glyphosate and AcCoA. Supernatants were analyzed for *N*-acetylgllyphosate. Many strains appeared to produce detectable product, but the *Bacillus licheniformis* strains catalyzed the greatest reproducible accumulation. *B. licheniformis* is a common benign saprophytic bacterium.

Position-specific iterated BLAST (PSI-BLAST) (16) search and analysis indicates that GAT shares similarity with enzymes of the GNAT or GCN5-like *N*-acetyltransferase superfamily (17, 18). GNAT superfamily enzymes are found in mammals, plants, fungi, algae, and bacteria. Members often lack pairwise sequence similarity, although their tertiary structures are similar. Some GNAT proteins are involved in regulating sporulation, transcription, metabolism, or detoxification, but most of them remain to be characterized. Although the native function of GAT remains unknown, it is unusual among characterized acetyltransferases in that it acetylates a secondary amine (glyphosate).

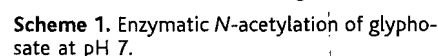


Fig. 1. Amino acid sequence of *B. licheniformis* GAT enzymes (26).

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The protein most closely related to GAT is YITI, a hypothetical *N*-acetyltransferase (NAT) protein predicted from the genomic sequence of *B. subtilis*. The *yitI* gene (GenBank accession Y09476) is 63% identical to *gat* and the hypothetical protein sequence is 59% identical and 66% similar to GAT. We used recombinant *E. coli* to produce YITI and found that it was capable of acetylating glyphosate but with lower efficiency than GAT. Similar hypothetical proteins (with GenBank accession numbers and the respective identity to *B. licheniformis* ST401 GAT) are encoded in *B. cereus* YITI (NP_830505, 49%); *B. anthracis* YITI (NP_654645, 48%); *Listeria innocua* NAT (CAC98097, 38%); *L. monocytogenes* NAT (AB1415, 39%); *Chloroflexus aurantiacus* NAT (ZP_00018467, 28%); *Vibrio parahaemolyticus* NAT (NP_799747, 26%); and *Zymomonas mobilis* NAT (CAA63806, 28%).

GAT enzymes are ~17 kD, are most active at pH 7, and require only an acetyl donor and glyphosate for activity. To evaluate GAT activity, we determined the k_{cat} , which is the rate constant of the catalytic reaction, and K_M , a measure of the affinity of the enzyme for the substrate. The K_M of GAT enzymes for AcCoA is 1 to 2 μ M (11), which strongly suggests that this is the native acetyl substrate. This tight binding property enables enzyme purification by CoA-Agarose affinity chromatography, and subsequent Superdex-75 gel filtration results in protein preparations of >95% purity (11). The three parental GAT enzymes are similar and have k_{cat} of 1.0 to 1.7 min^{-1} and K_M for glyphosate of 1.2 to 1.8 mM at pH 6.8 and 21°C (11). The ratio of k_{cat}/K_M is a measure of enzyme efficiency and specificity. The average k_{cat}/K_M ratio of

the GAT enzymes is 0.81 $\text{min}^{-1} \text{mM}^{-1}$ (11) and was used as the benchmark for improvement using directed evolution.

Although the GAT enzymes from bacilli can acetylate glyphosate, they do so poorly. Production of the GAT proteins in *E. coli* did not enable the cells to grow on medium supplemented with inhibitory concentrations of glyphosate. Similarly, *gat* expression in transgenic tobacco and *Arabidopsis* did not confer herbicide tolerance (19). Therefore, we used ST401, B6, and DS3 *gat* genes as parents for fragmentation-based multigene shuffling (20, 21) to create enzymes with higher efficiency and increased specificity for

glyphosate with the goal of developing herbicide-tolerant plants.

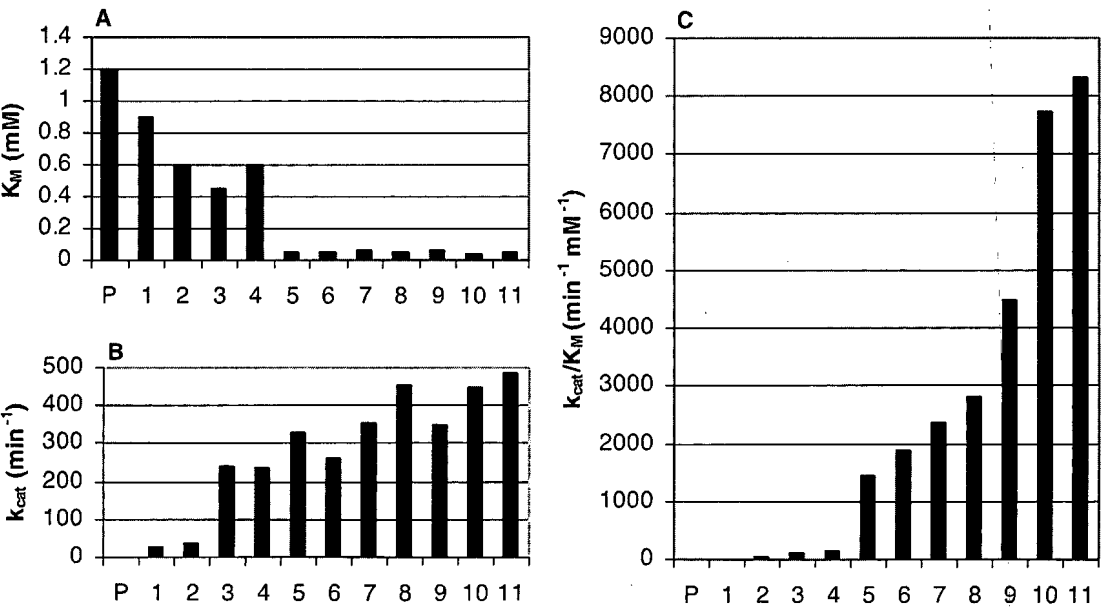
Libraries of shuffled gene variants were created, expressed in *E. coli*, and screened (11). In a primary high-throughput screen, supernatants from permeabilized *E. coli* cells carrying shuffled gene variants were assayed for the quantity of *N*-acetylglyphosate formed. Shuffled variants that specified the accumulation of more product than the parental controls were selected for further analysis. At each iteration of DNA shuffling, we screened about 5000 shuffled gene variants and analyzed 24 to 48 purified enzymes. Typically, 3 to 12 improved variants with a high

Table 1. Summary of directed evolution iterations for improvement of glyphosphate *N*-acetyltransferase activity. The complexity of each library is determined by the number of enzyme sequences possible given the diversity encoded by the parental genes (not counting mutations that arise by PCR).

Shuffling iteration	No. of parental genes	Library complexity	No. of variants screened	Amino acid changes in hits from original parents
1	3	6,144	5000 unselected	1–9
2	10	32,768	5000 unselected	1–9
3	12	2,048	5000 unselected	5–13
4	8	8,192	5000 unselected	6–15
5	3*	1.3×10^8	5000 selected on 1 mM glyphosate	17–26
6	10	3.4×10^7	5000 selected on 1 mM glyphosate	20–30
7	7	131,000	5000 selected on 1 mM glyphosate	20–27
8	6*	4.3×10^6	5000 selected on 5 mM glyphosate	21–34
9	8	131,000	5000 selected on 5 mM glyphosate	27–35
10	9	38,000	5000 selected on 5 mM glyphosate	28–37
11	10	147,456	5000 selected on 5 mM glyphosate	30–35

*Synthetic libraries for incorporation of natural diversity.

Fig. 2. Evolution of GAT activity. Parental and evolved GAT enzymes were purified and k_{cat} , K_M , and k_{cat}/K_M measured in 20 mM Hepes, pH 6.8, 10% ethylene glycol, 0.17 mM AcCoA, and ammonium glyphosate at 21°C (11). (A) The lowest K_M , (B) the highest k_{cat} , and (C) the highest k_{cat}/K_M ratios observed among the variants analyzed in each shuffling iteration. Although plateaus in k_{cat} and K_M occurred, the total catalytic efficiency and specificity of new GAT enzymes continued to increase (C).



k_{cat} , a low K_M , or a high ratio were chosen to be the parents for the next iteration (Table 1). Several GAT enzymes from the third iteration of gene shuffling showed ~100-fold improvement over the parent enzymes to $k_{cat}/K_M = 80 \text{ min}^{-1}\text{mM}^{-1}$, which was sufficient to allow growth of *E. coli* on 1 to 5 mM glyphosate in minimal agar medium (11). However, these gene variants were unable to confer glyphosate tolerance *in planta*.

Although we saw continuous improvement in k_{cat} values, the K_M appeared to plateau at 0.5 mM (Fig. 2). Efforts to elicit further decreases in K_M by random PCR mutagenesis of *gat* genes were unsuccessful. This impasse was addressed using two strategies: (i) a functional prescreen based on resistance of *gat*-expressing *E. coli* to glyphosate and (ii) a dramatic increase in the diversity available for recombination. The functional prescreen allowed the screening of more than 10^6 variants by eliminating those with low activity such that we picked only highly active variants to assay. To increase the genetic variance in

our shuffling population, we used information from natural genetic variability in related hypothetical proteins. We constructed a synthetic library (22) using the best GAT variant from the fourth iteration as a template and incorporated diversity from the predicted *B. subtilis* and *B. cereus* YITI sequences [fig. S2A, Table 1 (11)]. To incorporate the entire diversity present in these three molecules, an unachievable 10^{33} molecules would have been required. To limit the library size, we allowed two residue choices at only 27 sites (11). The introduced residues were evenly scattered throughout the sequences. This is but one of many possible libraries.

In screening this fifth-iteration library, we found one enzyme variant with a K_M of 0.05 mM glyphosate. The low K_M was attributed to a mutation having Ala at position 28 replaced by Val. However, this change caused a corresponding decrease in k_{cat} . In later iterations, Val²⁸ was excluded and we found new Ala²⁸ variants with low K_M and high k_{cat} . No single residue was identified that could ac-

count for the improvement. Altering the context of the entire protein through introduction of many new residues at once resulted in a complex solution to the problem. At the sixth iteration, we moved from the product accumulation assay to a more informative and rapid spectrophotometric assay (11) that allowed us to select enzymes with specific k_{cat} and K_M combinations. Two iterations of recombining the new diversity resulted in isolation of variants with increasing k_{cat} while maintaining the low K_M .

With each iteration of selection, some residues (present in all chosen parents) were fixed, leading to a decrease in the diversity available for recombination (Table 1). To keep the library population from reaching another functional plateau, we incorporated into the eighth-iteration library additional amino acid diversity from the more distantly related putative proteins of *L. innocua* and *Z. mobilis* [fig. S2B (11)]. We completed an additional three iterations of multigene shuffling to recombine the new diversity.

After 11 iterations of directed molecular evolution, the most efficient GAT variant (Fig. 3) had a k_{cat} of 416 min^{-1} and K_M of 0.05 mM glyphosate, resulting in a k_{cat}/K_M ratio of $8320 \text{ min}^{-1} \text{ mM}^{-1}$, nearly a 10,000-fold improvement over the parental enzymes. This protein is 76-79% identical to the original native GAT proteins. The evolved GAT enzyme is equally or more efficient than other characterized NAT enzymes (11, 23, 24). Over time, residues that had a positive effect on k_{cat} or K_M were selected; residues with negative effects were eliminated. Because of the number of varying sites in any given iteration of shuffling, it was not clear which residues accounted for the improvements. We substituted several amino acid changes present in improved variants into the parental or less active enzymes, but no simple combination of residues accounted for the efficiency of the improved variants. Our ability to improve enzyme activity by DNA shuffling and functional selection outpaced and outperformed attempts to predict sequence-function relations.

Our purpose in discovering and evolving the *gat* genes was to develop glyphosate-tolerant crop plants. To be effective, the GAT enzyme must convert the glyphosate to *N*-acetylgllyphosate before cell death from inhibition of EPSPS occurs. Like glyphosate, *N*-acetylgllyphosate is stable and is not metabolized in plants, but it is not herbicidal. The *gat* genes were introduced into *Arabidopsis* (19), tobacco (11), and maize (11, 25). In all cases, transformants were selected on glyphosate, and the presence and activity of the *gat* gene were confirmed by PCR and Western analysis. The transgenes were introduced into the nuclear genome, and the pro-

Fig. 3. Sequence analysis of the evolved 11th-iteration GAT enzyme with greatest k_{cat}/K_M for glyphosate. Blue residues represent the original diversity sites from the parental enzymes. Green residues were introduced in the fifth-iteration synthetic library and magenta residues in the eighth-iteration library. Orange residues arose by random mutagenesis as follows: V114A, 1st iteration; G37R, 2nd iteration; I135V, 2nd iteration; I132T, 3rd iteration; R47G, 5th iteration; Y31F, 6th iteration; I91V, 6th iteration; G38S, 9th iteration; L36T, 9th iteration; L106I, 10th iteration; Q67K, 11th iteration. The DNA sequence of this *gat* gene is given in GenBank AY597418.

```

      *      *      *      *      *      *      *      *      *      *      *      *      *      *      *      *
MIEVKPINAEDTY*LRHR*LRPNQ*YEA*NEESDL*TRSN*PHLGG*YGGK*LIS*ASPHQAE*
      *      *      *      *      *      *      *      *      *      *      *      *      *      *      *      *
HSEL*GGK*QYOLRG*ATLEGYREQAGS*SLV*HAEE*ILRKEGAD*MTWCNAR*TS*SGYV*K*
      *      *      *      *      *      *      *      *      *      *      *      *      *      *      *      *
LGFSEQC*VED*PP*GP*PHILMYK*RI*
      *      *      *      *      *      *      *      *      *      *      *      *      *      *      *      *
                                         60
                                         120
                                         146
```



Fig. 4. Maize plants expressing GAT-encoding transgenes. (A) Untransformed maize regenerants 10 days after 1X glyphosate spray. (B to D) Plants on the right are shown 10 days after glyphosate spray application. On the left are untreated T₀ plants. (B) Transformed maize T₀ plants expressing a fifth-iteration *gat* variant. Although the plants were tolerant to 4X glyphosate, they showed a setback in growth. (C) Many events expressing a seventh-iteration *gat* variant showed no symptoms or growth setback at 4X glyphosate compared to unsprayed controls. (D) Maize plants expressing 10th- and 11th-iteration *gat* genes were tolerant to 6X glyphosate with no visual damage or growth setback. 1X glyphosate = 26 oz/ac Roundup UltraMAX.

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teins were present in the cytosol. The specificity for glyphosate is high. Other common agrichemicals such as phosphinothricin, atrazine, and sulfonylureas are not acetylated by GAT (11).

Improved *gat* variants from the fifth iteration enabled the regeneration of glyphosate-tolerant transgenic tobacco plants. The plants were morphologically normal and fertile. To evaluate the tolerance profile of the tobacco plants, we selected T₁ plants that contained a segregating *gat* gene, using a glyphosate spray of 1 lb. acid equivalents per acre (ae/ac) and followed that with a dose-response regime using sprays of 2 to 24 lb. ae/ac. Untransformed plants showed severe symptoms or were killed by 1 lb. ae/ac (fig. S3A), whereas several transgenic GAT lines tolerated the highest dose sprayed (fig. S3B).

