

**SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO
DIRECCIÓN DE NUEVAS CREACIONES**

2020
Bogotá, D.C.,

Abogado
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SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO



No. 09-124408- -00006-0000

Fecha: 2012-09-19 17:27:14 Dep. 2020 DIR.NUEVASCR
Tra. 11 PATENTEIYII Eve: 378 FASENACIONALI
Act. 519 TRASLADOINFORME Folios: 12

ASUNTO	Radicación	09-124408
	Trámite	011
	Evento	378
	Actuación	519
	Oficio	16713
	Folios	012

Patente de Invención	☒	Patente de Modelo de Utilidad	☐
Vía Nacional	☒	Cap. I	☒
Vía PCT (fase nacional)	☒	Cap. II	☐

No. Solicitud Internacional PCT/EP2008/002667
Fecha de Presentación Internacional 31/Marzo/2008

Como resultado del Examen de Fondo realizado, con fundamento en el Artículo 45 de la Decisión 486 de la Comisión de la Comunidad Andina, se comunica:

1. Documentos estudiados

Descripción:	Folios 62 a 106	04/Noviembre/2009
Reivindicaciones:	Folios 121 a 132	04/Noviembre/2009
Dibujos:	Folios 134 a 136	04/Noviembre/2009
Prioridad:	EP 07075263,9	05/Abril/2007
	US 60/923,142	12/Abril/2007
	EP 07075299,3	23/Abril/2007

2. Análisis de la Prioridad

Se acepta la reivindicación de Prioridad porque esta solicitud fue presentada dentro del plazo establecido (Art. 9 D 486), y su contenido técnico corresponde con el de la prioridad reivindicada.

3. Objeto de la invención

Título de la solicitud: "Plantas de algodón con tolerancia a insectos y métodos para identificarlas"

El problema planteado en esta solicitud consiste en suministrar plantas tolerantes a insectos.

Para resolver este problema técnico la solicitud en estudio proporciona plantas que contienen un gen que codifica una proteína que le confiere tolerancia a insectos,

Expediente 09-124408 Primer Artículo 45

además de los métodos para identificar la presencia de material vegetal que comprende específicamente el evento de transformación.

El objeto de la presente Solicitud se relaciona con la Clasificación Internacional de Patentes (CIP): C12N15/82; A01H:5/00

4. No es una invención (Art 15 D 486 literales (b) – (f))

El objeto de las reivindicaciones 5, 8, 9 y 10 no se considera una invención, de acuerdo con el Art citado.

5. Excepción a la Patentabilidad (Art 20 D 486 literales (a) – (d))

El objeto de las reivindicaciones 1 a 4, 6 y 7 no es patentable de acuerdo con el Art citado.

6. De la Solicitud de Patente

Título

Se sugiere al solicitante modificar el título ya que las plantas no son objeto de patente según el Art. 20 literal c.

Reivindicaciones (Art 30 D 486, Art 22 PCT)

El capítulo en general se refiere al “evento de EE-GH5”, el cual es un nombre propio con el cual se designó una planta transformada, pero que no reemplaza las características técnicas en particular que deben definir la invención, dichas características técnicas se refieren a las secuencias completas de los genes que generan la resistencia.

El solicitante debe modificar las reivindicaciones 14, 15 y 16 ya que presentan una falsa dependencia con las reivindicaciones 2, 4 y 5 respectivamente.

Las reivindicaciones 27, 28, 31, 33 y 34 deben modificarse para no incluir porcentajes de homología en las secuencias de las sondas reclamadas ya que este tipo de definición no permite establecer la secuencia nucleótido exacta a la que se refiere.

El método de la reivindicación 11 no es claro porque no define claramente los pasos secuenciales para que una persona versada en la materia pueda reproducirlo (un método tiene mas de un paso), la caracterización del método se limita a un solo paso que comprende “la detección de una región específica para EE-GH5 con un cebador o una sonda específica, que reconoce específicamente la región flanqueadora 5' o 3' de EE-GH5”, sin embargo no define cual es la SEQ ID NO que caracteriza el evento EE-GH5, ni de los cebadores utilizados para su detección.

Las mismas observaciones son válidas para las reivindicaciones 12 a 14, 39, 44 en las cuales el solicitante además debe restringir la estructura de los cebadores a una longitud y secuencia específica, que tenga sustento en la descripción y que sea funcional para el método reclamado.

El método de la reivindicación 16 no es claro porque se caracteriza porque el método comprende amplificar un fragmento de aproximadamente 369 bp usando el protocolo de identificación de EE-GH5”, sin embargo no especifica cual es dicho protocolo, se recuerda al solicitante que debe caracterizar tanto el método como el protocolo por la serie de pasos secuenciales necesarios para realizar la invención.

Las reivindicaciones 17 a 19, no son claras por que no identifican los componentes de dicho kit de manera inequívoca, el solicitante debe restringir la estructura de los cebadores a una longitud y secuencia específica, que tenga sustento en la descripción y que sea funcional para el kit reclamado.

De la misma manera, para las reivindicaciones 21 a 23 el solicitante debe restringir la estructura de los cebadores a una longitud y secuencia específica, que tenga sustento en la descripción y que sea funcional.

El método de la reivindicación 26 debe especificar la SEQ ID NO del ácido nucleico y la sonda involucrada, además debe caracterizar el tipo de muestras biológicas en las que se puede aplicar el método, estas mismas observaciones aplican para las reivindicaciones 27 y 28.

La reivindicación 29 tal como está escrita reclama la misma materia que la reivindicación 17, un kit de cebadores que sonda específica es capaz de hibridarse específicamente con una región específica de EE-GH5. El solicitante debe eliminar la materia repetida o indicar la diferencia entre las dos reivindicaciones, indicando la estructura de los cebadores involucrados. Las mismas observaciones aplican para las reivindicaciones 30, 32, 33, 36, 37, 38.

Se sugiere al solicitante modifique el capítulo reivindicatorio de modo que las SEQ ID NO de los cebadores reclamados tengan una SEQ ID NO propia, y no que sean citadas a través de SEQ ID NO más largas que las contienen.

