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(73) Patenthaver: **Dow AgroSciences LLC, 9330 Zionsville Road, Indianapolis, IN 46268-1054, USA**
Sangamo Therapeutics, Inc., Point Richmond Tech Center , 501 Canal Blvd., Suite A100, Richmond, CA 94804, USA

(72) Opfinder: **BUTLER, Holly J., c/o Dow AgroSciences LLC, 9330 Zionsville Road, Indianapolis, Indiana 46268, USA**
CORBIN, David R., c/o Dow AgroSciences LLC, 9330 Zionsville Road, Indianapolis, Indiana 46268, USA
DOYON, Yannick, c/o Sangamo BioSciences Inc., Point Richmond Tech Center, 501 Canal Blvd. Suite A100, Richmond, California 94804, USA
GAO, Zhifang, c/o Dow AgroSciences LLC, 9330 Zionsville Road, Indianapolis, Indiana 46268, USA
SHUKLA, Vipula K., c/o Dow AgroSciences LLC, 9330 Zionsville Road, Indianapolis, Indiana 46268, USA
URNOV, Fyodor, c/o Sangamo BioSciences Inc., Point Richmond Tech Center, 501 Canal Blvd. Suite A100, Richmond, California 94804, USA
WORDEN, Sarah E., c/o Dow AgroSciences LLC, 9330 Zionsville Road, Indianapolis, Indiana 46268, USA

(74) Fuldmægtig i Danmark: **Chas. Hude A/S, H.C. Andersens Boulevard 33, 1780 København V, Danmark**

(54) Benævnelse: **MÅLRETTET INTEGRATION I ZP15-LOCUSET**

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DESCRIPTION

STATEMENT OF RIGHTS TO INVENTIONS

MADE UNDER FEDERALLY SPONSORED RESEARCH

[0001] Not applicable.

TECHNICAL FIELD

[0002] The present disclosure is in the field of plant genomic engineering, particularly targeted integration of a transgene into a plant *Zp15* gene.

BACKGROUND

[0003] Biotechnology has emerged as an essential tool in efforts to meet the challenge of increasing global demand for food production. Conventional approaches to improving agricultural productivity, *e.g.* enhanced yield or engineered pest resistance, rely on either mutation breeding or introduction of novel genes into the genomes of crop species by transformation. Both processes are inherently nonspecific and relatively inefficient. For example, conventional plant transformation methods deliver exogenous DNA that integrates into the genome at random locations. Thus, in order to identify and isolate transgenic lines with desirable attributes, it is necessary to generate thousands of unique random-integration events and subsequently screen for the desired individuals. As a result, conventional plant trait engineering is a laborious, time-consuming, and unpredictable undertaking. Furthermore the random nature of these integrations makes it difficult to predict whether pleiotropic effects due to unintended genome disruption have occurred. As a result, the generation, isolation and characterization of plant lines with engineered genes or traits has been an extremely labor and cost-intensive process with a low probability of success.

[0004] Targeted gene modification overcomes the logistical challenges of conventional practices in plant systems, and as such has been a long-standing but elusive goal in both basic plant biology research and agricultural biotechnology. However, with the exception of "gene targeting" via positive-negative drug selection in rice or the use of pre-engineered restriction sites, targeted genome modification in all plant species, both model and crop, has until recently proven very difficult. Terada et al. (2002) Nat Biotechnol 20(10):1030; Terada et al. (2007) Plant Physiol 144(2):846; D'Halluin et al. (2008) Plant Biotechnology J. 6(1):93.

[0005] Recently, methods and compositions for targeted cleavage of genomic DNA have been described. Such targeted cleavage events can be used, for example, to induce targeted mutagenesis, induce targeted deletions of cellular DNA sequences, and facilitate targeted recombination at a predetermined chromosomal locus. *See,* for example, United States Patent Publications 20030232410; 20050208489; 20050026157; 20050064474; and 20060188987, and International Publication WO 2007/014275.

[0006] U.S. Patent Publication No. 20080182332 describes use of non-canonical zinc finger nucleases (ZFNs) for targeted modification of plant genomes and U.S. Patent Application No. 12/284,888 describes ZFN-mediated targeted integration into a plant EPSPS locus.

[0007] However, there remain needs for compositions and methods for stable targeted integration into additional loci within a plant genome for establishing stable, heritable genetic modifications in the plant and its progeny.

SUMMARY

[0008] The present disclosure describes methods and compositions for expressing one or more products of an exogenous nucleic acid sequence (*i.e.* a protein or a RNA molecule) that has been integrated into a *Zp15* gene in a plant cell. The subject-matter of the invention is defined by the appended claims. As shown herein, the integration of one or more exogenous sequences at or near the *Zp15* locus does not appear to impair the ability of the host plant to regenerate, flower or produce seed and, optionally, allows heritable transmission of the exogenous sequence(s) over generations. The exogenous nucleic acid sequences can comprise, for example, one or more genes or cDNA molecules, or any type of coding or noncoding sequence, as well as one or more control elements (*e.g.,* promoters). For instance, herbicide tolerance genes can be integrated into this locus to produce crop plants with the desired herbicide resistance. Cells containing exogenous nucleic acids at or near the *Zp15* locus can also contribute to the gametophyte (germline) and therefore be transmitted to progeny in subsequent generations.

[0009] Integration of the exogenous nucleic acid sequence into a *Zp15* gene is facilitated by targeted double-strand cleavage of the genome in the selected *Zp15* locus. Cleavage is targeted to a *Zp15* gene through the use of fusion proteins comprising a DNA-binding domain, such as a meganuclease DNA-binding domain, a leucine zipper DNA-binding domain, a zinc finger protein (ZFP), or chimeric combinations of the aforementioned, which is engineered to bind a sequence within the selected *Zp15* locus, and a cleavage domain or a cleavage half-domain. Such cleavage stimulates integration of exogenous polynucleotide sequences at or near the cleavage site. Integration of exogenous sequences can proceed through both homology-dependent and homology-independent mechanisms.

[0010] In one aspect, described herein are engineered DNA-binding domains *(e.g.,* ZFPs, meganucleases, or leucine zippers) that bind to a target site in a *Zp15* gene. The DNA-binding

domain can comprise, for example, any of the engineered zinc finger DNA binding domains comprising the recognition helices shown in Table 1. Any of the DNA-binding domains described herein may further comprise a functional domain, for example a cleavage domain or cleavage half-domain. In some embodiments, the cleavage half-domain can be from a Type IIS restriction endonuclease such as *FokI* or *StsI*. In other embodiments, the cleavage domain can comprise a homing endonuclease, for example a homing endonuclease with a modified DNA-binding domain.

[0011] In another aspect, described herein are plants or seeds comprising an exogenous sequence integrated into the *Zp15* locus. In certain embodiments, the exogenous sequence is integrated into the gametophyte of the plant.

[0012] In another aspect, disclosed herein is a method for expressing the product of an exogenous nucleic acid sequence in a plant cell, the method comprising: introducing the one or more exogeneous nucleic acid sequences into the plant cell, wherein the exogenous nucleic acid sequences include one or more regions of homology with a *Zp15* gene; and cleaving a double-stranded sequence in an endogenous *Zp15* gene in the genome of the plant cell using at least one fusion protein comprising a cleavage domain and a zinc finger protein comprising the recognition helix regions as shown in a single row of Table 1, thereby resulting in integration of a polynucleotide comprising the one or more exogenous sequences into the genome of the cell, wherein the double-stranded cleavage is made by (a) expressing a first fusion protein in the cell, the first fusion protein comprising a first zinc finger binding domain and a first cleavage half-domain, wherein the first zinc finger binding domain has been engineered to bind to a first target site in or near a *Zp15* locus in the genome of the plant cell; and (b) expressing a second fusion protein in the cell, the second fusion protein comprising a second zinc finger binding domain and a second cleavage half domain, wherein the second zinc finger binding domain binds to a second target site in or near the *Zp15* locus in the genome of the plant cell, wherein the second target site is different from the first target site; and wherein binding of the first fusion protein to the first target site, and binding of the second fusion protein to the second target site, positions the cleavage half-domains such that the genome of the plant cell in or near the *Zp15* locus is cleaved and expressing a product of the one or more exogenous sequences.

[0013] The exogenous nucleic acid sequence may comprise a sequence encoding one or more functional polypeptides (*e.g.,* a cDNA), with or without one or more promoters and/or may produce one or more RNA sequences (*e.g.,* via one or more shRNA expression cassettes), which impart desirable traits to the plant. Such traits include, but are not limited to, herbicide resistance or tolerance; insect resistance or tolerance; disease resistance or tolerance (viral, bacterial, fungal, nematode); stress tolerance and/or resistance, as exemplified by resistance or tolerance to drought, heat, chilling, freezing, excessive moisture, salt stress; oxidative stress; increased yields; food content and makeup; physical appearance; male sterility; drydown; standability; prolificacy; starch quantity and quality; oil quantity and quality; protein quality and quantity; amino acid composition; and the like. Of course, any two or more exogenous nucleic acids of any description, such as those conferring herbicide, insect,

disease (viral, bacterial, fungal, nematode) or drought resistance, male sterility, drydown, standability, prolificacy, starch properties, oil quantity and quality, or those increasing yield or nutritional quality may be employed as desired. In certain embodiments, the nucleic acid sequence comprises a sequence encoding a herbicide resistance protein (*e.g.,* the *AAD-1* (aryloxyalkanoate dioxygenase) gene, the *AAD-12* gene, or the phosphinothricin acetyl transferase (*PAT*) gene) and/or functional fragments thereof. Expression of the integrated sequence can be driven by a promoter operably linked to the integrated sequence. Alternatively, the integrated sequence is promotorless and transcription is driven by the endogenous *Zp15* promoter.

[0014] In certain embodiments, the polynucleotide further comprises a second nucleotide sequence that is homologous to a second sequence in the *Zp15* gene. The second nucleotide sequence may be identical to the second sequence in the *Zp15* gene. Furthermore, in embodiments comprising first and second nucleotide sequences, the first nucleotide sequence may be identical to the first sequence in the *Zp15* gene and the second nucleotide sequence may be homologous but non-identical to a second sequence in the *Zp15* gene. In any of the methods described herein, the first and second nucleotide sequences flank the exogenous sequence. In certain embodiments, the polynucleotide is a plasmid. In other embodiments, the polynucleotide is a linear DNA molecule.

[0015] In another aspect, provided herein is a method of integrating one or more exogenous nucleic acid sequences into the genome of a plant cell, the method comprising: introducing the one or more exogeneous nucleic acid sequences into the plant cell, wherein the exogenous nucleic acid sequences include one or more regions of homology with a *Zp15* gene; and cleaving a double-stranded sequence in an endogenous *Zp15* gene in the genome of the plant cell using at least one fusion protein comprising a cleavage domain and a zinc finger protein comprising the recognition helix regions as shown in a single row of Table 1, thereby resulting in integration of a polynucleotide comprising the one or more exogenous sequences into the genome of the cell, wherein the double-stranded cleavage is made by (a) expressing a first fusion protein in the cell, the first fusion protein comprising a first zinc finger binding domain and a first cleavage half-domain, wherein the first zinc finger binding domain has been engineered to bind to a first target site in or near a *Zp15* locus in the genome of the plant cell; and (b) expressing a second fusion protein in the cell, the second fusion protein comprising a second zinc finger binding domain and a second cleavage half domain, wherein the second zinc finger binding domain binds to a second target site in or near the *Zp15* locus in the genome of the plant cell, wherein the second target site is different from the first target site; and wherein binding of the first fusion protein to the first target site, and binding of the second fusion protein to the second target site, positions the cleavage half-domains such that the genome of the plant cell in or near the *Zp15* locus is cleaved.

[0016] In certain embodiments, an exogenous sequence encoding a functional polypeptide is inserted into the *Zp15* gene.

[0017] In any of the methods described herein, the first and second cleavage half-domains

can be from a Type IIS restriction endonuclease, for example, *Fok*I or *Sts*I. Furthermore, in any of the methods described herein, at least one of the fusion proteins can comprise an alteration in the amino acid sequence of the dimerization interface of the cleavage half-domain, for example such that obligate heterodimers of the cleavage half-domains are formed.

[0018] In any of the methods described herein, the plant cell can comprise a monocotyledonous or dicotyledonous plant cell. In certain embodiments, the plant cell is a crop plant, for example maize.

BRIEF DESCRIPTION OF THE DRAWINGS

[0019]

Figure 1 depicts exemplary sequence analysis results of *Zp15* amplification products from maize HiII gDNA derived from cells subjected to transient expression of ZFN pair #25 (binding sites underlined) and reveals an 6 bp NHEJ insertion (bolded) at the expected cleavage site.

Figure 2 depicts exemplary sequence analysis results of *Zp15* amplification products from maize HiII gDNA derived from cells subjected to transient expression of ZFN pair #24 (binding sites underlined) and reveals a 3 bp deletion at the expected cleavage site.

Figure 3 is a schematic depicting the construct designated pDAB7489.

Figure 4 is a schematic depicting an exemplary herbicide-tolerance gene expression cassette encoding an *AAD gene*.

Figure 5 is a schematic depicting the construct designated pDAB7490.

Figure 6 depicts the alignment of a targeted integration (TI) event wherein the maize wild type (WT), *Zp15* donor fragment as well as the 5' and 3' border regions adjoining the integrated donor sequences are aligned.

Figure 7 is a schematic depicting the construct designated pDAB104101.

Figure 8 is a schematic depicting the *PAT* expression cassette.

Figure 9 is a schematic depicting the construct designated pDAB104107.

Figure 10 is a schematic depicting the construct designated pDAB104104.

Figure 11 is a schematic depicting the construct designated pDAB104105.

Figure 12 is a schematic depicting the construct designated pDAB104106.

Figure 13 is a schematic depicting the construct designated pDAB104100.

Figure 14 is a schematic depicting the construct designated pDAB104103.

Figure 15 is a schematic depicting the construct designated pDAB104102.

DETAILED DESCRIPTION

[0020] The present disclosure relates to methods and compositions for targeted integration (TI) into a plant *Zp15* gene, which lies on chromosome 6 in maize. Using fusion proteins comprising zinc finger-binding domains
and nuclease domains, an inserted (donor) sequence can be operably linked to an exogenous promoter or can be promoterless. If promoterless, transcription of the integrated open reading frame can occur from the endogenous *Zp15* gene promoter in the promoter-specified tissues. Use of a promoterless donor lowers the likelihood of random integration of the donor and/or the spurious activation of an endogenous gene by the promoter carried on the donor.

[0021] Compositions useful for targeted cleavage and recombination into a *Zp15* gene include fusion proteins comprising a cleavage domain (or a cleavage half-domain) and a zinc finger-binding domain, polynucleotides encoding these proteins and combinations of polypeptides and polypeptide-encoding polynucleotides. A zinc finger binding domain can comprise one or more zinc fingers (*e.g.,* 2, 3, 4, 5, 6, 7, 8, 9 or more zinc fingers), and can be engineered to bind to any sequence within a *Zp15* gene. The presence of such a fusion protein (or proteins) in a cell will result in binding of the fusion protein(s) to its (their) binding site(s) and cleavage within the endogenous *Zp15* gene.

General

[0022] Practice of the methods, as well as preparation and use of the compositions described herein employ, unless otherwise indicated, conventional techniques in molecular biology, biochemistry, chromatin structure and analysis, computational chemistry, cell culture, recombinant DNA and related fields as are within the skill of the art. These techniques are fully explained in the literature. *See,* for example, Sambrook et al. MOLECULAR CLONING: A LABORATORY MANUAL, Second edition, Cold Spring Harbor Laboratory Press, 1989 and Third edition, 2001; Ausubel et al., CURRENT PROTOCOLS IN MOLECULAR BIOLOGY, John Wiley & Sons, New York, 1987 and periodic updates; the series METHODS IN ENZYMOLOGY, Academic Press, San Diego; Wolffe, CHROMATIN STRUCTURE AND FUNCTION, Third edition, Academic Press, San Diego, 1998; METHODS IN ENZYMOLOGY, Vol. 304, "Chromatin" (P.M. Wassarman and A. P. Wolffe, eds.), Academic Press, San Diego, 1999; and METHODS IN MOLECULAR BIOLOGY, Vol. 119, "Chromatin Protocols" (P.B. Becker, ed.) Humana Press, Totowa, 1999.

Definitions

[0023] The terms "nucleic acid," "polynucleotide," and "oligonucleotide" are used interchangeably and refer to a deoxyribonucleotide or ribonucleotide polymer, in linear or circular conformation, and in either single- or double-stranded form. For the purposes of the present disclosure, these terms are not to be construed as limiting with respect to the length of a polymer. The terms can encompass known analogues of natural nucleotides, as well as nucleotides that are modified in the base, sugar and/or phosphate moieties (*e.g.,* phosphorothioate backbones). In general, an analogue of a particular nucleotide has the same base-pairing specificity; *i.e.,* an analogue of A will base-pair with T.

[0024] The terms "polypeptide," "peptide" and "protein" are used interchangeably to refer to a polymer of amino acid residues. The term also applies to amino acid polymers in which one or more amino acids are chemical analogues or modified derivatives of a corresponding naturally-occurring amino acids.

[0025] "Binding" refers to a sequence-specific, non-covalent interaction between macromolecules (*e.g.*, between a protein and a nucleic acid). Not all components of a binding interaction need be sequence-specific (*e.g.*, contacts with phosphate residues in a DNA backbone), as long as the interaction as a whole is sequence-specific. Such interactions are generally characterized by a dissociation constant (K_d) of 10^{-6} M^{-1} or lower. "Affinity" refers to the strength of binding: increased binding affinity being correlated with a lower K_d.

[0026] A "binding protein" is a protein that is able to bind non-covalently to another molecule. A binding protein can bind to, for example, a DNA molecule (a DNA-binding protein), an RNA molecule (an RNA-binding protein) and/or a protein molecule (a protein-binding protein). In the case of a protein-binding protein, it can bind to itself (to form homodimers, homotrimers, *etc.*) and/or it can bind to one or more molecules of a different protein or proteins. A binding protein can have more than one type of binding activity. For example, zinc finger proteins have DNA-binding, RNA-binding and protein-binding activity.

[0027] A "zinc finger DNA binding protein" (or binding domain) is a protein, or a domain within a larger protein, that binds DNA in a sequence-specific manner through one or more zinc fingers, which are regions of amino acid sequence within the binding domain whose structure is stabilized through coordination of a zinc ion. The term zinc finger DNA binding protein is often abbreviated as zinc finger protein or ZFP.

[0028] Zinc finger binding domains can be "engineered" to bind to a predetermined nucleotide sequence. Non-limiting examples of methods for engineering zinc finger proteins are design and selection. A designed zinc finger protein is a protein not occurring in nature whose design/composition results principally from rational criteria. Rational criteria for design include application of substitution rules and computerized algorithms for processing information in a database storing information of existing ZFP designs and binding data. See, for example, US Patents 6,140,081; 6,453,242; and 6,534,261; see also WO 98/53058; WO 98/53059; WO

98/53060; WO 02/016536 and WO 03/016496.

[0029] A "selected" zinc finger protein is a protein not found in nature whose production results primarily from an empirical process such as phage display, interaction trap or hybrid selection. See *e.g.,* US 5,789,538; US 5,925,523; US 6,007,988; US 6,013,453; US 6,200,759; WO 95/19431; WO 96/06166; WO 98/53057; WO 98/54311; WO 00/27878; WO 01/60970 WO 01/88197 and WO 02/099084.

[0030] The term "sequence" refers to a nucleotide sequence of any length, which can be DNA or RNA; can be linear, circular or branched and can be either single-stranded or double stranded. The term "donor sequence" refers to a nucleotide sequence that is inserted into a genome. A donor sequence can be of any length, for example between 2 and 10,000 nucleotides in length (or any integer value therebetween or thereabove), preferably between about 100 and 1,000 nucleotides in length (or any integer therebetween), more preferably between about 200 and 500 nucleotides in length.

[0031] A "homologous, non-identical sequence" refers to a first sequence which shares a degree of sequence identity with a second sequence, but whose sequence is not identical to that of the second sequence. For example, a polynucleotide comprising the wild-type sequence of a mutant gene is homologous and non-identical to the sequence of the mutant gene. In certain embodiments, the degree of homology between the two sequences is sufficient to allow homologous recombination therebetween, utilizing normal cellular mechanisms. Two homologous non-identical sequences can be any length and their degree of non-homology can be as small as a single nucleotide (*e.g.*, for correction of a genomic point mutation by targeted homologous recombination) or as large as 10 or more kilobases (*e.g.*, for insertion of a gene at a predetermined ectopic site in a chromosome). Two polynucleotides comprising the homologous non-identical sequences need not be the same length. For example, an exogenous polynucleotide (*i.e.,* donor polynucleotide) of between 20 and 10,000 nucleotides or nucleotide pairs can be used.

[0032] Techniques for determining nucleic acid and amino acid sequence identity are known in the art. Typically, such techniques include determining the nucleotide sequence of the mRNA for a gene and/or determining the amino acid sequence encoded thereby, and comparing these sequences to a second nucleotide or amino acid sequence. Genomic sequences can also be determined and compared in this fashion. In general, identity refers to an exact nucleotide-to-nucleotide or amino acid-to-amino acid correspondence of two polynucleotides or polypeptide sequences, respectively. Two or more sequences (polynucleotide or amino acid) can be compared by determining their percent identity. The percent identity of two sequences, whether nucleic acid or amino acid sequences, is the number of exact matches between two aligned sequences divided by the length of the shorter sequences and multiplied by 100. An approximate alignment for nucleic acid sequences is provided by the local homology algorithm of Smith and Waterman, Advances in Applied Mathematics 2:482-489 (1981). This algorithm can be applied to amino acid sequences by using the scoring matrix developed by Dayhoff, Atlas of Protein Sequences and Structure, M.O. Dayhoff ed., 5 suppl. 3:353-358, National

Biomedical Research Foundation, Washington, D.C., USA, and normalized by Gribskov, Nucl. Acids Res. 14(6):6745-6763 (1986). An exemplary implementation of this algorithm to determine percent identity of a sequence is provided by the Genetics Computer Group (Madison, WI) in the "BestFit" utility application. Suitable programs for calculating the percent identity or similarity between sequences are generally known in the art, for example, another alignment program is BLAST, used with default parameters. For example, BLASTN and BLASTP can be used using the following default parameters: genetic code = standard; filter = none; strand = both; cutoff = 60; expect = 10; Matrix = BLOSUM62; Descriptions = 50 sequences; sort by = HIGH SCORE; Databases = non-redundant, GenBank + EMBL + DDBJ + PDB + GenBank CDS translations + Swiss protein + Spupdate + PIR. Details of these programs can be found on the internet. With respect to sequences described herein, the range of desired degrees of sequence identity is approximately 80% to 100% and any integer value therebetween. Typically the percent identities between sequences are at least 70-75%, preferably 80-82%, more preferably 85-90%, even more preferably 92%, still more preferably 95%, and most preferably 98% sequence identity.

[0033] Alternatively, the degree of sequence similarity between polynucleotides can be determined by hybridization of polynucleotides under conditions that allow formation of stable duplexes between homologous regions, followed by digestion with single-stranded-specific nuclease(s), and size determination of the digested fragments. Two nucleic acid, or two polypeptide sequences are substantially homologous to each other when the sequences exhibit at least about 70%-75%, preferably 80%-82%, more preferably 85%-90%, even more preferably 92%, still more preferably 95%, and most preferably 98% sequence identity over a defined length of the molecules, as determined using the methods above. As used herein, substantially homologous also refers to sequences showing complete identity to a specified DNA or polypeptide sequence. DNA sequences that are substantially homologous can be identified in a Southern hybridization experiment under, for example, stringent conditions, as defined for that particular system. Defining appropriate hybridization conditions is within the skill of the art. See, *e.g., Sambrook et al., supra*; Nucleic Acid Hybridization: A Practical Approach, editors B.D. Hames and S.J. Higgins, (1985) Oxford; Washington, DC; IRL Press).

[0034] Selective hybridization of two nucleic acid fragments can be determined as follows. The degree of sequence identity between two nucleic acid molecules affects the efficiency and strength of hybridization events between such molecules. A partially identical nucleic acid sequence will at least partially inhibit the hybridization of a completely identical sequence to a target molecule. Inhibition of hybridization of the completely identical sequence can be assessed using hybridization assays that are well known in the art (*e.g.,* Southern (DNA) blot, Northern (RNA) blot, solution hybridization, or the like, see Sambrook, et al., Molecular Cloning: A Laboratory Manual, Second Edition, (1989) Cold Spring Harbor, N.Y.). Such assays can be conducted using varying degrees of selectivity, for example, using conditions varying from low to high stringency. If conditions of low stringency are employed, the absence of non-specific binding can be assessed using a secondary probe that lacks even a partial degree of sequence identity (for example, a probe having less than about 30% sequence identity with the target molecule), such that, in the absence of non-specific binding events, the secondary probe

will not hybridize to the target.

