

Brigard & Castro
Abogados

Apartado 3692

229
228
228

SUPERINDUSTRIA Y COMERCIO
Radicación : 02075735 00000003 Folios: 13
Fecha (HAB): 2003-03-27 16:53:44 S.A.
Trámite : 011 PLI-PHIMI 3/8 FASE NACI 329 COMPLET
Dependencia: 202V DIVISION DE NUEVAS CREACIONES 7976
(57.1) 3802700

AS: 88.855

E.Mail:bricas@brigardycastro.com.co

Señores

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

E. S. D.

REF: Expediente No.02 075.735

Solicitud de Patente FASE NACIONAL

a favor de YALE UNIVERSITY

Titulada: MÉTODO Y COMPOSICIÓN PARA REDUCIR O
ELIMINAR LA TRANSMISIÓN DE UN TRANSGENE

RAMIRO CASTRO DUQUE, vecino de Bogotá, portador de la tarjeta profesional No. 1003, obrando como apoderado de la sociedad YALE UNIVERSITY, me permito anexar al presente escrito los siguientes documentos:

1. Copia certificada por la Oficina de Patentes de Estados Unidos de la primera solicitud presentada para la misma invención, de la cual REIVINDICAMOS PRIORIDAD.

De la señora jefe atentamente,



RAMIRO CASTRO DUQUE

C.C. No. 170.322 de Bogotá

Código 340

Cra 7 No. 16-56 Piso 9

Bogotá, Mayo 27, 2003

CHP/hsr.

88-855
230
229

PA 984424

THE UNITED STATES OF AMERICA

TO ALL TO WHOM THESE PRESENTS SHALL COME:

UNITED STATES DEPARTMENT OF COMMERCE

United States Patent and Trademark Office

March 28, 2003

**THIS IS TO CERTIFY THAT ANNEXED HERETO IS A TRUE COPY FROM
THE RECORDS OF THE UNITED STATES PATENT AND TRADEMARK
OFFICE OF THOSE PAPERS OF THE BELOW IDENTIFIED PATENT
APPLICATION THAT MET THE REQUIREMENTS TO BE GRANTED A
FILING DATE UNDER 35 USC 111.**

APPLICATION NUMBER: 60/185,524

FILING DATE: February 28, 2000



**By Authority of the
COMMISSIONER OF PATENTS AND TRADEMARKS**

**M. K. HAWKINS
Certifying Officer**

02/28/00
JCS53 U.S. PTO
60/185524
02/28/00

A/PROY
JCS53 U.S. PTO
60/185524
02/28/00
230

Please type a plus sign (+) inside this box —>



PROVISIONAL APPLICATION FOR PATENT COVER SHEET

This is a request for filing a PROVISIONAL APPLICATION FOR PATENT under 37 CFR 1.53 (c)

INVENTOR(s)			
Given Name (first and middle, if any)		Family Name of Surname	RESIDENCE (CITY AND EITHER STATE OR FOREIGN COUNTRY)
Stephen	L.	Dellaporta	Branford, CT
Maria	A.	Moreno	Branford, CT
☐ Additional inventors are being named on the ___ separately numbered sheets attached hereto.			
TITLE OF THE INVENTION (280 characters max) Method for the Recovery of Dispersed, Stable and Independent Transposition Events			
CORRESPONDENCE ADDRESS Yale University, Office of Cooperative Research 155 Whitney Avenue PO Box 208336 New Haven CT 06520-8336			
Country	USA	Telephone	203-432-5446
		Fax	203-432-5449
ENCLOSED APPLICATION PARTS (check all that apply)			
☒	Specifications	No. of Pages	9
☒			
☐	Drawing(s)	No. of Sheets	
☐			
METHOD OF PAYMENT OF FILING FEES FOR THIS PROVISIONAL APPLICATION FOR PATENT (check one)			
☐	A check or money order is enclosed to cover the Provisional filing fees		
☒	The Commissioner is hereby authorized to charge filing fees and credit Deposit Account Number		
	25-0110	PROVISIONAL FILING FEE AMOUNT (\$)	\$75
The invention was made by an agency of the United States Government or under a contract with an agency of the United States Government.			
☒	No		
☐	Yes, the name of the U S Government agency and the Government contract number are.		

Respectfully submitted,

SIGNATURE

Henry S. Lowendoff

Date 28.Feb.00

TYPED or PRINTED NAME

Henry S. Lowendoff

REGISTRATION NO.