La reivindicación 41 debe ser modificada para eliminar la palabra “similar” que no permite definir claramente la materia reclamada, el solicitante debe proporcionar la estructura exacta de la molécula de ácido nucleico aislado. La misma observación aplica para las reivindicaciones 42 a 43.

7. Determinación del Estado de la Técnica

El estado de la técnica se determinó con base en la fecha de presentación de la solicitud de prioridad: 11/Junio/2007, y se encontraron los siguientes documentos que anticipan el objeto de esta solicitud:

Ítem	Documento	Título	Fecha de Publicación*
D1	WO03093484	Insect resistant plants and methods for making the same	13/noviembre/2003
D2	The Plant Journal 8(3), 457-463.	Efficient isolation and mapping of T-DNA insert junctions by thermal PCR Arabidopsis thaliana asymmetric interlaced.	1995

D1 divulga compuestos insecticidas que se derivan de *Bacillus thuringiensis*. Estas nuevas proteínas son designadas como Cry2Ae, Cry2Af, and Cry2Ag, la invención también divulga sus variantes.

http://worldwide.espacenet.com/publicationDetails/description?CC=WO&NR=02057664A2&KC=A2&FT=D&ND=3&date=20020725&DB=EPODOC&locale=en_EP

D2 el método de entrelazado térmico asimétrico (PCR TAIL) usado para la recuperación y el mapeo de secuencias genómicas que flanquean inserciones de ADN en *Arabidopsis thaliana*.

<http://www.ncbi.nlm.nih.gov/pubmed/7550382>

Nivel Inventivo (Art18 D486)

La reivindicación 11 consiste en “Un método para identificar el evento elite EE-GH5 en muestras biológicas, caracterizado porque comprende la detección de una región específica para EE-GH5 con un cebador o una sonda específica, que reconoce específicamente la región flanqueadora 5' o 3' de EE-GH5.”

Comparación de las características técnicas **esenciales**, con las del Estado de la Técnica:

Características esenciales	D1	D2
Método de identificación de EE-GH5 (cryIAb)	Secuencias de nucleótidos que codifican para Cry2Ae insertadas en algodón.	
Primer específico o sonda	Métodos de detección de la inserción de Cry2Ae, uso de PCR en dichos métodos (primers y sondas)	Métodos para el diseño de primer usando PCR anidada (3 primers)

Los documentos D1 y D2 se consideran el estado de la técnica cercano a la invención definida en la reivindicación 1 porque:

D1 divulga las secuencias de nucleótidos que codifican para cryIAb que se encuentra en el evento EE-GH5 (ver descripción de la presente invención página 63, ver resumen documento D1), además divulga métodos de detección de dicho gen usando PCR, por lo cual usa sondas y primers específicos para dicho gen (Ejemplo 3 página 20 líneas 20 y 21).

La diferencia entre la invención, definida en la reivindicación 1 y D1 consiste en que la invención se refiere a métodos que incluyen primer y sondas específicas para el evento EE-GH5 que contiene el gen para cryIAb, que no están divulgados previamente en D1.

El efecto que produce esta diferencia no resulta evidente puesto que ambos documentos se refieren el gen cryIAb, debido a que el solicitante no define correctamente el evento EE-GH5 se entiende que lo que cambia es el punto de inserción del mismo en el genoma de la planta de algodón, y por tanto el segmento de DNA flanqueante de la planta.

El problema técnico objetivo que resuelve la invención es encontrar primers y sondas específicas para el gen cryIAb que tiene secuencias flanqueantes no reportadas con anterioridad.

Sin embargo, para la persona del oficio normalmente versada en la materia resulta evidente usar otro sitio de inserción y saber que se obtendría una planta transgénica resistente según el gen insertado. La detección de este nuevo evento de inserción usa las mismas técnicas moleculares que el método reportado en D1, con primers específicos para el segmento flanqueante en el genoma de algodón.

Sin embargo, el diseño de primers es una técnica conocida en el estado de la técnica, y el diseño de dichos primers usando el método de entrelazado térmico asimétrico (PCR TAIL) usado para la recuperación y el mapeo de secuencias genómicas que flanquean inserciones de ADN, ya había sido previamente divulgado en D2, y corresponde a un proceso rutinario en el laboratorio

En consecuencia, la persona del oficio normalmente versada en la materia, incluiría primer y sondas específicos del gen cryIAb en el evento EE-HG5 diseñados usando las técnicas divulgadas en D2 en el método de detección de eventos en algodón divulgados en D1 para llegar así al objeto de la reivindicación 1 de la solicitud. Por lo cual se considera obvia.

La materia reclamada en las reivindicaciones 11 y dependientes 12 a 16, 26 a 28, 36 a 40 y 41 y las reivindicaciones referentes a kit de primer, primers y sondas 17 a 26, 29 a 35 y 45 carecen de nivel inventivo e incumplen el artículo 18 de la D486.

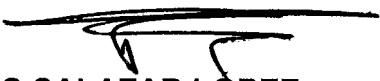
8. Conclusión

Los requisitos de Patentabilidad de la presente Solicitud están afectados así:

Ítem	Documento	Reivindicaciones afectadas	Requisito que afecta
D1	WO03093484	11 a 16 26 a 28, 36 a 40 y 41, 17 a 26, 29 a 35 y 45	Nivel Inventivo
D2	The Plant Journal 8(3), 457-463.	11 a 16 26 a 28, 36 a 40 y 41, 17 a 26, 29 a 35 y 45	Nivel Inventivo

En cumplimiento del artículo 45 de la Decisión 486 de la Comisión de la Comunidad Andina, se le indica que debe dar respuesta dentro del término de sesenta (60) días contados a partir de la fecha de notificación de la presente comunicación. En caso de no dar respuesta al presente requerimiento, o si a pesar de la respuesta subsistieran los impedimentos para la concesión, se denegará la patente.

Notifíquese el presente oficio conforme a lo dispuesto en el numeral 5.2, literal e), del capítulo quinto del título primero de la Circular Única No. 10 de 2001.