[0035] When utilizing a hybridization-based detection system, a nucleic acid probe is chosen that is complementary to a reference nucleic acid sequence, and then by selection of appropriate conditions the probe and the reference sequence selectively hybridize, or bind, to each other to form a duplex molecule. A nucleic acid molecule that is capable of hybridizing selectively to a reference sequence under moderately stringent hybridization conditions typically hybridizes under conditions that allow detection of a target nucleic acid sequence of at least about 10-14 nucleotides in length having at least approximately 70% sequence identity with the sequence of the selected nucleic acid probe. Stringent hybridization conditions typically allow detection of target nucleic acid sequences of at least about 10-14 nucleotides in length having a sequence identity of greater than about 90-95% with the sequence of the selected nucleic acid probe. Hybridization conditions useful for probe/reference sequence hybridization, where the probe and reference sequence have a specific degree of sequence identity, can be determined as is known in the art (see, for example, Nucleic Acid Hybridization: A Practical Approach, editors B.D. Hames and S.J. Higgins, (1985) Oxford; Washington, DC; IRL Press).

[0036] Conditions for hybridization are well-known to those of skill in the art. Hybridization stringency refers to the degree to which hybridization conditions disfavor the formation of hybrids containing mismatched nucleotides, with higher stringency correlated with a lower tolerance for mismatched hybrids. Factors that affect the stringency of hybridization are well-known to those of skill in the art and include, but are not limited to, temperature, pH, ionic strength, and concentration of organic solvents such as, for example, formamide and dimethylsulfoxide. As is known to those of skill in the art, hybridization stringency is increased by higher temperatures, lower ionic strength and lower solvent concentrations.

[0037] With respect to stringency conditions for hybridization, it is well known in the art that numerous equivalent conditions can be employed to establish a particular stringency by varying, for example, the following factors: the length and nature of the sequences, base composition of the various sequences, concentrations of salts and other hybridization solution components, the presence or absence of blocking agents in the hybridization solutions (e.g., dextran sulfate, and polyethylene glycol), hybridization reaction temperature and time parameters, as well as, varying wash conditions. The selection of a particular set of hybridization conditions is selected following standard methods in the art (see, for example, Sambrook, et al., Molecular Cloning: A Laboratory Manual, Second Edition, (1989) Cold Spring Harbor, N.Y.).

[0038] "Recombination" refers to a process of exchange of genetic information between two polynucleotides. For the purposes of this disclosure, "homologous recombination (HR)" refers to the specialized form of such exchange that takes place, for example, during repair of double-strand breaks in cells. This process requires nucleotide sequence homology, uses a "donor" molecule to template repair of a "target" molecule *(i.e.,* the one that experienced the double-strand break), and is variously known as "non-crossover gene conversion" or "short

tract gene conversion," because it leads to the transfer of genetic information from the donor to the target. Without wishing to be bound by any particular theory, such transfer can involve mismatch correction of heteroduplex DNA that forms between the broken target and the donor, and/or "synthesis-dependent strand annealing," in which the donor is used to resynthesize genetic information that will become part of the target, and/or related processes. Such specialized HR often results in an alteration of the sequence of the target molecule such that part or all of the sequence of the donor polynucleotide is incorporated into the target polynucleotide.

[0039] "Cleavage" refers to the breakage of the covalent backbone of a DNA molecule. Cleavage can be initiated by a variety of methods including, but not limited to, enzymatic or chemical hydrolysis of a phosphodiester bond. Both single-stranded cleavage and double-stranded cleavage are possible, and double-stranded cleavage can occur as a result of two distinct single-stranded cleavage events. DNA cleavage can result in the production of either blunt ends or staggered ends. In certain embodiments, fusion polypeptides are used for targeted double-stranded DNA cleavage.

[0040] A "cleavage domain" comprises one or more polypeptide sequences which possesses catalytic activity for DNA cleavage. A cleavage domain can be contained in a single polypeptide chain or cleavage activity can result from the association of two (or more) polypeptides.

[0041] A "cleavage half-domain" is a polypeptide sequence which, in conjunction with a second polypeptide (either identical or different) forms a complex having cleavage activity (preferably double-strand cleavage activity).

[0042] "Chromatin" is the nucleoprotein structure comprising the cellular genome. Cellular chromatin comprises nucleic acid, primarily DNA, and protein, including histones and non-histone chromosomal proteins. The majority of eukaryotic cellular chromatin exists in the form of nucleosomes, wherein a nucleosome core comprises approximately 150 base pairs of DNA associated with an octamer comprising two each of histones H2A, H2B, H3 and H4; and linker DNA (of variable length depending on the organism) extends between nucleosome cores. A molecule of histone H1 is generally associated with the linker DNA. For the purposes of the present disclosure, the term "chromatin" is meant to encompass all types of cellular nucleoprotein, both prokaryotic and eukaryotic. Cellular chromatin includes both chromosomal and episomal chromatin.

[0043] A "chromosome," is a chromatin complex comprising all or a portion of the genome of a cell. The genome of a cell is often characterized by its karyotype, which is the collection of all the chromosomes that comprise the genome of the cell. The genome of a cell can comprise one or more chromosomes.

[0044] An "episome" is a replicating nucleic acid, nucleoprotein complex or other structure comprising a nucleic acid that is not part of the chromosomal karyotype of a cell. Examples of episomes include plasmids and certain viral genomes.

[0045] An "accessible region" is a site in cellular chromatin in which a target site present in the nucleic acid can be bound by an exogenous molecule which recognizes the target site. Without wishing to be bound by any particular theory, it is believed that an accessible region is one that is not packaged into a nucleosomal structure. The distinct structure of an accessible region can often be detected by its sensitivity to chemical and enzymatic probes, for example, nucleases.

[0046] A "target site" or "target sequence" is a nucleic acid sequence that defines a portion of a nucleic acid to which a binding molecule will bind, provided sufficient conditions for binding exist. For example, the sequence 5'-GAATTC-3' is a target site for the Eco RI restriction endonuclease.

[0047] An "exogenous" molecule is a molecule that is not normally present in a cell, but can be introduced into a cell by one or more genetic, biochemical or other methods. "Normal presence in the cell" is determined with respect to the particular developmental stage and environmental conditions of the cell. Thus, for example, a molecule that is present only during embryonic development of muscle is an exogenous molecule with respect to an adult muscle cell. Similarly, a molecule induced by heat shock is an exogenous molecule with respect to a non-heat-shocked cell. An exogenous molecule can comprise, for example, a coding sequence for any polypeptide or fragment thereof, a functioning version of a malfunctioning endogenous molecule or a malfunctioning version of a normally-functioning endogenous molecule. Additionally, an exogenous molecule can comprise a coding sequence from another species that is an ortholog of an endogenous gene in the host cell.

[0048] An exogenous molecule can be, among other things, a small molecule, such as is generated by a combinatorial chemistry process, or a macromolecule such as a protein, nucleic acid, carbohydrate, lipid, glycoprotein, lipoprotein, polysaccharide, any modified derivative of the above molecules, or any complex comprising one or more of the above molecules. Nucleic acids include DNA and RNA, can be single- or double-stranded; can be linear, branched or circular; and can be of any length. Nucleic acids include those capable of forming duplexes, as well as triplex-forming nucleic acids. See, for example, U.S. Patent Nos. 5,176,996 and 5,422,251. Proteins include, but are not limited to, DNA-binding proteins, transcription factors, chromatin remodeling factors, methylated DNA binding proteins, polymerases, methylases, demethylases, acetylases, deacetylases, kinases, phosphatases, integrases, recombinases, ligases, topoisomerases, gyrases and helicases.

[0049] An exogenous molecule can be the same type of molecule as an endogenous molecule, *e.g.*, an exogenous protein or nucleic acid. For example, an exogenous nucleic acid can comprise an infecting viral genome, a plasmid or episome introduced into a cell, or a chromosome that is not normally present in the cell. Methods for the introduction of exogenous molecules into cells are known to those of skill in the art and include, but are not limited to, lipid-mediated transfer (*i.e.,* liposomes, including neutral and cationic lipids), electroporation, direct injection, cell fusion, particle bombardment, calcium phosphate co-precipitation, nanoparticle transformation, DEAE-dextran-mediated transfer and viral vector-mediated

transfer.

[0050] By contrast, an "endogenous" molecule is one that is normally present in a particular cell at a particular developmental stage under particular environmental conditions. For example, an endogenous nucleic acid can comprise a chromosome, the genome of a mitochondrion, chloroplast or other organelle, or a naturally-occurring episomal nucleic acid. Additional endogenous molecules can include proteins, for example, transcription factors and enzymes.

[0051] As used herein, the term "product of an exogenous nucleic acid" includes both polynucleotide and polypeptide products, for example, transcription products (polynucleotides such as RNA) and translation products (polypeptides).

[0052] A "fusion" molecule is a molecule in which two or more subunit molecules are linked, for example, covalently. The subunit molecules can be the same chemical type of molecule, or can be different chemical types of molecules. Examples of the first type of fusion molecule include, but are not limited to, fusion proteins (for example, a fusion between a ZFP DNA-binding domain and a cleavage domain) and fusion nucleic acids (for example, a nucleic acid encoding the fusion protein described *supra*). Examples of the second type of fusion molecule include, but are not limited to, a fusion between a triplex-forming nucleic acid and a polypeptide, and a fusion between a minor groove binder and a nucleic acid.

[0053] Expression of a fusion protein in a cell can result from delivery of the fusion protein to the cell or by delivery of a polynucleotide encoding the fusion protein to a cell, wherein the polynucleotide is transcribed, and the transcript is translated, to generate the fusion protein. Trans-splicing, polypeptide cleavage and polypeptide ligation can also be involved in expression of a protein in a cell. Methods for polynucleotide and polypeptide delivery to cells are presented elsewhere in this disclosure.

[0054] A "gene," for the purposes of the present disclosure, includes a DNA region encoding a gene product (see *infra*), as well as all DNA regions which regulate the production of the gene product, whether or not such regulatory sequences are adjacent to coding and/or transcribed sequences. Accordingly, a gene includes, but is not necessarily limited to, promoter sequences, terminators, translational regulatory sequences such as ribosome binding sites and internal ribosome entry sites, enhancers, silencers, insulators, boundary elements, replication origins, matrix attachment sites and locus control regions.

[0055] "Gene expression" refers to the conversion of the information, contained in a gene, into a gene product. A gene product can be the direct transcriptional product of a gene (*e.g.,* mRNA, tRNA, rRNA, antisense RNA, interfering RNA, ribozyme, structural RNA or any other type of RNA) or a protein produced by translation of a mRNA. Gene products also include RNAs which are modified, by processes such as capping, polyadenylation, methylation, and editing, and proteins modified by, for example, methylation, acetylation, phosphorylation, ubiquitination, ADP-ribosylation, myristilation, and glycosylation.

[0056] "Modulation" of gene expression refers to a change in the activity of a gene. Modulation of expression can include, but is not limited to, gene activation and gene repression.

[0057] "Plant" cells include, but are not limited to, cells of monocotyledonous (monocots) or dicotyledonous (dicots) plants. Non-limiting examples of monocots include cereal plants such as maize, rice, barley, oats, wheat, sorghum, rye, sugarcane, pineapple, onion, banana, and coconut. Non-limiting examples of dicots include tobacco, tomato, sunflower, cotton, sugarbeet, potato, lettuce, melon, soybean, canola (rapeseed), and alfalfa. Plant cells may be from any part of the plant and/or from any stage of plant development.

[0058] A "region of interest" is any region of cellular chromatin, such as, for example, a gene or a non-coding sequence within or adjacent to a gene, in which it is desirable to bind an exogenous molecule. Binding can be for the purposes of targeted DNA cleavage and/or targeted recombination. A region of interest can be present in a chromosome, an episome, an organellar genome (*e.g.,* mitochondrial, chloroplast), or an infecting viral genome, for example. A region of interest can be within the coding region of a gene, within transcribed non-coding regions such as, for example, leader sequences, trailer sequences or introns, or within non-transcribed regions, either upstream or downstream of the coding region. A region of interest can be as small as a single nucleotide pair or up to 2,000 nucleotide pairs in length, or any integral value of nucleotide pairs.

[0059] The terms "operative linkage" and "operatively linked" (or "operably linked") are used interchangeably with reference to a juxtaposition of two or more components (such as sequence elements), in which the components are arranged such that both components function normally and allow the possibility that at least one of the components can mediate a function that is exerted upon at least one of the other components. By way of illustration, a transcriptional regulatory sequence, such as a promoter, is operatively linked to a coding sequence if the transcriptional regulatory sequence controls the level of transcription of the coding sequence in response to the presence or absence of one or more transcriptional regulatory factors. A transcriptional regulatory sequence is generally operatively linked in *cis* with a coding sequence, but need not be directly adjacent to it. For example, an enhancer is a transcriptional regulatory sequence that is operatively linked to a coding sequence, even though they are not contiguous.

[0060] With respect to fusion polypeptides, the term "operatively linked" can refer to the fact that each of the components performs the same function in linkage to the other component as it would if it were not so linked. For example, with respect to a fusion polypeptide in which a ZFP DNA-binding domain is fused to a cleavage domain, the ZFP DNA-binding domain and the cleavage domain are in operative linkage if, in the fusion polypeptide, the ZFP DNA-binding domain portion is able to bind its target site and/or its binding site, while the cleavage domain is able to cleave DNA in the vicinity of the target site.

[0061] A "functional fragment" of a protein, polypeptide or nucleic acid is a protein, polypeptide or nucleic acid whose sequence is not identical to the full-length protein, polypeptide or nucleic acid, yet retains the same function as the full-length protein, polypeptide or nucleic acid. A functional fragment can possess more, fewer, or the same number of residues as the corresponding native molecule, and/or can contain one ore more amino acid or nucleotide substitutions. Methods for determining the function of a nucleic acid (*e.g.,* coding function, ability to hybridize to another nucleic acid) are well-known in the art. Similarly, methods for determining protein function are well-known. For example, the DNA-binding function of a polypeptide can be determined, for example, by filter-binding, electrophoretic mobility-shift, or immunoprecipitation assays. DNA cleavage can be assayed by gel electrophoresis. See Ausubel *et al., supra.* The ability of a protein to interact with another protein can be determined, for example, by co-immunoprecipitation, two-hybrid assays or complementation, both genetic and biochemical. See, for example, Fields et al. (1989) Nature 340:245-246; U.S. Patent No. 5,585,245 and PCT WO 98/44350.

Target sites

[0062] The described methods and compositions include fusion proteins comprising a cleavage half-domain and a zinc finger-binding domain in which the zinc finger-binding domain , by binding to a sequence in a plant *Zp15* locus directs the activity of the cleavage half-domain to the vicinity of the sequence and, hence, induces cleavage (namely a double stranded break) in *Zp15.* As set forth elsewhere in this disclosure, a zinc finger domain can be engineered to bind to virtually any desired sequence. Accordingly, one or more zinc finger-binding domains can be engineered to bind to one or more sequences in a plant *Zp15* gene. Expression of two fusion proteins, each comprising a zinc finger-binding domain and a cleavage half-domain, in a cell, effects cleavage in the *Zp15* gene.

[0063] Selection of a sequence in a *Zp15* for binding by a zinc finger domain *(e.g.,* a target site) can be accomplished, for example, according to the methods disclosed in co-owned US Patent No. 6,453,242 (Sept. 17, 2002), which also discloses methods for designing ZFPs to bind to a selected sequence. It will be clear to those skilled in the art that simple visual inspection of a nucleotide sequence can also be used for selection of a target site. Accordingly, any means for target site selection can be used in the methods described herein.

[0064] For ZFP DNA-binding domains, target sites are generally composed of a plurality of adjacent target subsites. A target subsite refers to the sequence (usually either a nucleotide triplet, or a nucleotide quadruplet that can overlap by one nucleotide with an adjacent quadruplet) bound by an individual zinc finger. *See,* for example, WO 02/077227. If the strand with which a zinc finger protein makes most contacts is designated the target strand "primary recognition strand," or "primary contact strand," some zinc finger proteins bind to a three base triplet in the target strand and a fourth base on the non-target strand. A target site generally has a length of at least 9 nucleotides and, accordingly, is bound by a zinc finger binding domain comprising at least three zinc fingers. However binding of, for example, a 4-finger

binding domain to a 12-nucleotide target site, a 5-finger binding domain to a 15-nucleotide target site or a 6-finger binding domain to an 18-nucleotide target site, is also possible. As will be apparent, binding of larger binding domains (*e.g.,* 7-, 8-, 9-finger and more) to longer target sites is also possible.

[0065] It is not necessary for a target site to be a multiple of three nucleotides. For example, in cases in which cross-strand interactions occur (see, *e.g.,* US Patent 6,453,242 and WO 02/077227), one or more of the individual zinc fingers of a multi-finger binding domain can bind to overlapping quadruplet subsites. As a result, a three-finger protein can bind a 10-nucleotide sequence, wherein the tenth nucleotide is part of a quadruplet bound by a terminal finger, a four-finger protein can bind a 13-nucleotide sequence, wherein the thirteenth nucleotide is part of a quadruplet bound by a terminal finger, *etc.*

[0066] The length and nature of amino acid linker sequences between individual zinc fingers in a multi-finger binding domain also affects binding to a target sequence. For example, the presence of a so-called "non-canonical linker," "long linker" or "structured linker" between adjacent zinc fingers in a multi-finger binding domain can allow those fingers to bind subsites which are not immediately adjacent. Non-limiting examples of such linkers are described, for example, in US Patent No. 6,479,626 and WO 01/53480. Accordingly, one or more subsites, in a target site for a zinc finger binding domain, can be separated from each other by 1, 2, 3,4, 5 or more nucleotides. To provide but one example, a four-finger binding domain can bind to a 13-nucleotide target site comprising, in sequence, two contiguous 3-nucleotide subsites, an intervening nucleotide, and two contiguous triplet subsites.

[0067] Distance between sequences (*e.g.*, target sites) refers to the number of nucleotides or nucleotide pairs intervening between two sequences, as measured from the edges of the sequences nearest each other.

[0068] In certain embodiments in which cleavage depends on the binding of two zinc finger domain/cleavage half-domain fusion molecules to separate target sites, the two target sites can be on opposite DNA strands. In other embodiments, both target sites are on the same DNA strand.

DNA-binding domains

[0069] A zinc finger-binding domain is used in the methods disclosed herein as the DNA-binding domain.

[0070] A zinc finger binding domain comprises one or more zinc fingers. Miller et al. (1985) EMBO J. 4:1609-1614; Rhodes (1993) Scientific American Feb.:56-65; US Patent No. 6,453,242. The zinc finger binding domains described herein generally include 2, 3, 4, 5, 6 or even more zinc fingers.

[0071] Typically, a single zinc finger domain is about 30 amino acids in length. Structural studies have demonstrated that each zinc finger domain (motif) contains two beta sheets (held in a beta turn which contains the two invariant cysteine residues) and an alpha helix (containing the two invariant histidine residues), which are held in a particular conformation through coordination of a zinc atom by the two cysteines and the two histidines.

[0072] Zinc fingers include both canonical C_2H_2 zinc fingers (*i.e.,* those in which the zinc ion is coordinated by two cysteine and two histidine residues) and non-canonical zinc fingers such as, for example, C_3H zinc fingers (those in which the zinc ion is coordinated by three cysteine residues and one histidine residue) and C_4 zinc fingers (those in which the zinc ion is coordinated by four cysteine residues). See also WO 02/057293 and also U.S. Patent Publication No. 20080182332 regarding non-canonical ZFPs for use in plants.

[0073] An engineered zinc finger binding domain can have a novel binding specificity, compared to a naturally-occurring zinc finger protein. Engineering methods include, but are not limited to, rational design and various types of selection. Rational design includes, for example, using databases comprising triplet (or quadruplet) nucleotide sequences and individual zinc finger amino acid sequences, in which each triplet or quadruplet nucleotide sequence is associated with one or more amino acid sequences of zinc fingers which bind the particular triplet or quadruplet sequence.

[0074] Exemplary selection methods, including phage display and two-hybrid systems, are disclosed in US Patents 5,789,538; 5,925,523; 6,007,988; 6,013,453; 6,410,248; 6,140,466; 6,200,759; and 6,242,568; as well as WO 98/37186; WO 98/53057; WO 00/27878; WO 01/88197 and GB 2,338,237.

[0075] Enhancement of binding specificity for zinc finger binding domains has been described, for example, in co-owned WO 02/077227.

[0076] Since an individual zinc finger binds to a three-nucleotide *(i.e.,* triplet) sequence (or a four-nucleotide sequence which can overlap, by one nucleotide, with the four-nucleotide binding site of an adjacent zinc finger), the length of a sequence to which a zinc finger binding domain is engineered to bind (e.g., a target sequence) will determine the number of zinc fingers in an engineered zinc finger binding domain. For example, for ZFPs in which the finger motifs do not bind to overlapping subsites, a six-nucleotide target sequence is bound by a two-finger binding domain; a nine-nucleotide target sequence is bound by a three-finger binding domain, *etc.* As noted herein, binding sites for individual zinc fingers (*i.e.,* subsites) in a target site need not be contiguous, but can be separated by one or several nucleotides, depending on the length and nature of the amino acids sequences between the zinc fingers (*i.e.,* the inter-finger linkers) in a multi-finger binding domain.

[0077] In a multi-finger zinc finger binding domain, adjacent zinc fingers can be separated by amino acid linker sequences of approximately 5 amino acids (so-called "canonical" inter-finger linkers) or, alternatively, by one or more non-canonical linkers. See, *e.g.*, co-owned US Patent

Nos. 6,453,242 and 6,534,261. For engineered zinc finger binding domains comprising more than three fingers, insertion of longer ("non-canonical") inter-finger linkers between certain of the zinc fingers may be desirable in some instances as it may increase the affinity and/or specificity of binding by the binding domain. See, for example, U.S. Patent No. 6,479,626 and WO 01/53480. Accordingly, multi-finger zinc finger binding domains can also be characterized with respect to the presence and location of non-canonical inter-finger linkers. For example, a six-finger zinc finger binding domain comprising three fingers (joined by two canonical inter-finger linkers), a long linker and three additional fingers (joined by two canonical inter-finger linkers) is denoted a 2x3 configuration. Similarly, a binding domain comprising two fingers (with a canonical linker therebetween), a long linker and two additional fingers (joined by a canonical linker) is denoted a 2x2 configuration. A protein comprising three two-finger units (in each of which the two fingers are joined by a canonical linker), and in which each two-finger unit is joined to the adjacent two finger unit by a long linker, is referred to as a 3x2 configuration.

[0078] The presence of a long or non-canonical inter-finger linker between two adjacent zinc fingers in a multi-finger binding domain often allows the two fingers to bind to subsites which are not immediately contiguous in the target sequence. Accordingly, there can be gaps of one or more nucleotides between subsites in a target site; *i.e.,* a target site can contain one or more nucleotides that are not contacted by a zinc finger. For example, a 2x2 zinc finger binding domain can bind to two six-nucleotide sequences separated by one nucleotide, *i.e.,* it binds to a 13-nucleotide target site. *See* also Moore et al. (2001a) Proc. Natl. Acad. Sci. USA 98:1432-1436; Moore et al. (2001b) Proc. Natl. Acad. Sci. USA 98:1437-1441 and WO 01/53480.

[0079] As mentioned previously, a target subsite is a three- or four-nucleotide sequence that is bound by a single zinc finger. For certain purposes, a two-finger unit is denoted a "binding module." A binding module can be obtained by, for example, selecting for two adjacent fingers in the context of a multi-finger protein (generally three fingers) which bind a particular six-nucleotide target sequence. Alternatively, modules can be constructed by assembly of individual zinc fingers. See also WO 98/53057 and WO 01/53480.