(if appropriate)

TELEPHONE

203-432-8929

DOCKET NUMBER:

CERTIFICATION UNDER 37 CFR § 1.10

I hereby certify that this correspondence and the documents referred to as attached therein are being deposited with the United States Postal Service on 28 Feb 2000, in an envelope as "Express Mail Post Office to Addressee" service under 37 C.F.R. § 1.10, Mailing Label Number EI 79186051923 addressed to :Box Provisional Application, Commissioner of Patents and Trademarks, Washington, D.C. 20531.

Henry S. Lowendoff
Henry S. Lowendoff

Title: Method for the Recovery of Dispersed, Stable and Independent Transposition Events

Inventors: Stephen L. Dellaporta and Maria A. Moreno

Home Address: 732 Leetes Island Rd, Branford, CT 06405-3317

Abstract

A method is described that permits the recovery of large number of dispersed and/or stable transpositions using a gametophytic semi-sterility trait to eliminate the genetic transmission of a specific chromosomal region. When this chromosomal region contains a transposon launching site, the "genetic sterility filter" imposed by this methodology greatly enriches for viable gametes containing unlinked transpositions and transposon that have recombined with the donor locus. Semi-sterility is genetically engineered using a pollen-specific promoter fused to a suicide gene or through the use of genetic mutations, such as deletions, that are unable to be transmitted through one of the sexes. The semi-sterility trait can also be physically linked to the transposase source to eliminate gametes containing a source of transposase. This arrangement prevents transmission of the transposase source to gametes, thereby stabilizing insertions in subsequent generations. All three components -- the suicide gene or mutation, the transposase source and the transposon -- can be engineered as a unit to provide a robust method of generating dispersed, stable transpositions. A further refinement of this invention is to limit transposase expression to floral development to prevent early somatic and vegetative transpositions. Control of transposase expression is achieved by fusing a florally expressed promoter to the transposase gene. Florally-controlled transposition means that transposons are stable in vegetative tissues; a trait that permits clonal propagation of stock plants without the concern of generating secondary transposition events. Moreover, each flower serves as an independent source of transpositions, decreasing the probability of recovering non-independent transpositions in progeny.

Background of the Invention

Transposable Elements

Transposons are genetic elements capable of transposition (movement) from a donor chromosomal site to a target site on the same chromosome or different chromosome. Transposons cause mutations by insertion into coding sequences, introns, and promoters, often completely eliminating target gene activity. Mutations caused by transposons can often be destabilized by subsequent excision of the transposon from the gene. One or more proteins, collectively referred to as "transposase", are required for the excision and integration of the transposon. Transposons that encode their own source of transposase are referred to as autonomous elements. Supplying transposase in trans can transpose transposons that do not encode transposase but contain terminal sequences, usually inverted repeats, required for transposition (non-autonomous elements).

Utility of Transposon and Insertion Mutations in Plants

Transposons have great utility in genetic analysis and functional genomic analysis of bacterial, fungal, plant and animal genomes. In plants, genetically engineered transposons have been successfully introduced into several species (for review see (Sundaresan 1996)) including rice (Izawa et al. 1997), tobacco, Arabidopsis, lettuce and several others. Several investigators have come up with ingenious methods to enhance the efficiency of recovering transposition events, including methods for positive selections for transposition (Fedoroff and Smith 1993; Honma et al. 1993), selection for unlinked elements (Sundaresan et al. 1995; Tissier et al. 1999), dispersed launching sites throughout a genome (Osborne et al. 1991; Knapp et al. 1994; Thomas et al. 1994; Cooley et al. 1996; van der Biezen et al. 1996; Takken et al. 1998) and adding features into elements such as enhancer and gene traps (for

reviews see (Sharknes 1990; Sundaresan et al. 1995)) and transcriptional activation (Fritze and Walden 1995; Kakimoto 1996; Kardailsky et al. 1999).

Even given the enormous progress in the utility of transposons, they still have limitations for functional genomic analysis. Transpositions are usually intrachromosomal, often within a short physical distance between donor and target sites. This limitation means that most new insertions occur in regions surrounding the donor site (launching site) with many fewer elements found dispersed randomly throughout the genome. Secondly, the process of transposition is not usually controlled leading to a great many somatic insertions, which are not transmitted to progeny, and developmentally early transpositions that can lead to non-concordance in germinally transmitted events. The inability to control transpositions somatically and temporally can result in a high background of false positive insertions in genes of interest due to the non-correspondence between somatic and germinal mutational events.