JOSÉ LUIS SALAZAR LÓPEZ
Director de Nuevas Creaciones (C)

El anterior requerimiento fue notificado por fijación en lista, de fecha 25 SET. 2012

Elaborado por: Sandra Carolina Crespo Cárdenas
Revisado Por: Consuelo Leguizamón Leguizamón 
Aprobado por: José Luis Salazar López
Expediente 09-124408 Primer Artículo 45

TECHNICAL ADVANCE

Efficient isolation and mapping of *Arabidopsis thaliana* T-DNA insert junctions by thermal asymmetric interlaced PCR

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Summary

Thermal asymmetric interlaced (TAIL-) PCR is an efficient technique for amplifying insert ends from yeast artificial chromosome (YAC) and P1 clones. Highly specific amplification is achieved without resort to complex manipulations before or after PCR. The adaptation of this method for recovery and mapping of genomic sequences flanking T-DNA insertions in *Arabidopsis thaliana* is described. Insertion-specific products were amplified from 183 of 190 tested T-DNA insertion lines. Reconstruction experiments indicate that the technique can recover single-copy sequences from genomes as complex as common wheat (1.5×10^{10} bp). RFLPs were screened using 122 unique flanking sequence probes, and the insertion sites of 26 T-DNA transgenic lines were determined on an RFLP map. These lines, whose mapped T-DNA insertions confer hygromycin resistance, can be used for fine-scale mapping of linked phenotypic loci.

Introduction

T-DNA and transposon insertion mutagenesis have become important tools for isolation of new genes and study of their function. In these studies it is usually necessary to recover genomic sequences flanking the insertions. So far several PCR-based methods have been described which can be used for such a purpose (for review, see Ochman *et al.*, 1993). These methods require a number of laborious manipulations before or after PCR. Recently we developed an efficient thermal asymmetric interlaced (TAIL-) PCR strategy for isolation of target DNA segments adjacent to known sequences, and used this method to isolate insert

end sequences from P1 and YAC clones (Liu and Whittier, 1995). This method requires neither special DNA manipulations before PCR nor laborious screening afterwards. It nevertheless yields specific products of high purity which can be used directly either as hybridization probes or as sequencing templates. TAIL-PCR utilizes a set of nested sequence-specific primers together with a shorter arbitrary primer of lower melting temperature. Annealing steps can be carried out at different temperatures so that both primers—or alternatively, only the specific primer—function well. Interspersing high and reduced stringency cycles enables target sequences to be amplified preferentially over nonspecific products.

In this study we describe the adaptation of this method to isolate genomic sequences flanking T-DNA insertions from transgenic lines of *Arabidopsis thaliana*. Those sequences detecting restriction fragment length polymorphisms (RFLPs) were used in turn to map the T-DNA insertion sites. In this manner we have assembled a collection of 26 plant lines with mapped antibiotic resistance markers. The usefulness of these plants for fine-scale mapping of phenotypic loci is described. In addition, reconstruction experiments were carried out to show that TAIL-PCR is highly sensitive and can be applied to more complex genomes.

Results

Amplification of T-DNA insertion junction DNA

Details of the TAIL-PCR strategy have been described (Liu and Whittier, 1995). The procedure used for this study and its principle are briefly explained in Figure 1, and detailed thermal cycler settings are listed in Table 1. TAIL-PCR utilizes three nested specific primers in successive reactions together with a shorter arbitrary degenerate (AD) primer so that the relative amplification efficiencies of specific and non-specific products can be thermally controlled. The specific primers and their annealing positions within the T-DNA region of the transfer vector pGDW32 (Wing *et al.*, 1989) are shown in Figure 2. We chose the pad g4 region (polyadenylation signals of T_L-DNA gene 4) rather than sequences more immediately adjacent to the right border to anchor our specific primers. T-DNA integration is not always precise, but we anticipated that loss of the

Received 24 February 1995; revised 19 May 1995; accepted 6 June 1995.

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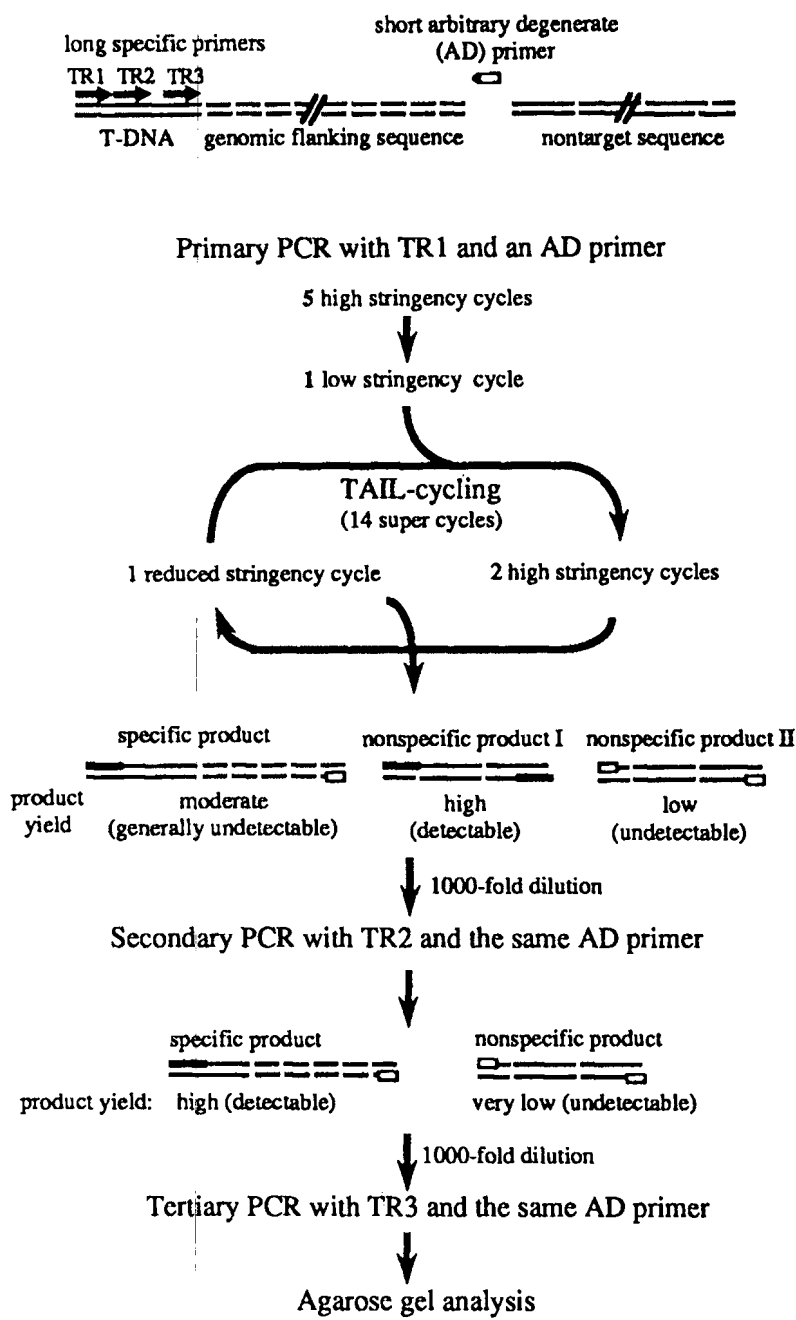