[0080] The cleavage domain portion of the fusion proteins described herein can be obtained from any endonuclease or exonuclease. Exemplary endonucleases from which a cleavage domain can be derived include, but are not limited to, restriction endonucleases and homing endonucleases. *See,* for example, 2002-2003 Catalogue, New England Biolabs, Beverly, MA; and Belfort et al. (1997) Nucleic Acids Res. 25:3379-3388. Additional enzymes which cleave DNA are known (e.g., S1 Nuclease; mung bean nuclease; pancreatic DNase I; micrococcal nuclease; yeast HO endonuclease; *see* also Linn et al. (eds.) Nucleases, Cold Spring Harbor Laboratory Press, 1993). Non limiting examples of homing endonucleases and meganucleases include I-*Sce*I, I-*Ceu*I, PI-*Psp*I, PI-*Sce,* I-*Sce*IV, I-*Csm*I, I-*Pan*I, I-*Sce*II, I-*Ppo*I, I-*Sce*III, I-*Cre*I, I-*Tev*I, I-*Tev*II and I-*Tev*III are known. *See* also U.S. Patent No. 5,420,032; U.S. Patent No. 6,833,252; Belfort et al. (1997) Nucleic Acids Res. 25:3379-3388; Dujon et al. (1989) Gene 82:115-118; Perler et al. (1994) Nucleic Acids Res. 22, 1125-1127; Jasin (1996) Trends Genet. 12:224-228; Gimble et al. (1996) J. Mol. Biol. 263:163-180; Argast et al. (1998) J. Mol. Biol.

280:345-353 and the New England Biolabs catalogue. One or more of these enzymes (or functional fragments thereof) can be used as a source of cleavage domains and cleavage half-domains.

[0081] Restriction endonucleases (restriction enzymes) are present in many species and are capable of sequence-specific binding to DNA (at a recognition site), and cleaving DNA at or near the site of binding. Certain restriction enzymes (*e.g.,* Type IIS) cleave DNA at sites removed from the recognition site and have separable binding and cleavage domains. For example, the Type IIS enzyme *Fok*I catalyzes double-stranded cleavage of DNA, at 9 nucleotides from its recognition site on one strand and 13 nucleotides from its recognition site on the other. See, for example, US Patents 5,356,802; 5,436,150 and 5,487,994; as well as Li et al. (1992) Proc. Natl. Acad. Sci. USA 89:4275-4279; Li et al. (1993) Proc. Natl. Acad. Sci. USA 90:2764-2768; Kim et al. (1994a) Proc. Natl. Acad. Sci. USA 91:883-887; Kim et al. (1994b) J. Biol. Chem. 269:31,978-31,982. Thus, in one embodiment, fusion proteins comprise the cleavage half-domain from at least one Type IIS restriction enzyme and one or more zinc finger binding domains, which may or may not be engineered.

[0082] An exemplary Type IIS restriction enzyme, whose cleavage domain is separable from the binding domain, is *Fok*I. This particular enzyme is active as a dimer. Bitinaite et al. (1998) Proc. Natl. Acad. Sci. USA 95: 10,570-10,575. Accordingly, for the purposes of the present disclosure, the portion of the FokI enzyme used in the described fusion proteins is considered a cleavage half-domain. Thus, for targeted double-stranded cleavage and/or targeted replacement of cellular sequences using zinc finger-*Fok*I fusions, two fusion proteins, each comprising a *Fok*I cleavage half-domain, can be used to reconstitute a catalytically active cleavage domain. Alternatively, a single polypeptide molecule containing a zinc finger binding domain and two *Fok*I cleavage half-domains can also be used. Parameters for targeted cleavage and targeted sequence alteration using zinc finger-*Fok*I fusions are provided elsewhere in this disclosure.

[0083] A cleavage domain or cleavage half-domain can be any portion of a protein that retains cleavage activity, or that retains the ability to multimerize (*e.g.,* dimerize) to form a functional cleavage domain.

[0084] Exemplary Type IIS restriction enzymes are described in co-owned International Publication WO 2007/014275.

[0085] To enhance cleavage specificity, cleavage domains may also be modified. In certain embodiments, variants of the cleavage half-domain are employed these variants minimize or prevent homodimerization of the cleavage half-domains. Non-limiting examples of such modified cleavage half-domains are described in detail in WO 2007/014275. See, also, Examples. In certain embodiments, the cleavage domain comprises an engineered cleavage half-domain (also referred to as dimerization domain mutants) that minimize or prevent homodimerization are known to those of skill the art and described for example in U.S. Patent Publication Nos. 20050064474 and 20060188987.

[0086] Amino acid residues at positions 446, 447, 479, 483, 484, 486, 487, 490, 491, 496, 498, 499, 500, 531, 534, 537, and 538 of *Fok*I are all targets for influencing dimerization of the *Fok*I cleavage half-domains. See, *e.g.,* U.S. Patent Publication Nos. 20050064474 and 20060188987; International Patent Publication WO 07/139898; Miller et al. (2007) Nat. Biotechnol. 25(7):778-785.

[0087] Additional engineered cleavage half-domains of *Fok*I that form obligate heterodimers can also be used in the ZFNs described herein. In one embodiment, the first cleavage half-domain includes mutations at amino acid residues at positions 490 and 538 of *Fok*I and the second cleavage half-domain includes mutations at amino acid residues 486 and 499.

[0088] In certain embodiments, the cleavage domain comprises two cleavage half-domains, both of which are part of a single polypeptide comprising a binding domain, a first cleavage half-domain and a second cleavage half-domain. The cleavage half-domains can have the same amino acid sequence or different amino acid sequences, so long as they function to cleave the DNA.

[0089] In general, two fusion proteins are required for cleavage if the fusion proteins comprise cleavage half-domains. Alternatively, a single protein comprising two cleavage half-domains can be used. The two cleavage half-domains can be derived from the same endonuclease (or functional fragments thereof), or each cleavage half-domain can be derived from a different endonuclease (or functional fragments thereof). In addition, the target sites for the two fusion proteins are preferably disposed, with respect to each other, such that binding of the two fusion proteins to their respective target sites places the cleavage half-domains in a spatial orientation to each other that allows the cleavage half-domains to form a functional cleavage domain, *e.g.*, by dimerizing. Thus, in certain embodiments, the near edges of the target sites are separated by 5-8 nucleotides or by 15-18 nucleotides. However any integral number of nucleotides or nucleotide pairs can intervene between two target sites (*e.g.*, from 2 to 50 nucleotides or more). In general, the point of cleavage lies between the target sites.

Fusion proteins

[0090] Methods for design and construction of fusion proteins (and polynucleotides encoding same) are known to those of skill in the art. For example, methods for the design and construction of fusion proteins comprising DNA-binding domains (e.g., zinc finger domains) and regulatory or cleavage domains (or cleavage half-domains), and polynucleotides encoding such fusion proteins, are described in co-owned U.S. Patents 6,453,242 and 6,534,261 and U.S. Patent Application Publications 2007/0134796 and 2005/0064474.

[0091] In certain embodiments, polynucleotides encoding the fusion proteins are constructed. These polynucleotides can be inserted into a vector and the vector can be introduced into a cell (see below for additional disclosure regarding vectors and methods for introducing

polynucleotides into cells).

[0092] In certain embodiments of the methods described herein, a zinc finger nuclease comprises a fusion protein comprising a zinc finger binding domain and a cleavage half-domain from the *Fok*I restriction enzyme, and two such fusion proteins are expressed in a cell. Expression of two fusion proteins in a cell can result from delivery of the two proteins to the cell; delivery of one protein and one nucleic acid encoding one of the proteins to the cell; delivery of two nucleic acids, each encoding one of the proteins, to the cell; or by delivery of a single nucleic acid, encoding both proteins, to the cell. In additional embodiments, a fusion protein comprises a single polypeptide chain comprising two cleavage half domains and a zinc finger binding domain. In this case, a single fusion protein is expressed in a cell and, without wishing to be bound by theory, is believed to cleave DNA as a result of formation of an intramolecular dimer of the cleavage half-domains.

[0093] In certain embodiments, the components of the fusion proteins (*e.g.,* ZFP-*Fok*I fusions) are arranged such that the zinc finger domain is nearest the amino terminus of the fusion protein, and the cleavage half-domain is nearest the carboxy-terminus. This mirrors the relative orientation of the cleavage domain in naturally-occurring dimerizing cleavage domains such as those derived from the *Fok*I enzyme, in which the DNA-binding domain is nearest the amino terminus and the cleavage half-domain is nearest the carboxy terminus. In these embodiments, dimerization of the cleavage half-domains to form a functional nuclease is brought about by binding of the fusion proteins to sites on opposite DNA strands, with the 5' ends of the binding sites being proximal to each other.

[0094] In additional embodiments, the components of the fusion proteins (*e.g.,* ZFP-*Fok*I fusions) are arranged such that the cleavage half-domain is nearest the amino terminus of the fusion protein, and the zinc finger domain is nearest the carboxy-terminus. In these embodiments, dimerization of the cleavage half-domains to form a functional nuclease is brought about by binding of the fusion proteins to sites on opposite DNA strands, with the 3' ends of the binding sites being proximal to each other.

[0095] In yet additional embodiments, a first fusion protein contains the cleavage half-domain nearest the amino terminus of the fusion protein, and the zinc finger domain nearest the carboxy-terminus, and a second fusion protein is arranged such that the zinc finger domain is nearest the amino terminus of the fusion protein, and the cleavage half-domain is nearest the carboxy-terminus. In these embodiments, both fusion proteins bind to the same DNA strand, with the binding site of the first fusion protein containing the zinc finger domain nearest the carboxy terminus located to the 5' side of the binding site of the second fusion protein containing the zinc finger domain nearest the amino terminus.

[0096] In certain embodiments of the described fusion proteins, the amino acid sequence between the zinc finger domain and the cleavage domain (or cleavage half-domain) is denoted the "ZC linker." The ZC linker is to be distinguished from the inter-finger linkers discussed above. *See, e.g.,* U.S. Patent Publications 20050064474A1 and 20030232410, and

International Patent Publication WO05/084190, for details on obtaining ZC linkers that optimize cleavage.

[0097] The present document describes a ZFN comprising a zinc finger protein having one or more of the recognition helix amino acid sequences shown in Table 1. Also described herein is a ZFP expression vector comprising a nucleotide sequence encoding a ZFP having one or more recognition helices shown in Table 1.

Targeted Integration

[0098] The described methods and compositions can be used to cleave DNA in a *Zp15* gene of plant cellular chromatin, which facilitates the stable, targeted integration of an exogenous sequence into the locus. As described herein, loss of function of endogenous *Zp15* genes is well tolerated by plant cells and sequences integrated within this gene are broadly transcribed and generate plants with germline modifications for heritable transmission of the integrated sequence. Accordingly, *Zp15* is a desirable site for targeted integration of exogenous sequences.

[0099] For targeted integration into *Zp15,* one or more DNA-binding domains (namely ZFPs) are engineered to bind a target site at or near the predetermined cleavage site, and a fusion protein comprising the engineered DNA-binding domain and a cleavage domain is expressed in a cell. Upon binding of the DNA-binding (i.e. zinc finger) portion of the fusion protein to the target site, the DNA is cleaved via a double-stranded break, near the target site by the cleavage domain.

[0100] The presence of a double-stranded break in the *Zp15* locus facilitates integration of exogenous sequences via homologous recombination. Thus, the polynucleotide comprising the exogenous sequence to be inserted into the *Zp15* gene will include one or more regions of homology with a *Zp15* gene to facilitate homologous recombination.

[0101] Any sequence of interest (exogenous sequence) can be introduced into a *Zp15* locus as described herein. Exemplary exogenous sequences include, but are not limited to any polypeptide coding sequence (*e.g.*, cDNAs), promoter, enhancer and other regulatory sequences (*e.g.*, interfering RNA sequences, shRNA expression cassettes, epitope tags, marker genes, cleavage enzyme recognition sites and various types of expression constructs. Such sequences can be readily obtained using standard molecular biological techniques (cloning, synthesis, etc.) and/or are commercially available.

[0102] In addition to the fusion molecules described herein, targeted replacement of a selected genomic sequence also involves the introduction of the replacement (or donor) sequence. The donor sequence can be introduced into the cell prior to, concurrently with, or subsequent to, expression of the fusion protein(s). The donor polynucleotide contains sufficient homology to *Zp15* to support homologous recombination (or homology-directed repair)

between it and the *Zp15* genomic sequence to which it bears homology. Approximately 25, 50, 100, 200, 500, 750, 1,000, 1,500, 2,000 nucleotides or more of sequence homology between a donor and a genomic sequence (or any integral value between 10 and 2,000 nucleotides, or more) will support homologous recombination therebetween. In certain embodiments, the homology arms are less than 1,000 basepairs in length. In other embodiments, the homology arms are less than 750 basepairs in length. See, also, U.S. Provisional Patent Application No. 61/124,047,

[0103] Donor sequences can range in length from 10 to 5,000 nucleotides (or any integral value of nucleotides therebetween) or longer. It will be readily apparent that the donor sequence is typically not identical to the genomic sequence that it replaces. For example, the sequence of the donor polynucleotide can contain one or more single base changes, insertions, deletions, inversions or rearrangements with respect to the genomic sequence, so long as sufficient homology with chromosomal sequences is present. Alternatively, a donor sequence can contain a non-homologous sequence flanked by two regions of homology. Additionally, donor sequences can comprise a vector molecule containing sequences that are not homologous to the region of interest in cellular chromatin. Generally, the homologous region(s) of a donor sequence will have at least 50% sequence identity to a genomic sequence with which recombination is desired. In certain embodiments, 60%, 70%, 80%, 90%, 95%, 98%, 99%, or 99.9% sequence identity is present. Any value between 1% and 100% sequence identity can be present, depending upon the length of the donor polynucleotide.

[0104] A donor molecule can contain several, discontinuous regions of homology to cellular chromatin. For example, for targeted insertion of sequences not normally present in a region of interest, said sequences can be present in a donor nucleic acid molecule and flanked by regions of homology to a gene sequence in the region of interest.

[0105] Donor molecules can also be inserted into the *Zp15* locus to serve as a reservoir for later use. For example, a donor molecule homologous to an endogenous gene, but containing a mutation of interest may be inserted in the *Zp15* locus. Next, ZFNs specific to the endogenous gene can be introduced which will cleave both the endogenous locus and the donor molecule in the *Zp15* locus which contains the mutation of interest. The resulting DSB in the genome can then become the integration site for the donor molecule released from the *Zp15* locus. In this way, the efficiency of targeted integration of a donor sequence at any region of interest can be greatly increased since the method does not rely on simultaneous uptake of both the nucleic acids encoding the ZFNs and those donor sequences.

[0106] Donor molecules can also be inserted into the *Zp15* locus to serve as a target site for subsequent insertions. For example, a donor molecule comprised of DNA sequences that contain recognition sites for additional ZFN designs may be inserted into the *Zp15* locus. Subsequently, additional ZFN designs may be generated and expressed in cells such that the original donor molecule is cleaved and modified by repair or homologous recombination. In this way, reiterative integrations of donor molecules may occur at the *Zp15* locus.

[0107] To simplify assays (*e.g.,* hybridization, PCR, restriction enzyme digestion) for determining successful insertion of the donor sequence, certain sequence differences may be present in the donor sequence as compared to the *Zp15* genomic sequence. Preferably, if located in a coding region, such nucleotide sequence differences will not change the amino acid sequence, or will make silent amino acid changes *(i.e.,* changes which do not affect the structure or function of the protein). The donor polynucleotide can optionally contain changes in sequences corresponding to the DNA-binding domain binding sites in the region of interest, to prevent cleavage of donor sequences that have been introduced into cellular chromatin by homologous recombination.

[0108] The donor polynucleotide can be DNA or RNA, single-stranded or double-stranded and can be introduced into a cell in linear or circular form. If introduced in linear form, the ends of the donor sequence can be protected (*e.g.,* from exonucleolytic degradation) by methods known to those of skill in the art. For example, one or more dideoxynucleotide residues are added to the 3' terminus of a linear molecule and/or self-complementary oligonucleotides are ligated to one or both ends. *See,* for example, Chang et al. (1987) Proc. Natl. Acad. Sci. USA 84:4959-4963; Nehls et al. (1996) Science 272:886-889. Additional methods for protecting exogenous polynucleotides from degradation include, but are not limited to, addition of terminal amino group(s) and the use of modified internucleotide linkages such as, for example, phosphorothioates, phosphoramidates, and O-methyl ribose or deoxyribose residues.

[0109] A polynucleotide can be introduced into a cell as part of a vector molecule having additional sequences such as, for example, replication origins, promoters and genes encoding antibiotic resistance. Moreover, donor polynucleotides can be introduced as naked nucleic acid, as nucleic acid complexed with an agent such as a nanoparticle, liposome or poloxamer, or can be delivered by bacteria or viruses (*e.g.*, *Agrobacterium, Rhizobium sp.* NGR234, *Sinorhizoboium meliloti, Mesorhizobium loti,* tobacco mosaic virus, potato virus X, cauliflower mosaic virus and cassava vein mosaic virus. *See, e.g.,* Chung et al. (2006) Trends Plant Sci. 11(1):1-4.

[0110] It appears that the presence of a double-stranded break in a cellular sequence, coupled with the presence of an exogenous DNA molecule having homology to a region adjacent to or surrounding the break, activates cellular mechanisms which repair the break by transfer of sequence information from the donor molecule into the cellular *(e.g.,* genomic or chromosomal) sequence; *i.e.,* by a processes of homology-directed repair, also known as "gene conversion." Applicants' methods advantageously combine the powerful targeting capabilities of engineered ZFPs with a cleavage domain (or cleavage half-domain) to specifically target paralogous genes such as *Zp15* genes such that cleavage of the target sequence produces a double-stranded break in the region of the genome where insertion of exogenous sequences is desired.

[0111] For alteration of a chromosomal sequence, it is not necessary for the entire sequence of the donor to be copied into the chromosome, as long as enough of the donor sequence is copied to effect the desired sequence alteration.

[0112] The efficiency of insertion of donor sequences by homologous recombination is inversely related to the distance, in the cellular DNA, between the double-stranded break and the site at which recombination is desired. In other words, higher homologous recombination efficiencies are observed when the double-stranded break is closer to the site at which recombination is desired. In cases in which a precise site of recombination is not predetermined (*e.g.*, the desired recombination event can occur over an interval of genomic sequence), the length and sequence of the donor nucleic acid, together with the site(s) of cleavage, are selected to obtain the desired recombination event. In cases in which the desired event is designed to change the sequence of a single nucleotide pair in a genomic sequence, cellular chromatin is cleaved within 10,000 nucleotides on either side of that nucleotide pair. In certain embodiments, cleavage occurs within 1,000, 500, 200, 100, 90, 80, 70, 60, 50, 40, 30, 20, 10, 5, or 2 nucleotides, or any integral value between 2 and 1,000 nucleotides, on either side of the nucleotide pair whose sequence is to be changed.

[0113] As detailed above, the binding sites for two fusion proteins, each comprising a zinc finger binding domain and a cleavage half-domain, can be located 5-8 or 15-18 nucleotides apart, as measured from the edge of each binding site nearest the other binding site, and cleavage occurs between the binding sites. Whether cleavage occurs at a single site or at multiple sites between the binding sites is immaterial, since the cleaved genomic sequences are replaced by the donor sequences. Thus, for efficient alteration of the sequence of a single nucleotide pair by targeted recombination, the midpoint of the region between the binding sites is within 10,000 nucleotides of that nucleotide pair, preferably within 1,000 nucleotides, or 500 nucleotides, or 200 nucleotides, or 100 nucleotides, or 50 nucleotides, or 20 nucleotides, or 10 nucleotides, or 5 nucleotide, or 2 nucleotides, or one nucleotide, or at the nucleotide pair of interest.

[0114] In certain embodiments, a homologous chromosome can serve as the donor polynucleotide. Thus, for example, correction of a mutation in a heterozygote can be achieved by engineering fusion proteins which bind to and cleave the mutant sequence on one chromosome, but do not cleave the wild-type sequence on the homologous chromosome. The double-stranded break on the mutation-bearing chromosome stimulates a homology-based "gene conversion" process in which the wild-type sequence from the homologous chromosome is copied into the cleaved chromosome, thus restoring two copies of the wild-type sequence.

[0115] Methods and compositions are also described that may enhance levels of targeted recombination including, but not limited to, the use of additional ZFP-functional domain fusions to activate expression of genes involved in homologous recombination, such as, for example, plant genes of the RAD54 epistasis group (*e.g., AtRad54, AtRad51),* and genes whose products interact with the aforementioned gene products. *See, e.g.,* Klutstein M, et al. Genetics. 2008 Apr;178(4):2389-97.

[0116] Similarly ZFP-functional domain fusions can be used, in combination with the methods and compositions disclosed herein, to repress expression of genes involved in non-

homologous end joining (e.g., Ku70/80, XRCC4, poly(ADP ribose) polymerase, DNA ligase 4). *See,* for example, Riha et al. (2002) EMBO 21:2819-2826; Freisner et al. (2003) Plant J. 34:427-440; Chen et al. (1994) European Journal of Biochemistry 224:135-142. Methods for activation and repression of gene expression using fusions between a zinc finger binding domain and a functional domain are disclosed, for example, in co-owned US Patents 6,534,261; 6,824,978 and 6,933,113. Additional repression methods include the use of antisense oligonucleotides and/or small interfering RNA (siRNA or RNAi) or shRNAs targeted to the sequence of the gene to be repressed.

[0117] As an alternative to or, in addition to, activating expression of gene products involved in homologous recombination, fusions of these protein (or functional fragments thereof) with a zinc finger binding domain targeted to *Zp15,* can be used to recruit these proteins (recombination proteins) to the region of interest, thereby increasing their local concentration and further stimulating homologous recombination processes. Alternatively, a polypeptide involved in homologous recombination as described above (or a functional fragment thereof) can be part of a triple fusion protein comprising a zinc finger binding domain, a cleavage domain (or cleavage half-domain) and the recombination protein (or functional fragment thereof). Additional proteins involved in gene conversion and recombination-related chromatin remodeling, which can be used in the aforementioned methods and compositions, include histone acetyltransferases (*e.g.*, Esalp, Tip60), histone methyltransferases (*e.g.,* Dot1p), histone kinases and histone phosphatases. *See, also,* Bhat et al. (1999) Plant J. 33:455-469.

[0118] Further increases in efficiency of targeted recombination, in cells comprising a zinc finger/nuclease fusion molecule and a donor DNA molecule, are achieved by blocking the cells in the G_2 phase of the cell cycle, when homology-driven repair processes are maximally active. Such arrest can be achieved in a number of ways. For example, cells can be treated with *e.g.*, drugs, compounds and/or small molecules which influence cell-cycle progression so as to arrest cells in G_2 phase. Exemplary molecules of this type include, but are not limited to, compounds which affect microtubule polymerization (*e.g.,* vinblastine, nocodazole, Taxol), compounds that interact with DNA (*e.g., cis*-platinum(II) diamine dichloride, Cisplatin, doxorubicin) and/or compounds that affect DNA synthesis (*e.g.,* thymidine, hydroxyurea, L-mimosine, etoposide, 5-fluorouracil). Additional increases in recombination efficiency are achieved by the use of histone deacetylase (HDAC) inhibitors (*e.g.,* sodium butyrate, trichostatin A) which alter chromatin structure to make genomic DNA more accessible to the cellular recombination machinery.

[0119] Additional methods for cell-cycle arrest include overexpression of proteins which inhibit the activity of the CDK cell-cycle kinases, for example, by introducing a cDNA encoding the protein into the cell or by introducing into the cell an engineered ZFP which activates expression of the gene encoding the protein. Cell-cycle arrest is also achieved by inhibiting the activity of cyclins and CDKs, for example, using RNAi methods (*e.g.,* U.S. Patent No. 6,506,559) or by introducing into the cell an engineered ZFP which represses expression of one or more genes involved in cell-cycle progression such as, for example, cyclin and/or CDK genes. See, *e.g.*, co- owned U.S. Patent No. 6,534,261 for methods for the synthesis of

engineered zinc finger proteins for regulation of gene expression.

[0120] Alternatively, in certain cases, targeted cleavage is conducted in the absence of a donor polynucleotide (preferably in S or G_2 phase), and recombination occurs between homologous chromosomes.

Expression vectors

[0121] A nucleic acid encoding one or more fusion proteins (i.e. ZFNs) as described herein can be cloned into a vector for transformation into prokaryotic or eukaryotic cells for replication and/or expression. Vectors can be prokaryotic vectors, *e.g.*, plasmids, or shuttle vectors, insect vectors, or eukaryotic vectors. A nucleic acid encoding a fusion protein can also be cloned into an expression vector, for administration to a plant cell.