Current Methods to Disperse Transpositions

Two methods are currently available to partially overcome the limitation of non-dispersed transpositions. One method makes use of first dispersing transposon launching sites throughout a genome. This method, however, requires a large number of starter lines, usually transgenic, to achieve good genome coverage. A second method to disperse transposition events is to employ a negative selectable marker(s), such as *iaaH*, *pehA*, *R404*, *CD*, to select against the donor site containing the transposon and/or transposase. Unlinked transpositions are recovered by the negative selection against progeny carrying the marker(s). Negative selections have been used in *Arabidopsis* to recovery unlinked *Ds* and *Spm* transpositions (Sundaresan et al. 1995; Tissier et al. 1999) and two negative selectable markers are based on proherbicide conversion (O'keefe et al. 1994; Dotson et al. 1996), a process theoretically amenable to soil selections.

Nevertheless, the use of negative selectable markers imposes serious limitations on the recovery of large numbers of independent transpositions. First, several of these markers require the use of tissue-culture based selection (Martien), a labor-intensive procedure that adds great expense and time to the process of recovering large numbers of dispersed transpositions. Moreover, negative selections are based on the elimination of progeny carrying linked elements or transposase, or both, by chemical selection. Progeny elimination can be problematic when seed number is limited. For instance, in a plant such as rice (*Oryza sativa*), a predominantly self-pollinating species, outcrossing is tedious and time-consuming, greatly limiting the number of progeny that can be readily obtained. Hence, if transposition rates are low and unlinked transpositions represent the minority of transposition events, it would be costly and impractical to use negative selections to recover dispersed transpositions. For instance, accounting for a rate of 1-5% transposition, 50% meiotic segregation, and only 30% unlinked transpositions, to recover 10,000 transpositions by outcrossing would require an estimated several million progeny plants. Finally, the use of negative selectable agents, such as proherbicides, can have a serious environmental impact and be costly due to the applications of chemicals that may be needed to select for large numbers of progeny carrying dispersed transpositions.

Methodology

Preface

The current invention describes the use of a technology that utilizes gametophytic "semi-sterility", such as pollen semi-sterility, to generate a "genetic sterility filter" that will eliminate gametes that inherit a specific chromosomal region. This chromosomal region may contain a launching site for a transposable element and/or transposase gene to have the effect of enriching for non-linked or dispersed transposition events throughout a genome. This methodology overcomes several current limitations of transposon mutagenesis

strategies that favors mostly localize over dispersed transpositions. The invention greatly improves on the current use of negative selectable markers to achieve transposon dispersion (Sundaresan et al. 1995; Tissier et al. 1999). Moreover, the use of the genetic sterility filter to eliminate transmission of a transposase source would, in effect, stabilize the newly transposed elements in progeny, thereby eliminating somatic or secondary transposition events that hamper mutation identification.

The nature of the semi-sterility trait and its associated transposon and/or transposase, may differ in details depending on the choice of suicide genes, promoters, transposon systems, and species. It is emphasized, however, that the same basic technology of semi-sterility can be used to recovery transpositions in many plants, both monocots and dicots, in such species such as maize, rice, soybeans, wheat, oats, barley, various trees and in non-plant systems, such as animals and fungi, that can be sexually propagated.

Semi-sterility

The semi-sterility trait is used to eliminate the products of meiosis (gametes) that carry a particular chromosomal region, such as a transposon launching site and/or transposase gene. This "genetic sterility filter" is used to enrich for unlinked (dispersed) transposed elements or elements that recombined from the launching site; it can also be used eliminate transmission of a transposase gene, thereby stabilizing transpositions in progeny. To achieve semi-sterility, two methods are described. One method relies on directing expression of a suicide gene to kill unwanted meiotic products. This method can be achieved by employing a specific promoter, such as a pollen-specific promoter, fused to a suicide gene, thereby killing only the products of meiosis that have inherited the gene fusion. For a single copy transgene in hemizygous condition, this would represent 50% of the gametes. Because pollen is produced in large excess, reducing pollen fertility by 50% will have no major consequence on subsequent seed production.

There are several excellent examples of suitable promoters to drive pollen-specific expression in plants. Pollen-specific promoters have been identified in many plant species such as maize, rice, tomato, tobacco, Arabidopsis, Brassica, and others (Chen et al. 1987; Siebertz et al. 1989; Sullivan et al. 1989; Bichler and Herrmann 1990; Guerrero et al. 1990; Twell et al. 1990; van Tunen et al. 1990; Albani et al. 1991; Kyoizuka et al. 1991; McCormick et al. 1991; Thorsness et al. 1991; Twell et al. 1991; Albani et al. 1992; Carpenter et al. 1992; Hamilton et al. 1992; Kim and An 1992; Kloeckener-Gruissem et al. 1992; Rommens et al. 1992; Marrs et al. 1993). Several other examples of pollen-specific promoters can be found in GenBank. For the present invention any promoter will be suitable given that the promoter drives gene transcription after meiosis I when homologous chromosome have separated into different cells. For instance, gene expression in the tetrad stage of meiosis II, the post-mitotic division of the microspore leading to pollen maturation, the mature pollen grain, or in the germinating pollen grain would, in theory, be suitable for the current invention.

