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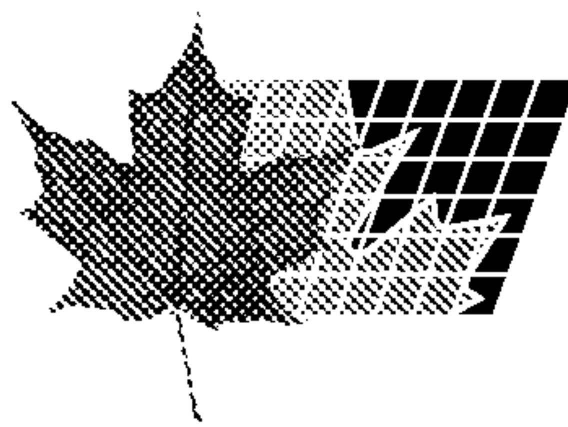

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(12) (19) (CA) **Demande-Application**

(21) (A1) **2,305,866**
(86) 1998/11/17
(87) 1999/05/27

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(51) Int.Cl.^{6} C12N 15/82, C12N 15/90, A01H 5/10, A01H 5/00
(30) 1997/11/18 (60/065,627) US
(30) 1997/11/18 (60/065,613) US

(54) **PROCEDE DE TRANSFORMATION STABLE ET DIRIGEE DE CELLULES EUCARYOTES**

(54) **A METHOD FOR DIRECTIONAL STABLE TRANSFORMATION OF EUKARYOTIC CELLS**

(57) Cette invention concerne des compositions et des procédés utilisés pour introduire des séquences nucléotidiques au niveau de sites cibles génomiques préférés dans un génome d' eucaryote. Les compositions comprennent des cassettes de transfert qui sont flanquées de sites de recombinaison non homologues. Le procédé consiste à transformer des cellules eucaryotes contenant des sites cibles à l'aide du procédé de transformation sans intégration. Le procédé assure l'intégration efficace de nucléotides dans des sites prédéterminés du génome et élimine l'intégration aléatoire d'ADN.

(57) The present invention is drawn to compositions and methods for introducing nucleotide sequences at preferred genomic target sites in a eukaryotic genome. The compositions comprise transfer cassettes which are flanked by nonhomologous recombination sites. The method involves transforming eukaryotic cells containing target sites utilizing non-integrating transformation methods. The method results in efficient integration of nucleotides into predetermined genetic locations and eliminates random DNA integration.

PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification [6] :
C12N 15/82, 15/90, A01H 5/00, 5/10

A1

(11) International Publication Number: WO 99/25854

(43) International Publication Date: 27 May 1999 (27.05.99)

(21) International Application Number: PCT/US98/24609

(22) International Filing Date: 17 November 1998 (17.11.98)

(30) Priority Data:
60/065,613 18 November 1997 (18.11.97) US
60/065,627 18 November 1997 (18.11.97) US

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(81) Designated States: AL, AM, AT, AT (Utility model), AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, CZ (Utility model), DE, DE (Utility model), DK, DK (Utility model), EE, EE (Utility model), ES, FI, FI (Utility model), GB, GE, GH, GM, HR, HU, ID, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SK (Utility model), SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published
With international search report.
Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(54) Title: A METHOD FOR DIRECTIONAL STABLE TRANSFORMATION OF EUKARYOTIC CELLS

(57) Abstract

The present invention is drawn to compositions and methods for introducing nucleotide sequences at preferred genomic target sites in a eukaryotic genome. The compositions comprise transfer cassettes which are flanked by nonhomologous recombination sites. The method involves transforming eukaryotic cells containing target sites utilizing non-integrating transformation methods. The method results in efficient integration of nucleotides into predetermined genetic locations and eliminates random DNA integration.

A METHOD FOR DIRECTIONAL STABLE TRANSFORMATION OF EUKARYOTIC CELLS

Field of the Invention

The invention relates to the genetic modification of eukaryotes. Particularly, the control of gene integration and expression in plants is provided.

Background of the Invention

Genetic modification techniques enable one to insert exogenous nucleotide sequences into an organism's genome. A number of methods have been described for the genetic modification of plants. All of these methods are based on introducing a foreign DNA into the plant cell, isolation of those cells containing the foreign DNA integrated into the genome, followed by subsequent regeneration of a whole plant. Unfortunately, such methods produce transformed cells that contain the introduced foreign DNA inserted randomly throughout the genome and often in multiple copies.

The random insertion of introduced DNA into the genome of host cells can be lethal if the foreign DNA happens to insert into, and thus mutate, a critically important native gene. In addition, even if a random insertion event does not impair the functioning of a host cell gene, the expression of an inserted foreign gene may be influenced by "position effects" caused by the surrounding genomic DNA. In some cases, the gene is inserted into sites where the position effects are strong enough to prevent the synthesis of an effective amount of product from the introduced gene. In other instances, overproduction of the gene product has deleterious effects on the cell.

Transgene expression is typically governed by the sequences, including promoters and enhancers, which are physically linked to the transgene. Currently, it is difficult to precisely modify the structure of transgenes once they have been introduced into plant cells. In many applications of transgene technology, it would

be desirable to introduce the transgene in one form, and then be able to modify the transgene in a defined manner. By this means, transgenes could be activated or inactivated where the sequences that control transgene expression can be altered by either removing sequences present in the original transgene or by inserting additional sequences into the transgene.