Figure 1. TAIL-PCR procedure for specific amplification of genomic sequence flanking a T-DNA insertion. Three PCR reactions are carried out sequentially to amplify target sequences using nested T-DNA-specific primers (shown as bold segments) on one side and an AD primer (shown as small open rectangles) on the other. One or more sites within the flanking sequence are adapted for annealing to the AD primer through a special low-stringency cycle. Even after creation of sites adapted for the AD primer, however, high-temperature annealing still favors the specific primer, resulting in a linear amplification of target molecules. To achieve adequate thermal asymmetry, the specific and AD primers were designed to have *T_m*s of 57–62°C and 44–46°C, respectively. By interspersing reduced-stringency cycles to allow AD priming, double-stranded molecules can be formed, and the preferential linear amplification of target molecules becomes logarithmic. In the secondary and tertiary reactions non-specific product I fails to be re-amplified and thus is not shown.

3' processing signals would sufficiently impair hygromycin phosphotransferase expression in simple insertions that such events would be rare or absent in our collection of antibiotic resistant transformed lines (An *et al.*, 1989; Ingelbrecht *et al.*, 1989). Sequences of the AD primers used are provided in Experimental procedures. In adapting this method from use on relatively simple *Escherichia coli* and yeast substrates, the most significant alteration was the omission of the 10 reduced-stringency (44°C annealing) PCR cycles prior to TAIL-cycling in the primary reaction. The supercycle number for TAIL-cycling was increased correspondingly. In addition, the annealing temperature in the high-stringency cycles was set 5°C higher than that in

the original protocol for P1 and YAC templates (Liu and Whittier, 1995). These alterations allow amplification of specific products from plant genomic DNA with lower backgrounds of non-specific products.

A library of T-DNA transformed *A. thaliana* (Wassilewskija ecotype), approximately 600 lines, was constructed by *Agrobacterium*-mediated *in planta* transformation (Chang *et al.*, 1994) with modifications (Oosumi and Whittier, unpublished). For amplification of genomic sequences flanking the T-DNA insertion, a total of 190 insertion lines were selected from this library whose segregation of hygromycin resistance was consistent with a single-locus T-DNA insertion. We developed a broadly applicable rapid mini-

Table 1. Cycle settings used for TAIL-PCR

Reaction	File no.	Cycle no.	Thermal settings ^a
Primary	1	1	93°C, 1 min, 95°C, 1 min
	2	5	94°C, 60 (30) sec; 62°C, 1 min; 72°C, 2.5 min
	3	1	94°C, 60 (30) sec; 25°C, 3 min, ramping to 72°C, over 3 min; 72°C, 2.5 min
	4	15 ^b	94°C, 30 (10) sec; 68°C, 1 min; 72°C, 2.5 min
	5	1	94°C, 30 (10) sec; 68°C, 1 min; 72°C, 2.5 min
Secondary	6	12 ^b	94°C, 30 (10) sec; 64°C, 1 min; 72°C, 2.5 min
			94°C, 30 (10) sec; 64°C, 1 min; 72°C, 2.5 min
			94°C, 30 (10) sec; 44°C, 1 min; 72°C, 2.5 min
	5	1	72°C, 5 min
	7	20	94°C, 60 (15) sec; 44°C, 1 min; 72°C, 2.5 min
Tertiary	5	1	72°C, 5 min
			72°C, 5 min

Note. The program files in each reaction were linked automatically.
^aDenaturation time settings used with the Perkin-Elmer Cetus DNA Thermal Cycler (original model) are indicated first, followed by a number in parentheses indicating the time setting used with the Perkin-Elmer GeneAmp PCR System 9600 model.
^bThese are nine-thermal-segment super cycles each consisting of two high-stringency and one reduced-stringency (see Figure 1).

preparation method to isolate genomic DNA from these *Arabidopsis* lines for PCR (see Experimental procedures). The primary TAIL-PCR produced several bands, and all these bands disappeared in the secondary TAIL-PCR in which TR1 was replaced by TR2 (Figure 3), indicating these bands were non-specific products primed at both ends by TR1. Desired specific products primed by TR1 at one end and the AD primer at the other end were not visible in the primary reactions. However, these specific products were amplified to detectable levels during the subsequent secondary TAIL-PCR. The amplification of non-specific products primed at both ends by the AD primer were suppressed to very low levels, and they were not detected in any reactions (Figure 3). The specificity of the tertiary PCR products was verified simply by the size shift of the secondary and tertiary PCR products; target products in the tertiary reactions were 64 bp smaller than those in the secondary reactions (see Figure 2 for the priming sites of TR2 and TR3).

The success rate for amplification of specific products (>250 bp) varied according to the AD primer used, from about 50% with AD1 to 70–80% with AD2 and AD3. Unsuccessful reactions were repeated using a different AD primer.