A second aspect of the semi-sterility trait is the directed expression of a suicide gene to kill unwanted meiotic products. Several examples of genes whose expression result in cell death have been described in the literature including the barnase (Goldman et al. 1994; Custers et al. 1997; Yazynin et al. 1999), tasselseed2 (DeLong et al. 1993), and the diphtheria toxin A gene (Day et al. 1995), to name a few. Because the suicide gene expression is confined to the post-meiosis I, only 50% of the gametes will be eliminated when the transgene is hemizygous and segregates normally in meiosis. The viable gametes will not have inherited the suicide gene. According to another aspect of the preceding invention, semi-sterility can also be achieved using antisense RNA technology () to inhibit expression of genes essential for pollen or egg viability. Alternatively, mutations that are incapable of transmission through one of the sexes, such as deletions that are not pollen-transmissible, can be used to achieve semi-sterility.

9 256
235

The aspect of the current invention to generating pollen semi-sterility differs from previous methods aimed at achieving male-sterility for hybrid seed production (patents). In the latter inventions, the aim is to achieve complete male sterility to facilitate the commercial production of hybrid seed. This usually involves expression of the suicide gene in the sporophyte or in all pollen. For instance, barnase expression in tapetal cells results in complete male sterility. In hybrid seed production, pollen semi-sterility would be insufficient to achieve the desired result, i.e. outcrossing of male-sterile (female) parent. In cases where the suicide gene and its associated elements are present in the genome in a single copy (in hemizygous condition), the suicide gene will be transmitted to only one-half of the meiotic products, resulting in an average of 50% semi-sterility, a rate of viable pollen production that is commercially unacceptable for hybrid seed production. For the present invention, the production of 50% viable pollen is necessary to recover dispersed transpositions or to prevent transposase expression. Hence, the sterility trait is used as a genetic filter to eliminate undesirable genomes. The present invention makes use of this "genetic sterility filter" to eliminate chromosomal region(s) containing a transposon launching site and/or transposase source.

Applications of semi-sterility in genomics

By physically linking the sterility trait to a transposon launching site and/or transposase source, the "genetic sterility filter" can be used to highly enrich for dispersed and/or stabilized transposition events without the use of chemicals and without the need to select against progeny containing linked transposition events and/or transposase source. For instance, when 50% pollen sterility is achieved, the remaining viable haploid genomes will have not inherited the suicide gene and its associated elements such as the transposon launching site and/or transposase gene because of normal homologous chromosome segregation and meiotic recombination. A fraction of these viable genomes will contain newly transposed elements, especially those elements that have assorted independently or recombined from the launching site with its associated suicide gene. In other words, this "genetic sterility filter" has eliminated gametes containing transposed elements that remained linked to the launching site and/or gametes containing a transposase gene. If the remaining viable pollen is used to fertilize ovules, either by controlled pollinations or by wind-pollination, a fraction of the resultant progeny will contain transposed elements that have recombined with the launching site. These progeny can readily be identified by the inclusion of a selectable or screenable marker inside the transposon, such as the *petunia*, *Arabidopsis*, or *Agrobacterium* CP4 EPSPS gene (Padgett et al. 1987) (Klee et al. 1987; Harrison et al. 1996; Hoef et al. 1998), encoding glyphosate (Roundup) resistance (Malik et al. 1989); and a variety of acetolactate synthase (ALS) genes, encoding resistance to sulfonylurea herbicides (Whitcomb 1999) such as the *Arabidopsis* multiherbicide-resistant gene, *csr1-4* (Mourad et al. 1994), or the *bar* gene from *Streptomyces* (Thompson et al. 1986), encoding resistance to phosphinotricin (Finale). In the case of the transmission of an autonomous element, such as *Ac*, progeny containing dispersed transposed *Ac* elements can be identified by classical genetic means such as transactivation of a *Ds*-induced reporter gene. It may also be possible to engineer the transposon with a pollen survival gene that permits only viable pollen that contain transposed elements, thereby completely eliminating the need for chemical selection or screens.