Therefore, it is essential to gain more control over foreign DNA integration into the nuclear genome of plant cells to expedite the efficient production of transgenic plants with stable and reliable expression of transgenic traits. Relatively low frequency and randomness of foreign DNA integration make genetic transformation a labor-intensive and unpredictable procedure. Multi-copy, random integrations of transforming DNA molecules frequently lead to aberrant expression of foreign genes, affect expression of endogenous genes, and provide transgenic organisms with unstable transgenic traits. All plant transformation procedures currently in use take advantage of biochemical pathway(s) involving random, illegitimate recombination to integrate foreign DNA. Illegitimate recombinations constitute the intrinsic property of a conventional genetic transformation process. As such, desired DNA integration events cannot be separated, or preferably selected for, from among any excessive random integrations, unless a different mechanism governs the integration of productive events.

One approach for gene targeting, which is extensively pursued, involves the use of DNA homologous recombination for integration of foreign DNA into pre-selected genomic locations. The process involves both productive (homologous, targeted) and non-productive (illegitimate, random) integrations. Innovative strategies have already been proposed to reduce, or eliminate random integration of targeting vectors. They include the use of negative selection markers to eliminate random integrations by selection against actively expressed foreign genes, excisions of randomly integrated copies of foreign genes by the use of site-specific recombinations, or identification and application of specific inhibitors of non-homologous recombinations such as poly-(ADP-ribosylation) inhibitors.

The basic problem with current gene targeting procedures, however, is that the efficiency of homologous recombination in somatic cells of higher eukaryotes is extremely low being about 1,000-, 1,000,000-fold less frequent than illegitimate, random integrations. Taking into account that random integrations are barely considered satisfactory in the conventional genetic transformation procedures, routine gene targeting is presently not practical, at least in plant genetic transformation systems. Therefore, methods to control targeting and integration of foreign genes into the genome are needed.

Summary of the Invention

Compositions and methods for introducing nucleotide sequences only at preferred genomic target sites are provided. The compositions comprise transfer cassettes which incorporate site-specific recombination sequences. The method involves transforming eukaryotic cells containing target sites utilizing transformation vectors which do not integrate genomic DNA, or integrate at very low frequency, unless provided with a site-specific integration system. The method results in efficient integration of nucleotides into predetermined genetic locations and minimizes or precludes random DNA integration.

Detailed Description of the Invention

Compositions and methods for introducing nucleotide sequences into predetermined genomic target sites in a plant genome is provided. The methods preclude the random integration of DNA into the genome. The methods use novel recombination sites in a gene targeting system which facilitates directional targeting of desired genes and nucleotide sequences into corresponding recombination sites previously introduced into the target genome. Methods for the production of transgenic plants containing specific recombination sites integrated in the plant genome are described in co-pending patent application entitled "Compositions and Methods for Genetic Modification of Plants" filed concurrently herewith and herein incorporated by reference. Methods for the site-specific integration of DNA into wild-type and mutant lox sites placed in the plant genome may also be found in Albert *et al.* (1995) *The Plant Journal* 7:649-659.

Generally, for targeted insertion of nucleotide sequences, two non-identical recombination sites are introduced into the target organism's genome establishing a target site for insertion of nucleotide sequences of interest. These recombination sites may flank other nucleotide sequences. Once a stable plant or cultured tissue is established a second construct, or nucleotide sequence of interest, flanked by corresponding recombination sites as those flanking the target site, is introduced into the stably transformed plant or tissues in the presence of a recombinase protein. This process results in exchange of the nucleotide sequences between any two identical recombination sites of the target site and the transfer cassette.

It is recognized that the transformed organism may comprise multiple target sites; *i.e.*, sets of non-identical recombination sites. In this manner, multiple manipulations of the target site in the transformed organism are available. By target site in the transformed organism is intended the DNA sequence that has been inserted into the transformed organism's genome and comprises the non-identical recombination sites.

Examples of recombination sites for use in the invention are known in the art and include *FRT* sites (See, for example, Schlake and Bode (1994) *Biochemistry* 33:12746-12751; Huang *et al.* (1991) *Nucleic Acids Research* 19:443-448; Paul D. Sadowski (1995) In Progress in Nucleic Acid Research and Molecular Biology vol. 51, pp. 53-91; Michael M. Cox (1989) *In Mobile DNA*, Berg and Howe (eds) American Society of Microbiology, Washington D.C., pp. 116-670; Dixon *et al.* (1995) 18:449-458; Umlauf and Cox (1988) *The EMBO Journal* 7:1845-1852; Buchholz *et al.* (1996) *Nucleic Acids Research* 24:3118-3119; Kilby *et al.* (1993) *Trends Genet.* 9:413-421: Rossant and Geagy (1995) *Nat. Med.* 1: 592-594; *Lox* Albert *et al.* (1995) *The Plant J.* 7:649-659: Bayley *et al.* (1992) *Plant Mol. Biol.* 18:353-361; Odell *et al.* (1990) *Mol. Gen. Genet.* 223:369-378; and Dale and Ow (1991) *Proc. Natl. Acad. Sci. USA* 88:10558-105620; Qui *et al.* (1994) *Proc. Natl. Acad. Sci. USA* 91:1706-1710; Stuurman *et al.* (1996) *Plant Mol. Biol.* 32:901-913; and Dale *et al.* (1990) *Gene* 91:79-85; all of which are herein incorporated by reference.)

By "target site" is intended a predetermined genomic location within the nucleus where the integration of a specific transformed nucleotide sequence is to occur. The target site of the invention is characterized by being flanked by non-identical recombination sites
5 corresponding to the non-identical recombination sites flanking the nucleotide sequence to be transformed into the cell, (the transfer cassette), and integrated into the genome. The non-identical recombination sites in combination with recombinase activity result in a recombination event between the non-identical recombination sites of the target site and the target cassette (the integrating sequence). This event produces an integrated nucleotide
10 sequence into the specified genomic location.