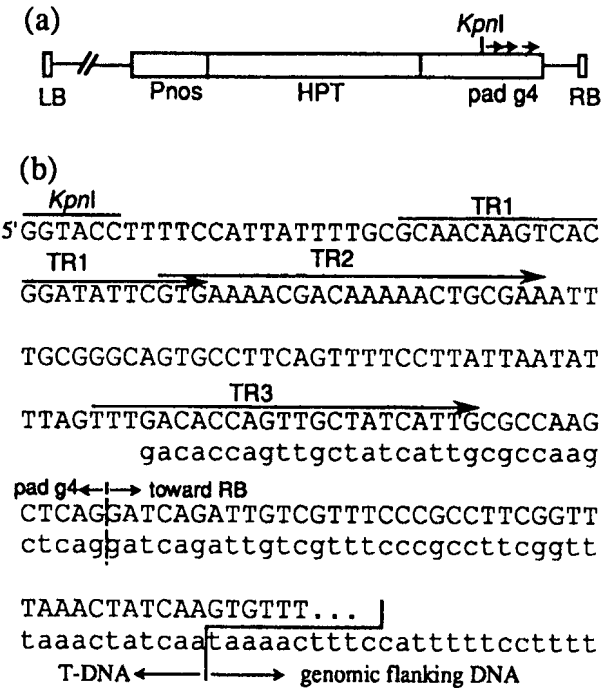


Figure 2. Specific primers used for TAIL-PCR and sequenced TAIL-PCR product.
(a) Schematic diagram of the T-DNA right border region of transfer vector pGDW32 (Wing *et al.*, 1989) indicating the position of the specific primers with respect to the right border (RB) and hygromycin phosphotransferase gene construct. Abbreviations: LB, RB, left and right T-DNA border, respectively; Pnos, nopaline synthase promoter; HPT, hygromycin phosphotransferase coding region; pad g4, polyadenylation signals of T-DNA gene 4.
(b) Nucleotide sequence of specific primers TR1, TR2 and TR3 and surrounding region. TR2 and TR3 are positioned 64 bases apart from each other to facilitate the confirmation of product specificity by size comparison. The sequence shown in lower case letters was determined by direct sequencing of the secondary amplification product of a T-DNA transformed line exhibiting altered leaf morphology (unpublished data). TR2 was utilized as the sequencing primer. Sequence alignment indicates the insertion junction site.

By these means, specific products were amplified in 183 of 190 lines attempted. We have previously shown with P1 and YAC clones (Liu and Whittier, 1995) that TAIL-PCR can yield two or more nested specific DNA fragments due to the annealing of AD primer at multiple sites downstream of the specific primers. Similarly, this phenomenon was observed again on amplification of plant genomic sequences flanking T-DNA (this study) and transposon insertions (unpublished results). On average, 2.2 fragments of flanking sequence were produced for each T-DNA insertion line, with as many as five fragments observed. Since our procedure for obtaining lines with single insertion sites did not preclude concatameric insertions (see below), it is probable that amplification from such lines would have yielded both internal T-DNA sequences and genomic junction sequences. Most of the amplified fragments ranged from about 300 bp to about 1 kb in size and some were even larger than 2 kb.

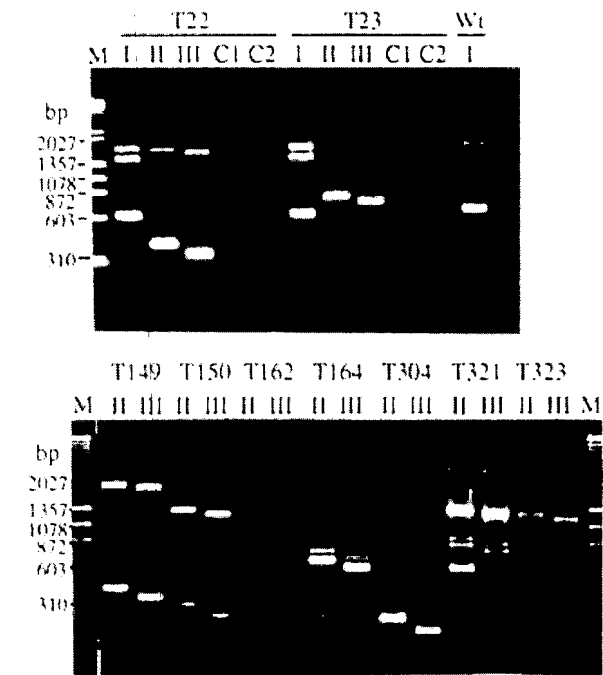


Figure 3. Agarose gel analysis of TAIL-PCR products amplified from T-DNA insertion lines. AD2 was used for lines T22, T23 and T304–T323 and AD3 for lines T149–T164. Lane designations I, II, and III indicate products of the primary, secondary, and complete tertiary reactions, respectively. Lanes designated C1 and C2 indicate control tertiary reactions lacking arbitrary or specific primer, respectively. ‘Wt’ is the parental Wassilewskija line from which the T-DNA insertion lines were derived. The similar patterns of visible primary reaction products among the parental and insertion lines confirm that these are not T-DNA insertion specific. The primary product patterns for T149–T323 were similar and therefore are not shown. All tertiary reactions were carried out as 20 consecutive cycles of reduced stringency (annealing temperature = 44°C). Size marker (M) is a mixture of λ -HindIII and ϕ X174-HaeIII digests.

Sequencing TAIL-PCR products directly

We directly sequenced secondary PCR products for two T-DNA tagged lines of interest by the thermal asymmetric cycle sequencing method (Liu *et al.*, 1993). The sequenced fragments contained junctions of T-DNA with other sequences, confirming their origin as flanking sequences. One of these sequencing gel profiles has been shown previously (Liu *et al.*, 1993) and partial sequence data obtained from a T-DNA insertion line exhibiting altered leaf morphology are given in Figure 2. The results showed in both cases that the right border sequence (RB) had been lost, and the T-DNA/genomic DNA junction sites were located between the pad g4 and RB sequences (Figure 2).