In addition to the transposon launching site, the suicide gene can be associated with the transposase source. In such cases, the "genetic sterility filter" would, in effect, enrich for dispersed elements while also eliminating the transmission of the transposase source to progeny. The simultaneous elimination of transposase would have the added benefit of stabilizing the transposed elements and preventing additional transpositions (secondary transpositions) from occurring in post-meiotic divisions or in progeny plants. It is also possible to have the transposase source and transposon launching sites in separate chromosomal regions, with one or the other associated with a suicide gene. For instances,

the launching site and the transposase source can be brought together in one genome on separate elements to achieve the same enrichment for dispersed transpositions. Moreover, in cases where localized transposition is desirable, i.e., to saturate a specific chromosomal region with insertions or to recover insertions in nearby genes of interest, the transposase source could be eliminated by the sterility filter method without necessarily eliminating linked transpositions.

10
25
236

Florally-Expressed Transposase

A second limitation using transposon for mutagenesis, gene-tagging and functional genomic analysis, is the lack of developmental and temporal control over the transposition process. In most cases, the transposase gene expression, under control of its own promoter, or under the control of constitutive promoters, is expressed during vegetative development of the plant. Vegetative expression of a transposase source leads to somatic transpositions. These transpositions are transmitted to progeny (germinal transpositions) only when these somatic cell lineages later produce megaspores or microspores. This is problematic for two reasons. One reason is that transpositions that occur during vegetative or early reproductive development can be clonally propagated and later transmitted into many gametes, resulting in a large number of non-independent elements recovered in progeny. This is undesirable when large numbers of independent transpositions are needed for functional genomic analysis or gene-tagging. Second, somatic transpositions often do not include lineages that give rise to gametes, such as those occurring in epidermal lineages or in terminal vegetative structures. These transpositions are never meiotically transmitted and therefore go unrecovered in progeny. This is problematic because these somatic events can be falsely identified in tissue DNA samples as mutations, yet are never recovered in progeny. This is especially problematic when mutational screens are PCR-based (examples: (McKinney et al. 1995; Tissier et al. 1999) (Krysan et al. 1996; Frey et al. 1997).

To minimize the problem of somatic transpositions, the transposase gene can be placed under the control of a floral-specific promoter that drives gene expression in subepidermal lineages of the flower that lead to the production of microspores and megaspores. Such promoters include those found in genes such as *agamous*, *apetala1*, *apetala2*, *apetala3*, *pistillate*, and their homologs found in other plant species such as maize, and rice. For example, the *apetala3* promoter drives expression of a reporter gene in petal and sepal primordial cells of the developing floral meristems. Transposase expression under the control of the *ap3* promoter will result in transpositions that are confined to floral development and when occurring in lineages that give rise to microspores, these events will be transmitted to the next generation. Control of transposition by this method has two effects: 1) it shuts down somatic transpositions; and 2) it leads to a large number of independent transpositions when pollen is derived from many different floral meristems. Hence, somatic tissue can be sampled without the concern of somatic or secondary transpositions, and each floral meristem becomes an independent source of transposition events.

- Albani, D., I. Altosaar, P.G. Arnison, and S.F. Fabijanski. 1991. A gene showing sequence similarity to pectin esterase is specifically expressed in developing pollen of *Brassica napus*. Sequences in its 5' flanking region are conserved in other pollen-specific promoters. *Plant Mol Biol* 16: 501-13.
- Albani, D., R. Sardana, L.S. Robert, I. Altosaar, P.G. Arnison, and S.F. Fabijanski. 1992. A *Brassica napus* gene family which shows sequence similarity to ascorbate oxidase is expressed in developing pollen. Molecular characterization and analysis of promoter activity in transgenic tobacco plants. *Plant J* 2: 331-42.
- Biehler, J. and R.G. Herrmann. 1990. Analysis of the promoters of the single-copy genes for plastocyanin and subunit delta of the chloroplast ATP synthase from spinach. *Eur J Biochem* 190: 415-26.