Fifth-iteration *gat* genes also allowed production of glyphosate-tolerant maize plants. T₀ plants were sprayed at the four-leaf stage with 104 oz./ac Roundup UltraMAX (4× field rate, equivalent to 3 lb. ae/ac glyphosate). The regenerants survived the treatment, but exhibited chlorotic banding and growth inhibition (Fig. 4B). Glyphosate tolerance improved with increases in the catalytic efficiency of GAT. With expression of seventh-iteration genes, nearly 50% of the maize regenerants showed no chlorotic banding and no growth inhibition (Fig. 4C). Most transformed plants expressing the best 10th and 11th round *gat* genes were tolerant to 6× glyphosate spray and showed no adverse symptoms (Fig. 4D). Efficacy trials of lines containing genes from several shuffling iterations are under way in the field to evaluate the commercial potential of this glyphosate tolerance trait.

Our approach shows that enzymes with useful yet insufficient activities can subsequently be improved by applying directed evolution until the desired activity is gained. DNA shuffling allowed us to develop crop plants exhibiting tolerance to the herbicide glyphosate, an important transgenic phenotype in global agriculture.

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27. We thank Y. Chen, T. Chen, L. Giver, C. Ivy, C. Kriebler, G. Wu, J. Wilkinson, A. Wong, N. Trinh, A. Madrigal, A. Umthun, P. Olsen, and W. Mehre for excellent technical assistance; J. Minshull, S. Govindarajan, R. Emig, and the Maxygen bioinformatics team; D. Dagarin and A. Wadley at Byotix for the glyphosate tolerance tests with *gat* tobacco.

Supporting Online Material

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Materials and Methods
Figs. S1 to S3
References

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Disulfide-Dependent Multimeric Assembly of Resistin Family Hormones

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Resistin, founding member of the resistin-like molecule (RELM) hormone family, is secreted selectively from adipocytes and induces liver-specific antagonism of insulin action, thus providing a potential molecular link between obesity and diabetes. Crystal structures of resistin and RELM β reveal an unusual multimeric structure. Each protomer comprises a carboxy-terminal disulfide-rich β -sandwich "head" domain and an amino-terminal α -helical "tail" segment. The α -helical segments associate to form three-stranded coiled coils, and surface-exposed interchain disulfide linkages mediate the formation of tail-to-tail hexamers. Analysis of serum samples shows that resistin circulates in two distinct assembly states, likely corresponding to hexamers and trimers. Infusion of a resistin mutant, lacking the intertrimer disulfide bonds, in pancreatic-insulin clamp studies reveals substantially more potent effects on hepatic insulin sensitivity than those observed with wild-type resistin. This result suggests that processing of the intertrimer disulfide bonds may reflect an obligatory step toward activation.

The increasing prevalence of obesity in Western societies is a cause of great medical concern (1, 2). Elevated body mass index

correlates with susceptibility to disease states including type II diabetes, coronary artery disease, hypertension, and dyslipidemias (3). Type II diabetes currently affects 17 million Americans (4) and has become a major public health problem. Nonetheless, the molecular basis for the link between obesity and insulin resistance—the hallmark of type II diabetes—remains unclear (5).

Insulin resistance in type II diabetes, and in several animal models of obesity-associated insulin resistance, can often be improved systemically by treatment with agonists for

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Monsanto Says DuPont Soybeans to Contain Unauthorized Genes

By Jack Kaskey - April 7, 2009 16:20 EDT

April 7 (Bloomberg) -- [Monsanto Co.](#), the world's biggest seed maker, said [DuPont Co.](#) failed to get a license to use technology for resisting Monsanto's Roundup weed killer in a new line of herbicide-resistant soybeans.

The companies are in dispute resolution over a plan by DuPont's Pioneer unit to combine Roundup Ready technology with DuPont genetics in Optimum GAT soybeans, Monsanto spokesman [Lee Quarles](#) said today. Monsanto doesn't license Roundup-resistant technology for use in so-called stacked soybean seeds, which combine multiple genetic changes, he said.

"Pioneer is not authorized to make this genetic combination," Monsanto said in an April 3 [regulatory filing](#). "We are seeking to prevent non-consensual use of our proprietary technology absent appropriate terms including compensation for providing access to such technology."

DuPont will notify the U.S. Department of Agriculture later this year that it is stacking Roundup Ready technology with other herbicide-resistant genetics in its Optimum GAT soybeans, Pioneer spokesman [Pat Arthur](#) said today.

Since 2005, DuPont has said its so-called GAT technology is an alternative to Roundup Ready seeds, which are unaffected by glyphosate herbicides, and would end royalty payments to Monsanto. DuPont, the No. 2 seed maker, won U.S. regulatory approval in July to sell Optimum GAT soybean seeds and plans to start commercial sales in 2011.

'Better Yield'

"Optimum GAT wasn't meeting our standards for glyphosate tolerance by itself," Arthur said. "With Optimum GAT stacked with Roundup Ready, we get better yield with more options for weed control."

Arthur declined to comment on the dispute with Monsanto, because DuPont doesn't discuss confidential business agreements.

The change doesn't require a second U.S. government approval, Arthur said.

DuPont fell \$1.12, or 4.4 percent, to \$24.32 at 4:01 p.m. in New York Stock Exchange composite trading. Monsanto, based in St. Louis, declined \$1.30, or 1.6 percent, to \$77.83.

More than 147 million acres globally were sown with Roundup Ready soybeans last year, including 72.5 million acres in the U.S., Monsanto said in an Oct. 8 report.

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Expression of single copies of a strongly expressed 35S transgene can be silenced post-transcriptionally

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Summary

The bacterial *UidA* gene cloned between the 35S promoter with a double enhancer and the terminator sequences of the pea *rbcS* 9C gene was introduced into tobacco plants. All 11 transformants carrying the transgene at a single locus showed silencing at each generation, but the timing of silencing occurred at different rates in the different transformants. Two plants showed high levels of *UidA* mRNA accumulation and GUS activity in young seedlings and a rapid decline of these levels during the first month of growth irrespective of the allelic state of the T-DNA. The other plants showed high levels of *UidA* mRNA accumulation and GUS activity in young seedlings and a slow decline of these levels during the first 4 months of growth in homozygous plants, whereas these levels decline only after 1 year of growth in hemizygous plants. Haploids showed the same kinetics of silencing as the homozygous plants from which they derived. Silencing correlated with a strong decrease of the steady-state level of *UidA* mRNA, while the transcription of the transgene in the nucleus was not affected. Since haploids and hemizygous plants derived from transformants carrying a single copy of the T-DNA show silencing, and since all the plants express the transgene at a very high level before the triggering of silencing, the results suggest that post-transcriptional silencing occurs through a dose effect and not through DNA–DNA interaction.

Introduction

High level of expression of a transgene coding for a product of interest is one of the main goals of plant biotechnology. However, silencing phenomena resulting from the introduction of transgenes expressed under the control of strong promoters, most often the 35S promoter of the cauliflower mosaic virus, have been described more and more frequently during the last 5 years (for review see Flavell, 1994; Matzke and Matzke, 1995). Silencing may affect the expression of the transgene alone, leading to a

plant devoid of its original interest. It may also affect the expression of homologous host genes, a phenomenon referred to as co-suppression that can have dramatic consequences for the survival of the plant if it involves a housekeeping gene or a defence-related gene.

Silencing can result from a block in gene expression either at the transcriptional level (Meyer *et al.*, 1993; Neuhauser *et al.*, 1994; Park *et al.*, 1996) or at the post-transcriptional level (de Carvalho *et al.*, 1992; de Carvalho-Niebel *et al.*, 1995; Dehio and Schell, 1994; Ingelbrecht *et al.*, 1994; Van Blockland *et al.*, 1994). The parameters that control the triggering of silencing remain unknown. Furthermore, up to now, it has not been possible to explain how and why silencing affects either all or only a fraction of the transformants carrying a given transgene. Model systems exhibiting silencing with high efficiency constitute valuable tools to identify the parameters controlling silencing. Silencing loci carrying transgenes able to trans-inactivate any other gene expressed under the control of a homologous promoter have been identified (Matzke *et al.*, 1989, 1994; Vaucheret, 1993, 1994). Similarly, silencing loci carrying transgenes able to trans-inactivate any other transgene expressing the same coding sequence have been identified (Hobbs *et al.*, 1993). Here, we describe an example of silencing that affects the entire homozygous progeny of all the 11 transformants carrying a 35S-*UidA* transgene and which leads, at each generation, to a strong decrease of the steady-state mRNA levels, without affecting transcription in the nucleus. This type of silencing can affect single copies of the transgene, that is, one copy in a haploid plant or one copy in a hemizygous plant. Since all the plants express the transgene at a very high level before the triggering of silencing, this post-transcriptional silencing effect is discussed in the context of a threshold mechanism.

Results

Introduction of genes 6b and 23b can lead to very high level of GUS activity

Genes 6b and 23b, consisting of the bacterial *UidA* gene with (23b) or without (6b) the Ω' leader sequence of the tobacco mosaic virus cloned between the 35S promoter with a double enhancer and the terminator sequences of the pea *rbcS* 9C gene of the binary vector pKYLX71-35S² (Maiti *et al.*, 1993), were introduced into a commercial tobacco variety (*Nicotiana tabacum* cv. Paraguay PBD6). GUS activity was measured in the leaves of the 11 primary

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Table 1. GUS activity in the leaves of hemizygous and homozygous mature plants

	L		H ^c								
	6b5	6b8	23b1	23b6	23b9	23b10	23b11	6b2	6b4	6b10	6b11
He	2.6	1.1	532	647	760	847	825	455	482	630	550
	4.7	1.4	597	687	857	945	1000	415	567	650	537
	2.5	0.9	615	757	887	692	875	367	460	607	522
Ho	0.4	0.6	3.2	0.6	2.2	7	12	17	30	0.7	35
	1	1.1	2.2	0.8	2.2	1.7	12	20	2.5	2	45
	0.6	0.5	7.2	1	1	3.7	11	25	27	1.5	ND

The self-progeny of each primary transformant (R₁ progeny) were sown *in vitro* on a medium containing kanamycin. Plants were transferred to the greenhouse after 1 month and grown under a 16 h light/8 h dark photoperiod. Proteins were extracted from the eighth leaf from the top of plants one week before flowering. GUS activity, determined for 4 µg of protein, is expressed in nmol MU min⁻¹ mg⁻¹. The allelic state of the T-DNA in each plant was determined by sowing the R₂ progeny on to kanamycin. Results are given for three plants at each allelic state. L, low expressers; H^c, high conditional expressers; He, hemizygous; Ho, homozygous; ND, not determined.

regenerants obtained, after transfer to the greenhouse. A wide range of variation was observed among independent mature transformants carrying 6b and 23b, with GUS activity reaching a maximum of 1000 nmol MU min⁻¹ mg⁻¹ protein.

Inactivation of gene expression occurs in all homozygous transformants

To monitor precisely the level of expression conferred by constructs 6b and 23b, transformants were allowed to self-fertilize and the R₁ progenies were sown on kanamycin-containing medium. Eleven transformants were generated, all exhibited a 3:1 segregation ratio (data not shown), suggesting that the T-DNA had inserted at a single locus. Kanamycin-resistant individuals from each progeny were transferred to the greenhouse. GUS activity was measured and mRNA accumulation was analysed by Northern blot in the eighth leaf from the top of the plants 1 week before flowering. After that, plants were allowed to self-fertilize and the subsequent R₂ progeny was sown on a kanamycin-containing medium to distinguish between homozygous and hemizygous plants. Results are summarized in Table 1 and Figure 1(a). The transformants can be grouped into two classes. One class comprises two transformants (6b5, 6b8) expressing a low level of GUS activity and steady-state *UidA* mRNA in both homozygous and hemizygous mature plants. This class will be further referred to as L plants (for low expressers). The other class comprises the nine remaining transformant lines, which express a high level of GUS activity and steady-state *UidA* mRNA when the plants are hemizygous for the T-DNA locus and a low level when the plants are homozygous for the T-DNA locus. This class will be further referred to as H^c plants (for high conditional expressers). Although the number of transformants analysed is not very large, these results show one atypical example of a silencing phenomenon which

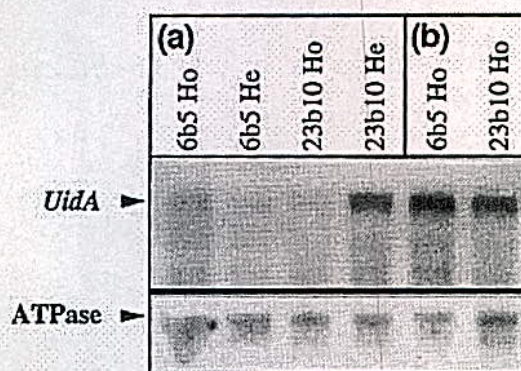


Figure 1. Detection of *UidA* steady-state mRNA in transgenic plants. (a) Detection of *UidA* steady-state mRNA in 4 month-old plants. RNA were extracted from the leaves of 4-month-old homozygous (Ho) and hemizygous (He) plants 6b5 (a L plant) and 23b10 (a H^c plant). (b) Detection of *UidA* steady-state mRNA in 7-day-old seedlings. RNA was extracted from 7-day-old seedlings of the homozygous plants 6b5 and 23b10. Four micrograms of total RNA were hybridized with the *UidA* coding sequence and mitochondrial ATPase β -subunit probes.

affects the entire homozygous progeny of all transformants carrying a given transgene.

Inactivation of gene expression can affect transformants carrying a single transgene copy

In order to determine if there was a relationship between the transgene copy number and the silencing phenomenon, the 11 transformants were characterized by Southern analysis. DNA was extracted, digested with *Hind*III (which cuts once in the T-DNA between the 35S promoter and the *UidA* gene), and probed with the 35S and the *UidA* probes. The hybridizations indicate that four H^c plants (23b1, 23b6, 23b10, 23b11) carry a single copy of the transgene (Figure 2). Conversely, the remaining H^c and L plants exhibit a complex pattern suggesting they carry more than one copy (data not shown). Additional digests with *Eco*RI and *Nco*I (which cut internal sites of the T-DNA) confirmed that the

four H^c plants (23b1, 23b6, 23b10, 23b11) carry actually a single and intact copy of the transgene (Figure 2). When GUS activity was determined late in the development (after 1 year of growth in the greenhouse), some hemizygous plants of the H^c group carrying multiple copies (6b10, 23b9) or a single copy (23b6, 23b10) of the transgene showed low activities comparable to those of homozygous plants (data not shown). These results therefore suggest that the triggering of this silencing phenomenon does not require the presence of more than one copy of the transgene per cell.