Large genome simulation

Although TAIL-PCR efficiently amplified insert end sequences of P1 and YAC clones (Liu and Whittier, 1995) and single-copy sequences from *Arabidopsis* genomic DNA (this study), other organisms have larger genomes so that amplification of single-copy sequences becomes technic-

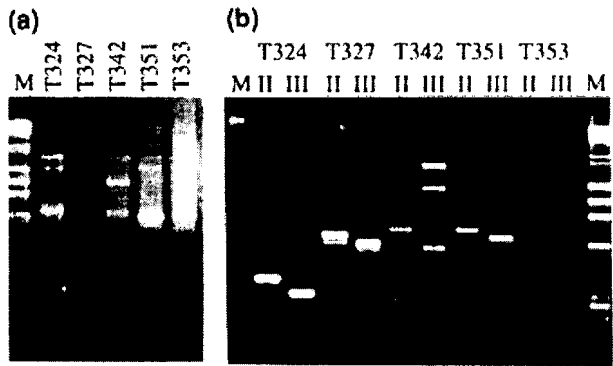


Figure 4. Reconstruction experiment simulating recovery of T-DNA flanking sequences from common wheat. Genomic DNAs from *Arabidopsis* T-DNA insertion lines and common wheat were mixed 1:150, and 100 ng of the mixed DNA samples were applied to the primary TAIL-PCR. All other parameters were as described. The arbitrary primer AD3 was used for all samples. (a) Primary TAIL-PCR products. (b) Secondary (II) and tertiary (III) PCR products. The size relationships between product bands indicate that all product bands in the tertiary reactions were the targeted sequences. The approximately 1250 and approximately 600 bp bands for T342 also possessed faint counterpart bands in the secondary reactions visible in the original photograph. The secondary reactions also produced non-specific products for lines T342, T351, and T353. No specific products were obtained from line T353.

ally more difficult. We did reconstruction experiments to test whether such genomes are amenable to TAIL-PCR. Genomic DNA isolated from T-DNA insertion lines of *Arabidopsis* (genome size, 10^8 bp) was mixed with genomic DNA of common wheat (*Triticum aestivum*, genome size, 1.5×10^{10} bp) in a 1:150 ratio to reconstruct a single-copy T-DNA insertion in the common wheat genome. Targeted products were amplified from four of five such reconstructed samples (Figure 4). This indicates that TAIL-PCR is very sensitive and can be applied to highly complex genomes. In contrast, inverse PCR is difficult to apply to genomes containing over 10^9 bp (Ochman *et al.*, 1993).

Mapping T-DNA insertion sites

Using the amplified T-DNA insertion flanking sequences as probes, RFLPs were screened between Columbia and Landsberg *erecta* ecotypes. The Southern hybridization patterns demonstrated that 75% of the insertion lines had T-DNA inserted in unique genomic sequences, 23% in moderately repeated sequences and 2% in tandemly repeated sequences. In addition, insertion-specific products from several lines failed to yield clear hybridization patterns with *Arabidopsis* genomic DNA, and most of these were not investigated further. Detailed analysis of two lines, however, revealed complex concatameric insertions in which no pad g4/RB sequence was present near a genomic DNA junction (Oosumi, Mitsukawa and Whittier, unpublished data). A total of 33 RFLPs were detected using 122 unique flanking sequence probes, and 26 of them showing clear polymorphism patterns were mapped on the