[0122] To express the fusion proteins (i.e. ZFNs), sequences encoding the fusion proteins are typically subcloned into an expression vector that contains a promoter to direct transcription. Suitable bacterial and eukaryotic promoters are well known in the art and described, *e.g.,* in Sambrook et al., Molecular Cloning, A Laboratory Manual (2nd ed. 1989; 3rd ed., 2001); Kriegler, Gene Transfer and Expression: A Laboratory Manual (1990); and Current Protocols in Molecular Biology (Ausubel et al., supra. Bacterial expression systems for expressing the ZFP are available in, e.g., *E. coli, Bacillus sp.,* and *Salmonella* (Palva et al., Gene 22:229-235 (1983)). Kits for such expression systems are commercially available. Eukaryotic expression systems for mammalian cells, yeast, and insect cells are well known by those of skill in the art and are also commercially available.

[0123] The promoter used to direct expression of a fusion protein-encoding nucleic acid depends on the particular application. For example, a strong constitutive promoter suited to the host cell is typically used for expression and purification of fusion proteins.

[0124] In contrast, when a fusion protein is administered *in vivo* for regulation of a plant gene (see, "Nucleic Acid Delivery to Plant Cells" section below), either a constitutive or an inducible promoter is used, depending on the particular use of the fusion protein. Non-limiting examples of plant promoters include promoter sequences derived from *A. thaliana* ubiquitin-3 (ubi-3) (Callis, et al., 1990, J. Biol. Chem. 265-12486-12493); *A. tumifaciens* mannopine synthase (Δmas) (Petolino et al., U.S. Patent No. 6,730,824); and/or Cassava Vein Mosaic Virus (CsVMV) (Verdaguer et al., 1996, Plant Molecular Biology 31:1129-1139). *See, also,* Examples.

[0125] In addition to the promoter, the expression vector typically contains a transcription unit or expression cassette that contains all the additional elements required for the expression of the nucleic acid in host cells, either prokaryotic or eukaryotic. A typical expression cassette thus contains a promoter operably linked, *e.g.,* to a nucleic acid sequence encoding the fusion protein, and signals required, *e.g.,* for efficient polyadenylation of the transcript, transcriptional termination, ribosome binding sites, or translation termination. Additional elements of the

cassette may include, *e.g.*, enhancers, heterologous splicing signals, and/or a nuclear localization signal (NLS).

[0126] The particular expression vector used to transport the genetic information into the cell is selected with regard to the intended use of the fusion proteins, *e.g.,* expression in plants, animals, bacteria, fungus, protozoa, *etc.* (see expression vectors described below). Standard bacterial and animal expression vectors are known in the art and are described in detail, for example, U.S. Patent Publication 20050064474A1 and International Patent Publications WO05/084190, WO05/014791 and WO03/080809.

[0127] Standard transfection methods can be used to produce bacterial, mammalian, yeast or insect cell lines that express large quantities of protein, which can then be purified using standard techniques (*see, e.g.,* Colley et al., J. Biol. Chem. 264:17619-17622 (1989); Guide to Protein Purification, in Methods in Enzymology, vol. 182 (Deutscher, ed., 1990)). Transformation of eukaryotic and prokaryotic cells are performed according to standard techniques (*see, e.g.,* Morrison, J. Bact. 132:349-351 (1977); Clark-Curtiss & Curtiss, Methods in Enzymology 101:347-362 (Wu et al., eds., 1983).

[0128] Any of the well known procedures for introducing foreign nucleotide sequences into such host cells may be used. These include the use of calcium phosphate transfection, polybrene, protoplast fusion, electroporation, ultrasonic methods (e.g., sonoporation), liposomes, microinjection, naked DNA, plasmid vectors, viral vectors, both episomal and integrative, and any of the other well known methods for introducing cloned genomic DNA, cDNA, synthetic DNA or other foreign genetic material into a host cell (*see, e.g.,* Sambrook *et al., supra*). It is only necessary that the particular genetic engineering procedure used be capable of successfully introducing at least one gene into the host cell capable of expressing the protein of choice.

Nucleic Acid Delivery to Plant Cells

[0129] As noted above, DNA constructs may be introduced into (e.g., into the genome of) a desired plant host by a variety of conventional techniques. For reviews of such techniques see, for example, Weissbach & Weissbach Methods for Plant Molecular Biology (1988, Academic Press, N.Y.) Section VIII, pp. 421-463; and Grierson & Corey, Plant Molecular Biology (1988, 2d Ed.), Blackie, London, Ch. 7-9.

[0130] For example, the DNA construct may be introduced directly into the genomic DNA of the plant cell using techniques such as electroporation and microinjection of plant cell protoplasts, or the DNA constructs can be introduced directly to plant tissue using biolistic methods, such as DNA particle bombardment (see, *e.g.,* Klein et al. (1987) Nature 327:70-73). Alternatively, the DNA construct can be introduced into the plant cell via nanoparticle transformation (*see, e.g.,* US Patent Application No. 12/245,685).

[0131] Alternatively, the DNA constructs may be combined with suitable T-DNA border/flanking regions and introduced into a conventional *Agrobacterium tumefaciens* host vector. *Agrobacterium tumefaciens-mediated* transformation techniques, including disarming and use of binary vectors, are well described in the scientific literature. See, for example Horsch et al. (1984) Science 233:496-498, and Fraley et al. (1983) Proc. Nat'l. Acad. Sci. USA 80:4803.

[0132] In addition, gene transfer may be achieved using non-Agrobacterium bacteria or viruses such as *Rhizobium sp.* NGR234, *Sinorhizoboium meliloti, Mesorhizobium loti,* potato virus X, cauliflower mosaic virus and cassava vein mosaic virus and/or tobacco mosaic virus, *See, e.g.,* Chung et al. (2006) Trends Plant Sci. 11(1):1-4.

[0133] The virulence functions of the *Agrobacterium tumefaciens* host will direct the insertion of a T-strand containing the construct and adjacent marker into the plant cell DNA when the cell is infected by the bacteria using binary T DNA vector (Bevan (1984) Nuc. Acid Res. 12:8711-8721) or the co-cultivation procedure (Horsch et al. (1985) Science 227:1229-1231). Generally, the *Agrobacterium* transformation system is used to engineer dicotyledonous plants (Bevan et al. (1982) Ann. Rev. Genet 16:357-384; Rogers et al. (1986) Methods Enzymol. 118:627-641). The *Agrobacterium* transformation system may also be used to transform, as well as transfer, DNA to monocotyledonous plants and plant cells. *See* U.S. Patent No. 5, 591,616; Hemalsteen et al. (1984) EMBO J 3:3039-3041; Hooykass-Van Slogteren et al. (1984) Nature 311:763-764; Grimsley et al. (1987) Nature 325:1677-179; Boulton et al. (1989) Plant Mol. Biol. 12:31-40; and Gould et al. (1991) Plant Physiol. 95:426-434.

[0134] Alternative gene transfer and transformation methods include, but are not limited to, protoplast transformation through calcium-, polyethylene glycol (PEG)- or electroporation-mediated uptake of naked DNA (see Paszkowski et al. (1984) EMBO J 3:2717-2722, Potrykus et al. (1985) Molec. Gen. Genet. 199:169-177; Fromm et al. (1985) Proc. Nat. Acad. Sci. USA 82:5824-5828; and Shimamoto (1989) Nature 338:274-276) and electroporation of plant tissues (D'Halluin et al. (1992) Plant Cell 4:1495-1505). Additional methods for plant cell transformation include microinjection, silicon carbide mediated DNA uptake (Kaeppler et al. (1990) Plant Cell Reporter 9:415-418), and microprojectile bombardment (see Klein et al. (1988) Proc. Nat. Acad. Sci. USA 85:4305-4309; and Gordon-Kamm et al. (1990) Plant Cell 2:603-618).

[0135] The described methods and compositions can be used to insert exogenous sequences into a predetermined location (*e.g.* a *Zp15* gene) in a plant cell genome. This is useful inasmuch as expression of an introduced transgene into a plant genome depends critically on its integration site. Accordingly, genes encoding, *e.g.*, herbicide tolerance, insect resistance, nutrients, antibiotics or therapeutic molecules can be inserted, by targeted recombination, into regions of a plant genome favorable to their expression.

[0136] Transformed plant cells which are produced by any of the above transformation techniques can be cultured to regenerate a whole plant which possesses the transformed genotype and thus the desired phenotype. Such regeneration techniques rely on manipulation

of certain phytohormones in a tissue culture growth medium, typically relying on a biocide and/or herbicide marker which has been introduced together with the desired nucleotide sequences. Plant regeneration from cultured protoplasts is described in Evans, et al., "Protoplasts Isolation and Culture" in Handbook ofPlant Cell Culture, pp. 124-176, Macmillian Publishing Company, New York, 1983; and Binding, Regeneration ofPlants, Plant Protoplasts, pp. 21-73, CRC Press, Boca Raton, 1985. Regeneration can also be obtained from plant callus, explants, organs, pollens, embryos or parts thereof. Such regeneration techniques are described generally in Klee et al. (1987) Ann. Rev. of Plant Phys. 38:467-486.

[0137] Nucleic acids introduced into a plant cell can be used to confer desired traits on essentially any plant. A wide variety of plants and plant cell systems may be engineered for the desired physiological and agronomic characteristics described herein using the nucleic acid constructs of the present disclosure and the various transformation methods mentioned above. In preferred embodiments, target plants and plant cells for engineering include, but are not limited to, those monocotyledonous and dicotyledonous plants, such as crops including grain crops (*e.g.,* wheat, maize, rice, millet, barley), fruit crops (*e.g.,* tomato, apple, pear, strawberry, orange), forage crops (*e.g.,* alfalfa), root vegetable crops (*e.g.,* carrot, potato, sugar beets, yam), leafy vegetable crops (*e.g.*, lettuce, spinach); flowering plants (*e.g.*, petunia, rose, chrysanthemum), conifers and pine trees (*e.g.,* pine fir, spruce); plants used in phytoremediation (*e.g.*, heavy metal accumulating plants); oil crops (*e.g.*, sunflower, rape seed) and plants used for experimental purposes (*e.g., Arabidopsis*). Thus, the disclosed methods and compositions have use over a broad range of plants, including, but not limited to, species from the genera Asparagus, Avena, Brassica, Citrus, Citrullus, Capsicum, Cucurbita, Daucus, Erigeron, Glycine, Gossypium, Hordeum, Lactuca, Lolium, Lycopersicon, Malus, Manihot, Nicotiana, Orychophragmus, Oryza, Persea, Phaseolus, Pisum, Pyrus, Prunus, Raphanus, Secale, Solanum, Sorghum, Triticum, Vitis, Vigna, and Zea.

[0138] One of skill in the art will recognize that after the exogenous sequence is stably incorporated in transgenic plants and confirmed to be operable, it can be introduced into other plants by sexual crossing. Any of a number of standard breeding techniques can be used, depending upon the species to be crossed.

[0139] A transformed plant cell, callus, tissue or plant may be identified and isolated by selecting or screening the engineered plant material for traits encoded by the marker genes present on the transforming DNA. For instance, selection can be performed by growing the engineered plant material on media containing an inhibitory amount of the antibiotic or herbicide to which the transforming gene construct confers resistance. Further, transformed plants and plant cells can also be identified by screening for the activities of any visible marker genes (e.g., the β-glucuronidase, luciferase, B or C1 genes) that may be present on the recombinant nucleic acid constructs. Such selection and screening methodologies are well known to those skilled in the art.

[0140] Physical and biochemical methods also may be used to identify plant or plant cell transformants containing inserted gene constructs. These methods include but are not limited

to: 1) Southern analysis or PCR amplification for detecting and determining the structure of the recombinant DNA insert; 2) Northern blot, S1 RNase protection, primer-extension or reverse transcriptase-PCR amplification for detecting and examining RNA transcripts of the gene constructs; 3) enzymatic assays for detecting enzyme or ribozyme activity, where such gene products are encoded by the gene construct; 4) protein gel electrophoresis, Western blot techniques, immunoprecipitation, or enzyme-linked immunoassays (ELISA), where the gene construct products are proteins. Additional techniques, such as *in situ* hybridization, enzyme staining, and immunostaining, also may be used to detect the presence or expression of the recombinant construct in specific plant organs and tissues. The methods for doing all these assays are well known to those skilled in the art.

[0141] Effects of gene manipulation using the methods disclosed herein can be observed by, for example, northern blots of the RNA (*e.g.*, mRNA) isolated from the tissues of interest. Typically, if the mRNA is present or the amount of mRNA has increased, it can be assumed that the corresponding transgene is being expressed. Other methods of measuring gene and/or encoded polypeptide activity can be used. Different types of enzymatic assays can be used, depending on the substrate used and the method of detecting the increase or decrease of a reaction product or by-product. In addition, the levels of polypeptide expressed can be measured immunochemically, *i.e.,* ELISA, RIA, EIA and other antibody based assays well known to those of skill in the art, such as by electrophoretic detection assays (either with staining or western blotting). As one non-limiting example, the detection of the AAD-1 and PAT proteins using an ELISA assay is described in U.S. Patent Application No. 11/587,893

[0142] The transgene may be selectively expressed in some tissues of the plant or at some developmental stages, or the transgene may be expressed in substantially all plant tissues, substantially along its entire life cycle. However, any combinatorial expression mode is also applicable.

[0143] The present disclosure also encompasses seeds of the transgenic plants described above wherein the seed has the transgene or gene construct. The present disclosure further encompasses the progeny, clones, cell lines or cells of the transgenic plants described above wherein said progeny, clone, cell line or cell has the transgene or gene construct.

[0144] Fusion proteins (i.e. ZFNs) and expression vectors encoding fusion proteins can be administered directly to the plant for gene regulation, targeted cleavage, and/or recombination. In certain embodiments, the plant contains multiple paralogous target genes. It is known that plants may contain multiple paralogous genes. Thus, one or more different fusion proteins or expression vectors encoding fusion proteins may be administered to a plant in order to target one or more *Zp15* genes in the plant.

[0145] Administration of effective amounts is by any of the routes normally used for introducing fusion proteins into ultimate contact with the plant cell to be treated. The ZFPs are administered in any suitable manner, preferably with acceptable carriers. Suitable methods of administering such modulators are available and well known to those of skill in the art, and,

although more than one route can be used to administer a particular composition, a particular route can often provide a more immediate and more effective reaction than another route.

[0146] Carriers may also be used and are determined in part by the particular composition being administered, as well as by the particular method used to administer the composition. Accordingly, there is a wide variety of suitable formulations of carriers that are available.

EXAMPLES

[0147] Below are examples of specific embodiments for carrying out the present disclosure. The examples are offered for illustrative purposes only, and are not intended to limit the scope of the present disclosure in any way.

[0148] Efforts have been made to ensure accuracy with respect to numbers used (*e.g.*, amounts, temperatures, etc.), but some experimental error and deviation should, of course, be allowed for.

Example 1: Identification and Characterization of the *Zp15* Target Locus

[0149] Based on publicly available genetic maps (Lawrence, C., et al. (2004) NAR 32:393-397; maize internet database) and the draft genome sequence of maize, the *Zp15* locus on the short arm of chromosome 6 was chosen as a target for modification using ZFNs based on location and information available about the *Zp15* gene. The genomic structure and sequence of a gene encoding 15kD beta zein (*Zp15*) from maize has been described and annotated in the public domain (Woo et al. (2001) Plant Cell 13(10): 2297-2313). The sequence for the *Zp15* gene is described in GenBank accession number AF371264.

[0150] The *Zp15* genomic sequence was used to query the TIGR and Maize GDB genome databases using BLAST algorithms. Several sequences with overlapping homology to *Zp15* including, but not limited to, two contigs, (AZM5_16782 and ZmGSStuc11-12-04.8785.1) and several ESTs (M72708, M13507, M12147, AY103640 and AF371264) were identified. Based on the sequence of these accessions as well as the *Zap15* sequence, multiple short oligonucleotides were designed for use as PCR primers using the Primer3 program (Rozen, S. and Skaletsky, H.J. (2000) Primer3 on the WWW for general users and for biologist programmers. In: Krawetz S, Misener S (eds.) Bioinformatics Methods and Protocols: Methods in Molecular Biology. Humana Press, Totowa, NJ, pp 365-386; also available on the internet).

[0151] These primers include, but are not limited to, the following forward orientation oligonucleotides:

P67F 5' - CGTATGAATTCATTGACAACC - 3' (SEQ ID NO:1)

P68F 5' - ATGATCTATCTGTAAATCC - 3' (SEQ ID NO:2)

P69F 5' - CGTCATGCAACGCAACATTCC - 3' (SEQ ID NO:3)

P73F 5' - AAGAACATCACAAGTTATGC - 3' (SEQ ID NO:4)

P74F 5' - TCATGTGGATCCAAGGCATC - 3' (SEQ ID NO:5)

[0152] In addition, the primers include, but are not limited to, the following reverse orientation oligonucleotides:

P70R 5' - ATGTGTGTCGTCTTACTGC - 3' (SEQ ID NO:6)

P71R 5' - CAGTAGTAGGGCGGAATG - 3' (SEQ ID NO:7)

P72R 5' - GGGCAGCTGGTACTG - 3' (SEQ ID NO:8)

P75R 5' - CTATAATCGATGTAGAGC - 3' (SEQ ID NO:9)

P76R 5' - CTATGCTTTGTCTATAGTCG - 3' (SEQ ID NO:10)

[0153] All oligonucleotide primers were synthesized by and purchased from Integrated DNA Technologies (IDT, Coralville, IA). Amplifications of gDNA from maize variety Hi-II were performed on a PCR thermal cycler using 30 ng gDNA. A 2,215 bp amplification fragment corresponding to the *Zp15* gene from Hi-II was isolated and cloned into the pCR2.1 plasmid (Invitrogen, Carlsbad, CA). Sequence analysis of this fragment revealed that the genomic structure of *Zp15* from maize variety Hi-II contains two exons and one small intron of 31 bp (SEQ ID NO:126). Designs for *Zp15*-targeted ZFN were focused on the coding regions of the *Zp15* gene from maize variety Hi-II.

Example 2: Design of Zinc Finger Nucleases Targeted to Maize *Zp15* Gene

[0154] In order to assemble expression vectors for ZFNs, a stepwise modular cloning scheme was devised that is applicable for any given pair of ZFN-encoding genes selected from the library archive or synthesized *de novo*.

[0155] *Zp15*-targeted ZFNs were first screened using the yeast assay screen as described in Doyon et al. (2008) Nature Biotechnology 26(6):702 and U.S. Patent Application No. 12/284,887. Briefly, the entire *Zp15* locus was introduced into the HO locus in the budding yeast genome in order to directly compare ZFN activity at different binding sites within the target gene; ZFNs were screened for their ability to induce a DSB in the reporter gene using a reporter assay (MEL1) as described in *Doyon et al.*

[0156] Based on the results of these proxy system assays, it was confirmed that various ZFN pairs tested were capable of inducing DSBs within *Zp15.*

[0157] Following yeast prescreening, the ZFN pairs were then subcloned into maize specific expression vectors. As described in U.S. Patent Publication No. 20080182332, a vector including redesigned and synthesized segments of a nuclear localization signal (NLS) derived from maize *op-2* and a *FokI* nuclease domain utilizing the maize codon-bias was modified with a single nucleotide insertion (C) downstream of the unique *Xho* I site to create an extra *Sac* I site. A similar vector was modified to include the 2A ribosomal stuttering sequence from *Thosea asigna* virus. The gene cassettes encoding ORFs of individual zinc-finger proteins were cloned into either of these vectors via *Kpn* I and *BamH* I restriction sites, and subsequently the two vectors were combined via *Bgl* II/*Xho* I restriction sites, yielding an intermediate construct that contained a cassette including 2 ZFN-encoding domains flanked by *Nco* I and *Sac* I restriction sites.

[0158] The *Nco* I/*Sac* I cassette from this intermediate construction was excised via restriction enzyme digestion and ligated into the plasmid backbone pDAB3872, which contains a promoter from the maize *ubiquitin-1* gene (Sharrock et al. (1992) Plant Mol Biol. 18(4):675) and terminator sequences from maize root preferential cationic peroxidase gene (US Patent No. 7,179,902).

[0159] The resulting plasmids include the ZFN genes, plus the relevant selectable markers for plasmid maintenance and flanking attL sites for convenient manipulation using the GATEWAY™ system from Invitrogen (Carlsbad, CA). Each of the ZFN constructs generated using this cloning scheme were transformed into E. *coli* DH5α cells and subsequently maintained under the appropriate selection.

[0160] Table 1 shows exemplary *Zp15*-targeted ZFNs that were used for targeted integration experiments into the *Zp15* locus. The DNA target sequence for the ZFN is shown in the second column (DNA target sites indicated in uppercase letters; non-contacted nucleotides indicated in lowercase), and the third through sixth columns show the amino acid sequence of the recognition region (amino acids -1 through +6, with respect to the start of the helix) of each of the zinc fingers (F1 through F4) in the protein. Also provided in the first column of Table 1 is an identification number for each protein.

Table 1

ZFN name	Target Site	F1	F2	F3	F4
11742	cgGGGCTGCAGGGCttg tacggcgctgg (SEQ ID NO:11)	DRSHLTR (SEQ ID NO:12)	RSDNLRE (SEQ 1D NO:13)	RSDVLSE (SEQ ID NO:14)	RSAHLSR (SEQ ID NO:15)
	gcAGGGGCAGGGCAtct gcattgcagag (SEQ ID NO:16)	QSGSLTR (SEQ ID	RSDHLTQ (SEQ ID	DRSHLTR (SEQ ID	RSDHLTQ (SEQ ID

ZFN name	Target Site	F1	F2	F3	F4
11743		NO:17)	NO:18)	NO:12)	NO:18)
11750	ctGAGGCAGCCGCAgtg cagcccgctgg (SEQ ID NO:19)	QSGDLTR (SEQ ID NO:20)	DRSDLSR (SEQ ID NO:21)	QSGDLTR (SEQ ID NO:20)	RSDNLTR (SEQ ID NO:22)
11753	acTCCGCGTAGGGGta cagcccgccggc (SEQ ID NO:23)	RSDHLSR (SEQ ID NO:24)	RSDNLTT (SEQ ID NO:25)	RSDDLTR (SEQ ID NO:26)	DSSDRKK (SEQ ID NO:27)
11754	acTCCGCGTAGGGGta cagcccgccggc (SEQ ID NO:23)	RSDHLSE (SEQ ID NO:29)	RNDNRKN (SEQ ID NO:30)	RSDDLTR (SEQ ID NO:26)	DSSDRKK (SEQ ID NO:27)
11755	agCCGCAGtGCAGCCc gctggcggcggc (SEQ ID NO:31)	DRSDLSR (SEQ ID NO:21)	QSSDLTR (SEQ ID NO:32)	RSDHLSE (SEQ ID NO:29)	TSSTRKT (SEQ ID NO:33)
11756	agCCGCAGtGCAGCCc gctggcggcggc (SEQ ID NO:31)	DRSDLSR (SEQ ID NO:21)	QSSDLTR (SEQ ID NO:32)	RSDHLSE (SEQ ID NO:29)	RSSTRKE (SEQ ID NO:35)
11757	ccTCAGGTACTCCGcgt aggggtacagc (SEQ ID NO:36)	RSDTLSE (SEQ ID NO:37)	ARSTRTN (SEQ ID NO:38)	QSSHLTR (SEQ ID NO:39)	QSADRTK (SEQ ID NO:40)
11758	gcCCGCTGGCGGCGgc gccctactacgc (SEQ ID NO:41)	RSDDLTR (SEQ ID NO:26)	RSDDLTR (SEQ ID NO:26)	RSDTLSA (SEQ ID NO:42)	RNQDRKT (SEQ ID NO:43)
11759	gcACTGCGGCTGCCtca ggtactccgcg (SEQ ID NO:44)	DRSDLSR (SEQ ID NO:21)	QSSDLRR (SEQ ID NO:45)	RSDDLTR (SEQ ID NO:26)	QSSDLTR (SEQ ID NO:32)
11760	cgCCGGGTGTGGGCag ccgagcgccatg (SEQ ID NO:46)	DRSHLSR (SEQ ID NO:47)	RSDALAR (SEQ ID NO:48)	QSSHLTR (SEQ ID NO:39)	RSDDRKT (SEQ ID NO:49)
11761	cgCCGGGTgTGGGCAg ccgagcgccatg (SEQ ID NO:46)	QSGSLTR (SEQ ID NO:17)	RSDHLTT (SEQ ID NO:51)	QSSHLTR (SEQ ID NO:39)	RSDDRKT (SEQ ID NO:49)
	gcCGGGTGTGGGCAgc cgagcgccatgt (SEQ ID NO:52)	QSGSLTR (SEQ ID	RSDHLTT (SEQ ID	RSDSLLR (SEQ ID	RSDNLRE (SEQ ID