A high level of GUS activity is recovered at each generation and then decreases during plant development

GUS activity was measured at a very early stage of development, that is, 6 days after germination, in the two L plants and in four representative H^c plants. Results (Figure 3) show that a very high level of GUS activity is reproducibly found at this stage. It is interesting to notice that GUS activity was reproducibly higher in L plants as compared with H^c plants, suggesting that a high level of expression could correlate with efficient silencing. In order to further explore the developmental aspect of *UidA* gene expression, GUS activity was measured at various stages of development of nine representative combinations, that is, an L plant (6b5) and two H^c plants (23b6 and 6b10) which were either self-fertilized, back-crossed with the wild-type line PBD6, or crossed one with another (Table 2). Results demonstrate clearly that the level of GUS activity is high in all the plants immediately after germination (day 7), irrespective of being homozygous or hemizygous, a result which is confirmed by the steady-state mRNA level (Figure 1b). The number of 6b5 plants expressing high GUS activity and high levels of *UidA* mRNA decreased very rapidly and reached zero after 1 month of growth. The data in Table 2 each correspond to one plant. The variation from plant to plant suggests that some of them have already undergone silencing whereas the others have not, and that silencing starts very early but not synchronously in the development. Silencing was somewhat more rapid in homozygous 6b5

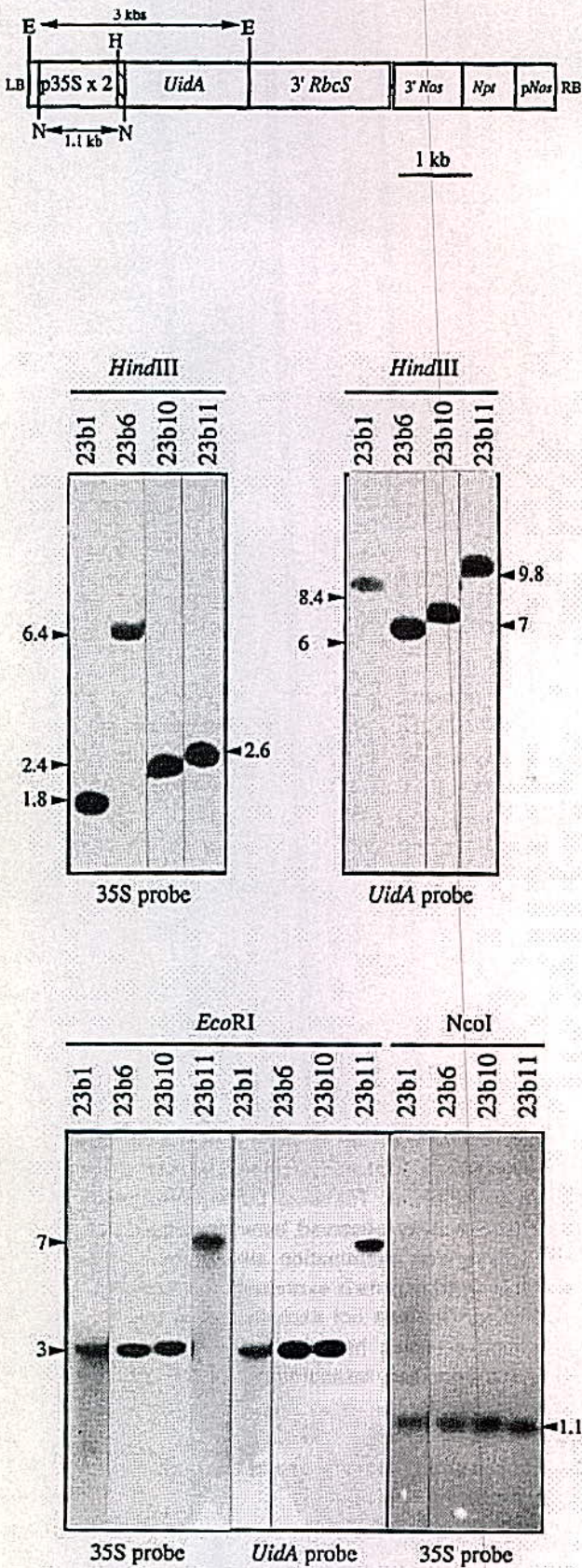


Figure 2. Detection of the T-DNA sequences inserted in the genome of the transformants. Five micrograms of genomic DNA was digested either by *HindIII*, *EcoRI* or *NcoI* and probed with either the 35S promoter or the *UidA* coding sequence. The physical map of the construct used for transformation is indicated above. H, *HindIII*; E, *EcoRI*; N, *NcoI*; RB, right border; LB, left border; *RbcS*, rubisco small subunit; *Nos*, nopaline synthase; *Npt*, neomycin phosphotransferase. Results of the hybridizations with *HindIII* digests indicate the presence of a single left border and of a single right border in the H^c plants 23b1, 23b6, 23b10 and 23b11, while results of the hybridizations with *EcoRI* and *NcoI* digests reveal the integrity of the 35S-*UidA* transgene. The presence of a 7 kb *EcoRI* fragment instead of the 3 kb in plant 23b11 suggests that the transfer of the T-DNA was interrupted between the *NcoI* and *EcoRI* sites at the left border, outside of the transgene.

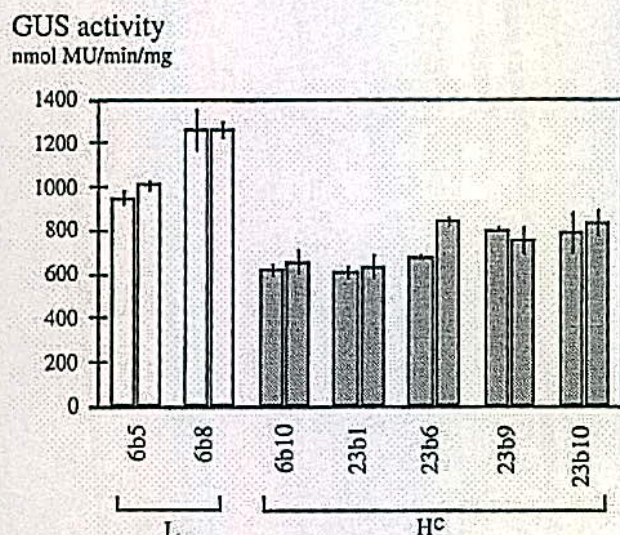


Figure 3. GUS activity in 6-day-old seedlings.

Homozygous plants of the R_2 progeny were back-crossed with wild-type plants. Seeds were germinated on filter discs steeped in water and grown under a 16 h light/8 h dark photoperiod (these conditions of culture were improved to avoid variation of GUS activity due to early inactivation). Proteins were extracted from 10 seedlings. GUS activity, determined with 0.5 μ g of protein, is expressed in $\text{nmol MU min}^{-1} \text{mg}^{-1}$. The means and standard deviation of five repetitions are given. Two assays were performed independently.

plants than in hemizygous ones. The number of plants displaying GUS activity at a high level also decreased very rapidly in hybrids between the 6b5 plant and either 23b6 or 6b10 plants, in a manner similar to hemizygous 6b5 plants. The number of plants expressing GUS activity at a high level decreased much more slowly in homozygous plants 23b6 or 6b10 and in hybrids between these plants. Conversely, no significant variation of activity was found in hemizygous plants (until it decreased late after 1 year of growth). Taken together, these results demonstrate that the decrease in GUS activity corresponds to a reversible silencing phenomenon, which occurs cyclicly at each generation. The timing of silencing occurs at different rates in the different transformants. The two L plants show very rapid inactivation whereas H^c plants show a slower inactivation. In addition, an epistatic effect of L plants on H^c plants is observed, since L plants can impose their timing of silencing on H^c plants. The weak but reproducible difference of GUS activity observed between L plants and H^c plants 6 days after germination also suggests that the more the *UidA* transgene is expressed, the more rapidly it is inactivated. One can not exclude that at this stage of development silencing has already been triggered in L plants, thus impeding measurement of the actual basal level of expression.

Silencing can affect a single copy of the UidA gene in haploid plants

Our results have shown that silencing was observed in hemizygous or homozygous L lines, in the homozygous

H^c lines and in some hemizygous H^c lines, even when they carry a single copy of the transgene. To further evaluate whether the number of copy of the transgene per genome is important for silencing, the time course of GUS activity was followed in haploid plants generated by anther culture from silenced homozygous H^c plants carrying either a single copy of the T-DNA (23b10) or multiple copies of the T-DNA (23b9) inserted at one locus (Table 3). Three weeks following their emergence, haploid plantlets exhibited a high level of GUS activity, indicating that fertilization is not required for resetting. Subsequently, GUS activity decreased in haploids derived from the silenced H^c plant carrying multiple copies of the T-DNA (23b9) but also in haploids derived from the silenced H^c plants carrying a single copy (23b10). These results confirm that the presence of two copies of the T-DNA is not required for the triggering of the silencing phenomenon described here. Haploids were also generated from hemizygous H^c plants of the R_1 progeny obtained by self-fertilization of primary regenerants carrying a single copy of the T-DNA (23b1, 23b6, 23b10). These haploids which derive from plants in which the expression of the *UidA* gene has never been silenced showed the same pattern of transgene inactivation during development as haploids derived from silenced homozygous 23b10 and 23b9 lines (data not shown). These results indicate that the triggering of silencing in haploids is not influenced by the state of the transgene in the plant from which they derived (active/hemizygous versus inactive/homozygous).

Molecular analysis of RNA transcription and steady-state RNA level

In order to define whether silencing occurred at the transcriptional or post-transcriptional level, Northern blots and nuclear run-off experiments were performed on four types of plants derived from transformant 23b6. Both nuclei and total RNA were prepared from the leaves of 4-month-old homozygous (silenced), hemizygous (non-silenced) or haploid (silenced) plants.

Nuclei and total RNA were also prepared from a hybrid between plant 23b6 and plant 271, a tobacco plant carrying a 'silencing locus' able to inactivate any transgene driven by the 35S promoter at the transcriptional level (Park *et al.*, 1996; Vaucheret, 1993, 1994). This 'silencing locus' carries two transgenes under the control of the 19S and 35S promoter, respectively. These transgenes are silent, and the locus acts as a general silencer for either 19S and 35S promoters, imprinting a silent state on any transgenes expressed from these promoters (Vaucheret, 1993). The silent state which is imprinted on to 'target' transgenes is somatically stable in the presence of the 'silencing locus' whereas it is metastable and progressively lost after the elimination of the 'silencing locus' (Vaucheret, 1994).

Table 2. Evolution of GUS activity in young seedlings and in mature plants.

Days after germination	7	12	15	21	27	32	40	54	3 months
Stage of development	2 cotyledons	1-2 leaves	2-3 leaves	3-4 leaves	4-5 leaves	5 leaves	5 leaves	5-6 leaves	1 week before flowering
6b5 Ho	807	52	9	7	ND	ND	0.2	3	14
	1300	82	6	7			8	7	10
L	817	42	17	6			6	8	7
	1100	62	6	8			7	4	7
	920	90	4.5	5			7	16	8
6b5 He	582	330	145	21	25	21	12	5	5
	847	350	267	23	23	22	6	3	7
L	1385	207	175	31	19	15	16	5	6
	852	420	292	24	15	20	6	3.5	9
	1400	422	215	23	14	14	11	6	17
6b10 Ho	1730	1425	1335	920	12	7	11	3	37
	905	1380	1265	2000	13	625		340	25
H ^c	1322	1570	1330	1825	11	17	1070	410	40
	1130	1350	1350	2400	1700	1172	130	20	40
	1557	2140	1780	1100	90	5	14	51	20
							8		
6b10 He	650	1030	905	755	1040	1037	845	990	665
	780	1117	875	812	1080	995	1012	1015	590
H ^c	907	1027	932	882	1085	1020	907	1010	687
	600	1195	977	1300	1180	852	920	1035	690
	660	1360	1150	1317	1127	1120	872	1190	672
23b6 Ho	1002	1927	1897	340	667	1370	1312	992	19
	907	2007	1430	1290	247	1262	1297	597	20
H ^c	1245	1932	1617	1312	1232	1222	1197	767	19
	1402	2387	2027	1915	1747	1282	1335	4.5	15
	1265		1817	1947	1645	1322	1427	5.5	15
23b6 He	827	1650	1452	782	1162	932	1247	995	802
	835	1312	1285	965	1302	1105	1257	1167	852
H ^c	945	1665	1612	1022	1257	1137	1020	1097	860
	817	1350	1352	775	1270	1002	907	1057	780
	1017	1775	1390	1482	1240	1065	955	1062	805
6b5 × 6b10	1297	107	95	5	ND	ND	2.2	3	10
Ho Ho	1377	127	15	7			2.2	2.5	16
	1500	187	172	9			2.5	1.5	14
L × H ^c	1105	110	92	11			6	3	9
	1717	315	102	6			2.2	1.5	11
6b5 × 23b6	1500	252	125	4	ND	ND	6.5	2.2	5
Ho Ho	1360	422	112	6			5	2.7	5
	1340	145	237	7			5	5.5	11
L × H ^c	1367	195	17	4.5			3	2.5	3
	1152	320	130	4.7			6	6.2	5
6b10 × 23b6	1515	1687	2042	1260	1692	395	1130	25	16
Ho Ho	1332	1785	2047	1235	1687	8	1137	4.2	16
	1152	1462	1685	1210	1795	1272	1112	2.7	20
H ^c × H ^c	1100	1432	1905	1000	1795	1377	1152	4.5	20
	1177	1822	1590	1002	1670	32	1230	3.2	25

Homozygous plants of the R₂ progeny were either self-fertilized (Ho), back-crossed with wild-type plants (He), or crossed one with each over (×). Seeds were sown *in vitro* and grown under a 16 h light/8 h dark photoperiod. Proteins were extracted from either the aerial part of the plant (day 7 and day 12) or from the first leaf from the top of five randomly chosen plants. Plants were transferred to the greenhouse and proteins were extracted from the first fully expanded leaf from the top of plants 1 week before flowering. GUS activity, determined on 1 µg of proteins (seedlings *in vitro*) or 4 µg of proteins (mature plants in the greenhouse), is given in nmol MU min⁻¹ mg⁻¹. Due to the small size of the seedlings and to the high frequency of harvesting, and to avoid a putative bias induced by the stress of cuttings, each plant was used for only one measurement. L, low expressers; H^c, high conditional expressers; ND, not determined.