To practice the methods of the invention, a transformed organism, particularly a plant, of interest containing a target site integrated into its genome is needed. The target site is characterized by being flanked by non-identical recombination sites. A targeting cassette is additionally required containing a nucleotide sequence flanked by corresponding
15 non-identical recombination sites as those sites contained in the target site of the transformed organism. A recombinase which recognizes the non-identical recombination sites and catalyzes site-specific recombination is required.

By non-identical recombination sites is intended that the flanking recombination sites are not identical. That is, one flanking recombination site may be a *FRT* site (SEQ ID
20 NO: 1 and 2) where the second recombination site may be a mutated *FRT* site (SEQ ID NOs:3, 4 and 5). The non-identical recombination sites used in the methods of the invention prevent or greatly suppress recombination between the two flanking recombination sites and excision of the nucleotide sequence contained therein. Accordingly, it is recognized that any suitable non-identical recombination sites may be
25 utilized in the invention, including *FRT* and mutant *FRT* sites, *FRT* and *lox* sites, *lox* and mutant *lox* sites, as well as other recombination sites known in the art.

By suitable non-identical recombination site implies that in the presence of active recombinase, excision of sequences between two non-identical recombination

sites occurs, if at all, with an efficiency considerably lower than the recombinationally-mediated exchange targeting arrangement of nucleotide sequences into the plant genome. Thus, suitable non-identical sites for use in the invention include those sites where the efficiency of recombination between the sites is low; 5 for example, where the efficiency is less than about 30 to about 50%, preferably less than about 10 to about 30%, more preferably less than about 5 to about 10%, even more preferably less than about 1%. .

As noted above, the recombination sites in the targeting cassette correspond to those in the target site of the transformed organism. That is, if the target site of 10 the transformed organism contains flanking non-identical recombination sites of *FRT* and a mutant *FRT*, the targeting cassette will contain the same *FRT* and mutant *FRT* non-identical recombination sites.

It is furthermore recognized that the recombinase, which is used in the invention, will depend upon the recombination sites in the target site of the 15 transformed organism and the targeting cassette. That is, if *FRT* sites are utilized, the FLP recombinase will be needed. In the same manner, where *lox* sites are utilized, the Cre recombinase is required. If the non-identical recombination sites comprise both a *FRT* and a *lox* site, both the FLP and Cre recombinase will be required in the plant cell.

20 The present invention utilizes nonintegrating vectors and methods of introducing transfer cassettes into the genome of the organism of interest. In this manner, efficient site specific integration of exogenous nucleotide sequences is promoted and random insertion is avoided. By efficient site specific DNA integration is intended the maximization of recombination events between the 25 introduced integrating sequence and the predetermined genomic target sites of transformed cells. That is, the methods prevent random DNA integration and insertion of DNA into sites other than the intended target site within the eukaryotic genome. Prevention of random integration is accomplished through the utilization of non-integrating nucleic acid molecules in association with the gene targeting 30 method set forth in the copending application disclosed above.

The methods of the invention can be used to target nucleotide sequences into any eukaryote. By eukaryote is intended to mean any higher eukaryotic organism, more specifically plants and even more specifically monocotyledonous plants.

Transient transformation methods for plants are available in the art and include DNA delivery systems which are capable of introducing nucleotide sequences into a eukaryotic cell, where these sequences either contain no homology to the genomic sequence of the target cell or have been modified in a way that precludes their own recombination or integration into the genome. Such non-integrative DNA delivery systems include the use of *Agrobacterium* for monocot, modified *Agrobacterium*-mediated T-DNA transfer for dicots, and viral vectors. These systems can effectively deliver DNA into plant cells without random integration. Thus, the nucleotide sequences are only or preferably able to insert at predetermined target sites and under suitable conditions such as those provided in copending application "Compositions and Methods for Genetic Modification of Plants". Thus, by non-integrating methods are intended methods of introducing nucleotide sequences into a cell without subsequent random integration or with minimum random integration. Random integration refers to integration or insertion of the nucleotide sequences at sites other than at corresponding target sites.

The development of plant virus gene vectors for expression of foreign genes in plants provides a means to provide high levels of gene expression within a short time. The benefits of virus-based transient RNA and DNA replicons include rapid and convenient engineering coupled with flexibility for expeditious application in various plant species. In this manner, autonomously replicating viruses offer numerous advantages for use as vehicles for transient expression of foreign genes, including their characteristic high levels of multiplication and concomitant levels of transient gene expression . Such viruses include but are not limited to Bromovirus, Caulimovirus, Furovirus, Geminivirus, Hordeivirus, Potexvirus, Tobamovirus, Tobravirus, Tombusvirus, Potyvirus, Comovirus, Alfamovirus, Dianthovirus, etc. See, for example, Ugaki *et al.*(1991) *Nucleic Acids Res.* 19:371-377; Timmermans *et al.* (1992) *Nucleic Acids Res.* 20:4047-4054; Louie, Raymond (1995)

Phytopathology 85:139-143; Scholthof *et al.* (1996) *Annu. Rev. Phytopathol.* 34:299-323, and the references cited therein, all of which are herein incorporated by reference.

Viral methods use viral vectors that replicate as extrachromosomal DNA, or RNA molecules. Shuttle vectors may be constructed that contain viral sequences critical to replication. Such vectors can be used to introduce transfer cassettes containing nucleotide sequences into plants and plant cells. Such vectors, which have included viral genomic DNA from the geminiviruses (wheat dwarf virus or maize streak virus) can be transformed into monocotyledonous plants and propagate in the plant cell nucleus to high copy numbers (Timmermans *et al.* (1992) *Nucleic Acids Res.* 20:4047-4054). Once viral particles are in the plant cell, they can accumulate to high copy numbers which will increase the probability that a recombination event will occur between the non-identical recombination sites flanking the target sequence, leading to a successful integration of the nucleotide sequence of interest.