ZFN name	Target Site	F1	F2	F3	F4
11762		NO:17)	NO:51)	NO:53)	NO:13)
11763	agTAGGGCGCCGCCgc cagcgggctgca (SEQ ID NO:54)	RSDNLTT (SEQ ID NO:25)	DRSDLSR (SEQ ID NO:21)	DRSHLTR (SEQ ID NO:12)	RSDNLTT (SEQ ID NO:25)
11766	tgTGGGCAGCCGAGcg ccatgttccagc (SEQ ID NO:55)	RSDNLAR (SEQ ID NO:56)	DRSDLSR (SEQ ID NO:21)	QSGSLTR (SEQ ID NO:17)	RSDHLTT (SEQ ID NO:51)
11767	tgTGGGCAGCCGAGcg ccatgttccagc (SEQ ID NO:55)	RSDNLSR (SEQ ID NO:58)	DNSTRKT (SEQ ID NO:59)	QSGSLTR (SEQ ID NO:17)	RSDHLTT (SEQ ID NO:51)
11768	cgGCGTAGTAGGGCgc cgccgccagcgg (SEQ ID NO:60)	DRSHLTR (SEQ ID NO:12)	RSDNLTT (SEQ ID NO:25)	RSDNLST (SEQ ID NO:61)	RSADLSR (SEQ ID NO:62)
11769	cgGCGTAGtAGGGCGc cgccgccagcgg (SEQ ID NO:60)	RSDDLTR (SEQ ID NO:26)	RSDHLTQ (SEQ ID NO:18)	RSDNLST (SEQ ID NO:61)	RSADLSR (SEQ ID NO:62)
11770	caGCCGCTCCGGCAac agtgctgccagc (SEQ ID NO:64)	QSGSLTR (SEQ ID NO:17)	RSDDRKT (SEQ ID NO:49)	QSSDLSR (SEQ ID NO:65)	DRSDLSR (SEQ ID NO:21)
11771	acATGGCGcTCGGCTg cccacacccggc (SEQ ID NO:66)	QSSDLSR (SEQ ID NO:65)	RNDDRKK (SEQ ID NO:67)	RSDDLTR (SEQ ID NO:26)	RSDALTQ (SEQ ID NO:68)
11772	tgGCAGCCCAGGGTctc aaccccatggc (SEQ ID NO:69)	QSSHLTR (SEQ ID NO:39)	RSDNLRE (SEQ ID NO:13)	DRSDLSR (SEQ ID NO:21)	QSSDLTR (SEQ ID NO:32)
11773	caGCTGCTGCTGCTgct gcatcagagct (SEQ ID NO:70)	QSSDLSR (SEQ ID NO:65)	QSSDLRR (SEQ ID NO:45)	QSSDLSR (SEQ ID NO:65)	QSSDLRR (SEQ ID NO:45)
11774	ctGCCCAGCTACCGcac caacccctgtg (SEQ ID NO:28)	RSDSLSA (SEQ ID NO:34)	DNSNRIK (SEQ ID NO:50)	RSDNLSE (SEQ ID NO:57)	ASKTRKN (SEQ ID NO:63)
	tgGTACTGGTAGAGtcc acccatggccg (SEQ ID NO:71)	RSDNLAR (SEQ ID	QSGSLTR (SEQ ID	RSDVLSE (SEQ ID	QSGSLTR (SEQ ID

ZFN name	Target Site	F1	F2	F3	F4
11775		NO:56)	NO:17)	NO:14)	NO:17)
11776	tgGTACTGGTAGAGtcc acccatggccg (SEQ ID NO:71)	RSDNLAR (SEQ ID NO:56)	QSGSLTR (SEQ ID NO:17)	RSDALSN (SEQ ID NO:72)	TSSARTT (SEQ ID NO:73)
11777	aaCCCCTGtGGCGTCtc cgctgccattc (SEQ ID NO:74)	DRSALSR (SEQ ID NO:75)	DRSHLAR (SEQ ID NO:76)	RSDTLSA (SEQ ID NO:42)	DRSTRTT (SEQ ID NO:77)
11778	gtGCGGTAGCTGGGca gctggtactggt (SEQ ID NO:78)	RSDHLSR (SEQ ID NO:24)	QSSDLRR (SEQ ID NO:45)	QSGALAR (SEQ ID NO:79)	RSDDLTR (SEQ ID NO:26)
11779	gtGCGGTAGCTGGGca gctggtactggt (SEQ ID NO:78)	RSDHLST (SEQ ID NO:80)	HSDTRKK (SEQ ID NO:81)	QSGALAR (SEQ ID NO:79)	RSDDLTR (SEQ ID NO:26)
11780	agGCGGGGcTTGACGa agttggaagccg (SEQ ID NO:82)	RSDSLSV (SEQ ID NO:83)	QNQHRIN (SEQ ID NO:84)	RSDHLSR (SEQ ID NO:24)	RSDDLTR (SEQ ID NO:26)
11781	aaATGGAAAAAACGcta aaattatgtgt (SEQ ID NO:85)	RSDDLSK (SEQ ID NO:86)	RNDHRKN (SEQ ID NO:87)	QRSNLVR (SEQ ID NO:88)	RSDALTQ (SEQ ID NO:68)
11782	ttGTGGTGCCAACGgga gccatgctcac (SEQ ID NO:89)	RSDTLSQ (SEQ ID NO:90)	QNATRIN (SEQ ID NO:91)	RSDALSR (SEQ ID NO:92)	RSDALAR (SEQ ID NO:48)
11783	ttGTGGTGCCAACGgga gccatgctcac (SEQ ID NO:89)	RSDTLSQ (SEQ ID NO:90)	QKATRIT (SEQ ID NO:93)	RSDALSR (SEQ ID NO:92)	RSDALAR (SEQ ID NO:48)
11784	ttGTGGTGcCAACGGga gccatgctcac (SEQ ID NO:89)	RSDHLSE (SEQ ID NO:29)	QNANRKT (SEQ ID NO:94)	RSDALSR (SEQ ID NO:92)	RSDALAR (SEQ ID NO:48)
11785	caATCACGCCGGTAgc ggggctagttat (SEQ ID NO:95)	QSGALAR (SEQ ID NO:79)	RSDDRKT (SEQ ID NO:49)	RSDTLSQ (SEQ ID NO:90)	DSSARKK (SEQ ID NO:96)

[0161] It will be apparent that ZFNs can be readily inserted into C2H2 or C3H backbones and

that a variety of sequences can be used to join the zinc finger protein and the cleavage domain. See, U.S. Patent Publication 20080182332, particularly Table 6, regarding such sequences,

Example 3: ZFN-mediated disruption of *Zp15* in maize cells

[0162] Induction ofDSB by the ZFN pairs was tested. ZFN pairs that are capable of efficiently producing DSB at the intended target site of the endogenous *Zp15* gene were identified. The error-prone nature of DSB repair by non-homologous end joining (NHEJ), which is known to generate small DNA deletions/insertions at the site of a ZFN-induced break, was utilized to select ZFN pairs which efficiently bound and cleaved the endogenous *Zp15* gene target site

[0163] ZFNs were transiently expressed in cultured maize cells and sequence analysis of the target locus at the predicted cleavage site was conducted. For example, a plasmid pDAB7468 encoding ZFN pair # 25 (11768 /11766 recognition helices shown above) was delivered via WHISKERS™ -mediated transformation into maize Hi-II cell cultures as described in U.S. Patent Application No. 12/001,939.

[0164] After either 24 or 72 hours of transient expression, the resulting disrupted ZFN target sequence was amplified from isolated genomic DNA and cloned into plasmid vector pCR2.1. The gDNA was subjected to restriction digestion using enzyme *Bsu36*I, followed by amplification of the *Zp15* target sequence and cloning of the PCR products into plasmid vector pCR2.1. Individual colonies of the cloned amplification product were analyzed by restriction digestions of plasmid DNA followed by agarose gel electrophoresis (cloned amplification products that displayed resistance to cleavage by restriction enzyme *Bsu36I* were considered to contain mutations that destroy the restriction site associated with the ZFN cleavage site).

[0165] Direct sequence analysis of 192 clones revealed a 6 bp insertion (Figure 1). In another example, plasmid pDAB7467 encoding ZFN pair # 24 (11753 /11750 recognition helices shown above) was delivered directly into maize cell cultures and after either 24 or 72 hours of transient expression, the ZFN target sequence was amplified and cloned into plasmid vector pCR2.1. Direct sequence analysis of 192 clones revealed a 3 bp deletion (Figure 2) at the precise cleavage site. The insertion and deletion described here are the outcome of NHEJ repair of an induced DSB at the target site and indicate that ZFNs 24 and 25 have cleavage activity at the endogenous *Zp15* locus in maize cells.

[0166] The same process was performed using ZFN 25 or 28, but instead of screening colonies by restriction enzyme digestion, 192 independent clones were directly sequenced. A 6 bp insertion was detected (Figure 2, top).

[0167] Taken together, these data demonstrate that a transient exposure to the ZFNs is sufficient to induce a targeted DSB at the *Zp15* locus in cultured maize cells.

Example 4: Targeted Integration into *Zp15* locus

[0168] In order to test whether designed ZFNs with cleavage activity at *Zp15* could drive integration of exogenous sequences, we constructed donor DNA molecules carrying an autonomous gene cassette encoding an exemplary exogenous herbicide resistance gene, *AAD-1* from *Sphingomonas herbicidovorans* (ATCC 700291). *AAD-1* encodes the enzyme aryloxyalkanoate dioxygenase and confers resistance to aryloxyphenoxypropionate herbicides (international patents WO 2005/107437, WO2008141154 A2). One of skill in the art will appreciate that other exogenous nucleic acids could be similarly incorporated into donor DNA molecules, including but not limited to other herbicide tolerance genes such as the related *AAD-12* gene. In this herbicide tolerance gene donor, the promoter sequence is derived from *O. sativa* actin (GenBank accession numbers S44221 and X63830) and terminator sequences are derived from *Z. mays* L. lipase (GenBank Accession Number L35913).

A. Donor DNA molecule construction

[0169] Donor constructs containing regions of homology to *Zp15* were generated as follows. A plasmid backbone containing homology flanks for the *Zp15* gene was engineered to allow for the integration of any donor DNA sequence into the corresponding target site of the *Zp15* gene. The plasmid backbone exemplified here originated with the base plasmid vector pBC SK(-) phagemid (3.4 kbp) (Stratagene, La Jolla, CA). There were four steps to this process.

[0170] First, the base plasmid was prepared by linearizing 3 μg pBC SK(-) using the *Spe* I and *Sal* I (New England Biolabs, Beverly, MA) restriction endonucleases. The 3.3 kbp *Spe* I/*Sal* I digested subcloning vector, pBC SK(-) was gel-excised and purified according to the manufacturer's directions using QIAQUICK Gel Extraction Kit (QIAGEN Inc., Valencia, CA).

[0171] Second, the 5'- & 3'-homology flanks were isolated from *Zp15* using the following oligonucleotide primers that were synthesized by Integrated DNA Technologies, Inc. (Coralville, IA):

Zp15HRDonorNotI(205)F: 5'- GCGGCCGCATGCAAGAGCTGTTGATC -3' (SEQ ID NO:97)

Zp15HRDonorMfeI(1025)R: 5'-CAATTGCCGGCGTAGTAGGGCGCCGCCGCCAGC -3' (SEQ ID NO:98)

Zp15HRDonorMfeI(1025)F: 5'-CAATTGGTGTGGGCAGCCGAGCGCCATGTTCCAG - 3' (SEQ ID NO:99)

Zp15HRDonorSalI(2270)R 5' - GTCGACCGATACTGATGCGGACCGTCCACCTTGTC - 3' (SEQ ID NO:100).

[0172] PCR amplification reactions were carried out using reagents provided with the LA TAQ PCR kit (TaKaRa Biotechnology Inc. Otsu, Shiga, Japan). The PCR reaction cocktail consisted of: 5 uL 10X LA PCR™ Buffer II ($Mg2^+$), 20 ng double-stranded template (Hi-II maize genomic DNA), 10 pmol forward oligonucleotide primer, 10 pmol reverse oligonucleotide primer, 8 uL dNTP mix (2.5 mM each dNTP), 33.5 uL H_2O, 0.5 uL (2.5 units) *TaKaRa LA Taq*™ DNA polymerase, 1 drop of mineral oil. The primers Zp15HRDonorNotI(205)F and Zp15HRDonorMfeI(1025)R were used for a reaction and the primers Zp15HRDonorMfeI(1025)F and Zp15HRDonorSalI(2270)R were used for the second reaction. PCR reactions were performed using a Perkin-Elmer Cetus, 48-sample DNA thermal cycler (Norwalk, CT) under the following cycle conditions: 94°C, 4 min/1 cycle; 98°C 20 sec, 65°C 1 min, 68°C 1 min/30 cycles; 72°C, 5 min/1 cycle; 4°C/hold. Fifteen (15) µl of each PCR reaction was electrophoresed and amplified fragments were visualized with UV light and fragment sizes estimated by comparison with 1 kbp DNA ladder. Expected plasmid clones were diagnosed by the presence of DNA fragments of 825 bp for the 5'-fragment or 1,250 bp for the 3'-fragment. These fragments were gel-excised and purified according to manufacturer's directions using QIAquick Gel Extraction Kit (QIAGEN Inc., Valencia, CA). Purified fragments were then cloned into pCR2.1 plasmid using TOPO TA CLONING® Kit and transformed into ONE SHOT® TOP10 Chemically competent *E. coli* cells (Invitrogen Life Technologies, Carlsbad, CA) according to manufacturer's protocol.

[0173] Individual colonies containing the 825 bp 5'-fragment or the 1,250 bp 3'-fragment were identified and confirmed via restriction enzyme digestion and sequencing data. Colonies containing the 825 bp 5'-fragment were confirmed via a restriction enzyme digestion of *Mfe* I and *Not* I (New England Biolabs, Beverly, MA). Colonies containing the 1,250 bp 3'-fragment were identified and confirmed via restriction enzyme digestion using *Sal* I (New England Biolabs, Beverly, MA). Expected plasmid clones were diagnosed by the presence of inserted DNA fragments of 825 bp (5'-fragment) or 1,250 bp (3'-fragment) in addition to the 3.9 kbp pCR®2.1 vector. Double-stranded sequencing reactions of plasmid clones were performed as described by manufacturer using CEQ™ DTCS-Quick Start Kit (Beckman-Coulter, Palo Alto, CA). Reactions were purified using Performa DTR Gel Filtration Cartridges (Edge BioSystems, Gaithersburg, MD) as described by manufacturer protocols. Sequence reactions were analyzed on a Beckman-Coulter CEQ™ 2000 XL DNA Analysis System and nucleotide characterization performed using SEQUENCHER™ version 4.1.4 (Gene Codes Corporation, Ann Arbor, MI). The sequences of the 5'- and 3'-homology fragments from *Zp15* are indicated in SEQ ID NO:127 and SEQ ID NO:128.

[0174] Third, the 3'-homology flank was ligated into the base plasmid as follows. Clones that contained the correct 3'-homology flank sequence were digested with restriction enzymes and a DNA fragment was gel-excised and purified using the QIAquick Gel Extraction Kit (QIAGEN Inc., Valencia, CA). These fragments were ligated into a purified base plasmid, previously digested with *Spe* I/*Sal* I (see above), at a 1:5 vector:insert ratio using 500 units T4 DNA Ligase (Invitrogen Life Technologies, Carlsbad, CA) in a reaction volume of 20 uL under conditions of 16 hr incubation in a 16°C water bath. Five (5) uL of the ligation reaction was

subsequently transformed into *E. coli* One SHOT® Top 10 Chemically Competent Cells, (Invitrogen Life Technologies, Carlsbad, CA) and plated onto media containing antibiotic selection. Putative colonies were isolated and digested with the *Spe* I and *Sal* I restriction enzymes (New England Biolabs, Beverly, MA) to identify clones which contained the ligated 3'-fragment.

[0175] Fourth, the 5'-homology flank was ligated into the plasmid containing the 3'-homology flank. The plasmid containing the 3'- homology flank, described above in step three, was digested with the *Mfe* I and *Not* I (New England Biolabs, Beverly, MA) restriction endonucleases for 1 hr at 37°C. The *Not* I/*Mfe* I digested the plasmid containing the 3' -homology flank was gel-excised and purified according to the manufacturer's directions using QIAquick Gel Extraction Kit (QIAGEN Inc., Valencia, CA).

[0176] Isolated fragments of the 5'-homology flank donor generated by restriction enzyme digestion using the *Mfe* I and *Not* I restriction enzymes were produced and ligated with the plasmid containing the 3'-homology flank in a 20 uL ligation reaction using a 1:5 vector:insert ratio and 500 units T4 DNA Ligase (Invitrogen Life Technologies, Carlsbad, CA). Ligation reactions were incubated for 16 hr in a 16°C water bath.

[0177] Following the ligation, 5 uL of the ligation reaction was transformed into MAX EFFICIENCY® DH5α™ Chemically Competent Cells (Invitrogen Life Technologies, Carlsbad, CA) as per the manufacturer's recommendations. Individual colonies were selected and plasmid DNA was isolated and digested with the *Not* I restriction enzyme (New England Biolabs, Beverly, MA) to identify plasmids which contained an integrated fragment of the 5'-homology flank donor. The resulting plasmid was given the name pDAB7489 (Figure 3).

[0178] An herbicide-tolerance gene expression cassette comprising a plant transcriptional unit (PTU) containing promoter, herbicide tolerance gene, and polyadenylation (polyA) termination sequences was constructed. The promoter sequence is derived from *O. sativa* actin 1 (McElroy et al. (1990) Plant Cell 2, 163-171; GenBank Accession S44221 and GenBank Accession X63830). The herbicide-tolerance gene comprised the *AAD-1* (aryloxyalkanoate dioxygenase) gene, which confers resistance to aryloxyphenoxypropionate herbicides (WO 2005/107437). The version of the gene utilized was version #3, which includes a codon optimized sequence for expression in plants. The terminator sequences are derived from Z. *mays* L. lipase (maize lipase cDNA clone of GenBank Accession Number L35913). This maize sequence comprises the 3' untranslated region/transcription terminator region for the *AAD-1* gene). The herbicide tolerance gene expression cassette is shown in Figure 4.

[0179] To generate this cassette, the following oligonucleotide primers were synthesized by Integrated DNA Technologies, Inc. (Coralville, IA) under conditions of standard desalting and diluted with water to a concentration of 0.125 ug/uL: OsActAad1v.3ZmLipMfeIF 5' -CAATTGGTCATTCATATGCTTGAGAAGAG-3' (SEQ ID NO:101) OsActAad1v.3ZmLipMfeIR 5' -CAATTGAGCACTTAAAGATCTTTAGAAG-3' (SEQ ID NO:102)

[0180] PCR amplification reactions were carried out using the LA TAQ PCR Kit (*TaKaRa* Biotechnology Inc., Otsu, Shiga,, Japan). The PCR reaction cocktail comprised: 5 uL 10X LA PCR™ Buffer II ($Mg2^+$), 20 ng double-stranded template (pDAB3878 plasmid DNA), 10 pmol forward oligonucleotide primer, 10 pmol reverse oligonucleotide primer, 8 uL dNTP mix (2.5 mM each), 33.5 uL H_2O, 0.5 uL (2.5 units) *TaKaRa LA Taq*™ DNA polymerase, 1 drop of mineral oil. PCR reactions were performed using a Perkin-Elmer Cetus, 48-sample DNA thermal cycler (Norwalk, CT) under the following cycle conditions 94°C, 4 min/1 cycle; 98°C 20 sec, 55°C 1 min, 68°C 3 min/30 cycles; 72°C, 5 min/1 cycle; 4°C/hold. Fifteen (15) µl of each PCR reaction was electrophoresed at 100 V for 1 hr in a 1.0% TAE agarose gel supplemented with 0.5% ethidium bromide. Amplified fragments were visualized with UV light and fragment size estimated by comparison with 1 kbp DNA ladder.

[0181] Expected PCR products were diagnosed by the presence of a DNA fragment of 2.7 kbp (*AAD*-1 PTU). This fragment was gel-excised and purified according to manufacturer's directions using QIAquick Gel Extraction Kit (QIAGEN Inc., Valencia, CA). Purified fragments were then cloned into pCR2.1 plasmid using TOPO TA Cloning® Kit (with pCR®2.1 vector) and One Shot® TOP10 Chemically competent *E. coli* cells (Invitrogen Life Technologies, Carlsbad, CA) according to manufacturer's protocol.

[0182] Individual colonies were selected, plasmid DNA was isolated, and digested with the restriction enzyme *Mfe* I (New England Biolabs, Beverly, MA). Expected plasmid clones were diagnosed by the presence of an inserted DNA fragment of 2,674 bp (*AAD*-1 PTU) in addition to the 3.9 kbp pCR®2.1 vector. Double-stranded sequencing reactions of plasmid clones were performed as described by the manufacturer using CEQ™ DTCS-Quick Start Kit (Beckman-Coulter, Palo Alto, CA). Reactions were purified using Performa DTR Gel Filtration Cartridges (Edge BioSystems, Gaithersburg, MD) as described by manufacturer protocols. Sequence reactions were analyzed on a Beckman-Coulter CEQ™ 2000 XL DNA Analysis System and nucleotide characterization performed using SEQUENCHER™ version 4.1.4 (Gene Codes Corporation, Ann Arbor, MI).

[0183] Restricted fragment from a clone that contained the correct 2,674 bp sequence was gel-excised and purified according to the manufacturer's directions using QIAquick Gel Extraction Kit (QIAGEN Inc., Valencia, CA). This fragment was then combined in a ligation reaction with purified pDAB7489 (plasmid backbone) which had been digested with restriction enzyme *Mfe* I and subsequently dephosphorylated. Ligation was carried out under the following conditions: 1:5 vector:insert ratio and 500 units T4 DNA Ligase (Invitrogen Life Technologies, Carlsbad, CA) in a reaction volume of 20 uL under conditions of 16 hr incubation in a 16°C water bath. Five (5) uL of the ligation reaction was subsequently transformed into 50 µl *E. coli* MAX EFFICIENCY® DH5α™ Chemically Competent Cells, (Invitrogen Life Technologies, Carlsbad, CA) and plated under selection conditions described by the manufacturer.

[0184] Individual colonies were selected, plasmid DNA was isolated, and digested with the restriction enzyme *Mfe* I (New England Biolabs, Beverly, MA). The expected plasmid clones

contained DNA fragments 2,674 bp (*AAD*-1 PTU) and 5,413 bp (pDAB7489 vector). The resulting plasmid was named pDAB7490 (Figure 5).

[0185] Embryogenic cell cultures of maize variety Hi-II (Armstrong et al. (1991) Maize Genet Coop Newsletter 65:92-93) were generated, maintained and subjected to simultaneous transformation of plasmids encoding ZFN24 and donor molecule pDAB7490. The transformation and selection of callus tissue and subsequent regeneration of transformants is described in U.S. Patent Application No. 12/001,939.
For additional guidance regarding the transformation and selection protocol *see* Petolino et al. (2000) Plant Cell Rept. 19:781-786. Following anthesis, plants were either self-pollinated or outcrossed to maize variety DAS5XH751. Resulting progeny seed were harvested and dried. The regeneration of callus into intact, fertile maize plants is described in U.S. Patent Application No. 12/001,939, particularly Example 22.

B. Targeted integration of the *AAD-1* gene cassette into the *Zp15* locus

[0186] Of the herbicide-tolerant events containing an integrated donor DNA molecule encoding an herbicide-tolerance gene cassette, it is expected that some proportion of said events are the product of targeted integration of donor DNA into the site of the ZFN-induced double-stranded break. In order to differentiate these targeted integration events from those derived from random integration of the herbicide-tolerance gene cassette, a PCR-based genotyping strategy using a combination of genome-specific and subsequent genome-specific plus donor-specific PCR primers was utilized.