- X 1238
237
- Carpenter, J.L., S.E. Ploense, D.P. Snustad, and C.D. Silflow. 1992. Preferential expression of an alpha-tubulin gene of *Arabidopsis* in pollen. *Plant Cell* 4: 557-71.
- Chen, C.H., K.K. Oishi, B. Kloeckener-Gruissem, and M. Freeling. 1987. Organ-specific expression of maize *Adh1* is altered after a Mu transposon insertion. *Genetics* 116: 469-77.
- Cooley, M.B., A.P. Goldsbrough, D.W. Still, and J.I. Yoder. 1996. Site-selected insertional mutagenesis of tomato with maize *Ac* and *Ds* elements. *Mol Gen Genet* 252: 184-94.
- Custers, J.B., M.T. Oldenhof, J.A. Schrauwen, J.H. Cordewener, G.J. Wullems, and M.M. van Lookeren Campagne. 1997. Analysis of microspore-specific promoters in transgenic tobacco. *Plant Mol Biol* 35: 689-99.
- Day, C.D., B.F. Galgoci, and V.F. Irish. 1995. Genetic ablation of petal and stamen primordia to elucidate cell interactions during floral development. *Development* 121: 2887-95.
- DeLong, A., A. Calderon-Urrea, and S.L. Dellaporta. 1993. Sex determination gene *TASSELSEED2* of maize encodes a short-chain alcohol dehydrogenase required for stage specific floral organ abortion. *Cell* 74: 757-768.
- Dotson, S.B., M.B. Lanahan, A.G. Smith, and G.M. Kishore. 1996. A phosphonate monoester hydrolase from *Burkholderia caryophylli* PG2982 is useful as a conditional lethal gene in plants. *The Plant Journal* 10: 383-392.
- Fedoroff, N.V. and D.L. Smith. 1993. A versatile system for detecting transposition in *Arabidopsis*. *Plant J.* 3: 273-289.
- Frey, M., P. Chomet, E. Glawischnig, C. Stettner, S. Grun, A. Winklmaier, W. Eisenreich, A. Bacher, R.B. Meeley, S.P. Briggs, K. Simcox, and A. Gierl. 1997. Analysis of a chemical plant defense mechanism in grasses. *Science* 277: 696-699.
- Fritze, K. and R. Walden. 1995. Gene activation by T-DNA tagging. *Methods Mol Biol* 44: 281-94.
- Goldman, M.H., R.B. Goldberg, and C. Mariani. 1994. Female sterile tobacco plants are produced by stigma-specific cell ablation. *Embo J* 13: 2976-84.
- Guerrero, F.D., L. Crossland, G.S. Smutzer, D.A. Hamilton, and J.P. Mascarenhas. 1990. Promoter sequences from a maize pollen-specific gene direct tissue-specific transcription in tobacco. *Mol Gen Genet* 224: 161-8.
- Hamilton, D.A., M. Roy, J. Rueda, R.K. Sindhu, J. Sanford, and J.P. Mascarenhas. 1992. Dissection of a pollen-specific promoter from maize by transient transformation assays. *Plant Mol Biol* 18: 211-8.
- Harrison, L.A., M.R. Bailey, M.W. Naylor, J.E. Ream, B.G. Hammond, D.L. Nida, B.L. Burnette, T.E. Nickson, T.A. Mitsky, M.L. Taylor, R.L. Fuchs, and S.R. Padgett. 1996. The expressed protein in glyphosate-tolerant soybean, 5-enolpyruvylshikimate-3-phosphate synthase from *Agrobacterium* sp. strain CP4, is rapidly digested in vitro and is not toxic to acutely gavaged mice. *J Nutr* 126: 728-40.
- Hoef, A.M., E.J. Kok, E. Bouw, H.A. Kuiper, and J. Keijer. 1998. Development and application of a selective detection method for genetically modified soy and soy-derived products. *Food Addit Contam* 15: 767-74.
- Honma, M.A., B.J. Baker, and C.S. Waddell. 1993. High-frequency germinal transposition of *DsALS* in *Arabidopsis*. *Proc Natl Acad Sci U S A* 90: 6242-6.
- Izawa, T., T. Ohnishi, T. Nakano, N. Ishida, H. Enoki, H. Hashimoto, K. Itoh, R. Terada, C. Wu, C. Miyazaki, T. Endo, S. Iida, and K. Shimamoto. 1997. Transposon tagging in rice. *Plant Mol Biol* 35: 219-29.
- Kakimoto, T. 1996. CKI1, a histidine kinase homolog implicated in cytokinin signal transduction. *Science* 274: 982-5.
- Kardailsky, I., V.K. Shukla, J.H. Ahn, N. Dagenais, S.K. Christensen, J.T. Nguyen, J. Chory, M.J. Harrison, and D. Weigel. 1999. Activation tagging of the floral inducer *FT*. *Science* 286: 1962-5.