Table 3. Evolution of GUS activity in haploids plants

Weeks after emergence	3	4	5	6	7	8	9	11	15	19
23b9-1	1550	1480	1560	830	32	24	6	3	10	ND
23b9-2	1135	1790	1482	27	6	22	2	13	10	ND
23b9-3	1550	1725	1547	902	127	17	3	6	9	ND
23b9-4	1280	1722	1847	1750	500	610	80	5	16	ND
23b9-5	1457	1852	1490	440	8	17	15	11	8	ND
23b9-6	1347	1570	370	10	11	7	7	8	12	ND
23b9-7	875	65	172	11	22	18	11	4	8	ND
23b9-8	1377	1157	47	10	13	12	10	4	9	ND
23b9-9	1137	520	25	7	8	8	2	7	7	ND
23b9-10	1415	1350	1060	162	8	11	11	3	12	ND
23b9-11	1385	1135	1510	1430	550	535	327	8	16	ND
23b9-12	1460	1462	1312	147	6	15	15	2	9	ND
23b9-13	1452	912	30	8	8	14	6	5	4	ND
23b9-14	1477	1392	1507	1382	542	755	472	16	9	ND
23b9-15	1442	750	25	7	9	7	13	3	ND	ND
23b9-16	1212	1155	50	18	6	8	9	12	6	ND
23b9-17	32	55	12	7	7	7	8	8	9	ND
23b9-18	1245	1327	1327	1450	710	640	482	15	12	ND
23b10-1	1400	1582	665	95	22	17	3	1	7	ND
23b10-2	1420	1740	1805	1960	125	145	2	5	7	ND
23b10-3	1100	1597	1470	1422	330	40	2	4	13	ND
23b10-4	1250	2035	2012	1882	490	760	642	823	7	ND
23b10-5	1255	1207	870	20	35	25	2	18	7	ND
23b10-6	1277	1572	1175	28	11	19	8	3	9	ND
23b10-7	1102	1510	1815	1660	437	747	182	8	10	ND
23b10-8	ND	1182	1085	1385	410	585	400	233	383	6
23b10-9	507	305	10	20	8	62	3	7	14	ND
23b10-10	1012	232	18	3	7	18	2	2	12	ND
23b10-11	1265	1335	1267	1122	200	62	3	5	13	ND
23b10-12	1217	1907	1410	1617	465	895	280	5	10	ND
23b10-13	1325	1820	1665	1087	55	27	9	11	11	ND
23b10-14	1130	1800	1720	1680	762	762	610	800	47	ND

Haploids plants were generated by *in vitro* anther culture from H^c plants carrying either a single copy (23b10) or multiple copies (23b9). Plants were transferred to the greenhouse between weeks 6 and 7. Proteins were extracted from the first leaf from the top of the plants. GUS activity, determined on 4 µg of protein, is expressed in nmol MU min⁻¹ mg⁻¹. ND, not determined.

Results of the run-off experiments, Northern blots and GUS activity determinations are shown in Figure 4. The absence of *Uida* mRNA and GUS activity in the hybrid involving plant 271 correlates with the absence of transcription of the 35S-*Uida* transgene. Conversely, the strong reduction of *Uida* mRNA and GUS activity in the homozygous and haploid plants as compared with the hemizygous plant does not correlate with a reduction of transcription. Indeed, the transcriptional signal is similar in the homozygous and haploid plants, and a little lower in the hemizygous plant. This is consistent with the allelic state of these three plants, since the ratio between the transgene dose and the genome copy is identical between homozygous and haploid plants (one transgene per haploid genome), whereas this ratio is halved in the hemizygous plants. These results indicate that the reversible silencing which affects transformants 6b and 23b at each generation occurs at the post-transcriptional level, as opposed to the tran-

scriptional silencing directed by the unlinked 35S silencing locus of plant 271.

Discussion

A strongly expressed 35S-Uida transgene shows high susceptibility to silencing

Many cases of silencing have been described in transgenic plants during the last 5 years (for review see Flavell, 1994; Matzke and Matzke, 1995). In this paper, we show that the transgenes 6b and 23b, consisting of the bacterial *Uida* gene cloned between the 35S promoter with a double enhancer and the terminator sequences of the pea *rbcS* 9C gene of the binary vector pKYLX71-35S² (Maiti *et al.*, 1993), were silenced in the entire homozygous progeny of all 11 transgenic tobacco plants analysed. It had been shown previously by Hobbs *et al.* (1990, 1993) that the

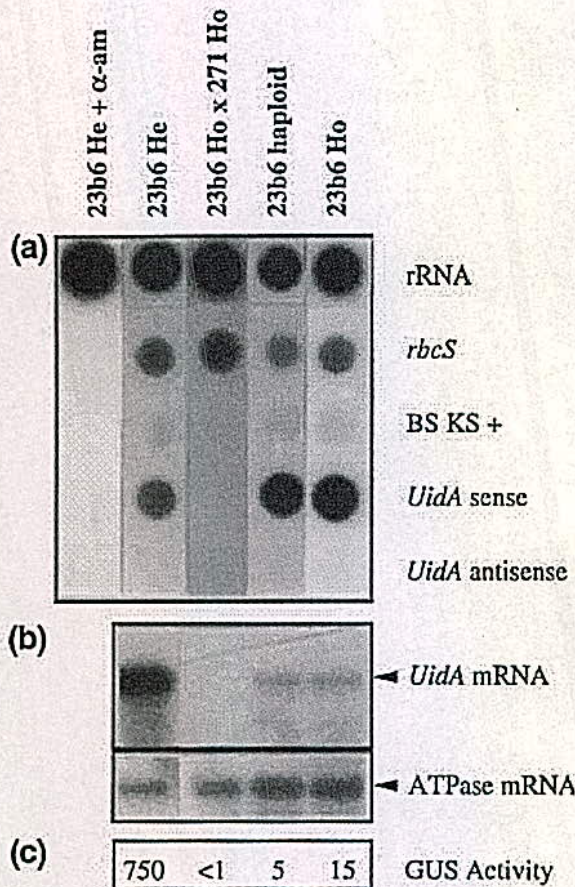


Figure 4. Transcriptional analysis of silencing. Run-off experiments (a) Northern blot analysis (b) and GUS activity (c) determination were performed on the leaves of 4-month-old homozygous (silenced), hemizygous (non-silenced) or haploid (silenced) plants 23b6 or on the leaves of a hybrid between plant 23b6 and plant 271, a tobacco plant carrying a silencing locus able to inactivate at the transcriptional level any transgene driven by the 35S promoter. (a) In run-off experiments, labelled RNA were hybridized to 2 µg linearized plasmid containing gene-specific sequences: *rbcS* and ribosomal DNA (rRNA); and 2 µg strand-specific single-stranded DNAs prepared from Bluescript BS KS (+/-): *UidA* sense and antisense, BS KS+ (as control); α-am, α-Amanitin. (b) For Northern blot analysis, 4 µg of total RNA were hybridized with the *UidA* coding sequence and mitochondrial ATPase β-subunit probes. (c) GUS activity, determined with 4 µg of protein, is expressed in nmol MU min⁻¹mg⁻¹.

bacterial *UidA* gene cloned between the 35S promoter and the terminator sequences of the *Agrobacterium tumefaciens nos* gene, carried by the binary vector pBI 121, when introduced into *N. tabacum* cv. Petit Havana SR1, was silenced in some transformants. The maximal level of GUS activity conferred by constructs 6b and 23b in plants of *N. tabacum* cv. Paraguay PBD6 was approximately 50-fold higher than the maximal level conferred by the pBI 121 construct in plants of *N. tabacum* cv. Petit Havana SR1 or Xanthi XHFD8 (Hobbs *et al.*, 1990; Jefferson *et al.*, 1987; Vaucheret *et al.*, 1992). It is therefore possible that the higher level of expression of the *UidA* gene in our experi-

ments is responsible for the amplification of the silencing phenomenon to all transformants.

Initial levels of UidA expression seem to correlate with rates of silencing

Transformants carrying either the pBI 121 construct (Hobbs *et al.*, 1993) or the constructs 6b or 23b can be classified in two groups. One group (referred to as L plants, in this work, as in Hobbs' work) always exhibits a low level of GUS activity in the leaves of mature plants and shows similar trans-silencing effects in the two systems described. The low level of GUS activity of these L plants was also found in hybrids involving any other plant expressing the *UidA* gene irrespective of the promoter used to direct its expression (data not shown). Conversely, the expression of other reporter genes under the control of the 35S promoter was not affected when brought into the presence of the *UidA* gene expressed under the control of the 35S promoter of our L plants (data not shown), suggesting that the T-DNA of L plants is able to trans-inactivate only genes expressing the same coding sequence. The second group (referred to as H plants in Hobbs' study or H^c plants in this work) behave differently. In the case of the pBI 121 transformants, a high level of GUS activity was always found in both homozygous and hemizygous H plants as well as in hybrids between H plants. In contrast, a high level of GUS activity was found only in hemizygous H^c plants carrying constructs 6b or 23b. GUS activity was low in homozygous H^c plants, in hybrids between H^c plants, and in hemizygous H^c plants much later in development. In addition, silencing occurred very rapidly in homozygous L plants, rapidly in hemizygous L plants and in hybrids between L and H^c plants, whereas the timing of silencing was slow in homozygous H^c plants and in hybrids between different homozygous H^c plants.

The results summarized in Table 4 indicate that the rate of silencing seems to correlate with the initial level of expression of the transgene. These results suggest that in all the transformants, the strength of the 35S promoter with a double enhancer gives rise to a level of gene expression, close to a threshold level beyond which silencing is triggered. This could explain why silencing occurred in some cases (H^c lines) only in homozygous plants, expressing twice as many transgenes as hemizygous plants.

A single copy of the transgene can undergo silencing in hemizygous or haploid plants

Results obtained with construct pBI 121 revealed that L plants carried two copies of the T-DNA per locus whereas H plants carried only a single copy of the T-DNA, suggesting that silencing correlates with the copy number of the T-

Table 4. Correlation between the timing of silencing and the initial level of expression of the transgene in the 11 transgenic lines carrying T-DNA at a single locus

	Clones	Transgene copy number	Initial GUS activity in 6-days-old seedlings (in nmol MU min ⁻¹ mg ⁻¹)	Rates of silencing		
				He	Ho	Ha
L	6b5	>1	950–1000	+++	++++	ND
	6b8	>1	1250	+++	++++	ND
	23b6	1	650–750	-/+	++	++
	23b9	>1	700–800	-/+	++	++
	23b10	1	700–800	-/+	++	++
Hc	23b11	1	800–1000	-/+	++	ND
	6b10	>1	650	-/+	++	ND
	23b1	1	550	-	+	++
	6b2	≥1	400–450	-	+	++
	6b4	≥1	450–550	-	+	ND
	6b11	≥1	550	-	+	ND

Gus activity is expressed in nmol MU min⁻¹ mg⁻¹. Ho, homozygous plant; He, hemizygous plant; Ha, haploid plant; L, low expressers; Hc, high conditional expressers. +++, 100% of the plants are silenced within the first 2 weeks of growth *in vitro*. ++, 100% of the plants are silenced within the first 2 months of growth *in vitro*. +, 100% of the plants are silenced in the greenhouse before flowering. -/+, some plants are silenced after more than 1 year of growth in the greenhouse. -, none of the plants are silenced in the greenhouse. ND, not determined.

DNA or with the structure of the insertion (Hobbs *et al.*, 1993). In the case of constructs 6b and 23b, we found that L plants carry multiple copies of the T-DNA inserted at one locus whereas H^c plants carry either a single or multiple copies (Table 4). Since we observed that silencing can affect hemizygous or haploid plants derived from H^c plants carrying a single copy of the T-DNA, our results suggest that neither two copies per locus, nor two copies per cell are actually required to trigger silencing. Previous results have shown that either haploid plants or hemizygous plants with a single transgene copy can exhibit co-suppression of glucanase or nitrate reductase host genes and transgenes, respectively (de Carvalho-Niebel *et al.*, 1995; Palauqui and Vaucheret, 1995). However, in these cases, the presence of the endogenous gene did not allow us to test whether a single gene copy per cell was susceptible to silencing, a result which was clearly demonstrated with the 35S-*Uida* transgene. However, one can not exclude that the presence of a high copy number of the transgene can increase the susceptibility to silencing and/or its timing.

Uida gene silencing is a post-transcriptional event and is reversible

Several hypotheses have been formulated to explain silencing in transgenic plants. These models often implicate a block of transcription or a post-transcriptional control of RNA accumulation (for review see Flavell, 1994; Matzke and Matzke, 1995). Silencing of single or multiple copies of the maize *A1* gene driven by the 35S promoter in the petals of transgenic petunia plants results from a block in

the transcription of the transgene and correlates with the methylation of cytosines within the 35S promoter sequence (Linn *et al.*, 1990; Meyer *et al.*, 1993, 1994). In the case of constructs 6b and 23b, no methylation was found at the two *Hpa*I sites of the 35S promoter with a double enhancer (data not shown), suggesting that promoter methylation is not involved in this silencing process. In addition, the strong reduction of *Uida* mRNA and GUS activity observed in homozygous and haploid H^c plants as compared with hemizygous plants did not correlate with a reduction of transcription. In contrast, *de novo* methylation of the promoter and an absence of transcription of the 35S-*Uida* transgene were observed when these plants were crossed with plant 271 carrying a '35S silencing locus' (Park *et al.*, 1996; Vaucheret, 1993; this work). Therefore, these results indicate that silencing of the 35S-*Uida* transgene in our plants results from a post-transcriptional block.

Post-transcriptional co-suppression of homologous transgenes and host genes (de Carvalho *et al.*, 1992; de Carvalho-Niebel *et al.*, 1995; Van Blockland *et al.*, 1994) and post-transcriptional silencing of reporter transgenes (Dehio and Schell, 1994; Ingelbrecht *et al.*, 1994) have been reported before. We suggest that the post-transcriptional silencing phenomenon described here is similar to these phenomena. In some of these cases, as in ours, silencing was reversed at each generation (de Carvalho *et al.*, 1992; Dehio and Schell, 1994) and occurred at much higher frequency in homozygous plants as compared with hemizygous plants. We showed that a high level of GUS activity was found at the cotyledon stage in L and H^c homozygous plants, suggesting that silencing is fully reversible and that

it is triggered at each generation during the development of the plants. Resetting does not require fertilization since a high activity was always found in young haploid plantlets (generated by anther culture from a silenced plant). In addition, silencing was found to be reversible by crossing homozygous H^c plants with wild-type plants and by segregating the two loci in the progeny of hybrids between H^c and L plants (data not shown).

High level of expression up to a threshold may trigger silencing

At least three hypotheses have been formulated to explain the post-transcriptional silencing of transgenes and homologous host genes.