Agrobacterium-mediated gene transfer exploits the natural ability of *Agrobacterium tumefaciens* to transfer DNA into plant cells. *Agrobacterium* is a plant pathogen that transfers a set of genes encoded in a region called T-DNA of the Ti plasmid into plant cells at wound sites. The typical result of gene transfer is a tumorous growth called a crown gall in which the T-DNA is stably integrated into a host chromosome. The ability to cause crown gall disease can be removed by deletion of the genes conferring tumorigenesis in the T-DNA without loss of DNA transfer and integration. The DNA to be transferred is attached to border sequences that define the end points of an integrated T-DNA.

Agrobacterium-based transformation methods may also be used in the invention. The *Agrobacterium* system can be used to introduce transfer cassettes into monocotyledonous plant cells to take advantage of the inability of T-DNA to efficiently integrate into the genome of monocot plants. It is known that in nature *Agrobacterium* does not transform monocots. Thus, supervirulent strains of *Agrobacterium* have been developed to utilize *Agrobacterium* as a vector to

transform monocots. The present invention takes advantage of the ability of the *Agrobacterium* system to introduce transfer cassettes into monocot cells without the ability to direct incorporation of the transferred sequence into the monocot genome. It has been demonstrated that the *Agrobacterium* system can be used to transfer DNA from the bacteria to the plant cell. See, for example Grimsley *et al.* (1988) *BioTechnology* 6:185-189; Dasgupta *et al.* (1991) *J. Gen. Virol.* 72:1215-1221; and the references cited therein.

It is further recognized that *Agrobacterium* based transfer systems may be modified such that the *Agrobacterium* directs introduction and transient expression of the transferred DNA (in this instance the transfer cassette), but is unable to direct efficient integration of T-DNA into the genome of the plant. Such modified *Agrobacterium* systems are available in the art. See, for example, Narasimhulu *et al.* (1996) *The Plant Cell* 8:873-886, herein incorporated by reference. Narasimhulu *et al.* demonstrate that the C-terminal nuclear localization signal of the VirD2 protein is not essential for nuclear uptake of T-DNA and further show that the ϖ domain of VirD2 is required for efficient integration of T-DNA into the plant genome. Thus, mutations into this region will allow introduction and transient expression of the transfer cassette but avoid unwanted random insertion. For example, a nonpolar transposon insertion into the C-terminal coding region of virD2 resulted in only slightly decreased production of mRNA, although this insertion resulted in the loss of the nuclear localization sequence the ϖ region from VirD2 protein and rendered the bacterium avirulent. Thus, the modified *Agrobacterium* is particularly beneficial for use in dicots.

The non-integrating transformation methods can be used to introduce the transfer cassettes into any plant cell. In this manner, genetically modified plants, plant cells, plant tissue, seed, and the like can be obtained. Transformation protocols may vary depending on the type of plant or plant cell, i.e. monocot or dicot, targeted for transformation.

Once the transfer cassettes have been introduced into the plant, the flanking non-identical recombination sites of transfer cassettes recombine with corresponding

sites of the target within the plant genome. The cells having a modified genome may be grown into plants in accordance with conventional approaches. See, for example, McCormick *et al.* (1986) *Plant Cell Reports, 5*:81-84. These regenerated plants may then be pollinated with either the same transformed strain or different strains, and the resulting hybrid having the desired phenotypic characteristic identified. Two or more generations may be grown to ensure that the subject phenotypic characteristic is stably maintained and inherited and then seeds harvested to ensure the desired phenotype or other property has been achieved.

Because of the use of non-integrating means of introducing transfer cassettes provided herein, the plants of the invention may be distinguishable from other transformation methods as the modified plants of the invention will contain nucleotide sequences of interest inserted into the plant genome only or substantially at target sites. By substantially at target sites, is intended that target cassettes are inserted into the genome only about five times at non-target sites, preferably less than about three times, more preferable about one time or less.

It is recognized that the methods of the invention can additionally be used in other eukaryotic cells for efficient insertion of nucleotide sequences of interest, including mammalian cells. In this manner, target sites can be introduced into a cell line and non-integrating methods used to introduce transfer cassettes into the cells. This provides an efficient means of introducing genes of interest into animals, particularly agricultural animals.

Viral means of introducing DNA into mammalian cells are known in the art. In particular, a number of vector systems are known for the introduction of foreign or native genes into mammalian cells. These include SV40 virus (See, e.g., Okayama *et al.* (1985) *Molec. Cell Biol. 5*:1136-1142); Bovine papilloma virus (See, e.g., DiMaio *et al.* (1982) *Proc. Natl. Acad. Sci. USA 79*:4030-4034); adenovirus (See, e.g., Morin *et al.* (1987) *Proc. Natl. Acad. Sci. USA 84*:4626; Yifan *et al.* (1995) *Proc. Natl. Acad. Sci. USA 92*:1401-1405; Yang *et al.* (1996) *Gene Ther. 3*:137-144; Tripathy *et al.* (1996) *Nat. Med. 2*:545-550; Quantin *et al.* (1992) *Proc. Natl. Acad. Sci. USA 89*:2581-2584; Rosenfeld *et al.* (1991) *Science*