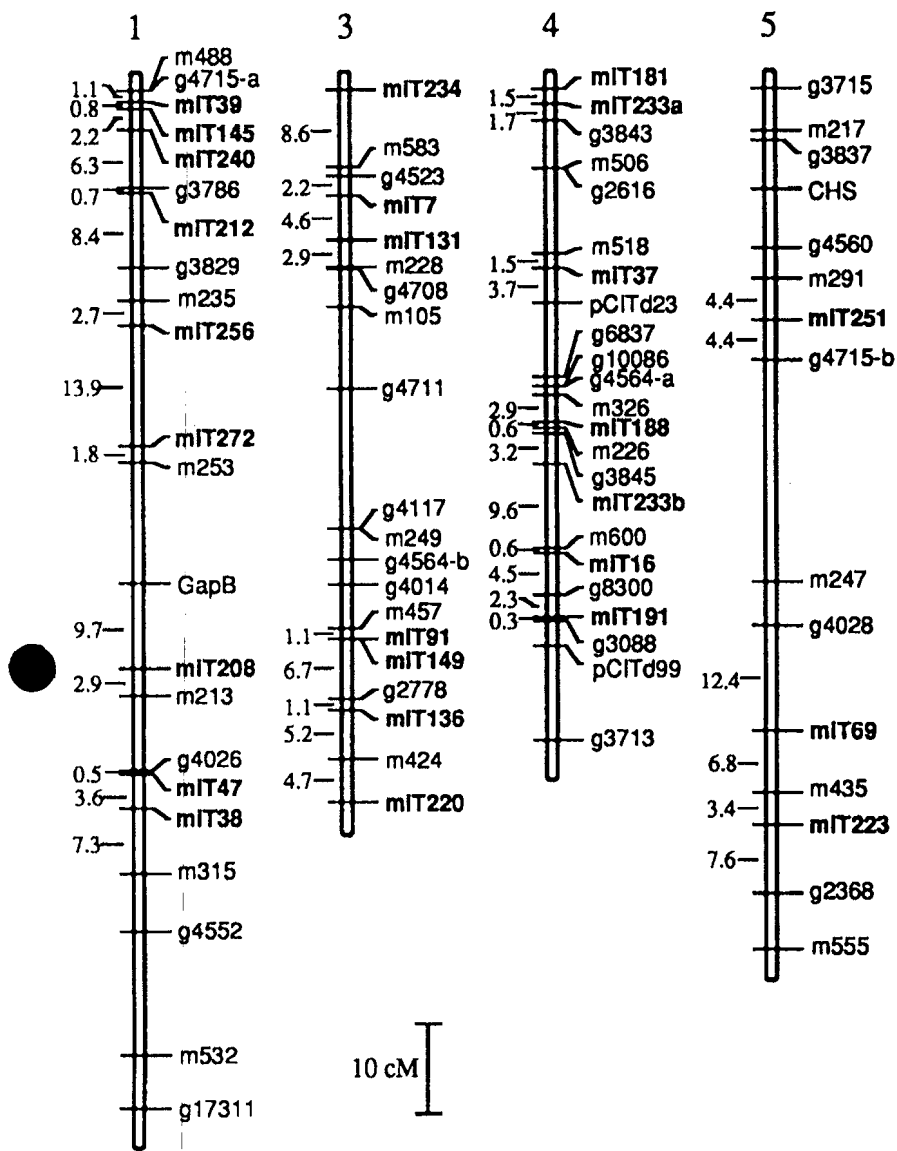


Figure 5. Genetic map positions of the T-DNA flanking sequences amplified from T-DNA insertion lines. These sites are designated 'mitT'. The other sites prefaced with 'm' or 'g' are markers first described by Chang *et al.* (1988) or Nam *et al.* (1989), respectively, and remapped by Lister and Dean (1993). Map distances are shown in centimorgans. Chromosome 2 is omitted for lack of mapped T-DNA insertions.

Arabidopsis chromosomes using a set of 100 recombinant inbred lines (Lister and Dean, 1993). Figure 5 shows the map positions of these markers relative to 54 others (Chang *et al.*, 1988; Nam *et al.*, 1989) which have been remapped by Lister and Dean (1993). The flanking sequence markers were mapped to chromosomes 1, 3, 4 and 5, but none were found on chromosome 2.

Discussion

Recovery of insertion flanking sequences in gene tagging programs is a prerequisite for gene isolation and molecular genetic analysis of putative tagged lines. This paper describes the use of TAIL-PCR for efficient recovery and mapping of genomic sequences flanking T-DNA insertions in *Arabidopsis*, and establishment of a collection of 26 *Arabidopsis* lines with mapped antibiotic selectable markers. As discussed previously (Liu and Whittier, 1995),

this method has advantages over other methods with respect to simplicity, specificity, efficiency, speed, and sensitivity. The mapping strategy employed here is also useful in transposon tagging systems, both in their establishment and in the subsequent recovery of tagged genes. Owing to the propensity of transposons such as *Ds* to transpose short distances, knowledge of the donor site locations would be a great aid in attempts to target chromosomal regions for tagging. Using this TAIL-PCR method, genomic sequences flanking transposon insertions in *Arabidopsis* and rice have been successfully recovered in this and other laboratories (Yanai *et al.*, unpublished; Fedoroff, Ishiguro, Shimamoto, personal communications). In obtaining insertion flanking sequences for cloning tagged genes or for RFLP mapping, it may not matter whether the insertions are simple or concatameric, provided at least one anchoring T-DNA sequence exists near a genomic DNA junction. Some of the probes we created

may have contained internal T-DNA sequences obtained from concatameric insertions; we did not test this routinely. Unless the junction region fails to be amplified entirely, a clear hybridization pattern with *Arabidopsis* DNA will be obtained. We did occasionally get insertion-specific product without obtaining genomic junction DNA. In the two cases we examined thoroughly, the concatameric insertions were complex with no genomic DNA junction occurring near the pad g4/RB sequence.

The mapped flanking sequences indicate the chromosomal insertion positions of the corresponding T-DNAs. No insertions were mapped to chromosome 2. Although unexpected, there is little reason to suspect an underlying bias in T-DNA insertion or RFLP distribution. In a recent mapping study of embryo lethal mutations, Franzmann *et al.* (1995) mapped four of 26 T-DNA insertions to this chromosome by traditional crossing. Using short, random genomic clones as probes, we have also found 14 of 120 RFLPs to lie on this chromosome (unpublished results). These latter two ratios are roughly consistent with genetic map distance accorded this chromosome in recent studies (Franzmann *et al.*, 1995; Hauge *et al.*, 1993; Lister and Dean, 1993).

The amplified flanking sequences can be used as normal RFLP markers. For example, we used the miT191 flanking sequence mapped on chromosome 4 (see Figure 5) as the starting point in a chromosome walk (Liu *et al.*, 1995). More importantly, the mapped T-DNAs can serve as antibiotic resistance markers for selection of recombination events following genetic crosses. This is especially important for map-based gene isolation where the precision of mapping is crucial to success. This precision is determined by two factors: (i) the density of available molecular markers and (ii) the occurrence of recombination events near the gene of interest. The number of such informative events is generally limited by how many cross-progeny it is feasible to analyze. However, informative recombination events can be enriched in the analyzed population if the gene of interest lies near a mapped antibiotic resistance marker. Following a cross between the homozygous (recessive) mutant and a homozygous antibiotic-resistant line, informative recombination events are enriched in the F_2 generation by selecting individuals exhibiting both antibiotic resistance and the homozygous mutant phenotype. The *Arabidopsis* lines with mapped antibiotic selectable markers established in this study should be useful for such fine-scale mapping in map-based gene isolation efforts.

Experimental procedures

DNA isolation

Rapid mini-preparation of genomic DNA from the T-DNA insertion lines was done as follows: 2–3 leaves (30–50 mg) stored frozen in a 1.5 ml microfuge tube were ground in the same tube in the

presence of 50 μ l isolation buffer (0.3 M NaCl, 50 mM Tris-HCl pH 7.5, 20 mM EDTA, 2% sarkosyl, 0.5% SDS, 5 M urea and 5% phenol, modified from Shure *et al.*, 1983) using a disposable grinder (Treff, Cat# 34400) driven by a variable speed electric drill set at about 200–300 r.p.m. Another 100 μ l isolation buffer and 100 μ l phenol/chloroform/isoamyl alcohol (25:24:1) were added to the tube, mixed, and centrifuged at 15 000 r.p.m. (ca. 11 000 *g*) for 5 min in a microfuge. Two volumes of ethanol (room temperature) were added to the supernatant and DNA was recovered by centrifugation at 12 000 r.p.m. (ca. 7000 *g*) for 5 min. The pellets were washed with 0.5 ml of 70% ethanol (room temperature) and dissolved in 50 μ l TE buffer containing RNase (20 μ g ml⁻¹). For RFLP mapping, large-scale DNA isolation was from liquid-cultured roots. Surface-sterilized seeds (20–30) of each parent (Columbia or Landsberg *erecta*) or recombinant inbred line (Lister and Dean, 1993) were cultured in 300 ml flasks (one line per flask) containing 70 ml of 1/2 $\times$ Gamborg's B-5 medium (pH 5.7) supplemented with 1% sucrose and 1/1000 $\times$ HYPONEX at 25°C for 18–20 days with one change of medium on the 14th or 15th day. The roots were ground in liquid nitrogen with a mortar and pestle. The ground tissue was mixed in 4 volumes of isolation buffer as described above and extracted twice with phenol/chloroform. DNA was recovered by ethanol precipitation and dissolved in TE buffer containing RNase (20 μ g ml⁻¹). DNA concentrations were determined by fluorescence with Hoechst 33258 dye (Labarca and Paigen, 1980).