- (i) One hypothesis involves the production of unintended antisense RNA by the transgene (Grierson *et al.*, 1991). In run-off experiments we did not detect any antisense RNA of the *Uida* transgene in the silenced plants (Figure 4). Moreover, this hypothesis does not fit with efficient silencing in homozygous plants but not in hemizygous plants since the ratio between sense and antisense RNA is assumed to remain the same in both types of plants. However, one cannot exclude that a minimum of antisense RNA molecules, or small complementary RNA (cRNA), found only in silenced transformants, might be required to trigger silencing. Dougherty and Parks postulate that small cRNA could be synthesized by an RNA-dependent RNA-polymerase leading to the formation of cRNA-target mRNA complexes which could be recognized and then degraded (Dougherty and Parks, 1995).
- (ii) It has also been hypothesized that the initial event that ultimately leads to silencing might be an interaction and pairing between homologous copies of a gene (Jorgensen, 1992). According to this hypothesis, haploid plants derived from H^c plants carrying a single copy of the *Uida* transgene should not be silenced. However, these plants are indeed silenced in our experiments, indicating that a single copy per cell is sufficient to trigger silencing. Therefore, this result indicates clearly that silencing of the *Uida* gene is neither due to allelic nor ectopic interaction between homologous sequences present in several T-DNAs.
- (iii) A third hypothesis is based on the fact that overexpression of the product of a gene up to a threshold level can trigger the specific degradation of this product (Dehio and Schell, 1994; Meins, 1989) and it is assumed that the target product could be the RNA. This hypothesis is supported by some studies on plant resistance mechanisms underlying pathogen-derived resistance. The theory of pathogen-derived resistance proposes that resistance genes can be derived from a patho-

gene's own genetic material. Indeed, some transgenic plants carrying a transgene homologous to a viral gene are resistant to the virus due to a cytoplasmic-based post-transcriptional silencing event which inhibits the accumulation of both the transgene RNA and the viral RNA (Lindbo *et al.*, 1993; Mueller *et al.*, 1995; Smith *et al.*, 1994). In some cases, silencing of the transgene was established prior to infection by the virus, and the plants are immune. In some other cases, silencing is triggered after infection by the virus, a process called recovery. These results suggest that the amount of RNA produced by the transgene alone might be either sufficient to reach the threshold level which triggers silencing or below this level, and that the replication of viral RNA in the early stages of the infection might be required to reach this threshold level. According to this hypothesis, the very high level of expression conferred by constructs 6b and 23b carrying a 35S promoter with a double enhancer, at the early stage of development, might be responsible for the efficient silencing found in each transformant. Conversely, the limited silencing effect found in only some transformants carrying a transgene expressed under the control of a 35S promoter with a single enhancer copy (de Carvalho *et al.*, 1992; Dehio and Schell, 1994; Hobbs *et al.*, 1993; Ingelbrecht *et al.*, 1994; Van Blokland *et al.*, 1994) might be attributed to the lower level of expression conferred by this promoter.

Plants expressing transgenes at a high level are likely to be desired by plant breeders and it is the reason why strong promoters have been looked for and used in a lot of transgenic experiments. The results reported in this paper show that a high level of expression of the *Uida* gene in transgenic tobacco plants induces a post-transcriptional silencing phenomenon, suggesting that strong promoters might, in some cases, lead to silencing of the transgene they drive. Since silencing of the *Uida* gene occurs in the progeny of the 11 tobacco plants we analysed, our genetic material provides us with a unique model system to undertake further experiments aimed at an understanding of the molecular mechanisms underlying transgenes post-transcriptional silencing.

Experimental procedures

Plant material

Seeds of tobacco (*N. tabacum* cv. Paraguay PBD6) were provided by René Delon, Institut du tabac, Bergerac, France.

Plasmids

Constructs 6b and 23b, built in pKYLX71-35S², a plant transformation vector allowing inserts to be cloned between a 35S promoter

with a duplicated enhancer and a *rbcs3'* region (Maiti *et al.*, 1993) have already been described (Elmayan and Tepfer, 1994, 1995).

Transformation

Plasmids were introduced by triparental mating into a disarmed strain of *Agrobacterium tumefaciens*, C58C1(pMP90) (Koncz and Schell, 1986). These strains were used to transform leaf discs as described by Horsch *et al.* (1985).

Genetic analysis

Primary transformants (the R_0 generation) were allowed to self-fertilize giving R_1 progeny. Surface-sterilized seeds were sown *in vitro* on medium B (Bourgin *et al.*, 1979) supplemented with 50 mg l⁻¹ kanamycin. Plants were allowed to self-fertilize, giving R_2 progeny. Homozygous individuals were identified as giving 100% kanamycin-resistant and GUS-positive progeny when tested at the seedling level.

Haploids

Anthers from young buds were surface-sterilized and incubated *in vitro* on medium B supplemented with activated 1% charcoal, 0.1 mg l⁻¹ naphthalen acetic acid and 0.2 mg l⁻¹ kinetin.

Growth conditions

Plants were grown at 23°C under conditions of a 16 h light/8 h dark photoperiod, 70% humidity and 120 µE m⁻² lighting.

Molecular analysis

Genomic DNA and RNA were extracted from leaves as previously described (Vaucheret *et al.*, 1992). Southern blots were performed with the coding sequence of the *UidA* gene or with the 35S promoter of the cauliflower mosaic virus as probes. Northern blots were performed with the coding sequence of the *UidA* gene and a tobacco mitochondrial ATPase β-subunit as probes.

Isolation of nuclei and nuclear run-off transcription assays

Nuclei were isolated from leaf tissue essentially as described by Luthe and Quatrano (1980) with three exceptions: (i) leaves were homogenized in Honda buffer containing 2.5% Triton X-100 and the nuclear pellet was dissolved in Honda without Triton; (ii) most nuclei were found in the pellet after centrifugation in a discontinuous gradient of Percoll; (iii) purified nuclei were washed and resuspended in 20 mM Hepes pH 7.2, 5 mM MgCl₂, 2 mM dithiothreitol, 30% glycerol. Nuclei from 10 g tobacco leaf tissue were resuspended in ±1 ml and stored in 0.25 ml aliquots at -80°C.

Nuclear run-off and nascent RNA hybridizations were performed according to Hagen and Guilfoyle (1985). One hundred microlitres of 3 × transcription buffer (plus heparine) were added to 200 µl nuclear suspension (10⁷ nuclei) with 250 µCi of [α-³²P]UTP (3000 Ci mmol⁻¹, NEN). Labelled RNA was resuspended in 1.5 ml hybridization buffer. Pre-hybridization, hybridization and washing were as previously described (Vaucheret *et al.*, 1992).

Single-stranded DNAs were prepared from Bluescript recombinant using the M13K07 phage helper as described by Sambrook

et al. (1989). Samples of 2 µg of the single-stranded DNAs (in 100 µl 10 × SSC) were applied to a Hybond N filter by dot blotting (Schleicher and Schuell SRC 96 minifold I dot blotter).

GUS assays

GUS assays were performed essentially as described by Jefferson (1987). Soluble proteins were extracted from leaves of mature plants grown in the greenhouse or from young seedlings grown *in vitro* as described above, in phosphate buffer (50 mM NaPO₄ pH 7, 10 mM Na₂EDTA, 40 mM β-mercaptoethanol) without detergent. Either 1 or 4 µg of soluble protein were incubated with 1 mM 4-methyl umbelliferyl-β-D-glucuronide (MUG) in a 200 µl final reaction mixture. Fluorescence was measured at 2 min intervals for 30 min with a Fluoroscan II fluorimeter (Labsystems). Fluorescence of a solution of 4-methyl umbelliferone (MU) in reaction buffer was used as a standard.

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Transgene integration into the same chromosome location can produce alleles that express at a predictable level, or alleles that are differentially silenced

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In an effort to control the variability of transgene expression in plants, we used Cre-lox mediated recombination to insert a *gus* reporter gene precisely and reproducibly into different target loci. Each integrant line chosen for analysis harbors a single copy of the transgene at the designated target site. At any given target site, nearly half of the insertions give a full spatial pattern of transgene expression. The absolute level of expression, however, showed target site dependency that varied up to 10-fold. This substantiates the view that the chromosome position can affect the level of gene expression. An unexpected finding was that nearly half of the insertions at any given target site failed to give a full spatial pattern of transgene expression. These partial patterns of expression appear to be attributable to gene silencing, as low *gus* expression correlates with DNA methylation and low transcription. The methylation is specific for the newly integrated DNA. Methylation changes are not found outside of the newly inserted DNA. Both the full and the partial expression states are meiotically heritable. The silencing of the introduced transgenes may be a stochastic event that occurs during transformation.

[Key Words: Epigenetics; gene silencing; methylation; Cre-lox; site-specific recombination]

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Different levels of gene expression from independent transformants are common with plant or animal transformation systems. The variation in transgene expression has been attributed to several factors, including differences with respect to chromosome location, copy number, and transgene construct fidelity (Matzke and Matzke 1998; Kooter et al. 1999). Similar epigenetic phenomena are also associated with nontransgenic loci, such as position-effect variegation (PEV) and paramutation (Hollick et al. 1997; Wakimoto 1998).

Chromosome locations that promote transgene expression are not well characterized but are thought to be transcriptionally active regions of euchromatin. The heterochromatin state is thought to be inaccessible to transcription factors and often correlates with cytosine hypermethylation and histone hypoacetylation (Ng and Bird 1999). A consequence of this chromosome architec-

ture is that transgenes that randomly integrate in the proximity of heterochromatin can show variable levels of expression (Dobie et al. 1996; Iglesias et al. 1997). For both transgenic and nontransgenic loci, repeat sequences have been implicated in gene silencing (Selker 1999). In *Drosophila*, plants, and mice, experiments with variable transgene repeats at the same locus support this hypothesis (Assaad et al. 1993; Sabl and Henikoff 1996; Garrick et al. 1998). A proposed role for the repeat sequences is to induce DNA-DNA interactions that lead to changes in chromatin conformation and epigenetic silencing (Matzke and Matzke 1998).

The effect of repeat sequences on transgene expression in plants has led to the term homology-dependent gene silencing (HDGS) (Kooter et al. 1999). Similar phenomena have also been reported in *Drosophila* (Pal-Bhadra et al. 1997), mice (Garrick et al. 1998), and fungi (Cogoni and Macino 1999). HDGS can occur at the level of transcription (Meyer et al. 1993; Park et al. 1996) or post-transcription (English et al. 1997). Transcriptional HDGS (TGS) is associated with homology in promoter regions (Park et al. 1996). In post-transcriptional HDGS (PTGS), mRNA transcripts are destabilized by dsRNA formed from complementary RNA (crRNA) of the silenced gene (Waterhouse et al. 1998; Grant 1999). The crRNA might be produced by endogenous promoters ad-

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jacent to the transgene or read-through transcription of inverted repeats in a complex transgene [Kooter et al. 1999]. Alternatively, aberrant transcripts may be produced that stimulate the production of cRNA by RNA-dependent RNA polymerase [Jorgensen et al. 1999]. In *Caenorhabditis elegans* and *Drosophila*, the descriptions of RNA interference (RNAi) have substantiated the view that dsRNA can act as a potent and specific inhibitor of gene expression [Sharp 1999].

In recent years, the integration of DNA through use of recombinase-mediated site-specific integration systems has been described [Fukushige and Sauer 1992; Albert et al. 1995; Golio et al. 1997]. Common features of these systems are that (1) the DNA inserts into a designated site in the genome; (2) the DNA inserts often as a single copy; and (3) the integrated molecule is usually free of rearrangements often seen with random DNA insertions. Hence, a commonly held belief is that recombinase-mediated DNA integration would produce consistent expression of the introduced DNA.

In this study, we find that a full spatial pattern of transgene expression can be obtained reproducibly through specific integration into a designated genomic site. Moreover, the absolute level of full expression is dependent on the site of integration. Contrary to expectation, however, we also recover a large percentage of integration events that give a partial spatial pattern of transgene expression. These events are not site dependent and appear to be due to a heritable form of gene silencing.

Results

Site-specific placement of a reporter gene

The experimental design was to insert pEL1 into 35S-*lox-cre* plant lines (Fig. 1A), in which 35S is the 35S RNA promoter from cauliflower mosaic virus, *cre* is the coding region for Cre recombinase, and *lox* is the 34-bp recombination site incorporated into the leader sequence of the transcript. This was achieved by transforming leaf mesophyll protoplasts, derived from 35S-*lox-cre* plants, with the pEL1 plasmid that contains *lox-hpt* and *Cp-gus*, in which *hpt* is the hygromycin phosphotransferase coding region, *gus* is the β -glucuronidase coding region, and *Cp* is the commelina yellow mottle virus promoter [Medberry et al. 1992]. Cre-mediated integration of pEL1 into the genomic 35S-*lox-cre* target confers a hygromycin resistant (*Hyg^R*) phenotype through a 35S-*lox-hpt* linkage, and abolishes *cre* transcription through displacement of the 35S promoter. Selection is not imposed on the linked *Cp-gus* reporter gene. Table 1 lists the five hemizygous 35S-*lox-cre* plant lines used as target lines.

Molecular analysis of integrant plants

A number of putative integrants from each experiment were analyzed by PCR for the presence of the expected 35S-*lox-hpt* junction. This junction was found in 77 of 81

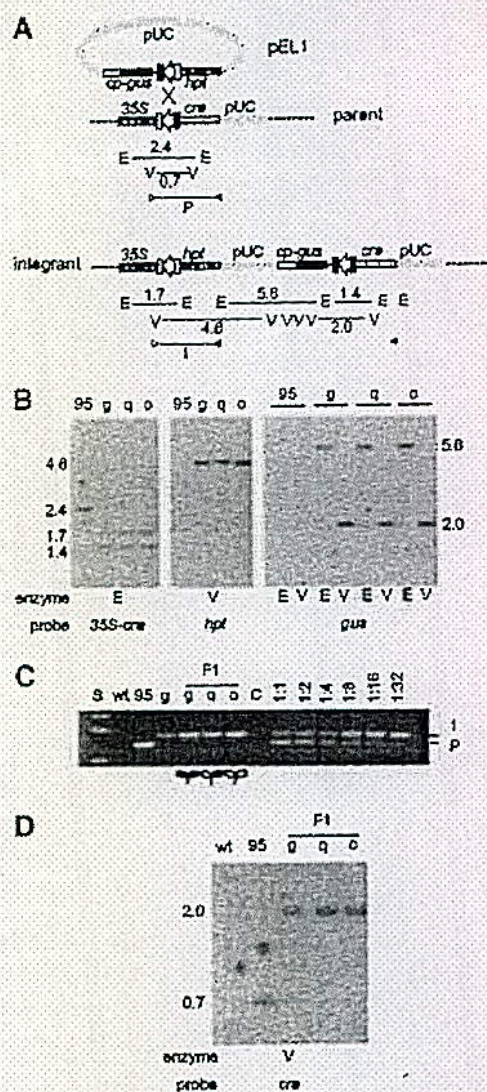


Figure 1. Precise and stable integration of transgene. (A) Schematic map of the genomic target and the structure produced through integration of pEL1. The *lox* site is depicted by an open arrow with flanking rectangles that represent the 8-bp core and the flanking 13-bp Cre-binding sites, respectively. (Solid rectangle) Mutant sequence; when present on both sides of the core sequence, the site recombinates less efficiently [Albert et al. 1995]. Not shown are *nos3'* terminator sequences of *cre*, *hpt*, and *gus*. Endonuclease sites shown: E, EcoRI; V, EcoRV. Fragment sizes, in kilobases, correspond to those next to the gels in (B,D). (B) Representative Southern blots of parent 95 and integrant T0 plants, 95g, 95q, and 95o, that show single precise copy pEL1 integration. (C) PCR products specific to parental [P] or F1 integrant [I] DNA detected from individual seedlings. Primers corresponding to 35S (open arrowhead) and *nos* terminator (solid arrowhead) are shown in A. Primer-only control lane is C. Lanes after C are reconstruction experiments of 95 and 95g DNA mixed, respectively, at ratios from 1:1 to 1:32. Expression of *gus* is depicted pictorially; solid and open leaves represent active and inactive *gus* expression, respectively. (D) Southern blot showing stable integration of pEL1 in integrant lines.