- Kim, Y. and G. An. 1992. Pollen-specific expression of the *Arabidopsis thaliana* alpha 1-tubulin promoter assayed by beta-glucuronidase, chloramphenicol acetyltransferase and diphtheria toxin reporter genes. *Transgenic Res* 1: 188-94.
- Klee, H.J., Y.M. Muskopf, and C.S. Gasser. 1987. Cloning of an *Arabidopsis thaliana* gene encoding 5-enolpyruvylshikimate-3-phosphate synthase: sequence analysis and manipulation to obtain glyphosate-tolerant plants. *Mol Gen Genet* 210: 437-42.
- Kloeckener-Gruissem, B., J.M. Vogel, and M. Freeling. 1992. The TATA box promoter region of maize *Adh1* affects its organ-specific expression. *Embo J* 11: 157-66.
- Knapp, S., Y. Larondelle, M. Rossberg, D. Furtek, and K. Theres. 1994. Transgenic tomato lines containing Ds elements at defined genomic positions as tools for targeted transposon tagging. *Mol Gen Genet* 243: 666-73.
- Krysan, P.J., J.C. Young, F. Tax, and M.R. Sussman. 1996. Identification of transferred DNA insertions within *Arabidopsis* genes involved in signal transduction and ion transport. *Proc. Natl. Acad. Sci. USA* 93: 8145-8150.
- Kyozuka, J., H. Fujimoto, T. Izawa, and K. Shimamoto. 1991. Anaerobic induction and tissue-specific expression of maize *Adh1* promoter in transgenic rice plants and their progeny. *Mol Gen Genet* 228: 40-8.
- Malik, J., G. Barry, and G. Kishore. 1989. The herbicide glyphosate. *Biofactors* 2: 17-25.
- Marrs, K.A., E.S. Casey, S.A. Capitant, R.A. Bouchard, P.S. Dietrich, I.J. Mettler, and R.M. Sinibaldi. 1993. Characterization of two maize HSP90 heat shock protein genes: expression during heat shock, embryogenesis, and pollen development. *Dev Genet* 14: 27-41.
- McCormick, S., D. Twell, G. Vancanneyt, and J. Yamaguchi. 1991. Molecular analysis of gene regulation and function during male gametophyte development. *Symp Soc Exp Biol* 45: 229-44.
- McKinney, E.C., E.C. Ali, N. Traut, K.A. Feldmann, and R.B. Meagher. 1995. Sequence-based identification of T-DNA insertion mutations in *Arabidopsis*: actin mutants *act2-1* and *act4-1*. *Plant J* 8: 613-622.
- Mourad, G., G. Haughn, and J. King. 1994. Intragenic recombination in the CSR1 locus of *Arabidopsis*. *Mol Gen Genet* 243: 178-84.
- O'keefe, D.P., J.M. Tepperman, C. Dean, K.J. Leto, D.L. Erbes, and T. Odell. 1994. Plant expression of a bacterial cytochrome P450 that catalyzes activation of a sulfonylurea pro-herbicide. *Plant Physiol* 105: 473-482.
- Osborne, B.I., C.A. Corr, J.P. Prince, R. Hehl, S.D. Tanksley, S. McCormick, and B. Baker. 1991. Ac transposition from a T-DNA can generate linked and unlinked clusters of insertions in the tomato genome. *Genetics* 129: 833-44.
- Padgett, S.R., Q.K. Huynh, J. Borgmeyer, D.M. Shah, L.A. Brand, D. Biest Re, B.F. Bishop, S.G. Rogers, R.T. Fraley, and G.M. Kishore. 1987. Bacterial expression and isolation of *Petunia hybrida* 5-enol-pyruvylshikimate-3-phosphate synthase. *Arch Biochem Biophys* 258: 564-73.
- Rommens, C.M., M.J. van Haaren, A.S. Buchel, J.N. Mol, A.J. van Tunen, H.J. Nijkamp, and J. Hille. 1992. Transactivation of Ds by Ac-transposase gene fusions in tobacco. *Mol Gen Genet* 231: 433-41.
- Sharknes, W.C. 1990. Entrapment vectors: a new tool for mammalian genetics. *Biotechnology* 8: 827-831.
- Siebertz, B., J. Logemann, L. Willmitzer, and J. Schell. 1989. cis-analysis of the wound-inducible promoter *wun1* in transgenic tobacco plants and histochemical localization of its expression. *Plant Cell* 1: 961-8.
- Sullivan, T.D., J.W. Schiefelbein, Jr., and O.E. Nelson, Jr. 1989. Tissue-specific effects of maize bronze gene promoter mutations induced by Ds1 insertion and excision. *Dev Genet* 10: 412-24.
- Sundaresan, V. 1996. Horizontal spread of transposon mutagenesis: new uses for old elements. *Trends Plant Sci.* 1: 184-190.