252:431-434; Wagner (1992) *Proc. Natl. Acad. Sci. USA 89*:6099-6103; Curiel *et al.* (1992) *Human Gene Therapy 3*:147-154; Curiel (1991) *Proc. Natl. Acad. Sci. USA 88*:8850-8854; LeGal LaSalle *et al.* (1993) *Science 259*:590-599); Kass-Eisler *et al.* (1993) *Proc. Natl. Acad. Sci. USA 90*:11498-11502); adeno-associated virus (See, e.g., Muzyczka *et al.* (1994) *J. Clin. Invest. 94*:1351; Xiao *et al.* (1996) *J. Virol. 70*:8098-8108); herpes simplex virus (See, e.g., Geller *et al.* (1988) *Science 241*:1667; Huard *et al.* (1995) *Gene Therapy 2*:385-392; U.S. Patent No. 5,501,979); retrovirus-based vectors (See, for example, Curran *et al.* (1982) *J. Virol. 44*:674-682; Gazit *et al.* (1986) *J. Virol. 60*:19-28; Miller, A.D. (1992) *Curr. Top. Microbiol. Immunol. 158*:1-24; Cavanaugh *et al.* (1994) *Proc. Natl. Acad. Sci. USA 91*:7071-7075; Smith *et al.* (1990) *Molecular and Cellular Biology 10*:3268-3271); herein incorporated by reference. See also, Wu *et al.* (1991) *J. Biol. Chem. 266*:14338-14342; Wu and Wu (*J. Biol Chem.* (1988)) *263*:14621-14624; Wu *et al.* (1989) *J. Biol. Chem. 264*:16985-16987; Zenke *et al.* (1990) *Proc. Natl. Acad. Sci. USA 87*:3655-3659; Wagner *et al.* (1990) *87*:3410-3414.

Standard techniques for the construction of the vectors of the present invention are well-known to those of ordinary skill in the art and can be found in such references as Sambrook *et al.*, Molecular Cloning: A Laboratory Manual, 2nd ed. (Cold Spring Harbor, New York, 1989). A variety of strategies are available for ligating fragments of DNA, the choice of which depends on the nature of the termini of the DNA fragments and which choices can be readily made by those of skill in the art.

The following examples are offered by way of illustration not by way of limitation.

EXPERIMENTAL

Example 1. Creation of novel non-identical *FRT* sites

DNA fragments containing novel *FRT* sequences are constructed either by synthesizing, annealing and ligating complementary oligonucleotides or by creating

primers for PCR amplification of a DNA product containing the new *FRT* sequence near the 5' end of the PCR product. The newly constructed *FRT* product includes flanking restriction sites useful for cloning into plant expression units. In general, the 5' end is flanked by an NheI site and a terminal NcoI site. The NcoI site includes the bases ATG, which are advantageously used in newly developed vector constructs as the recognition sequence to initiate an open reading frame. In sequence-based constructs designated noATG/*FRT*, the NheI site is used for cloning thereby eliminating the upstream ATG in the process. At the 3' end of the *FRT* sequence, a restriction site is included enabling unique identification of the individual spacer sequences. As specific examples, the wild type *FRT* site (designated *FRT*1 here, SEQ ID NO: 2) is cloned with a flanking BglII site, the *FRT*5 site (spacer TTCAAAAG) has a ScaI site, the *FRT*6 site (SEQ ID NO: 4, spacer TTCAAAAA) has an AatII site, and the *FRT*7 site (SEQ ID NO: 5) spacer TTCAATAA) has an SpeI site. The outermost flanking restriction site is an XhoI site and is used to clone a gene of interest into the open reading frame.

The structures and sequences of the *FRT* sites as designed and/or used in the present invention example are depicted below with positions of restriction sites, repeats and spacer regions indicated.

FRT1 (SEQ ID NO: 2)

NcoI NheI Repeat 1 Repeat 2 Spacer Inverted Repeat BglII XhoI

5′ CCATGGCTAGC GAAGTTCCTATTCC GAAGTTCCTATTC TCTAGAAA GTATAGGAACTTC AGATCTCGAG

FRT5 (SEQ ID NO: 3)

NcoI NheI Repeat 1 Repeat 2 Spacer Inverted Repeat ScaI XhoI

5′ CCATGGCTAGC GAAGTTCCTATTCC GAAGTTCCTATTC TTCAAAAG GTATAGGAACTTC AGTACTCGAG

FRT6 (SEQ ID NO: 4)

NcoI NheI Repeat 1 Repeat 2 Spacer Inverted Repeat AatII XhoI

5′ CCATGGCTAGC GAAGTTCCTATTCC GAAGTTCCTATTC TTCAAAAA GTATAGGAACTTC AGACGTCCTCGAG

FRT7 (SEQ ID NO: 5)

NcoI NheI Repeat 1 Repeat 2 Spacer Inverted Repeat SpeI XhoI

5´ CCATGGCTAGC GAAGTTCCTATTCC GAAGTTCCTATTCTTCAATAA GTATAGGAACTTCACTAGTTCTCGAG

Example 2. Creation of *Agrobacterium* plant transformation vectors containing novel non-identical FRT sites for dicots.

Bacterial Strains and Growth Conditions

Escherichia coli strains are grown at 37°C on Luria-Bertani medium (Maniatis, *et al.*(1982) Molecular Cloning: A Laboratory Manual (Cold Spring Harbor, NY: Cold Spring Harbor Laboratory)) and *Agrobacterium tumefaciens* strains at 30°C on AB-sucrose minimal medium (Lichtenstein, *et al.* (1986) Genetic engineering of plants, in DNA Cloning: A Practical Approach, Vol. 2, D.M. Glover, ed. (Oxford, UK: IRL Press), pp. 67-119) containing the appropriate antibiotics. Antibiotic concentrations (μg/mL) are as follows: ampicillin, 100; kanamycin, 20 for *E. coli*; carbenicillin, 100; kanamycin, 100; spectinomycin, 100; rifampicin, 10 for *Agrobacterium*.