Table 1. Expression of site-specific integrants

Parent line	Transformation date	Integrand line	GUS pattern in leaves {0%–100%, n = 10}	GUS activity in leaves (pmole/min/mg, n = 3)
<i>nt35S-loxP-cre.5</i>	4/26/95	95g	100	81
		95e ^a	100	98
		95f ^a	100	67
	5/5/95	95h	100	98
		95s ^a	100	27
	5/23/95	95o	0	N.D.
		95q	10	N.D.
95p ^a		5	N.D.	
<i>nt35S-loxP-cre.11</i>	5/5/95	911e	100	533
	5/23/95	911c	30	N.D.
		911h	0	N.D.
		911f ^a	30	N.D.
		911b ^a	0	N.D.
		911d	5	N.D.
<i>nt35-loxP-cre3</i>	6/2/95	93b	10	N.D.
	6/8/95	93a	0	N.D.
		93g	100	111
		93f ^a	100	49
<i>nt35S-lox66-cre.5</i>	8/31/95	75c	5	N.D.
<i>nt35S-lox76-cre.A</i>	8/31/95	75d	45	N.D.
		85h	100	845
		85d ^a	100	743

N.D., not determined.

^aPotential sibling of another line from the same transformation.

candidates. The four plants that failed to yield a PCR product may be due to *hpt* fusion to an endogenous promoter in the genome. The 77 plants were analyzed by Southern hybridization. DNA treated with *EcoRI* was probed with a 35S-*cre* fragment. An intact left and right junction was confirmed in 73 of the 77 plants through detection of the 1.7-kb 35S-*lox-hpt* and the 1.4-kb *gus-lox-cre* band, and the loss of the 2.4-kb parental 35-*lox-cre* fragment (Fig. 1B). With *EcoRV*-treated DNA, a *gus*-specific probe hybridized to only one 2.0-kb *gus-lox-cre* fragment in 35 of the 73 plants (Fig. 1B). The presence of one or more additional bands in the other plants indicated more than a single copy of *Cp-gus* DNA, whether situated in tandem at the same site, or elsewhere in the genome.

These 35 plants were examined by *hpt*-specific hybridization to *EcoRV*-treated DNA that should detect a 4.6-kb 35S-*lox-hpt*-pUC backbone fragment (Fig. 1B). They were also examined by *gus*-specific hybridization to *EcoRI*-cleaved DNA that should reveal a 5.6-kb pUC-*Cp-gus* fragment (Fig. 1B). Fragments of the predicted sizes were found in 22 of the 35 single-copy integrant plants. Together, the detection of all five (three *EcoRI* and two *EcoRV*) overlapping fragments shows a contiguous arrangement of the pEL1 insertion as illustrated in Figure 1A. The failure to detect fragments of the expected sizes could be attributable to either of two events: (1) random integration of pEL1 followed by recombination between the two *lox* sites; in these occurrences, the 35S-*lox-hpt* and *gus-lox-cre* junctions are found, but they are not contiguous; and (2) site-specific integration of pEL1, fol-

lowed by DNA rearrangement, such as insertion or deletion between the two *lox* junctions, such that the expected-size bands are not found.

Reporter gene expression pattern

Table 1 lists the 22 plants found with a precise single-copy integration of pEL1. For the plants from the same transformation experiment, each was regenerated from a different cluster of immobilized cells. The chances are remote for any two plants to be derived from the same protoplast during the brief incubation period before imbedding the protoplasts in agarose. Nonetheless, this possibility could not be entirely ruled out. Therefore, although we do not believe this could be the case, we yield to the possibility that eight lines might be sibling clones of the other 14 representative lines (Table 1, as indicated).

The primary transformants (T0) of the lines listed in Table 1 were crossed to wild-type tobacco to generate F₁ progeny hemizygous for *Cp-gus*. Seedlings were germinated in the presence of hygromycin, and GUS enzyme activity was examined at the two-leaf stage by histochemical staining. Among the progeny of any given plant line, the staining pattern is highly consistent; hence, sibling seedlings are alike. The seedlings of different integrant lines, however, differ considerably. In four of the target sites, one to two integrant lines show the expression pattern previously reported for the *Cp* promoter, namely, expression throughout the plant in vascular tissue (Medberry et al. 1992). These lines have been desig-

nated as having a 100% value with regard to the pattern of *Cp-gus* expression. When quantified by enzyme activity, however, each locus has a distinct GUS activity level associated with the 100% *gus* pattern (Table 1). In other integrant lines, seedlings showed staining in only some tissue, as though the reporter gene has been silenced. These lines have been assigned a numeric value to indicate the approximate percentage of blue staining observed (Table 1). Figure 2 shows the typical staining patterns of three independent integrants at the same locus. The three lines 95g, 95q, and 95o have served as representative lines for detailed analysis.

In the silenced lines 95q and 95o, the blue patches of staining have sharp boundaries that define blue sectors. Typical sectors have boundaries that either divide a leaf in half (Fig. 2B) or follow a boundary between the midvein and the margin (Fig. 2E,F). The smaller sectors are still quite large (Fig. 2H,I), suggesting that the reversion of *Cp-gus* silencing occurs early during leaf development. These sectors are similar to clonal L2 sectors because they extend to the leaf margin. Less common L3-like sectors are also seen (Fig. 2G). These patterns suggest a clonal origin as they are similar to clonal sectors in tobacco (Poethig 1987).

When F_1 seedlings are grown with or without selection for *hpt* or *nptII*, the degree of GUS expression does not

change (data not shown). This indicates that the silencing phenomenon is not related to expression of linked marker genes. In F_2 hemizygous seedlings, obtained from outcross to wild type as either male or female donor, the sectoring patterns are very similar to the F_1 seedlings. The only minor difference was that in 95o, small blue sectors can be seen in ~25% of the F_2 cotyledons, whereas this was not seen with the F_1 cotyledons unless the F_1 seedlings were treated with 5-azacytidine (see below). This is an indication that there is some, but not significant, reactivation of *Cp-gus* in the F_2 generation. Siblings that show an all-blue pattern (as in 95g) were not observed among more than 100 F_2 seedlings of 95q or 95o. This can be interpreted to indicate that either cells representing the blue sectors in 95q and 95o are not progenitors of the germ line, or that the transgene is reset to the "off" state in the gamete. Because a limited number of flowers were outcrossed to produce seed, we cannot rule out the possibility that flowers with "blue" germ line sectors existed but were not selected for outcrossing. The observations made with F_2 seedlings were also found in the F_3 and F_4 generation. Therefore, the silencing phenomenon is meiotically transmissible at high fidelity, through either male or female gametes.

The integrated DNA is stable

With 95q and 95o, the mosaic patterns of expression suggest that the *Cp-gus* transgene is active or inactive, respectively, in the blue or white sectors. The possibility that this phenotype could be due to instability of the integrated DNA was a consideration. Excision may be caused, for example, by residual Cre activity. To determine if this could be the cause, we used PCR to assay for the presence of the integration (35S-*lox-hpt*) or excision (35S-*lox-cre*) junctions. Both *hpt* and *cre* transcripts use the terminator sequence of the nopaline synthase gene (*nos3'*). Primers corresponding to 35S and *nos3'* can amplify specific 35S-*lox-hpt* and 35S-*lox-cre* junction products. The sensitivity of the PCR assay is shown in Figure 1C. When genomic DNA from the parent and the T0 integrant lines were mixed in known ratios, the parental junction was visible in as low as 1 parent to 32 integrant DNA. Should the colorless phenotype observed in 95o be caused by excision of pEL1 from the genome, the assay would detect the excision-specific parental junction. Of the five representative F_1 plants of 95g, 95q, or 95o analyzed by PCR, none showed the band representing the excision junction. In contrast, all showed the band representing the integration junction.

Genomic DNA prepared from pools of 10 F_1 seedlings was also subjected to Southern analysis with a *cre*-specific probe (Fig. 1D). Although the *gus-lox-cre* integration junction was detected in all three lines, a band corresponding to the 35S-*lox-cre* excision junction was not observed. Both the PCR and the Southern data support the conclusion that the integrated DNA is stable. Therefore, the inactivity of the *Cp-gus* transgene is not attributable to excision of pEL1 from the target site.

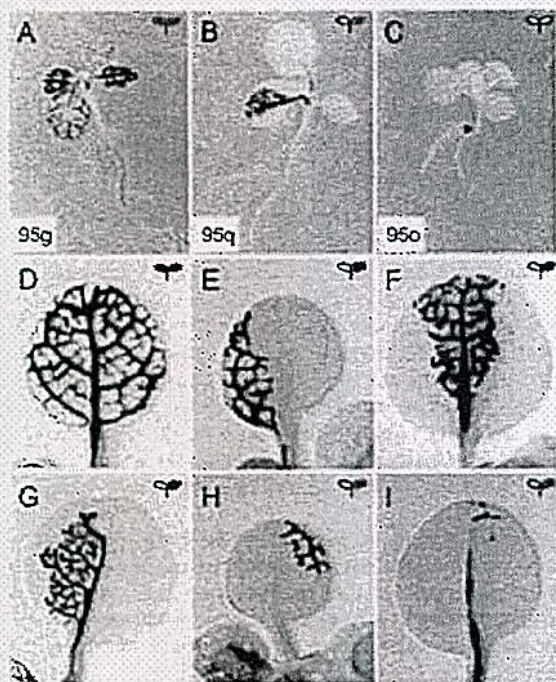


Figure 2. Independent integrants at the same locus show different spatial patterns of *gus* expression. All seedlings are stained for GUS activity at the two-leaf stage. At the 95 locus, the 95g line has a vascular specific expression characteristic of the *Cp* promoter (A,D). The 95q line shows sectoring of *gus* expression (B,E-H), and the 95o line rarely shows *gus* expression (C).

Activation and developmental maintenance of silenced transgene

The pattern of small blue sectors seen in large colorless areas but not vice versa suggested that the blue sectors represent an activation of gene expression from an initial inactive state. To examine if the blue sectors were caused by the switching on of the transgene, as opposed to switching off the transgene in the colorless sectors, we stained the F₁ seedlings at different stages after germination. The 95g line showed staining in the embryo a day after germination and the blue staining was restricted to the vascular tissue by day 11 (Fig. 3A-C3). In contrast, lines 95q and 95o did not show *Cp-gus* expression in the seedlings until days 9 to 11. This is when early leaf sectors in 95q and the first sign of blue staining near the shoot apical meristem in 95o could be seen (Fig. 3D,E). This is consistent with the interpretation that the *Cp-gus* transgene in the silenced lines was not initially expressed but was switched on later in development.

To examine if the expression pattern is maintained through development, we stained the second to last leaves before the inflorescence of fully grown plants for GUS activity. The expression pattern was similar to those seen at the two-leaf seedling stage (Fig. 3F-H). It appears that each line not only has a particular frequency at which *Cp-gus* expression switches from an off to an on state, but that the frequency is maintained through organismal development.

Steady state mRNA and reporter gene activity

We examined if the silencing of *Cp-gus* in 95o and 95q is related to the steady state level of *Cp-gus* transcripts. RNA was prepared from a pool of 10 F₁ seedlings of 95g, 95q, and 95o grown on hygromycin selection, and from the 95 parent line grown on kanamycin selection. Indeed, the level of *Cp-gus* mRNA correlated to the amount of GUS staining. 95g stained blue and *Cp-gus*

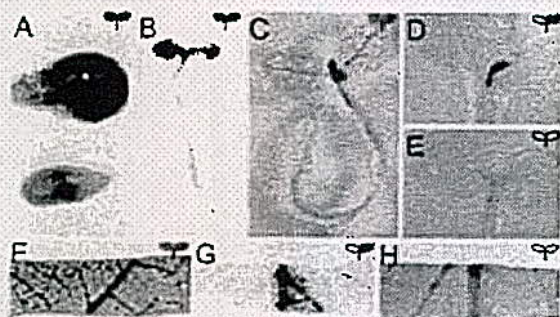


Figure 3. Expression pattern during early stages of seedling development (A-E) and in mature leaves (F-H). Expression in 95g is observed at 3 (A), 7 (B), and 11 (C) days after germination. By day 11, expression is detected in the leaves of 95q (D), but not 95o (E). Expression patterns in mature leaves (adjacent to the inflorescence) of 95g (F), 95q (G), and 95o (H) are consistent with those observed in seedlings.

Site-specific transgene expression

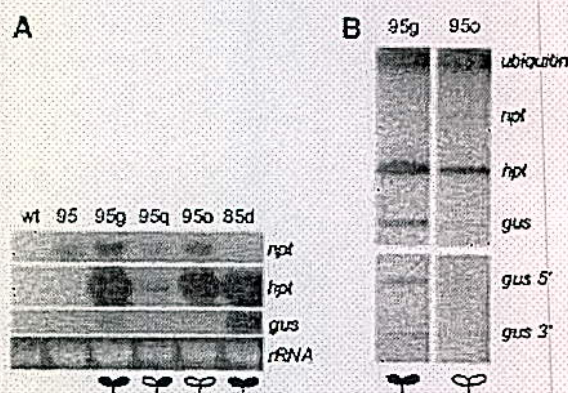


Figure 4. Gene silencing acts at the level of transcription. (A) Steady state mRNA of parental and integrant plants. Probes for *nptII*, *hpt*, and *gus* mRNA were used in Northern blots. Loading control is large ribosomal RNA stained with ethidium bromide. (B) Run-on transcription from leaf nuclei of active and inactive lines. Radioactive transcripts were used to probe target DNA specific for the full-length *ubiquitin*, *nptII*, *hpt*, and *gus* genes. In an independent experiment, 5'- and 3'-specific regions of the *gus* gene were used as target DNA (bottom). The run-on transcripts from the 95g and 95o nuclei were hybridized to the target DNA and the signals from each filter were normalized relative to the ubiquitin signal.

transcript was detected by Northern analysis, whereas, 95q and 95o showed little GUS staining and *Cp-gus* transcripts were not detected (Fig. 4A). This correlation can be extended between loci because the level of *gus* transcript for 85d is significantly higher than 95g, similar to the GUS enzyme activity measured for the two lines (Table 1).