Primers

The specific primers TR1, TR2 and TR3 (Figure 2) have melting temperatures (T_m s) of 61°C, 57°C and 57°C, respectively, as calculated with the formula: $69.3 + 0.41(\%GC) - 650/L$ (Mazars *et al.*, 1991), where L is primer length. In addition, three arbitrary degenerate (AD) primers (15- or 16-mers) were designed and synthesized: 5'-NTCGA(G/C)T(A/T)T(G/C)G(A/T)GTT-3' (AD1, 64-fold degeneracy, average T_m = 45.3°C), 5'-NGTCGA(G/C)(A/T)GANA(A/T)GAA-3' (AD2, 128-fold degeneracy, average T_m = 46.6°C), and 5'-(A/T)GTGNAG(A/T)ANCANAGA-3' (AD3, 256-fold degeneracy, average T_m = 45.3°C).

PCR procedure

Primary TAIL-PCR reactions (20 μ l) contained 1 $\times$ PCR buffer (10 mM Tris-HCl pH 8.3, 50 mM KCl, 1.5 or 2.0 mM MgCl₂, 0.001% gelatin), 200 μ M each of dNTPs, about 20 ng of genomic DNA, 0.8 unit of AmpliTaq polymerase (Perkin-Elmer), 0.15–0.2 μ M TR1 and a given AD primer (2 μ M for AD1, 3 μ M for AD2 or 4 μ M for AD3). Empirically, these concentrations of AD primers were found to yield good results, and their different optimal concentrations non-linearly reflect their degeneracy levels. Primary TAIL-PCR was executed as summarized in Table 1 in Perkin-Elmer thermal cyclers, the original model or GeneAmp PCR System 9600. Aliquots (1 μ l) from 50-fold dilutions of the primary PCR products were applied directly to secondary TAIL-PCR reactions (20 μ l) containing 1 $\times$ PCR buffer, 0.6 unit of AmpliTaq, 200 μ M each of dNTPs, 0.2 μ M TR2 and the same AD primer used in the primary reaction (1.5 μ M for AD1, 2.0 μ M for AD2 and AD3). For samples to be sequenced directly without purification (see below), the concentration of dNTPs was reduced to 25 μ M. The reduced dNTP concentration did not interfere with product amplification. After amplification (Table 1), the secondary TAIL-PCR products (1 μ l aliquots of 10-fold dilutions) were re-amplified in 100 μ l tertiary reactions of 20 reduced-stringency cycles (Table 1). Components and their

concentrations were the same as in the secondary reaction except that TR3 replaced TR2. Amplified products from the reactions with TR2 and TR3 were analyzed by agarose gel electrophoresis. A difference in product size consistent with primer positions was used as the criterion for judging product to be insertion-specific.

DNA sequencing

Unpurified secondary TAIL-PCR products for several samples were directly sequenced using the thermal asymmetric cycle sequencing method (Liu and Whittier, 1995; Liu *et al.*, 1993). The same specific primer, TR2 (unlabeled) was used for sequencing as in the immediately preceding amplification reaction, eliminating the need to remove carried over primers.

RFLP mapping

For use as hybridization probes, the amplified flanking sequence products were purified by ultrafiltration through spun-filter cups (SUPREC-02, Takara Shuzo), or by electrophoresis on 1.2% low-melting agarose gels (FMC SeaPlaque). In the latter case, agarose gel slices containing recovered DNA were equilibrated with 3.3 mM Tris-HCl pH 7.5, 0.33 mM EDTA (1/3 × TE) for 10 min, digested with β-agarase I (New England Biolabs), then directly used for Southern hybridization probe labeling. With these probes RFLPs were screened between the *Arabidopsis* ecotypes Columbia and Landsberg *erecta* using five restriction endonucleases (*Bam*HI, *Dra*I, *Eco*RI, *Eco*RV and *Hind*III). The digested DNAs were separated on 0.8% agarose gels (1.5–2 µg per lane) and alkaline-transferred to Hybond-N+ membranes (Amersham). Southern hybridization was conducted overnight at 65°C in a solution (Liu *et al.*, 1992) containing 5 × SSC, 0.1% sodium *N*-lauroyl sarcosinate, 0.02% SDS, 0.5% Blocking Reagent (Boehringer Mannheim), and 5% dextran sodium sulfate (optional). After washing for 1 h at 65°C in 0.1 × SSC and 0.1% SDS with one change of solution, hybridization signals were detected using a BAS-2000 Bio-image analyzer (Fuji Film). Using a set of 100 recombinant inbred lines (Lister and Dean, 1993), the map positions of the flanking sequences detecting RFLPs were determined relative to the RFLP markers (Chang *et al.*, 1988; Nam *et al.*, 1989) remapped by Lister and Dean (1993). Linkage analysis was done with the MapMaker program (Lander *et al.*, 1987).

Acknowledgments

We thank Caroline Dean and Clare Lister for providing recombinant inbred lines of *Arabidopsis* and RFLP segregation data. This research is conducted as a part of the Industrial Technology Development Promotion Program of the Research Institute of Innovative Technology for the Earth (RITE) for the global environmental problems, supported by the Ministry of International Trade and Industry of Japan.

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