Construction of pBISN1 and Its Derivatives

To construct the transferred (T)-DNA binary vectors, one can clone an EcoRI-SalI fragment of pCNL65 (Liu *et al.* (1992) *Plant Mol. Biol.* 20:1071-1087), containing a β-glucuronidase *gusA* gene with the ST-LS1 second intron (Vancanneyt *et al.*(1990) *Mol. Gen. Genet.* 220:245-250), into pBluescript SK+ (Stratagene). This plasmid is digested with XhoI (upstream of the *gusA* gene), the overhanging ends filled in, using the Klenow fragment of DNA polymerase I and nucleotide triphosphates, and the *gusA*-intron gene using *Sac*I is released. The *gusA* gene (lacking an intron) from pE1120 (Ni *et al.*(1995) *Plant J.* 7:661-676) is removed by using *Sma*I and *Sac*I and replaced with the *gusA*-intron gene fragment described above. The final plasmid will contain T-DNA border repeat sequences, a nopaline synthase-neomycin phosphotransferase II gene for selection of kanamycin-resistant transgenic plants, and a *gusA*-intron gene under the regulation of the promoter from pE1120.

Based on the design of FRT sites as described above, various methods such as PCR, mutagenesis and/or other standard cloning protocols can be used to introduce the FRT sites into desired locations in the plasmid above during the vector creation process. Example methods are described in a co-pending patent application entitled "Compositions and Methods for Genetic Modification of Plants" filed concurrently herewith and herein incorporated by reference.

The plasmid described above is placed into an IncW replicon as described by Narisasimhulu *et al.* (1996) *The Plant Cell* 8:873-886, herein incorporated by reference.

The plasmid is mobilized into *Agrobacterium* strains, using a triparental mating procedure (Figurski and Helinski (1979) *Proc. Natl. Acad. Sci. USA* 76:1648-1652) and the mobilizing plasmid pRK2013 (Ditta *et al.* (1980) *Proc. Natl. Acad. Sci. USA* 77:7347-7351). The trans-conjugants are selected on AB-sucrose minimal medium containing rifampicin and kanamycin or rifampicin and spectinomycin. Alternatively, the *Agrobacterium* binary system as described by Bevan, M. (1984) *Nucl. Acids Res. 12*:8711-8721; herein incorporated by reference.

Growth and Infection of Plant Cells and Determination of GUS Activity

Nicotiana tabacum BY-2 cells are propagated in Murashige and Skoog medium (Gibco BRL) containing 3% sucrose, 1 μg/mL thiamine, 0.2 μg/mL 2,4-D, and 370 μg/mL KH_2PO_4. *Zea mays* Black Mexican Sweet (BMS) cells are propagated in Murashige and Skoog medium containing 2% sucrose, 2 μg/mL 2,4-D, 0.2 mg/mL myoinositol, 0.13 mg/mL L-asparagine, 0.13 μg/mL nicotinic acid, and 0.25 μg/mL each of thiamine, pyridoxine, and pantothenic acid. The cultures are shaken at 140 rpm at 25°C in continuous light.

To infect plant cells, virulence (*vir*) gene activity is induced in *Agrobacterium* with acetosyringone. *Agrobacterium* cells are grown to a density of 2×10^9 cells per mL (A = 100, using a Klett-Summerson spectrophotometer, red filter) in AB-sucrose medium. The cells are centrifuged at 10,000g, suspended at a

concentration of 1 x 10 ^{9} cells per mL (A = 50) in induction medium (AB salts, 0.5% glucose, 2mM sodium phosphate, 50 mM Mes, pH 5.6, 50μM acetosyringone), and incubated with gentle shaking at 25°C for 14 to 18 hr. After washing the bacterial cells in plant culture medium, plant cells are inoculated with
5 induced *Agrobacterium* (~20 bacterial cells per plant cell, except where noted otherwise) and cocultivated at 25°C with shaking at 140 rpm for various periods of time. Most of the bacteria is washed off by centrifugation of the cocultivation mixture at 300 rpm (model GLC-2 clinical centrifuge; Beckman Sorvall, Newtown, CT) for 2 min. The plant cell pellet is suspended and washed once more in plant
10 culture medium and then resuspended in culture containing either 100 μg/mL timentin or 200 μg/mL cefotaxime. To collect plant cells for isolation of RNA, the cells are washed three times, as described above, in plant culture medium. RNA is extracted from these cells either directly after harvesting (either of the two methods listed below) or after freezing in liquid nitrogen and storage at -70°C (TRIzol
15 reagent [Gibco BRL] extraction method).

The percentage of cells expressing GUS activity is determined by incubating the cells in GUS histochemical staining solution (50 mM NaH_2PO_4, 10 mM Na_2; EDTA, 0.3 M mannitol, 20% methanol, and 1 mM 5-bromo-4-chloro-3-indolyl β-D-glucuronic acid [X-gluc] overnight at 37°C (Kosuge *et al.*(1990) *Plant Sci.*
20 70:133-140).

Example 3. Creation of *Agrobacterium* plant transformation vectors containing novel non-identical *FRT* sites for monocots.