To determine whether this observation is *Cp-gus* specific, or is characteristic of the locus, we measured the transcript levels of other transgenes of the integration locus. The *nptII* gene was used as the selectable marker for the first parental {35S-*lox-cre*} transgene. It therefore is present in both the parent and integrant lines. The *hpt* and *gus* genes are present only in the integrant lines as they were introduced through pEL1. The transcript levels of *nptII* are rather similar among the integrant and parent lines, whereas large differences are observed for *hpt* (Fig. 4A). The steady state level of *hpt* mRNA in 95q is lower than in 95g, consistent with that observed for *Cp-gus*. However, in the 95o line, the *hpt* transcript level is similar to that found in 95g, whereas the *Cp-gus* transcript is not detectable. Therefore, the data for *nptII* and *hpt* expression levels are inconsistent with a locus-wide silencing effect.

Nuclear run-on experiments were conducted to examine the rate of transcription (Fig. 4B). Compared with 95g, the transcription initiation rates of the *gus* and *hpt* genes in 95o are reduced by 10- and 3-fold, respectively, when compared with the ubiquitin gene standard. There is relatively low transcription from *nptII*, but the two lines appear to show similar rates. This supports an interpretation that the silencing phenomenon observed is attributable to an inhibition of transcription.

Gene dosage and allelic interactions

Genetic interactions between alleles of a locus that show HDGS have been reported [English et al. 1997; Vaucheret et al. 1997]. In those instances, a positive gene dosage from an allelic or a nonallelic locus often correlates inversely with gene activity. In addition, it is common in HDGS for a silencing locus to act in trans to inactivate an otherwise actively expressing homologous gene [English et al. 1997].

When *gus* staining patterns of the self-fertilized T1 seedlings were examined, a third of the Hyg^R 95g seedlings showed a more rapid rate of staining. Because this is the proportion of Hyg^R seedlings expected to be homozygous for *Cp-gus*, this suggests that a higher level of expression may be attributable to the higher transgene dosage. When hemizygous 95g was crossed to hemizygous 95o, the Hyg^R progeny pool should be comprised of the genotypes 95g/+, 95o/+, and 95g/95o in a ratio of 1:1:1. When the Hyg^R progeny were analyzed, 85% ($n = 15$) had the all-blue phenotype characteristic of 95g. Similarly, in the cross between hemizygous 95g and hemizygous 95q, 76% ($n = 49$) had the 95g phenotype. A few of the blue plants do have paler leaves, so we cannot entirely rule out that a limited interaction can occur between the alleles. To examine for possible nonallelic interactions, we also crossed hemizygous 95g to hemizygous 911d. Of the Hyg^R progeny, 60% ($n = 20$) showed the all-blue phenotype. In sum, the data do not suggest that the silenced alleles can exert a dominant negative effect on the nonsilenced alleles.

Cp-gus methylation correlates with silencing

Methylation sensitive and insensitive enzymes were used to probe the extent of DNA methylation in active and silenced *Cp-gus* transgenes at the different loci. The genomic DNA was first cleaved with methylation insensitive enzymes *EcoRV* and *EcoRI* into defined 5' (880-bp) and 3' (1080-bp) fragments, and then further cleaved with methylation-sensitive enzymes *HpaII*, *Sau3A*, or *BglII* (Fig. 5). A methylation signature of small bands is detected by Southern blotting with either the *Cp-gus* 5' fragment or a *gus* 3' fragment. Analysis of the different size bands infers the methylation state of specific cytosines in the target sequence.

The 95o line is almost fully methylated at the restriction sites we assayed including sites in the promoter, coding region, and the 3' untranslated region (UTR) (Figs. 5,6). Very few of the sites are methylated in the 95g line. The 95q line, with sectors of GUS expression, was partially methylated in the promoter and the coding region but was fully methylated in the 3' UTR. Analysis of the promoter and coding region of *Cp-gus* was extended to the 93 and 911 loci. Lines that show active (93g and 911c) or relatively inactive (93b and 911b) *Cp-gus* expression also have a correlation between cytosine methylation in *Cp-gus* and low GUS activity (Fig. 5). For the 911 locus, hypermethylation in the *Cp* promoter and the *gus* coding region was detected in the 911b silenced allele. At the 93

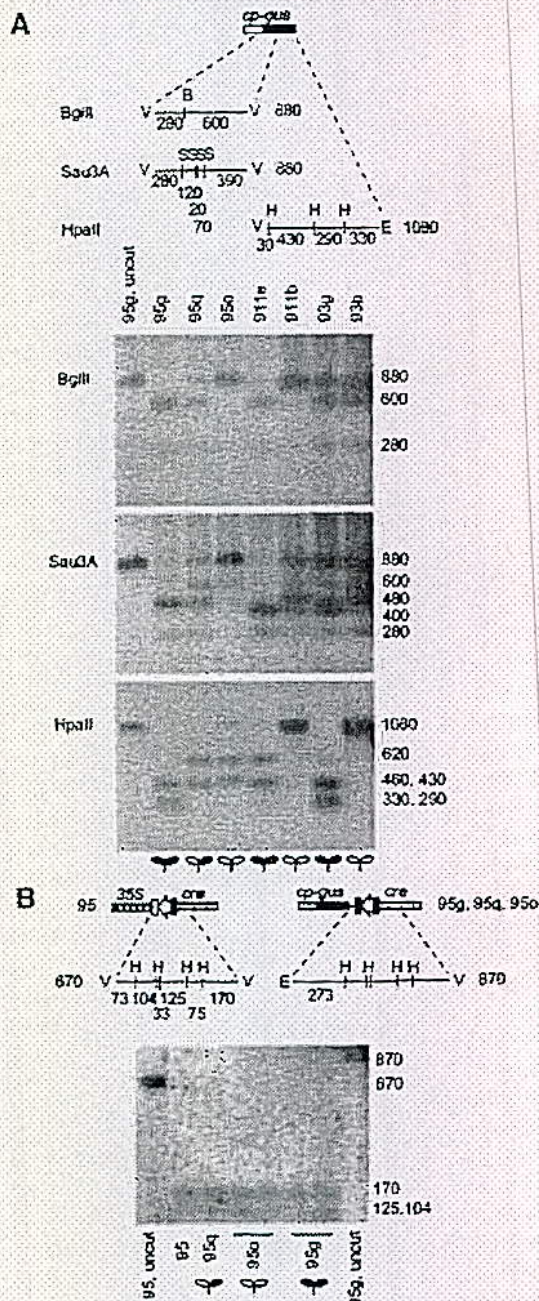


Figure 5. Methylation analysis of active and inactive integrant lines at the 95, 93, and 911 target sites. (A) Predicted DNA fragments, in basepairs, of *Cp-gus* from cleavage with *EcoRV* and *EcoRI* and either *BglII*, *Sau3A*, or *HpaII*. Southern blots reveal the fragments hybridizing to a *Cp-gus* 5' fragment probe (for *BglII* and *Sau3A* panels) or a *gus* 3' fragment probe (for *HpaII* panel). Leftmost control lanes were not cleaved with *BglII*, *Sau3A*, or *HpaII*. Numbers next to autoradiograms correspond to sizes of predicted fragments (bp). Endonuclease sites shown are as follows: B, *BglII*; E, *EcoRI*; H, *HpaII*; S, *Sau3A*; V, *EcoRV*. (B) Predicted DNA fragments, in basepairs, of *lox-cre* after cleavage with *EcoRV*, *EcoRI*, and *HpaII* and detected by hybridization to a *cre* probe. Outermost control lanes were not cleaved with *HpaII*.

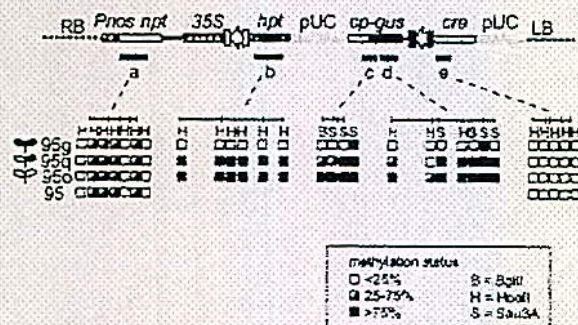


Figure 6. Methylation patterns in parental and integrant lines. Summary of methylation analysis at specific restriction sites in 95 and integrant lines 95g, 95q, and 95o. Specific probes (a, b, c, d, e) represent *nptII*, *hpt*, *Cp-gus* 5' fragment, *gus* 3' fragment and *cre*, respectively. (RB and LB) the positions of the T-DNA right border and left border, respectively. Endonuclease sites are as follows: S, *Sau3A*; B, *BglII*; H, *HpaII*.

locus, the *Cp* promoter and 5' region of the *gus* gene are partially methylated in both 93 alleles, but differences were detected in the middle of the *gus* gene. In this region, the silenced allele 93b showed complete methylation whereas the expressed allele 93g is unmethylated. The analysis of three different loci all correlate hypermethylation to a silenced state, especially at the middle of the *gus* coding region.

Methylation is restricted to integrant DNA

To determine the extent of methylation beyond *Cp-gus*, we used probes for *nptII*, *hpt*, and *cre* to compare the methylation state throughout the 95 locus. Despite a positive correlation between hypermethylation and *Cp-gus* silencing, the *hpt* steady state mRNA data are inconsistent. When *hpt* was used to probe the methylation of *HpaII* restriction sites in 95q and 95o, those sites were fully methylated (Fig. 6). As the plants are *Hyg^R*, both lines express *hpt*, but the 95o line accumulates a much higher level of *hpt* steady state mRNA (Fig. 4A). A lower level of methylation might have been expected for the 95o *hpt* gene, but there are examples of genes that express normally despite high levels of methylation (Luff et al. 1999).

For the *nptII* and *cre* probes, we also analyzed the 95 parental line to determine the level of methylation at the locus before transgene integration. Few of the *HpaII* restriction sites were methylated in the *cre* (Figs. 5,6) or *nptII* (Fig. 6) gene in either the parental or the integrant lines. This suggests that the low level of methylation in the 95 parent is maintained in the new transgenic lines, despite the variability of methylation seen among the newly introduced DNA.

Chromatin and methylation inhibitors partially activate expression

To test whether silenced states can reverse by manipulating the extent of DNA methylation, we treated the

silenced 95o line with 5-azacytidine. This drug causes the genome to become hypomethylated. Plants were grown on a nontoxic concentration of 5-azacytidine and then stained for GUS activity. Approximately 18% of the total leaf area stained for GUS activity in sectoring patterns similar to 95q (Fig. 7B-E). This suggests that by hypomethylating the genome it is possible to reactivate *Cp-gus* from its silenced state. We also tested sodium butyrate, a deacetylase inhibitor, which perturbs the formation of chromatin. This inhibitor was also found to reactivate the *Cp-gus*, although not as well as 5-azacytidine. Again, sectoring was seen to increase and the area of leaves that stained was approximately 4% (Fig. 7F-H). It is possible that the reactivation of *Cp-gus* is not efficient due to insufficient application of the drugs. Higher concentrations, however, could not be used due to their toxicity to the tobacco seedlings. Although we did not see complete reactivation of *Cp-gus*, these experiments suggest that cytosine methylation and histone acetylation have a role in the silencing phenomenon. This substantiates the view that the correlation of methylation with gene silencing is not coincidental.

Discussion

This study was designed to test two hypotheses. First, we sought to determine whether placing a transgene into the same genomic target site would yield a reproducible level of expression. Second, we wanted to know if each target site might confer a distinct level of expression for independent integrant lines. The experimental strategy was designed to remove many of the variables that have been implicated to cause gene silencing in other sys-

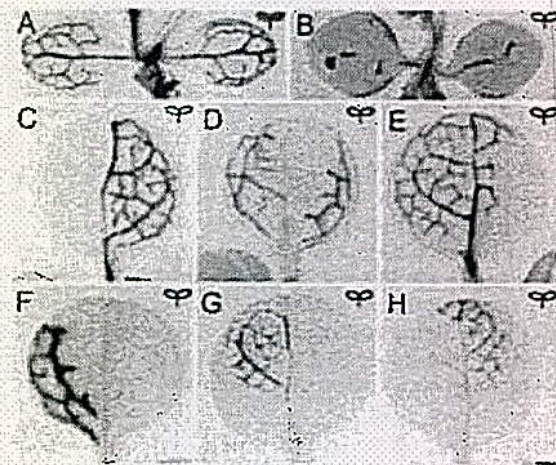


Figure 7. Reactivation of *Cp-gus* expression in 95o by DNA methylation and acetylation inhibitors. (A) GUS activity from untreated cotyledons from the active line 95g. (B) Na butyrate-treated cotyledons from the inactive line 95o shows slight reactivation of expression. (C-H) Leaves of 95o treated with 5-azacytidine (C-E) or Na butyrate (F-H). Seedlings were grown on agar with 0.6 mM Na butyrate or 19 mg/L 5-azacytidine.

tems, namely, the generation of inverted repeats, multiple transgene copies, and variable chromosome locations.

The data are consistent with both hypotheses. In three target loci in which two or more full spatial pattern lines were found (loci 95, 93, and 85), their levels of *Cp-gus* expression, as measured by enzyme activity, are quite similar to each other. In four target loci in which at least one full spatial pattern line was found, the *Cp-gus* expression is distinct for each target loci. Full expression at the 85 locus is close to 10-fold higher than that of the 95 locus. Therefore, the chromosome position is important for the level of transgene expression, and that reproducible insertion into the same chromosome position is capable of generating the same expression level.

What is most surprising is that gene silencing can also be obtained at a high frequency, resulting in a mosaic pattern of gene expression. This was not reported in other Cre-lox site-specific targeting experiments using mammalian cell lines (Fukushige and Sauer 1992; Feng et al. 1999). These groups assayed a marker gene at two and six genomic targets, respectively, and reported that transgene expression is reproducible at each site. Although the silencing effect we observed could reflect differences between plants and animals, it is also quite possible that silencing mechanisms in cultured cell lines behave quite differently from those in a differentiated whole organism. Several studies in mice have targeted transgenes to either the *hprt* or *pim-1* loci by homologous recombination (Jasin et al. 1996). In some cases, variable expression of the transgene was observed, and this was postulated to be a position effect attributable to promoter occlusion. Further studies in transgenic mice have led to targeted integration with more stable expression, although few integration events have been studied in detail and variable expression from independent insertions remains a possibility (Jasin et al. 1996; Wallace et al. 2000).

Genetic changes unlikely

The behavior of the silenced loci is inconsistent with a simple mutation theory. If a mutation had occurred to render the gene nonfunctional, then one would have to postulate that each silenced line has a distinct reversion rate. For example, the 95q and 95o lines would each have a unique and heritable reversion rate that occurs at a particular time during leaf development. A temporally nonrandom reversion rate that is also heritable places a severe limitation on a genetic change theory.