[0187] Differential genotyping of targeted versus random-integration of the *AAD-1* transgene in all herbicide-tolerant transformed events was carried out using PCR-based assays specific to the *Zp15* locus and *AAD-1* gene. In the examples presented here, all oligonucleotide primers were synthesized by Integrated DNA Technologies, Inc. (Coralville, IA) under conditions of standard desalting and diluted with water to a concentration of 100 μM. The following set of forward and reverse oligonucleotide primers were designed to anneal to gDNA sequences specific for the *Zp15* gene target that lie outside the boundaries of the donor DNA sequences:

HB501f: 5'- AAGGTCCCAAATCTGAGGCATACTGTTGCT -3' (SEQ ID NO:103)

HB502r: 5'- GAGGTCCTATGCTTTGTCTATAGTCGGCAG -3' (SEQ ID NO:104)

[0188] A second set of forward and reverse oligonucleotide primers were also designed to anneal to gDNA sequence specific for the *Zp15* gene target outside the boundaries of the donor DNA sequences, yet nested within the first pair:

HB503 f 5'- GGCATACTGTTGCTGCCCTGCTGGAA -3' (SEQ ID NO:105)

HB504r 5'- GACACCTATAATCGATGTAGAGCCGAAGAG -3' (SEQ ID NO:106)

[0189] Forward and reverse oligonucleotide primers were additionally designed to anneal specifically to donor DNA corresponding to coding region of the *AAD-1* herbicide-tolerance gene:

HB505f 5'- AGTCCACCCCAGTGATCTCAGCACCA -3' (SEQ ID NO:107)

HB506f 5'- AGTGGCTGGACAGCTATTCTCTCAAAGCGT -3' (SEQ ID NO:108)

HB507r 5'- ACGCTTTGAGAGAATAGCTGTCCAGCCACT -3' (SEQ ID NO:109)

HB508r 5'- TGGTGCTGAGATCACTGGGGTGGACT -3' (SEQ ID NO:110)

[0190] Two distinct primary amplification reactions were carried out utilizing primers that bind in the *Zp15* genomic region and the donor molecule, giving rise to an amplicon that spans the boundary of integration between genome and donor. The first reaction focused on the 5'-boundary between genome and donor and used primer set HB501f and HB507r. The second reaction focused on the 3'-boundary between donor and genome and used primer set HB505f and HB502r. Genomic DNA was isolated from the transformed maize Hi-II events. Primary PCR amplification reactions were carried out using reagents provided by the LA TAQ PCR Kit (*TaKaRa* Biotechnology Inc., Otsu, Shiga, Japan). The PCR reaction cocktail consisted of: 2.5 µl 10X La Taq PCR™ Buffer, 40-200 ng double-stranded genomic DNA template, 10 µM forward oligonucleotide primer, 10 µM reverse oligonucleotide primer, 2 µl dNTP mix (2.5 mM each), 16.25 µl H_2O, 0.25 µl (1.25 units) *LA Taq*™ DNA polymerase. PCR reactions were performed using a Bio-Rad, 96-sample DNA Engine Tetrad2, Peltier Thermal Cycler (Hercules, CA) under the following cycle conditions: 94°C, 2 min/1 cycle; 94°C 30 sec, 62°C 30 sec, 68°C 5 min/30 cycles; 4°C/hold.

[0191] The primary PCR reaction products were subsequently diluted 1:100 in H_2O and used as template DNA for two distinct secondary PCR reactions. The secondary reactions also utilize primers that bind in the *Zp15* genomic region and the donor molecule, giving rise to an amplicon that spans the boundary of integration between genome and donor. The identity of the specific primers determines whether the amplification is focused on either the 5'- or 3'-boundary between genome and donor. The first reaction focused on the 5'-boundary between genome and donor and used primer sets HB503 f and HB508r. The second reaction focused on the 3'-boundary between donor and genome and used primer set HB506f and HB504r. Both reactions consisted of the following: 2.5 µl 10X La Taq PCR™ Buffer, 1 µl template [1:50 dilution of 1° PCR reaction], 10 µM forward oligonucleotide primer, 10 µM reverse oligonucleotide primer, 2 µl dNTP mix (2.5 mM each), 16.25 µl H_2O, 0.25 µl (1.25 units) *LA Taq*™ DNA polymerase. PCR reactions were performed using a Bio-Rad, 96-sample DNA

Engine Tetrad2, Peltier Thermal Cycler (Hercules, CA) under the following cycle conditions: 94°C, 2 min/1 cycle; 94°C 30 sec, 62°C 30 sec, 68°C 5 min/30 cycles; 4°C/hold. Expected PCR amplicon fragments of 2,180 bp for the 5'-boundary or 2,980 bp for the 3'- boundary were observed.

[0192] These fragments were gel-excised and purified according to manufacturer's directions using QIAquick Gel Extraction Kit (QIAGEN Inc., Valencia, CA). Purified fragments were subsequently cloned into pCR2.1 plasmid using TOPO TA CLONING® Kit (with pCR®2.1 vector) and ONE SHOT® TOP10 Chemically competent *E. coli* cells (Invitrogen Life Technologies, Carlsbad, CA) according to manufacturer's protocol.

[0193] Individual colonies were selected, plasmid DNA was isolated and digested with the restriction enzyme *Eco* RI (New England Biolabs, Beverly, MA). Expected plasmid clones were diagnosed by the presence of inserted DNA fragments of the appropriate size in addition to the 3.9 kbp pCR®2.1 vector.

[0194] Double-stranded sequencing reactions of plasmid clones were performed and nucleotide characterization and alignments were performed using SEQUENCHER™ version 4.1.4 (Gene Codes Corporation, Ann Arbor, MI).

[0195] Selected sequence data derived from a targeted integration event (event #147) of the *AAD-1 donor* gene cassette inserted into the *Zp15* target gene is shown in the alignment of Figure 6.

[0196] Primary PCR products amplification focused on either the 5'- or 3'-boundary between genome and donor were subjected to secondary amplification also focused on either the 5'- or 3'- boundary between genome and donor. Alignment of cloned fragments corresponding to these secondary amplification products with the wild-type *Zp15* genomic sequence as well as the expected sequence of a targeted integration event clearly indicates that the precise integration of donor DNA at the target site has occurred. Nucleotide sequence of the *Zp15* genomic locus, the genome/donor boundary, nucleotide sequence of the donor regions corresponding to *Zp15* homology flanks and nucleotide sequence of the herbicide tolerance cassette were all preserved in multiple cloned PCR products derived from this event. Therefore, this event represents a genome in which homology-driven repair of a ZFN-mediated double-stranded break and targeted integration of a donor DNA at a specific gene target has occurred. In Figure 6, we show sequence alignment data derived from a single representative isolated transformed maize callus (event #147).

[0197] Additional transformed events representing unique targeted integration occurrences have been obtained, demonstrating that the methods taught herein are reproducible in maize callus.

Example 5: Targeted Integration of PAT into *Zp15* locus

[0198] As a further exemplification of methods for targeted integration of selected exogenous polynucleotides into targeted loci disclosed herein, additional DNA donor molecules carrying an autonomous gene cassette encoding *PAT* were designed and constructed to test the integration of an exogenous donor sequence within the endogenous *Zp15* target locus. ZFNs with specific cleavage activity of the endogenous *Zp15* gene target were deployed to create a DSB at this locus. Donor DNA molecules carrying an autonomous gene cassette encoding *PAT,* from *Streptomyces viridochromogenes,* and flanking sequences homologous to *Zp15* were subsequently integrated into the ZFN induced DSB of the *Zp15* target. *PAT* encodes the enzyme phosphinothricin acetyl transferase and confers resistance to the herbicidal compound phosphinothricin (PPT) by acetylation (U.S. Patent No. 5,633,434). Phosphinothricin is the active ingredient of the herbicide LIBERTY, BASTA and IGNITE. The *PAT* coding sequence was constructed as a plant transcription unit (PTU) and contained a promoter sequence derived from *O. sativa* actin (GenBank accession numbers S44221 and X63830) and a terminator sequences derived from *Z. mays* L. lipase (GenBank accession number L35913).

A. Donor DNA molecule construction

[0199] An *Zp*15 donor construct containing regions of homology to *Zp15* was generated synthetically as follows. A *Zp15* homology region comprising nucleotides 4595-5346 (5' - homology sequence; SEQ ID NO:111) and nucleotides 21-796 (3' - homology sequence; SEQ ID NO:112) from pDAB7489 was designed. This homology region included an *Mfe* I cloning sites between the 5' and 3' - homologous elements and *Not* I restriction sites at the 5' and 3' ends. This DNA sequence (SEQ ID NO:113) was synthesized and inserted into the kanamycin resistant ColE1 type plasmid, pMK at the *Sac* I and *Kpn* I cloning sites (Gene Art Ag, Regensburg, Germany). The resulting donor plasmid was designated pDAB104101 (Figure 7) and contains homology flanks for the *Zp15* gene to allow for the integration of any DNA sequence of interest into the corresponding target site of the *Zp15* gene.

[0200] An herbicide-tolerance gene expression cassette comprising a complete PTU containing promoter, herbicide tolerance gene, and poly adenylation (polyA) termination sequences was constructed. The promoter sequence is derived from *O. sativa* actin 1 (McElroy et al. (1990) Plant Cell 2:163-171). GenBank Accession S44221 and GenBank Accession X63830). The herbicide-tolerance gene was *PAT.* The terminator sequences are derived from *Z. mays* L. lipase (maize lipase cDNA clone of GenBank Accession Number L35913). This maize sequence comprises the 3' untranslated region/transcription terminator region for the *PAT* gene. The *PAT* herbicide tolerance gene expression cassette is shown in Figure 8.

[0201] The *PAT* gene cassette was amplified from a plasmid pDAB102256 by PCR using primers synthesized by Integrated DNA Technologies, Inc. (Coralville, IA):

DC001 5' - CCAGTGCAATTGGGTCATTCATATGCTTGAGAAG - 3' (SEQ ID NO:114)

DC002 5' - CCAGTGCAATTGAATTCAGCACTTAAAGATCTTTAG - 3' (SEQ ID NO:115)

[0202] PCR amplification reactions were carried out using PHUSION™ DNA Polymerase (New England Biolabs, Beverly, MA) under the following cycle conditions: 98°C, 30sec/1 cycle; 98°C 10 sec, 60°C 20 sec, 72°C 45 sec/9 cycles; 98°C 10 sec, 72°C 60 sec/24 cycles; 72°C, 10 min/1 cycle; 4°C/hold. The PCR reaction was analyzed by electrophoresis in a 1.0% TAE agarose gel.

[0203] Expected PCR products were diagnosed by the presence of a DNA fragment of 2.3 kbp (*PAT* PTU). This fragment was excised and purified from the gel according to manufacturer's directions using QIAQUICK™ Gel Extraction Kit (QIAGEN Inc., Valencia, CA). Purified fragments were then cloned into pCR-Blunt II-TOPO vector using the Zero Blunt TOPO PCR Cloning Kit and ONE SHOT® TOP10 Chemically competent *E. coli* cells (Invitrogen Life Technologies, Carlsbad, CA)

[0204] Individual colonies were picked and plasmid DNA was isolated and subjected to plasmid DNA restriction enzyme digestion and sequence analysis. The cloned *PAT* inserts were sequenced to demonstrate the identity and sequence fidelity of the cloned PCR products (SEQ ID NO:116). One such plasmid clone was designated pDAB104107 (Figure 9) and was subsequently used as the source of the *PAT gene* cassette for insertion into the new *Zp15* homology donor vectors.

[0205] The 2.3 kbp *PAT* gene fragment was recovered from pDAB104107 by digestion with *Mfe I* followed by gel electrophoresis, excision and purification. The *Zp15* homology donor plasmid pDAB104101 was also digested with *Mfe* I and gel purified. Ligation of the *PAT* gene fragment into pDAB104101 yielded clones in which the *PAT gene* was inserted at the *Mfe* I site in either of two orientations with respect to the *Zp15* gene sequences as determined by differential restriction enzyme digestion. pDAB104104 (Figure 10) comprised the *PAT* gene inserted in the same transcriptional orientation as the *Zp15* gene. pDAB104105 (Figure 11) comprised the *PAT* gene inserted in the opposite orientation relative to the *Zp15* gene.

B. Additional donor DNA molecule construction

[0206] Another donor construct containing regions of homology to *Zp15* was generated in which the *Zp15* 3'- homology sequence in pDAB7489 was altered by truncation while the *Zp15* 5'- homology sequence in pDAB7489 remained the same. The truncated 3' homology region was generated from pDAB7489 by PCR using primers synthesized by Integrated DNA Technologies, Inc. (Coralville, IA):

DC003 5' - CTAATCGTCGACTCGTCAAGCCCCGCCTTTAAAT - 3' (SEQ ID NO:117)

DC004 5' - CTAATCCAATTGGTGTGGGCAGCCGAGCG - 3' (SEQ ID NO:118)

[0207] PCR amplification reactions were carried out using PHUSION HOT START DNA Polymerase (New England Biolabs, Beverly, MA) under the following cycle conditions: 98°C, 30s/1 cycle; 98°C 10 sec, 72°C 15 sec/33 cycles; 72°C, 5 min/1 cycle; 4°C/hold. The PCR reaction was subjected to electrophoresis in a 1.0% TAE agarose gel.

[0208] Expected PCR products were diagnosed by the presence of a DNA fragment of 0.8 kbp (*Zp15* 3' -homology). This fragment was gel-excised and purified according to manufacturer's directions using QIAquick Gel Extraction Kit (QIAGEN Inc., Valencia, CA). Purified fragments were then cloned into pCR-Blunt II-TOPO vector using the Zero Blunt TOPO PCR Cloning Kit and One Shot® TOP10 Chemically competent *E. coli* cells (Invitrogen Life Technologies, Carlsbad, CA) according to manufacturer's protocol.

[0209] Individual colonies were picked and subjected to plasmid DNA isolation and restriction enzyme digestion confirmation. *Zp15* 3' homology inserts were sequenced to demonstrate the identity and sequence fidelity of the cloned PCR products (SEQ ID NO:119). One such plasmid clone was designated pDAB104106 (Figure 12) and was subsequently used as the source of the new *Zp15* 3' homology sequence for substitution into pDAB7489 to create a new *Zp15* homology donor vector.

[0210] pDAB7489 was digested sequentially with *Mfe* I and *Sal* I and the 4.2 kbp vector fragment was gel purified. pDAB104106 was also digested with *Mfe* I and *Sal* I and the 0.8 kbp fragment comprising the truncated *Zp15* 3' homology sequence was gel purified. Ligation and transformation of these gel purified fragments yielded clones in which the truncated *Zp15* 3' homology sequence was substituted for the original *Zp15* 3' homology sequence. This plasmid was designated pDAB104100 (Figure 13) and was used as the recipient for the *PAT* gene.

[0211] The *PAT* gene was removed from pDAB 104107 by digestion with *Mfe* I. After gel electrophoresis, the 2.3 kbp *PAT gene* fragment was gel purified. The *Zp15* homology donor plasmid pDAB104100 was also digested with *Mfe* I and gel purified. Ligation of the *PAT* gene into pDAB104100 yielded clones in which the *PAT gene* was inserted at the *Mfe* I site in either of two orientations with respect to the *Zp15* gene as determined by differential restriction enzyme digestion. pDAB104103 (Figure 14) comprised the *PAT gene* inserted in the same transcriptional orientation as the *Zp15* gene. pDAB104102 (Figure 15) comprised the *PAT* gene inserted in the opposite orientation relative to the *Zp15* gene.

C. Transformation of maize and recovery of *Zp15*-targeted *PAT* insertions.

[0212] Embryogenic cell cultures of maize variety Hi-II (Armstrong et al. (1991) Maize Genet Coop Newsletter 65:92-93) were generated, maintained and subjected to simultaneous transformation of plasmids encoding ZFN24 and donor molecule. Donor molecules include

those described here; pDAB104102, pDAB104103, pDAB104104, and pDAB104105. The transformation and selection of callus tissue and subsequent regeneration of transformants is described in U.S. Patent Application No. 12/001,939, particularly Example 19.

[0213] For additional guidance regarding the transformation and selection protocol *see* Petolino et al. (2000) Plant Cell Rept. 19:781-786. Following anthesis, plants can be either self-pollinated or outcrossed to a maize variety such as DAS5XH751. Resulting progeny seed can be harvested and dried, and plants from these seed can be analyzed to demonstrate the heritability of the targeted integration events. The regeneration of callus into intact, fertile maize plants is described in U.S. Patent Application No. 12/001,939, particularly Example 22.

D. Identification of *Zp15*-targeted *PAT* insertions

[0214] *Zp15*-targeted *PAT* insertions in transformed callus tissue are detected by PCR. Template genomic DNA is extracted from callus tissue via well known and commonly used methods such as the Plant DNEASY Kit (QIAGEN Inc., Valencia, CA) or the method of Dellaporta (Dellaporta et al., (1983) Plant Mol. Biol. Rep. 1;19-21). Use of PAT specific primers in conjunction with the *Zp15* flanking sequence primers already used to detect *AAD-1* targeted integration into the *Zp15* locus results in the amplification of the *PAT* targeted insertion junctions in the 5' and 3' *Zp15* homology regions. The *PAT* specific primers can be:

DC013 5' - CAATCGTAAGCGTTCCTAGCCTTCCAG - 3' (SEQ ID NO:120)

DC014 5' - CTGGAAGGCTAGGAACGCTTACGATTG - 3' (SEQ ID NO:121)

[0215] Specifically, primers HB501f or HB503f in the genomic region flanking the donor DNA 5' *Zp15* homology sequence are used in conjunction with primer DC013 (SEQ ID NO:120) in the PAT protein coding region to detect *PAT* - *Zp15* 5' insert junctions when the donor DNAs have the *PAT* gene in the direct orientation relative to the *Zp15* gene. Likewise, primers HB501 f or HB503f are used in conjunction with primer DC014 (SEQ ID NO:121) in the PAT protein coding region to detect *PAT- Zp15 5'* insert junctions when the donor DNAs have the *PAT* gene in the indirect orientation relative to the *Zp15* gene. For detection of *PAT* - *Zp15* 3' insert junctions, primers HB502r or HB504r are used in conjunction with primer DC013 (SEQ ID NO:120) to detect insert junctions when the donor DNAs have the *PAT* gene in the indirect orientation relative to the *Zp15* gene. Likewise, primers HB502r or HB504r are used in conjunction with primer DC014 (SEQ ID NO:121) to detect *PAT* - *Zp15* 3' insert junctions when the donor DNAs have the *PAT* gene in the direct orientation relative to the *Zp15* gene.

[0216] PCR amplification reactions are carried out using PHUSION HOT START DNA Polymerase (New England Biolabs, Beverly, MA) under the following cycle conditions: 98°C, 30s/1 cycle; 98°C 10 sec, 72°C 15 sec/33 cycles; 72°C, 5 min/1 cycle; 4°C/hold. PCR products are resolved and identified using TAE agarose gel electrophoresis. The expected gel fragment

sizes for the PCR products from *PAT - Zp15* targeted integration events in transgenic callus generated using the different *PAT - Zp15* donor DNAs are as follows:

HB501f/HB503f + DC013 (5') = 2.6 kbp (pDAB104103), 2.6 kbp (pDAB104104)

HB501f/HB503f + DC014 (5') = 1.6 kbp (pDAB104105), 1.7 kbp (pDAB104102)

HB502r/HB504r + DC013 (3') = 3.2 kbp (pDAB104105), 3.2 kbp (pDAB104102)

HB502r/HB504r + DC014 (3') = 1.2 kbp (pDAB104103), 1.2 kbp (pDAB104104)

[0217] The PCR products comprising the 5' and 3' PAT - *Zp15* targeted integration junctions are cloned and sequenced using standard methods known by a person skilled in the art. For example, the PCR products are purified from the agarose gel and cloned into pCR-BluntII TOPO plasmid using TOPO BLUNT CLONING® Kit and ONE SHOT® TOP 10 Chemically competent *E. coli* cells (Invitrogen Life Technologies, Carlsbad, CA) according to manufacturer's protocol.

[0218] The cloned integration junctions are then sequenced to demonstrate that the *PAT* gene is inserted into the maize genome at the *Zp15* locus by homologous recombination via the 5' and 3' *Zp15* homology sequences that are incorporated into the donor transformation vectors. In addition to the TOPO vector specific primers M13forward and M13reverse, PAT gene cassette specific primers are used to obtain complete sequence of the targeted integration clones. PCR Primers SEQ ID NO:120 and SEQ ID NO:121 which are specific to the PAT protein coding sequence are also used as sequencing primers. In addition, other primers can also be used for sequencing. These include but are not limited to those that are specific to the rice actin promoter element of the PAT cassette:

DC-S1 5'-CCAACTGGACAATAGTCTCCAC-3' (SEQ ID NO:122)

DC-S2 5'-CATCGCCACTATATACATACC-3' (SEQ ID NO:123)

and those that are specific to the PAT protein coding sequence:

DC-S3 5'-CGTCTCAATGTAATGGTTAACG-3' (SEQ ID NO:124)

DC-S4 5'-GCCCAGCGTAAGCAATACCAG-3' (SEQ ID NO:125)

Example 6: Heritability of *AAD-1* targeted integration at the *Zp15* locus

[0219] A transgenic callus event carrying the *AAD-1* gene cassette targeted to the *Zp15* locus was generated (Event 138). Event 138 T_0 plants were regenerated and crossed as females to

DAS5XH751 males. Resulting T_1 seed was planted and T_1 plants were grown.

[0220] T_1 plants were analyzed by PCR to demonstrate the occurrence of the *AAD-1 - Zp15* targeted integration. PCR amplification reactions were carried out using PHUSION HOT START DNA Polymerase (New England Biolabs, Beverly, MA) under the following cycle conditions: 98°C, 30s/1 cycle; 98°C 10 sec, 72°C 15 sec/33 cycles; 72°C, 5 min/1 cycle; 4°C/hold. PCR reactions were analyzed by electrophoresis in 1.0% TAE agarose gels. Genomic DNA was extracted from a T_1 plant and used as template DNA in PCR reactions with nested primers designed to detect the *AAD-1 Zp15* 5' integration junction. Primary and secondary PCR reactions were conducted using the same primers used before in the analysis of callus event 147. For primary PCR, primers HB501f and HB507r were used. Primary PCR yielded a band at the expected size of 2.2 kbp. An aliquot of the primary PCR reaction was diluted 1:100 and used as template in secondary PCR using nested primers HB503f and HB508r. Secondary PCR also yielded a band at the expected size of 2.2 kbp.

[0221] The 2.2 kbp secondary PCR product was cloned into pCR-Blunt II-TOPO using the ZERO BLUNT TOPO PCR Cloning Kit and ONE SHOT TOP10 Chemically competent *E. coli* cells (Invitrogen Life Technologies, Carlsbad, CA) according to manufacturer's protocol. The cloned DNA was sequenced using flanking vector specific primers (M13forward and M13 reverse). The sequence was found to be identical to that expected for a targeted integration of *AAD-1* at the *Zp15* locus.

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Patentkrav

1. Fremgangsmåde til integrering af en eller flere exogene nucleinsyresekvenser i genomet af en plantecelle, hvilken fremgangsmåde omfatter:
at indføre den ene eller de nævnte flere exogene nucleinsyresekvenser i plantecellen, hvor de exogene nucleinsyresekvenser indbefatter en eller flere regioner, der er homologe med et Zp15-gen; og
at spalte en dobbeltstrenget sekvens i et endogent Zp15-gen i genomet af plantecellen ved at anvende mindst et fusionsprotein, der omfatter et spaltningsdomæne og et zinkfingerprotein, som omfatter genkendelseshelixregionerne som vist i en enkelt række i tabel 1, hvilket resulterer i integration af et polynucleotid, der omfatter den ene eller de nævnte flere exogene sekvenser i cellens genom, hvor den dobbeltstrengede spaltning udføres ved

(a) at udtrykke et første fusionsprotein i cellen, hvilket første fusionsprotein omfatter et første zinkfingerbindende domæne og et første spaltningshalvdomæne, hvor det første zinkfingerbindende domæne er blevet konstrueret til at binde til et første målsted i eller nær et Zp15-locus i genomet af plantecellen; og

(b) at udtrykke et andet fusionsprotein i cellen, hvilket andet fusionsprotein omfatter et andet zinkfingerbindende domæne og et andet spaltningshalvdomæne, hvor det andet zinkfingerbindende domæne binder til et andet målsted i eller nær Zp15-locuset i genomet af plantecellen, hvor det andet målsted er forskelligt fra det første målsted; og hvor binding af det første fusionsprotein til det første målsted og binding af det andet fusionsprotein til det andet målsted placerer spaltningshalvdomænerne således, at genomet af plantecellen i eller nær Zp15-locuset spaltes.

2. Fremgangsmåden ifølge krav 1, hvilken fremgangsmåde yderligere omfatter at udtrykke et produkt af den ene eller de nævnte flere exogene sekvenser.

3. Fremgangsmåde ifølge krav 1 eller 2, hvor spaltningshalvdomænerne forekommer naturligt eller ikke-naturligt.

4. Fremgangsmåde ifølge et hvilket som helst af kravene 1 til 3, hvor den ene eller de nævnte flere exogene nucleinsyresekvenser omfatter en kodende sekvens, en regulatorisk sekvens eller et målsted for et DNA-bindingsdomæne.