Agrobacterium-mediated DNA transfer to maize is roughly as efficient as it is to dicotyledenous plants in different, but functionally equivalent agroinfection systems. See, Grimsley *et al.*(1987) Agroinfection, p. 87-107. *In: Plant DNA Infectious Agents*. Hohn and Schell (Eds.)Springer, New York and Vienna. This observation questions the definition of the host/parasite interaction, since the steps up to and including DNA transfer do seem to occur in a plant that does not produce tumors.

EXPERIMENTAL PROTOCOL

Plasmid constructions, bacterial strains and media. Construction of the transferred (T)-DNA binary vectors including incorporation of FRT sites is essentially as described in Example 2. Plasmids are maintained in *Escherichia coli* strain DHl (Maniatis and Sambrook (1982) Molecular Cloning: A Laboratory Manual. (Cold Spring Harbor, NY: Cold Spring Harbor Laboratory)) at 37°C or in *A. tumefaciens* strain C58 (Holsters *et al.* (1980) *Plasmid* 3:212-230) at 28°C. The strain C58(pTiC58,pEAP37) carrying a dimer of MSV genomes in the T-DNA of a binary vector has been described (Grimsley *et al.* (1987) *Nature* 325:177-179). C58(pGV3850::pEAP25) is constructed by (i) cutting pMSV12 (Grimsley *et al.* (1987) *supra*) at its unique SalI site, (ii) cutting pEAP1, a 7.6kb large mobilizable plasmid encoding bacterial resistances for ampicillin and kanamycin and a kanamycin resistance gene expressed in plants with SalI, (iii) ligating (i) + (ii) to produce a plasmid, PHMI, which could be selected in *E. coli* by ampicillin, kanamycin and chloramphenicol resistance, and (iv) mobilization (Rogers *et al.*(1986) *Meth. Enzymol.* 118:627-640) of the plasmid PHMI to C58(pGV3850) (Zambryski *et al.*(1983) *EMBO J.* 2:2143-2150) producing C58(pGV3850::PHMI). Restriction enzyme digestions and ligations are done under conditions recommended by the manufacturer (Biofinex, Switzerland). Prior to inoculation, strains of *Agrobacterium* are streaked out on YEB (Grimsley *et al.*(1986) *Proc. Natl. Acad. Sci. USA*, 83:3282-3286) plates solidified with 1.5% agar and

supplemented with 100μg/ml rifampicin and 25μg/ml kanamycin and allowed to grow for 48h. A single colony is used to inoculate 10 ml of liquid YEB medium in a 100ml Erlenmeyer flask supplemented with antibiotics as previously. Growth is continued with shaking at 200 r.p.m. for 24h, then 500μl of this culture is used to
5 inoculate a similar flask and growth continued for a further 20h. This procedure yields a final density of viable *Agrobacterium* cells in the region of 10^9/ml (estimated by plating). The cells are then harvested by centrifugation and resuspended in an equal volume of 10mM $MgSO_4$ without antibiotics; such a suspension is subsequently referred to as undiluted or 10^0 dilution; for experiments
10 involving a dilution series 10mM $MgSO_4$ was also used as the diluent.

Growth of plants: Maize seeds for 10-day old plants are sown in pots in a phytotron in a 12 hour light/dark cycle at 25°C in a light intensity of about 10000 lux (Sylvania 215W fluorescent lamps type F96T12/CW/VHO) then moved to the BL3 containment laboratory immediately prior to inoculation; subsequent growth
15 conditions have been described (Grimsley *et al.*(1987) *Nature* 325:177-179). Three-day old seedlings are prepared by (i) sterilization by stirring for 20min in 0.7% calcium hypochlorite solution, (ii) washing three times (stirring for 20min each time) in sterile distilled water (iii) preparing 9cm diameter presterilized Petri dishes with 3 sheets of sterile 8.5cm diameter Macherey-Nagel (Germany) filter
20 paper in the bottom and *ca.* 10ml of sterile water per dish, (iv) putting *ca.* 20 seeds into each geranium dish, and (v) incubating in the dark at 28°C for 3 days, or until the distance between the scutellar node and the apical tip of the coleoptiles is 1-2 cm.

Inoculation of plants: For injections, a 50μl or a 100μl Hamilton syringe
25 fitted with a 0.4mm diameter disposable needle is loaded with the bacterial suspension avoiding trapped air bubbles. Between inoculations with different bacterial strains the needle is discarded and the syringe flushed out 3 times with 100% ethanol and 3 times with sterile distilled water. 10-day old plants are inoculated by (i) abrasion of an upper leaf, applying 20μl of suspension, and
30 rubbing in with carborundum powder until the leaf appears wet all over, (ii)

injection of 10μl of bacterial suspension into the central part of the plant either just above the first leaf blade, or 1cm below the first leaf blade, or at the base of the plant, in the meristematic region where adventitious roots later begin to appear. Three-day old seedlings are injected with 10μl of bacterial suspension in different ways by (i) pushing the needle down through the apical tip of the coleoptile to the coleoptilar node, (ii) injecting 2mm below the apical tip of the coleoptile, (iii) 2 mm above the coleoptilar node, (iv) at the coleoptilar node, (iv) 2mm below the coleoptilar node, (v) at the scutellar node, and by pushing the needle up through the primary root to a region close to the scutellar node. Ten μl is used as a standard inoculum of bacterial suspension, but only 1-2μl routinely remains in the inoculation site, the rest is forced out, usually coming out from the point of entry of the inoculating needle. Following inoculation seedlings are planted immediately in damp soil, incubated as before (Grimsley *et al.*(1987) *Nature* 325:177-179), and observed daily for the appearance of symptoms of viral infection, characterized by the appearance of yellow spots and/or stripes at the base of new leaves.