Cp-gus silencing compared with other examples of HDGS

The silencing of the *Cp-gus* reporter gene shares some of the characteristics of HDGS (Kooter et al. 1999). It correlates with hypermethylation of the transgene, there is meiotic transmission of the phenotype, and clonal sectors appear stochastically reminiscent of metastable al-

leles. Both PTGS and TGS can produce metastable alleles (Meyer et al. 1993; Davies et al. 1997), but in the case of PTGS, the sectors are nonclonal (Jorgensen 1995). In some cases, a silenced nonclonal sector can be transmitted throughout the plant by a systemic mechanism (Palauqui et al. 1997; Voinnet et al. 1998). Because the phenomenon we describe has clonal sectors, and that the nuclear run-on experiments showed reduced *Cp-gus* transcription, it is most likely TGS.

Nearly all TGS phenomena are attributed to an interaction between gene copies. Nonetheless, there are a few examples of a TGS-like phenomenon with single-copy transgenes. In one instance, the silencing of a single-copy transgene was attributed to its recognition as a nonplant sequence (Meyer and Heidmann 1994). In a second example, the transgene silencing was attributed to a position effect that influences chromatin structure (Iglesias et al. 1997).

The transgene in this study is a single hemizygous copy maintained by outcrossing to wild-type plants and thus would fall into this rare class of single-copy TGS.

Methylation confined to integrant DNA

The nonplant sequence model proposes a cellular scanning mechanism that recognizes foreign motifs in a transgene such as GC-rich, bacterial-like or unusual transgene/host boundary sequences (Matzke and Matzke 1998). This model is difficult to reconcile with our data. The *Cp-gus* is inserted precisely into a target transgene; therefore, no new plant/plasmid junctions are created from the integration. Moreover, only the new transgene DNA, and not the old transgene DNA, is subjected to hypermethylation. This is particularly striking when the silenced alleles of *Cp-gus* are highly methylated, and yet the adjacent *cre* gene does not change in methylation state. If a coordinated scanning mechanism can identify foreign DNA, either it does not achieve this by recognizing nonplant sequences or the nonplant sequences in our lox target lines are masked, perhaps by chromatin.

The position-effect model is similar to the stochastic silencing of PEV that is attributable either to the spread of heterochromatin into the gene or to the locus being looped into a heterochromatic complex (Wakimoto 1998). Either effect can silence other genes in the region as they are also incorporated into the heterochromatin (Dorer and Henikoff 1997). This model does not adequately explain the variegation we observe in our tobacco loci for two reasons. First, a spread of heterochromatin into the transgene should correlate with methylation across the 95 locus. Contrary to expectation, we observe an island of methylation that is specific to the integrant DNA. The possibility that integrant DNA has looped into a heterochromatic complex would also be unlikely because *hpt* and *nptII* are expressed. Note that *nptII* had not been used for selection after pEL1 integration. Second, because we observed gene silencing in most loci, the chromosome position-induced effect would have to be nearly ubiquitous throughout the genome.

Silenced alleles do not trans-inactivate

Epigenetic *trans*-inactivation and paramutation have been described in *Drosophila* and in maize (Dorer and Henikoff 1997; Hollick et al. 1997). In these instances, a silencer allele converts an active homolog to an inactive state, and the modification is meiotically stable after segregation of the *trans*-acting allele. Transgene analysis in both *Drosophila* and plants reveals that some silenced transgenes also behave in this manner. The transmission of the silenced state from one allele to another is believed to be facilitated by DNA pairing or chromatin interaction and are promoted by repetitive DNA (Hollick et al. 1997; Kooter et al. 1999). Most of the silencer alleles have gene duplications or complex structures (Jakowitsch et al. 1999; Luff et al. 1999). For the rare single-copy silencers, nearby repetitive sequences have been suggested to be the cause. In the case of the single-copy *Pl'-mah* gene in maize, its paramutagenic effect may be attributable to adjacent sequences of a transposable element (Hollick et al. 1997). Similarly, for the single-copy maize *A1* transgene in petunia (Meyer et al. 1993), its ability to *trans*-inactivate may be explained by adjacent genomic elements. In this study, the silenced *Cp-gus* alleles 95o, 95q, or 911d did not affect the expression of the active 95g allele. This may suggest insufficient repetitive DNA elements that can cause silencing either adjacent to, or within, the transgenic loci.

The *nos-nptII*, *35S-hpt*, *Cp-gus*, and *cre* genes are unique in the integrant loci and the tobacco genome; however, there are some direct repeat sequences in the locus. These include four *nos* terminator sequences 3' of the *nptII*, *hpt*, *gus*, and *cre* genes and two pUC sequences, one in the integrant pEL1 plasmid and one 3' of the *cre* gene in the target site. Potentially, these repetitive DNA might account for the silencing phenomenon we observe; however, there are several reasons why we do not consider this a likely interpretation. First, we do not see evidence for *trans*-inactivation that is commonly attributed to DNA-DNA interactions. Second, methylation and silencing induced by somatic pairing is usually restricted to sequences with homology (Luff et al. 1999; Melquist et al. 1999). In this study, the *gus* and *hpt* genes can be highly methylated despite being a single-copy sequence. If the *nos* or pUC repeats can induce methylation in *hpt* and *gus*, then *cre* should also be susceptible. However, this is not the case. Third, if the *nos* and pUC repeats can induce silencing of *Cp-gus*, we would expect the *Cp-gus* lines that show full expression to be susceptible to self-induced silencing. However, somatic or germinal silencing of the alleles that show full spatial patterns of expression was not observed. Therefore, all of the above suggest that the direct repeats do not play a key role in gene silencing at these loci.

The *cre* DNA is not associated with gene silencing

The strategy used in this study relies on integrating pEL1 between 35S and *cre*. We found no evidence of excision of the integrated molecule, which is consistent with the

intended inactivation of *cre* transcription. Nonetheless, the presence of the *cre* coding region at the target construct is a common denominator in the integrant lines. The possibility of a *cre* effect on gene silencing, however, has been ruled out in a different set of integration experiments. One aspect of these experiments results in pEL1 integration into a number of 35S-*lox-luc* target lines, in which *luc* is the firefly luciferase gene (H. Albert et al., in prep.). The Cre recombinase was provided by transient expression, and *cre* DNA was not incorporated into the genome. F₁ hemizygous seedlings with a single, precise integration of pEL1 at the target site likewise showed patterns of gene silencing as those described in Table 1. Therefore, the presence of *cre* DNA is not associated with the *Cp-gus* silencing phenomenon.

Silenced alleles meiotically heritable

Cubas et al. (1999) described a natural epiallele affecting the flower morphology of toadflax that is stable over many generations. The methylation pattern is not fixed because some revertant flowers are detected and these are shown to have lost their methylation. The role of methylation during development is still poorly understood, but it is clear that methylation patterns can become stable. The silenced *Cp-gus* alleles are relatively stable and can be maintained at least to the F₄ generations. Occasionally, we see reversions at the somatic level for each of our silenced alleles. The frequency of reversion is comparable to epialleles of the *SUPERMAN* and *PAI* genes in *Arabidopsis* (Jacobsen 1999).

Possible mechanisms for specific methylation of new DNA

To account for the silencing of *Cp-gus*, a mechanism must be compatible with three observations. First, the silencing is independent of the integration locus. Second, the degree of methylation differs among independent transformation events. Third, the new methylation states are confined to the newly introduced DNA.

We propose that there is an initial imprinting step for newly introduced DNA. This imprinting is transient and inefficient, leading to a variety of methylation patterns on the new DNA. Because active alleles are not susceptible to silencing, we believe the methyltransferase activity that maintains a methylation state during development and meiosis is distinct from this initial imprinting process. We present several models that fit the criteria, although our data do not distinguish among them.

Model 1 The imprinting activity may be induced by environmental stress associated with tissue culture growth. Some somaclonal variants derived from tissue culture are associated with changes in DNA methylation (Phillips et al. 1994). The integrant DNA may be more susceptible to this methyltransferase activity before it incorporates into chromatin.

Model 2 Extrachromosomal DNA may mimic viral DNA and induce a defense response during transformation. The DNA virus CaMV can be recognized and methylated in tobacco cells as an extrachromosomal entity [Tang and Leisner 1998]. Similarly, some plasmid molecules may be methylated before integration, resulting in a silenced allele.

Model 3 DNA cruciform junctions, similar to those formed during transposon and site-specific recombination, enhance animal DNA methyltransferase activity. During DNA recombination into the genome, the integration intermediate may be a more susceptible target for de novo methylation [Bestor and Tycko 1996].

Model 4 A fourth scenario is an RNA-DNA interaction instigated by transient expression from the episomal plasmid. Transiently produced RNA may induce de novo methylation of plasmid DNA that has integrated into the genome. This mechanism is similar to a model of RNA-induced TGS proposed by Wassenecker and Pelissier [1998] and can account for both the specificity and transient period of the imprinting activity. Further evidence for such a mechanism is that a 35S-*Pro* transgene producing RNA homologous to the *nos* promoter can specifically silence another gene driven by the *nos* promoter [Mette et al. 1999].

To conclude, our results show that a transgene can be delivered into a specific chromosome position and be expressed at a predicted level. It seems likely that target sites can be found such that the chromosomal context will yield a higher and more consistent level of transgene expression. However, regardless of the chromosome position, there is the probability of methylation-associated silencing. A future challenge is to design a transformation strategy that is not susceptible to transgene-specific imprinting during the transformation process.

Materials and methods

Plant transformation

Plasmid pEL1 was made by ligating a *Sa*I to *Bam*HI *Cp-gus* fragment from pCoYMV-GUS [Medberry et al. 1992] to *Eco*RI- and *Bam*HI-cleaved pED112 [Albert et al. 1995], with *S*all and *Eco*RI ends filled by DNA polymerase I [Klenow fragment]. Polyethylene glycol-mediated transformation of tobacco protoplasts and the four target lines *nt35S-loxP-cre.5*, *nt35S-loxP-cre.3*, *nt35S-lox66-cre.5*, and *nt35S-lox76-cre.A* have been described previously [Albert et al. 1995]. A new line used in this study, *nt35S-loxP-cre.11*, likewise contains a single copy of the target site (data not shown).

Plant analyses

Tobacco seedlings were grown on 1/2 Murashige-Skoog medium with 3% sucrose, and when necessary with either 20 µg/mL hygromycin or 50 µg/mL kanamycin. The seedlings were selected at the two-leaf stage and stained for GUS enzyme activity in 2.5 mg/mL X-glucuronidase, 100 mM phosphate buffer (pH 7.4), 1.0 mM EDTA, 10% Triton X-100, and 5 mM ferri/ferrocyanide for 24 h. Tissues were then cleared in 70% ethanol

and photographed using a Nikon SMZ-2T microscope. Gus activity was assayed using standard procedures.

Primers for PCR were as follows: 35S, 5'-GTTTCATTCA TTGGAGAGG-3' and *nos* 3', 5'-CAAATGTTTGAACGATC TGC-3'. Probes for hybridization were amplified by PCR and purified from agarose with the GeneClean II kit (BIO101). Sources for the probes were *nptII* from pBIN19; *hpt*, *Cp-gus* 5' fragment, and *gus* 3' fragment from pEL1; and 35S-*cre* and *cre* 5' fragment from pMM23. Primers used were as follows: *nptII*, 5'-ATGATTGAACAAGATGG-3' and 5'-CTCGATGCGATG TTTCGCTT-3' or 5'-CGCTCAGAAGAAGTCCA-3'; *hpt*, 5'-ACCGCGACGTCTGTGAGAA-3' and 5'-CAAATGTTTGA ACGATCTGC-3'; *Cp-gus* 5' fragment, 5'-GTCATCAATGA CATCATCAC-3' and 5'-TGATATCGTCCACCCAG-3'; *gus* 3' fragment, 5'-GGTCAGTGGCAGTGAAG-3' and 5'-TGTAGC CGACGATGGTG-3'; 35S-*cre*, 5'-AGATCACCATTGGACGAC 3' and 5'-CTAATCGCCATCTCCAGCA-3'; *cre* 5' fragment, 5'-ATGTCCAATTACTGACCGT-3' and 5'-CTTTAACCCT GATCCTG-3'.

Standard conditions were used for PCR and hybridization analyses. Tobacco genomic DNA was isolated from two leaf seedlings or from the top leaves of a mature tobacco plant. Ten micrograms of DNA was cleaved with endonucleases, separated by agarose electrophoresis, and transferred to a positively charged nylon membrane. Membranes were hybridized to probes made radioactive by the random primed DNA labeling kit (Boehringer Mannheim) at 65°C in a rotating hybridization oven as recommended by the manufacturer (250 mM NaPO₄ at pH 7.4, 1 mM EDTA, 7% SDS). For methylation analysis, the DNA fragments were separated by a 1.5% agarose gel. RNA was isolated by grinding plant tissue with liquid nitrogen. Extraction buffer (100 mM Tris-HCl at pH 8.5, 100 mM NaCl, 20 mM EDTA, 1% w/v sarkosyl) was added, and the solution was cleaned with phenol/chloroform extraction, ethanol precipitation, and resuspended in water. An equal volume of 4 M LiCl was added followed by an incubation on ice for 3 h and centrifuged. The pellet containing the RNA was washed with 70% ethanol and resuspended in water. Five micrograms of RNA was separated by a 1.3% agarose gel with 0.25 M formaldehyde and 1× MOPS buffer (pH 7.0). The RNA was blotted to nylon membrane and hybridized using the same conditions as for Southern analysis.

Run-on transcription

Nuclei were isolated from tobacco leaves [Vorst et al. 1993] except for the following modifications: (1) Dark-grown plants were used to reduce starch granules; (2) grinding of the leaf tissue in liquid nitrogen was omitted; (3) nuclei were passed through a discontinuous Percoll gradient after the first wash at 3000g for 20 min. The nuclei that partitioned in the 80% Percoll and sucrose cushion were used. Run-on experiments were conducted with 5×10^6 nuclei in 100 µL of buffer containing 25 mM Tris-HCl (pH 7.5), 75 mM NH₄Cl, 7.5 mM MgCl₂, 0.5 mM each of ATP, CTP, and GTP, 1 mM DTT, 40 U RNA guard, and 150 µCi UTP (3000 Ci/mmol). The reaction was mixed every 5 min for a total incubation of 30 min at 30°C. DNA was then degraded for 10 min at 37°C with 10 U RNase free DNase, followed by 15 min at 37°C with 10 µg of proteinase K, 1% SDS, 10 mM EDTA (pH 8.0). Radioactive transcripts were purified twice by phenol:chloroform (1:1) extraction, precipitated with ethanol, and resuspended in hybridization buffer.

Hybridization of radioactive nuclear run-on transcripts to test DNA on slot blot filters was performed as described [Vorst et al. 1993], and the signals were quantified using a Molecular Dynamics STORM 860 detection system. The test DNA was pro-

duced by PCR using the same primers described above, and the ubiquitin gene DNA was made using PCR primers (Universal -20), (Reverse Primer), from the plasmid pSKUB, a gift from P. Quail. The test DNA was quantified by UV spectrophotometry, denatured in 500 mM NaOH, 1.5 M NaCl for 10 min at 37°C and 1 min at 100°C, and applied to nylon membrane as 2 µg per slot.

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