- 23
239
- Sundaresan, V., P. Springer, T. Volpe, S. Haward, J.D. Jones, C. Dean, H. Ma, and R. Martienssen. 1995. Patterns of gene action in plant development revealed by enhancer trap and gene trap transposable elements. *Genes Dev* 9: 1797-810.
- Takken, F.L., D. Schipper, H.J. Nijkamp, and J. Hille. 1998. Identification and Ds-tagged isolation of a new gene at the Cf-4 locus of tomato involved in disease resistance to *Cladosporium fulvum* race 5. *Plant J* 14: 401-11.
- Thomas, C.M., D.A. Jones, J.J. English, B.J. Carroll, J.L. Bennetzen, K. Harrison, A. Burbidge, G.J. Bishop, and J.D. Jones. 1994. Analysis of the chromosomal distribution of transposon-carrying T-DNAs in tomato using the inverse polymerase chain reaction. *Mol Gen Genet* 242: 573-85.
- Thompson, C.J., N.R. Movva, R. Tizard, R. Crameri, J.E. Davies, M. Lauwereys, and J. Botterman. 1986. Characterization of the herbicide-resistant gene *bar* from *Streptomyces hygroscopicus*. *EMBO J.* 6: 2519-2523.
- Thorsness, M.K., M.K. Kandasamy, M.E. Nasrallah, and J.B. Nasrallah. 1991. A Brassica S-locus gene promoter targets toxic gene expression and cell death to the pistil and pollen of transgenic *Nicotiana*. *Dev Biol* 143: 173-84.
- Tissier, A.F., S. Marillonnet, V. Klimyuk, K. Patel, M.A. Torres, G. Murphy, and J.D.G. Jones. 1999. Multiple independent defective Suppressor-Mutator transposon insertions in *Arabidopsis*: a tool for functional genomics. *The Plant Cell* 11: 1841-1852.
- Twell, D., J. Yamaguchi, and S. McCormick. 1990. Pollen-specific gene expression in transgenic plants: coordinate regulation of two different tomato gene promoters during microsporogenesis. *Development* 109: 705-13.
- Twell, D., J. Yamaguchi, R.A. Wing, J. Ushiba, and S. McCormick. 1991. Promoter analysis of genes that are coordinately expressed during pollen development reveals pollen-specific enhancer sequences and shared regulatory elements. *Genes Dev* 5: 496-507.
- van der Biezen, E.A., B.F. Brandwagt, W. van Leeuwen, H.J. Nijkamp, and J. Hille. 1996. Identification and isolation of the FEEBLY gene from tomato by transposon tagging. *Mol Gen Genet* 251: 267-80.
- van Tunen, A.J., L.A. Mur, G.S. Brouns, J.D. Rienstra, R.E. Koes, and J.N. Mol. 1990. Pollen- and anther-specific chi promoters from petunia: tandem promoter regulation of the chiA gene. *Plant Cell* 2: 393-401.
- Whitcomb, C.E. 1999. An introduction to ALS-inhibiting herbicides. *Toxicol Ind Health* 15: 231-9.
- Yazynin, S., H. Lange, T. Mokros, S. Deyev, and H. Lemke. 1999. A new phagemid vector for positive selection of recombinants based on a conditionally lethal barnase gene. *FEBS Lett* 452: 351-4.

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Verified Statement (Declaration) Claiming Small Entity
Status (37 CFR 1.9(f) and 1.27(d) - Non-Profit Organization

Applicants: Stephen L. Dellaporta and Maria A. Moreno

Filing Date: 28 February 2000

For: **Method for the Recovery of Dispersed, Stable and Independent
Transposition Events**

I hereby declare that I am an official empowered to act on behalf of the nonprofit organization identified below:

Yale University, Yale Station, New Haven, Connecticut 06520

Type of Organization: University or other institution of higher education.

I hereby declare that the University identified above qualifies as a nonprofit organization as defined in 37 CFR 1.9(e) for purposes of paying reduced fees under Section 41(a) and (b) of Title 35, United States Code with regard to the invention entitled: **Method for the Recovery of Dispersed, Stable and Independent Transposition Events**

by inventor(s) JStephen L. Dellaporta and Maria A. Moreno, described in the specification enclosed.

I hereby declare that rights under contract or law have been conveyed to and remain with the nonprofit organization identified above and/or there is an obligation under contract or law by the inventor(s) to convey rights to the nonprofit organization identified above with regard to the invention.

I acknowledge the duty to file, in this application or patent, notification of any change in status resulting in loss of entitlement to Small entity status prior to paying, or at the time of paying, the earliest of the issue fee or any maintenance fee due after the date on which status as a small entity is no longer appropriate (37 CFR 1.28(b)).

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application, any patent issuing thereon, or any patent to which this verified statement is directed.

Date:

28 February 2000

Name of person signing:

Henry S. Lowendorf
Henry S. Lowendorf

Title in Organization:

Associate Director, Office of Cooperative Research

Address of person signing:

Office of Cooperative Research
155 Whitney Avenue
PO Box 208336
New Haven, CT 06520-8336