Histology: Plant pieces containing the site of injection are collected, fixed in Carnoy's fluid (60% ethanol, 30% chloroform, 10% glacial acetic acid) overnight, dehydrated in a series of 50%, 75% and 100% ethanol, and then prepared for the infiltration of paraffin wax in a series of 25%, 50%, 75% and 100% xylene in ethanol (at least 30 min is allowed for each of the serial steps). Finally they are embedded in paraffin at 65°C and cut with a microtome into slices of 15-35μm depending upon the size of the plant pieces. All procedures are carried out according to Sass (Sass, J.E. (1958). Botanical Microtechnique, p. 14-54. The Iowa State University Press, Ames, Iowa.).

All publications and patent applications mentioned in the specification are indicative of the level of those skilled in the art to which this invention pertains. All publications and patent applications are herein incorporated by reference to the same extent as if each individual publication or patent application was specifically and individually indicated to be incorporated by reference.

Although the foregoing invention has been described in some detail by way of illustration and example for purposes of clarity of understanding, it will be obvious that certain changes and modifications may be practiced within the scope of the appended claims.

SEQUENCE LISTING

<110> Baszczynski, Christopher L.

Lyznik, Leszek A.

Gordon-Kamm, William J.

<120> A Method for Directional Stable Transformation of

Eukaryotic Cells

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What is claimed is:

1. A method for targeting the insertion of nucleotide sequences of interest to a specific chromosomal site within the genome of a plant cell, said method comprising:

transforming said plant cell with a transfer cassette, said transfer cassette comprising said nucleotide sequence of interest and said transfer cassette is flanked by or comprises non-identical recombination sites;

wherein said plant genome comprises a target site comprising non-identical recombination sites which correspond to the flanking sites of said transfer cassette; and,

providing a recombinase that recognizes and implements recombination at the non-identical recombination sites; wherein said transfer cassettes are introduced into said plant cell by a non-integrating transformation method.

2. The method of claim 1, wherein said nucleotide sequence of interest is flanked by said non-identical recombination sites.

3. The method of Claim 1, wherein said non-integrating transformation method is an Agrobacterium-mediated method.

4. The method of Claim 1, wherein said plant cell is a monocotyledonous cell.

5. The method of Claim 4, wherein said monocotyledonous cell is a maize cell.

6. A modified plant made by the method of Claim 4.

7. Seed of the plant of Claim 6.

8. A modified plant made by the method of Claim 5.

9. Seed of the plant of Claim 8.

10. The method of Claim 1, wherein said non-integrative transformation
5 method comprises a modified *Agrobacterium*-mediated method.

11. The method of Claim 10, wherein said *Agrobacterium*-mediated method contains a modified VirD2 gene.

10 12. The method of Claim 11, wherein said plant cell is a dicotyledonous plant cell.

13. A modified plant made by the method of Claim 12.

15 14. Seed of the plant of Claim 13.

15. The method of Claim 1, wherein said non-integrating transformation method is a virus based method.

20 16. The method of Claim 1, wherein said non-identical recombination sites are selected from the group consisting of *FRT*, mutant *FRT*, *LOX*, and mutant *LOX* sites.

17. The method of Claim 1, wherein said sites are a *FRT* site and a mutated
25 *FRT* site.

18. The method of Claim 1 wherein said recombinase is provided by genetically transforming said plant with an expression cassette containing a nucleotide sequence encoding said recombinase.

30

19. The method of Claim 18, wherein said recombinase is FLP.

20. The method of Claim 19, wherein said FLP has been synthesized using maize preferred codons.

5 21. The method of Claim 17, wherein said mutant *FRT* site is *FRT*5 (SEQ ID NO: 3), *FRT* 6 (SEQ ID NO: 4) or *FRT* 7 (SEQ ID NO: 5).

22. A plant whose genome has been modified by introducing into said plant a transfer cassette, said transfer cassette comprising a nucleotide sequence of interest and
10 said transfer cassette is flanked by or comprises non-identical recombination sites; wherein said plant genome comprises a target site comprising non-identical recombination sites which correspond to the non-identical recombination sites of said transfer cassette; and, providing a recombinase that recognizes and implements recombination at the non-identical recombination sites; wherein said transfer cassette is
15 introduced into said plant cell by a non-integrating transformation methods.

23. The plant of claim 22, wherein said nucleotide sequence of interest is flanked by said non-identical recombination sites.

20 24. The plant of Claim 22, wherein said non-integrating transformation method is an *Agrobacterium*-mediated method.

25. The plant of Claim 24, wherein said plant cell is a monocotyledonous cell.

25

26. The plant of Claim 25, wherein said monocotyledonous cell is a maize cell.

27. Seed of the plant of Claim 22.

30

28. Seed of the plant of Claim 24.

29. Seed of the plant of Claim 25.

30. Seed of the plant of Claim 26.

5

31. The plant of Claim 25, wherein said *Agrobacterium*-mediated method contains a modified VirD2 gene.

32. The plant of Claim 31, wherein said plant cell is a dicotyledonous plant

10 cell.

33. Seed of the plant of Claim 32.

34. The plant of Claim 22, wherein said non-integrating transformation

15 method is a virus based method.

35. The plant of Claim 22, wherein said non-identical recombination sites are selected from the group consisting of *FRT*, mutant *FRT*, *LOX*, and mutant *LOX* sites.

20

36. The plant of Claim 22, wherein said non-identical recombination sites are a *FRT* site and a mutated *FRT* site.

37. The plant of Claim 22, wherein said recombinase is provided by

25 genetically transforming said plant with an expression cassette containing a nucleotide sequence encoding said recombinase.

38. The plant of Claim 37, wherein said recombinase is FLP.

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