5. Fremgangsmåden ifølge krav 4, hvor den kodende sekvens koder for et produkt, der bibringer: herbicidresistens; herbicidtolerance insektresistens; insekttolerance; sygdomsresistens; sygdomstolerance; stresstolerance; stressresistens; en ændring af oxidativ stress; øgede udbytter af olie; en ændring i fødeindhold og sammensætning; en ændring i fysisk udseende; mandlig sterilitet; nedtørring; stå evne; frugtbarhed; en ændring i stivelsesmængde eller kvalitet: en ændring i oliekvaliteten; en ændring i proteinkvalitet eller mængde; en ændring i aminosyresammensætning eller kombinationer deraf.

6. Fremgangsmåde ifølge et hvilket som helst af kravene 1 til 5, hvor polynucleotidet yderligere omfatter nucleotidsekvenser, som er homologe med sekvenser i Zp15-locuset.

7. Fremgangsmåden ifølge krav 6, hvor de homologe nucleotidsekvenser flankerer den exogene sekvens.

8. Fremgangsmåde ifølge et hvilket som helst af kravene 1 til 7, hvor polynucleotidet yderligere omfatter en promotor.

9. Fremgangsmåde ifølge et hvilket som helst af kravene 1 til 8, hvor en eller flere af de integrerede exogene sekvenser overføres til afkom i efterfølgende generationer.

10. Fremgangsmåde ifølge et hvilket som helst af kravene 1 til 9, hvor plantecellen er en enkimbladet plantecelle.

11. Fremgangsmåde ifølge krav 10, hvor plantecellen er en majscelle.

12. Plante- eller plantedel, der omfatter en eller flere exogene sekvenser, som er integreret i et Zp15-locus ved fremgangsmåden ifølge krav 1.

13. Frø, der omfatter en eller flere exogene sekvenser, som er integreret i et Zp15-locus ved fremgangsmåden ifølge krav 1.

DRAWINGS

```
Zp15genomic: GTGCAGCCCGCTGGCGGCGCCCTACTACGCCG:::::GGTGTGGGCAGCCGAGCGCCATGTTCCAGCCGCTC

Plate7 B10:  GTGCAGCCCGCTGGCGGCGCCCTACTACGCCGGCAATTGGTGTGGGCAGCCGAGCGCCATGTTCCAGCCGCTC (+6)
```

11768 11766

Figure 1

```
Zp15 Genomic ATGGGCGCCGCGGCGGGCTGTACCCCTACGCGGAGTACCTGAGGCAGCCGCAGTGCAGCCCGCTGGCGGCGGCGCCCT

Plate6 C12   ATGGGCGCCGCGGCGGGCTGTACCCCTACGCGGAG:::CTGAGGCAGCCGCAGTGCAGCCCGCTGGCGGCGGCGCCCT (Δ3)
```

11753 11750

Figure 2

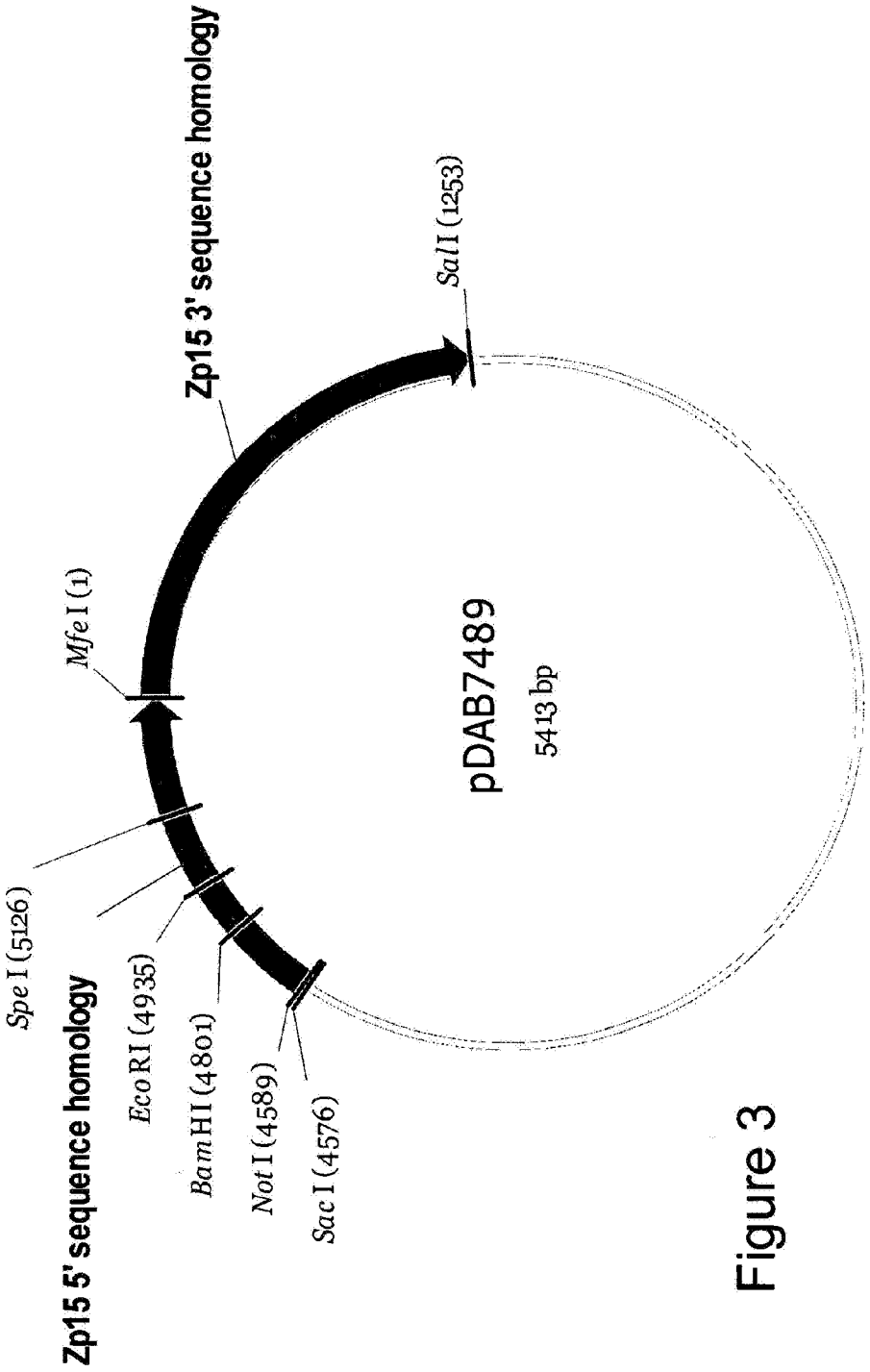
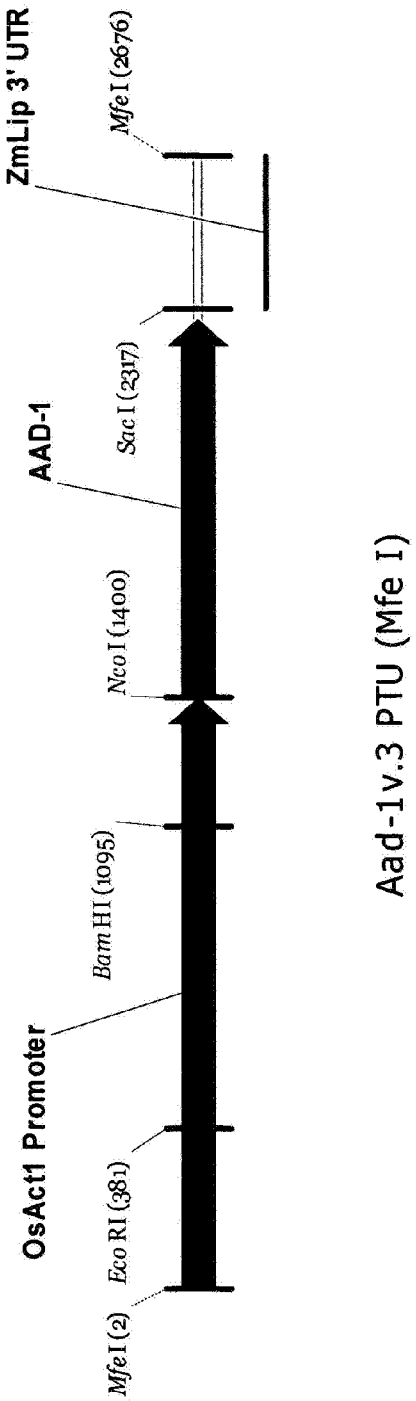

Zp15 3' sequence homology
SalI (1253)
MfeI (1)
pDAB7489
5413 bp
SpeI (5126)
Zp15 5' sequence homology
EcoRI (4935)
BamHI (4801)
NotI (4589)
SacI (4576)

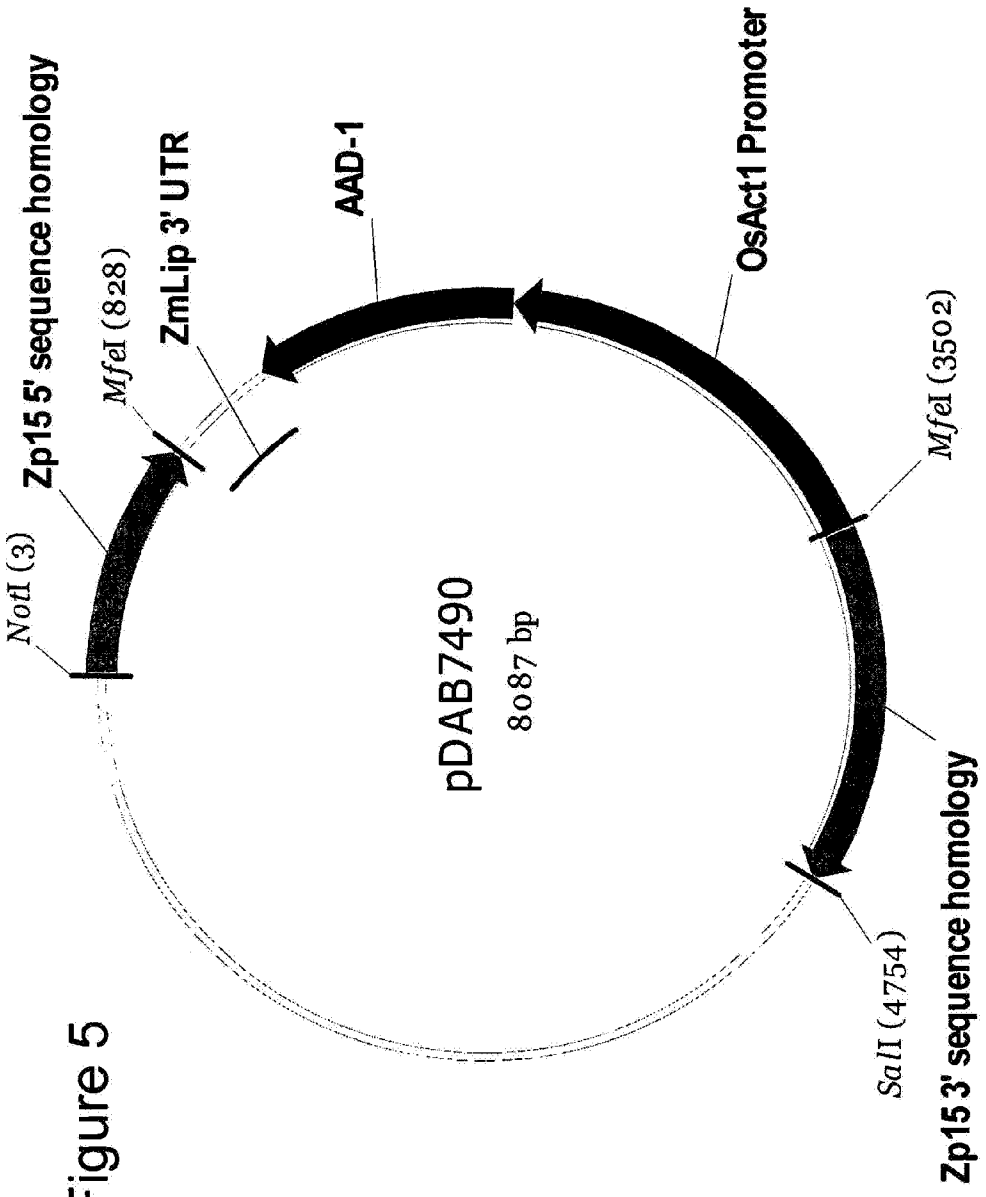
Figure 3



Aad-1v.3 PTU (Mfe I)

2680 bp

Figure 4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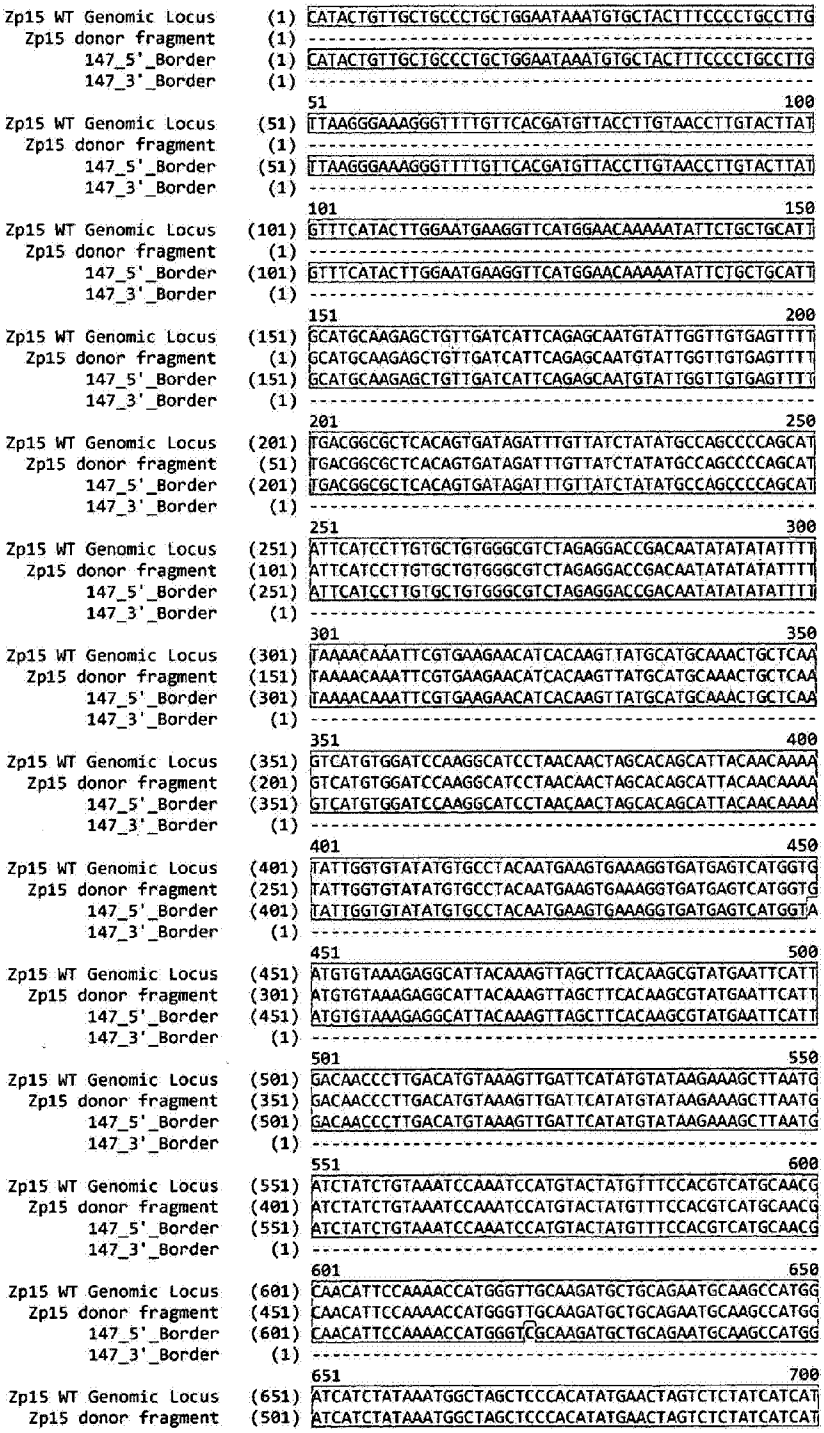
MfeI (828)
ZmLip 3' UTR
AAD-1
OsAct1 Promoter
MfeI (3502)
NotI (3)
pDAB7490
8087 bp
SalI (4754)
Zp15 3' sequence homology

Figure 5

FIGURE 6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Zp15 donor fragment (1) ----------
147_5'_Border (1) CATACTGTTGCTGCCCTGCTGGAATAAATGTGCTACTTTCCCCTGCCTTG
147_3'_Border (1) ----------
51 100
Zp15 WT Genomic Locus (51) TTAAGGGAAAGGGTTTTGTTCACGATGTTACCTTGTAACCTTGTACTTAT
Zp15 donor fragment (1) ----------
147_5'_Border (51) TTAAGGGAAAGGGTTTTGTTCACGATGTTACCTTGTAACCTTGTACTTAT
147_3'_Border (1) ----------
101 150
Zp15 WT Genomic Locus (101) GTTTCATACTTGGAATGAAGGTTCATGGAACAAAAATATTCTGCTGCATT
Zp15 donor fragment (1) ----------
147_5'_Border (101) GTTTCATACTTGGAATGAAGGTTCATGGAACAAAAATATTCTGCTGCATT
147_3'_Border (1) ----------
151 200
Zp15 WT Genomic Locus (151) GCATGCAAGAGCTGTTGATCATTCAGAGCAATGTATTGGTTGTGAGTTTT
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147_3'_Border (1) ----------
201 250
Zp15 WT Genomic Locus (201) TGACGGCGCTCACAGTGATAGATTTGTTATCTATATGCCAGCCCCAGCAT
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147_3'_Border (1) ----------
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147_3'_Border (1) ----------
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147_3'_Border (1) ----------
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147_3'_Border (1) ----------
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147_3'_Border (1) ----------
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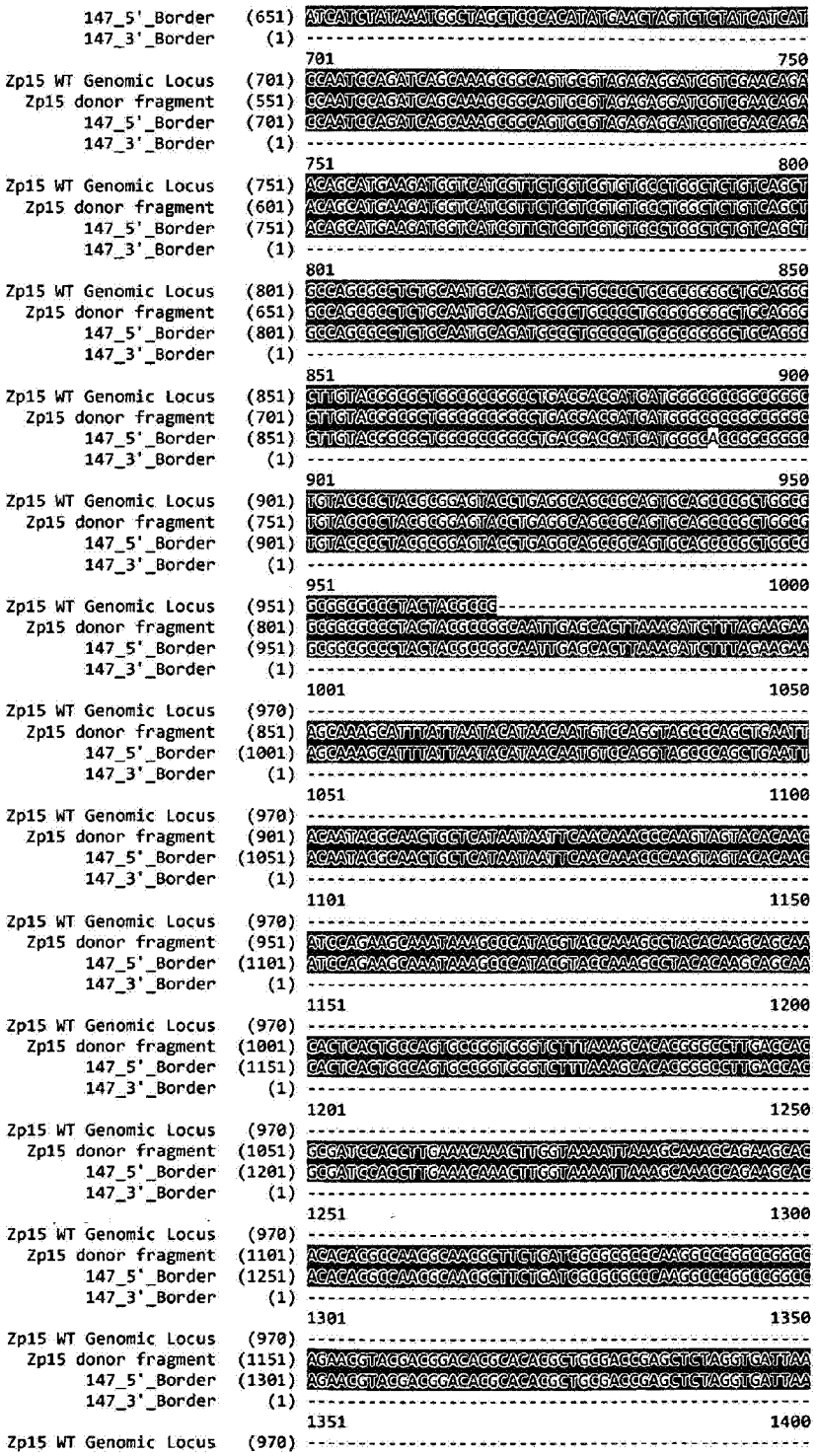
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147_3'_Border (1) ----------
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147_3'_Border (1) ----------
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147_5'_Border (901) TGTACCCCTACGCGGAGTACCTGAGGCAGCCGCAGTGCAGCCCGCTGGCG
147_3'_Border (1) ----------
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147_3'_Border (1) ----------
1001 1050
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147_3'_Border (1) ----------
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147_3'_Border (1) ----------
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147_3'_Border (1) ----------
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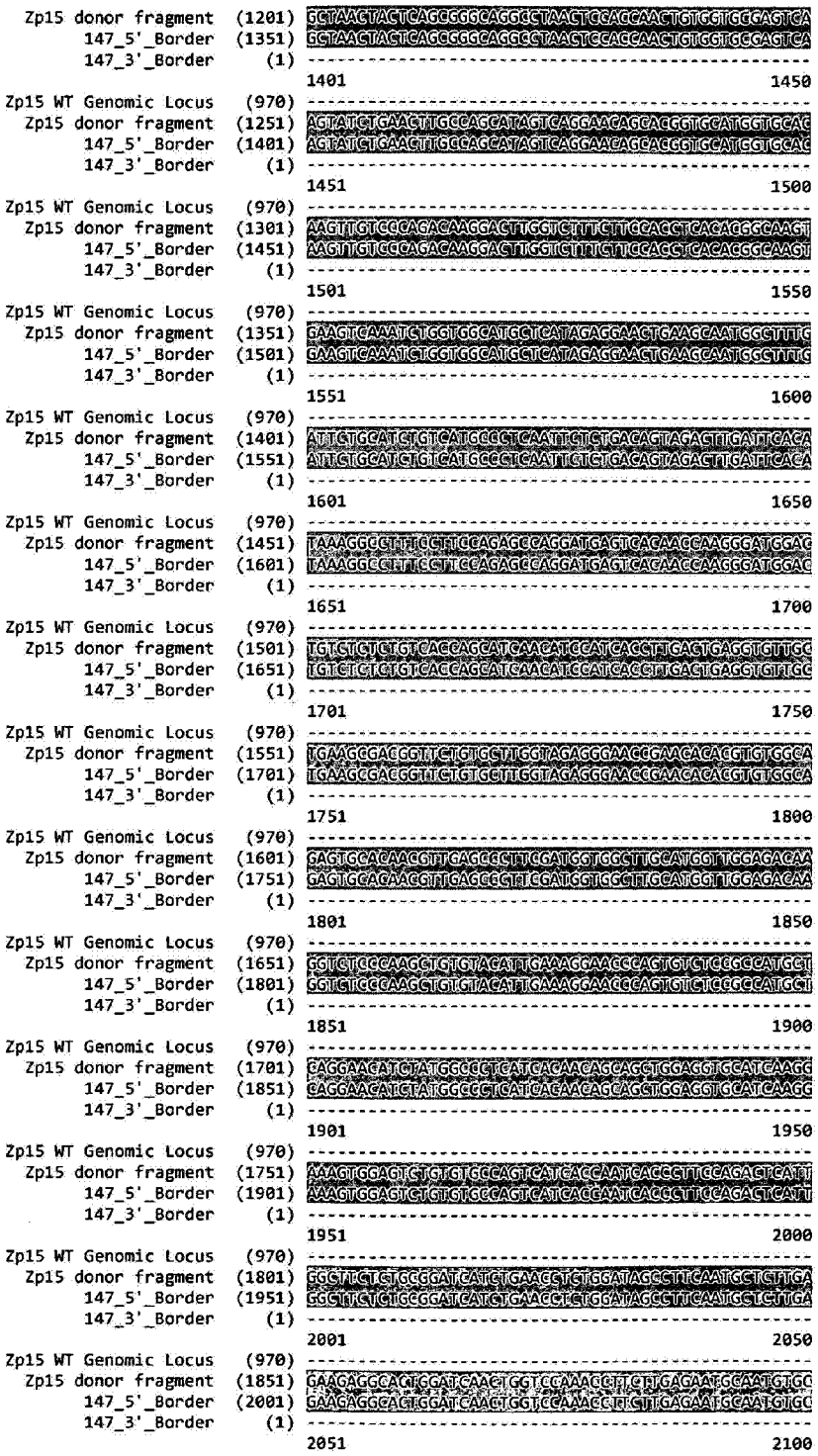
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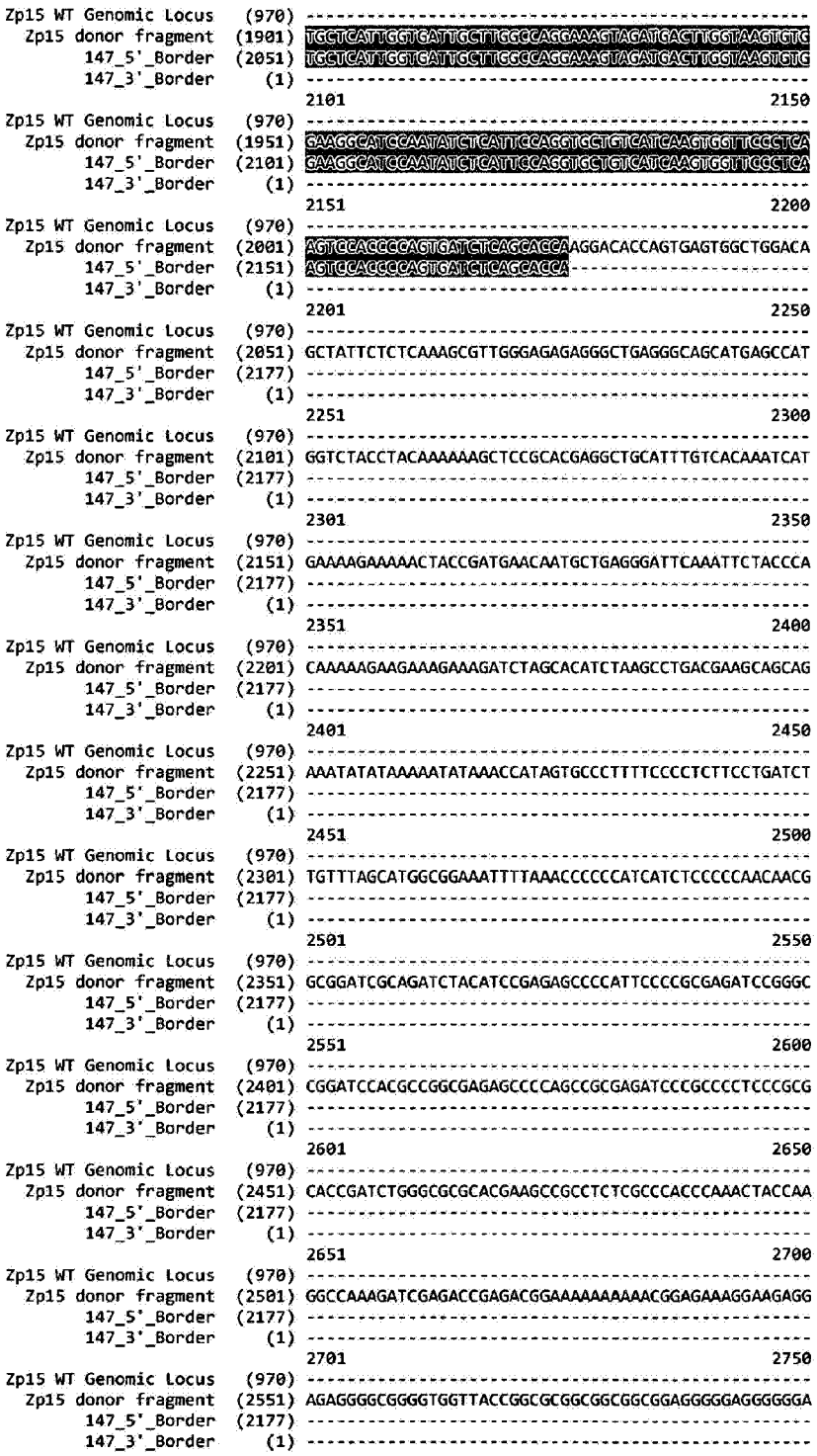
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2101 2150
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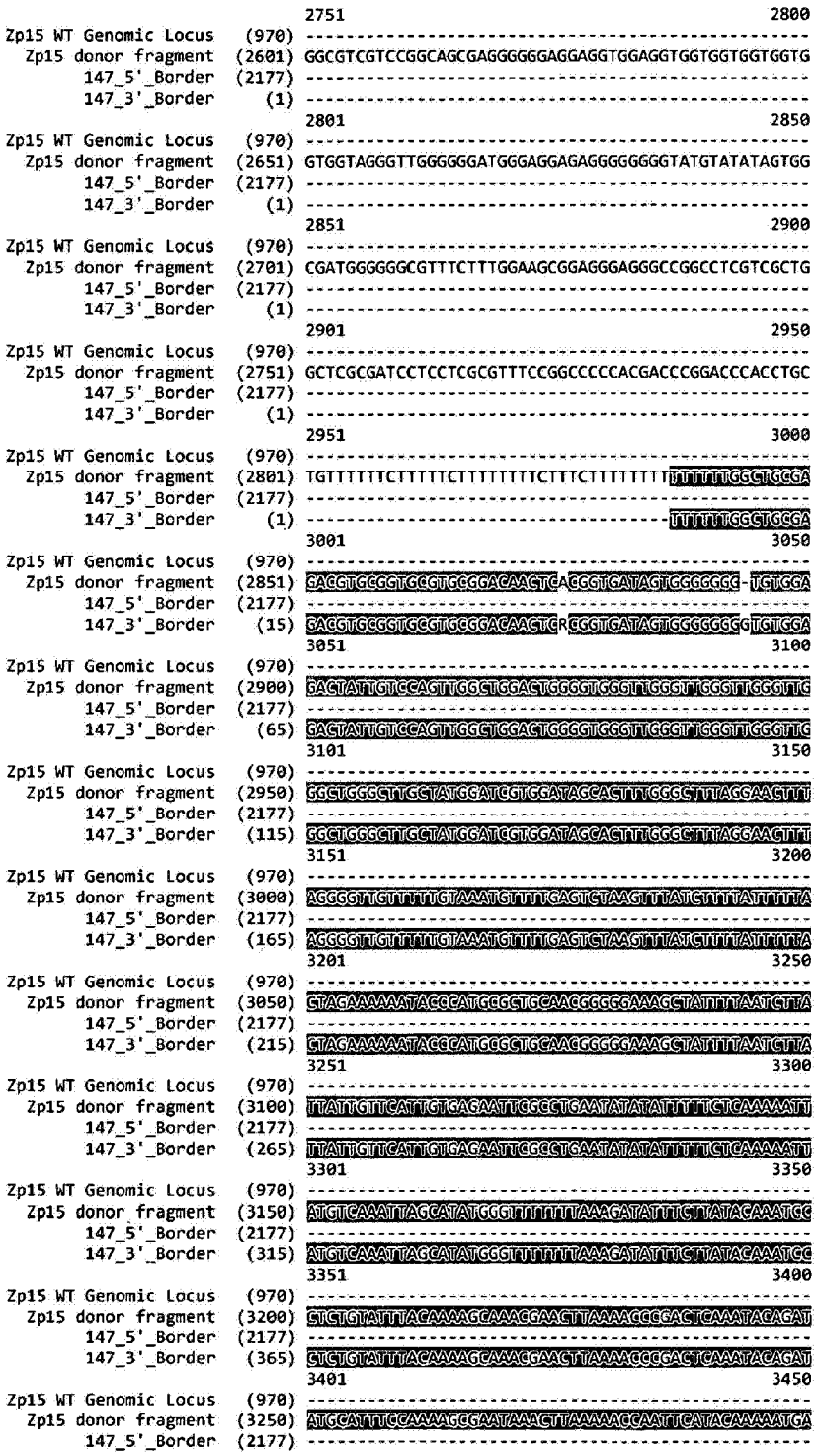
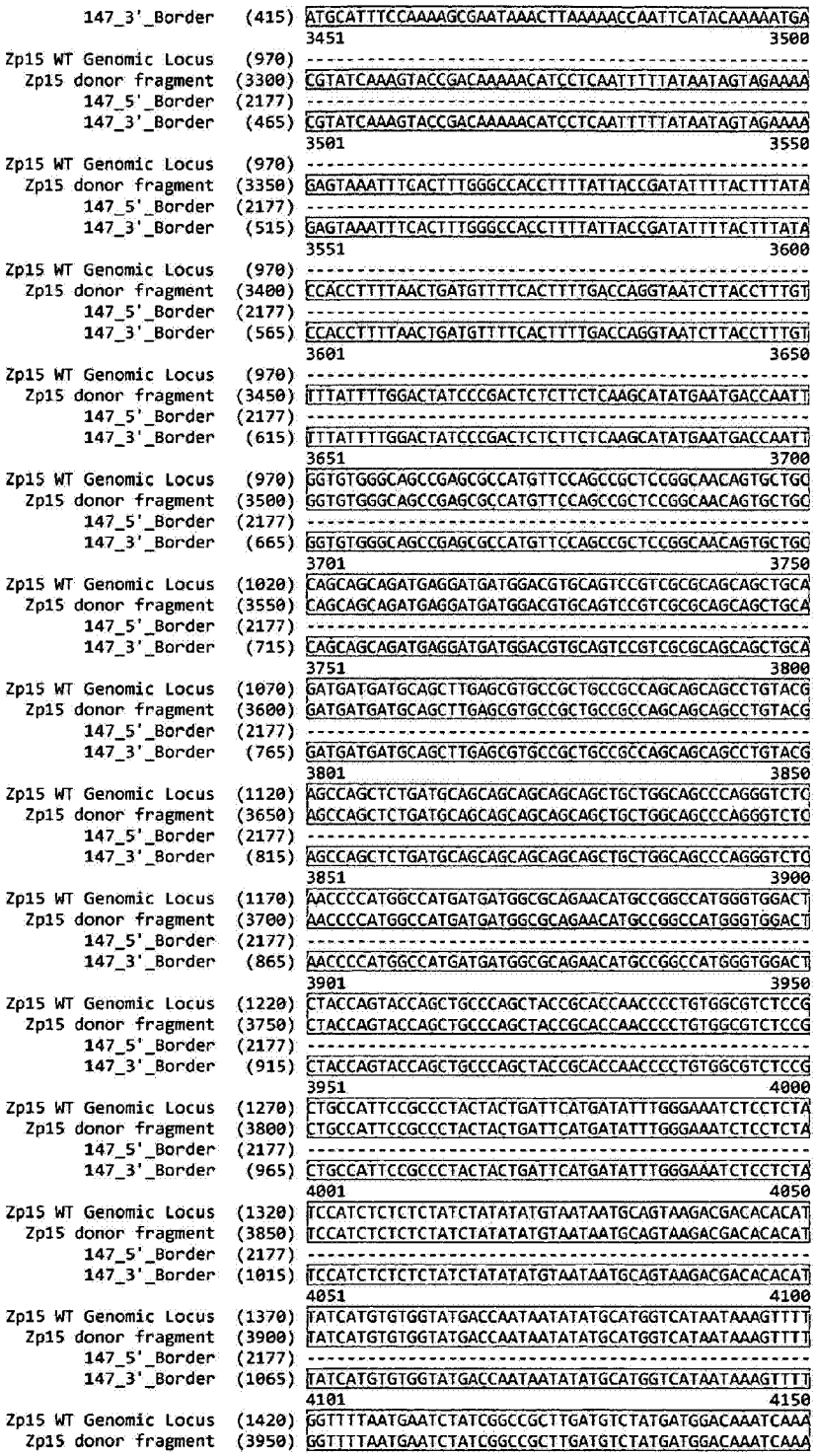
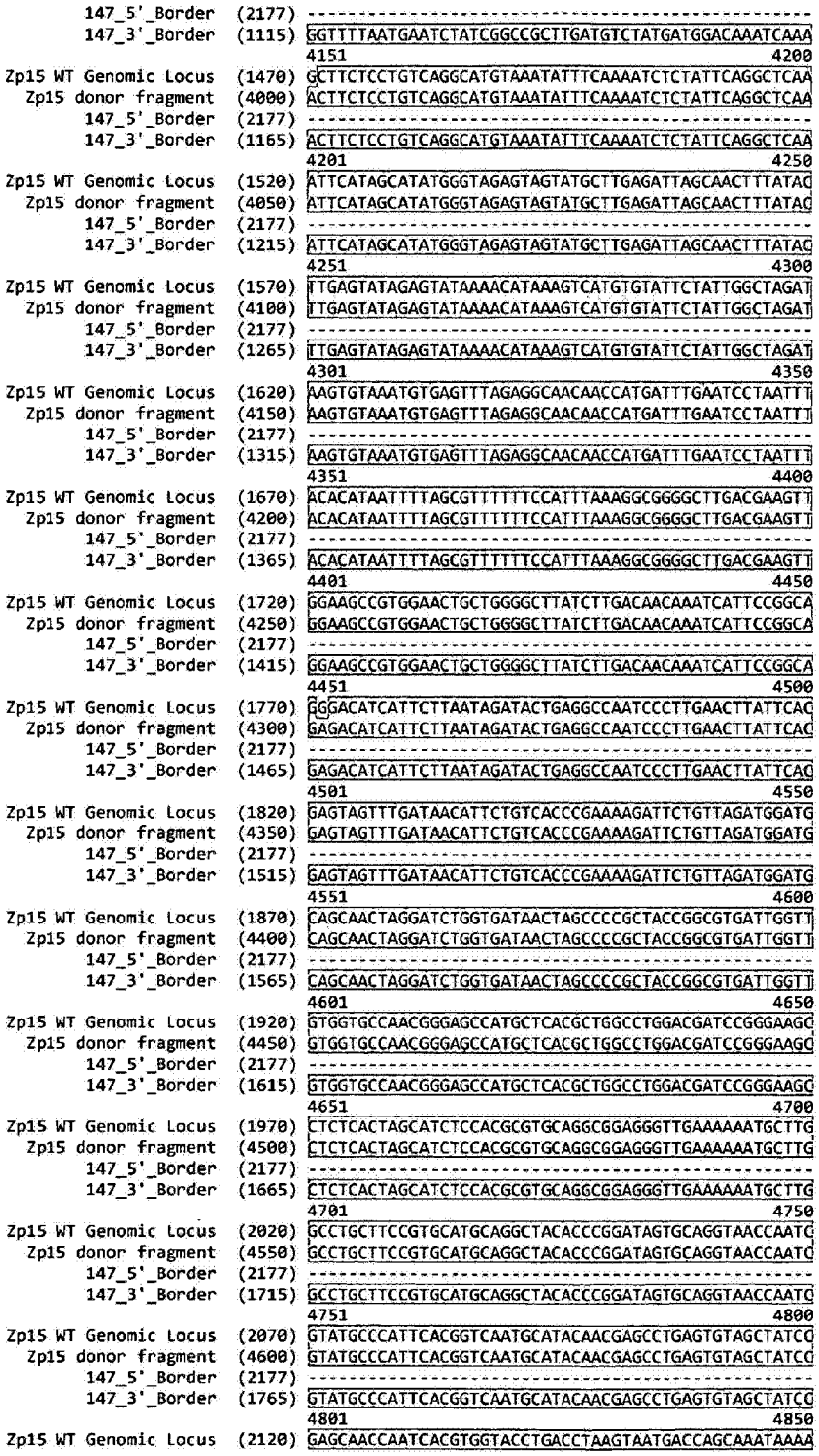
Zp15 WT Genomic Locus (970)
Zp15 donor fragment (2201) CAAAAAGAAGAAAGAAAGATCTAGCACATCTAAGCCTGACGAAGCAGCAG
147_5'_Border (2177)
147_3'_Border (1)
2401 2450
Zp15 WT Genomic Locus (970)
Zp15 donor fragment (2251) AAATATATAAAAATATAAACCATAGTGCCCTTTTCCCCTCTTCCTGATCT
147_5'_Border (2177)
147_3'_Border (1)
2451 2500
Zp15 WT Genomic Locus (970)
Zp15 donor fragment (2301) TGTTTAGCATGGCGGAAATTTTAAACCCCCCATCATCTCCCCCAACAACG
147_5'_Border (2177)
147_3'_Border (1)
2501 2550
Zp15 WT Genomic Locus (970)
Zp15 donor fragment (2351) GCGGATCGCAGATCTACATCCGAGAGCCCCATTCCCCGCGAGATCCGGGC
147_5'_Border (2177)
147_3'_Border (1)
2551 2600
Zp15 WT Genomic Locus (970)
Zp15 donor fragment (2401) CGGATCCACGCCGGCGAGAGCCCCAGCCGCGAGATCCCGCCCCTCCCGCG
147_5'_Border (2177)
147_3'_Border (1)
2601 2650
Zp15 WT Genomic Locus (970)
Zp15 donor fragment (2451) CACCGATCTGGGCGCGCACGAAGCCGCCTCTCGCCCACCCAAACTACCAA
147_5'_Border (2177)
147_3'_Border (1)
2651 2700
Zp15 WT Genomic Locus (970)
Zp15 donor fragment (2501) GGCCAAAGATCGAGACCGAGACGGAAAAAAAAAAACGGAGAAAGGAAGAGG
147_5'_Border (2177)
147_3'_Border (1)
2701 2750
Zp15 WT Genomic Locus (970)
Zp15 donor fragment (2551) AGAGGGGCGGGGTGGTTACCGGCGCGGCGGCGGCGGAGGGGGAGGGGGGA
147_5'_Border (2177)
147_3'_Border (1)


2751 2800
Zp15 WT Genomic Locus (970)
Zp15 donor fragment (2601) GGCGTCGTCCGGCAGCGAGGGGGGAGGAGGTGGAGGTGGTGGTGGTG
147_5'_Border (2177)
147_3'_Border (1)
2801 2850
Zp15 WT Genomic Locus (970)
Zp15 donor fragment (2651) GTGGTAGGGTTGGGGGGATGGGAGGAGAGGGGGGGGTATGTATATAGTGG
147_5'_Border (2177)
147_3'_Border (1)
2851 2900
Zp15 WT Genomic Locus (970)
Zp15 donor fragment (2701) CGATGGGGGGCGTTTCTTTGGAAGCGGAGGGAGGGCCGGCCTCGTCGCTG
147_5'_Border (2177)
147_3'_Border (1)
2901 2950
Zp15 WT Genomic Locus (970)
Zp15 donor fragment (2751) GCTCGCGATCCTCCTCGCGTTTCCGGCCCCCACGACCCGGACCCACCTGC
147_5'_Border (2177)
147_3'_Border (1)
2951 3000
Zp15 WT Genomic Locus (970)
Zp15 donor fragment (2801) TGTTTTTTCTTTTTCTTTTTTTTCTTTCTTTTTTTTTTTTTTTGGCTGCGA
147_5'_Border (2177)
147_3'_Border (1) TTTTTTGGCTGCGA
3001 3050
Zp15 WT Genomic Locus (970)
Zp15 donor fragment (2851) GACGTGCGGTGCGTGCGGACAACTCACGGTGATAGTGGGGGGG-TGTGGA
147_5'_Border (2177)
147_3'_Border (15) GACGTGCGGTGCGTGCGGACAACTCRCGGTGATAGTGGGGGGGGTGTGGA
3051 3100
Zp15 WT Genomic Locus (970)
Zp15 donor fragment (2900) GACTATTGTCCAGTTGGCTGGACTGGGGTGGGTTGGGTTGGGTTGGGTTG
147_5'_Border (2177)
147_3'_Border (65) GACTATTGTCCAGTTGGCTGGACTGGGGTGGGTTGGGTTGGGTTGGGTTG
3101 3150
Zp15 WT Genomic Locus (970)
Zp15 donor fragment (2950) GGCTGGGCTTGCTATGGATCGTGGATAGCACTTTGGGCTTTAGGAACTTT
147_5'_Border (2177)
147_3'_Border (115) GGCTGGGCTTGCTATGGATCGTGGATAGCACTTTGGGCTTTAGGAACTTT
3151 3200
Zp15 WT Genomic Locus (970)
Zp15 donor fragment (3000) AGGGGTTGTTTTTTGTAAATGTTTTGAGTCTAAGTTTATCTTTTATTTTTA
147_5'_Border (2177)
147_3'_Border (165) AGGGGTTGTTTTTTGTAAATGTTTTGAGTCTAAGTTTATCTTTTATTTTTA
3201 3250
Zp15 WT Genomic Locus (970)
Zp15 donor fragment (3050) CTAGAAAAAATACCCATGCGCTGCAACGGGCGAAAGCTATTTTAATCTTA
147_5'_Border (2177)
147_3'_Border (215) CTAGAAAAAATACCCATGCGCTGCAACGGGCGAAAGCTATTTTAATCTTA
3251 3300
Zp15 WT Genomic Locus (970)
Zp15 donor fragment (3100) TTATTGTTCATTGTGAGAATTCGCCTGAATATATATTTTTCTCAAAAATT
147_5'_Border (2177)
147_3'_Border (265) TTATTGTTCATTGTGAGAATTCGCCTGAATATATATTTTTCTCAAAAATT
3301 3350
Zp15 WT Genomic Locus (970)
Zp15 donor fragment (3150) ATGTCAAATTAGCATATGGGTTTTTTTAAAGATATTTCTTATACAAATCC
147_5'_Border (2177)
147_3'_Border (315) ATGTCAAATTAGCATATGGGTTTTTTTAAAGATATTTCTTATACAAATCC
3351 3400
Zp15 WT Genomic Locus (970)
Zp15 donor fragment (3200) CTCTGTATTTACAAAAGCAAACGAACTTAAAACCCGACTCAAATACAGAT
147_5'_Border (2177)
147_3'_Border (365) CTCTGTATTTACAAAAGCAAACGAACTTAAAACCCGACTCAAATACAGAT
3401 3450
Zp15 WT Genomic Locus (970)
Zp15 donor fragment (3250) ATGGATTTCGCAAAAGCGAATAAACTTAAAAACGCAATTCATACAAAAATGA
147_5'_Border (2177)


147_3'_Border (415) ATGCATTTCCAAAAGCGAATAAACTTAAAAACCAATTCATACAAAAATGA
3451 3500
Zp15 WT Genomic Locus (970) ----------
Zp15 donor fragment (3300) CGTATCAAAGTACCGACAAAAACATCCTCAATTTTTATAATAGTAGAAAA
147_5'_Border (2177) ----------
147_3'_Border (465) CGTATCAAAGTACCGACAAAAACATCCTCAATTTTTATAATAGTAGAAAA
3501 3550
Zp15 WT Genomic Locus (970) ----------
Zp15 donor fragment (3350) GAGTAAATTTCACTTTGGGCCACCTTTTATTACCGATATTTTACTTTATA
147_5'_Border (2177) ----------
147_3'_Border (515) GAGTAAATTTCACTTTGGGCCACCTTTTATTACCGATATTTTACTTTATA
3551 3600
Zp15 WT Genomic Locus (970) ----------
Zp15 donor fragment (3400) CCACCTTTTAACTGATGTTTTCACTTTTGACCAGGTAATCTTACCTTTGT
147_5'_Border (2177) ----------
147_3'_Border (565) CCACCTTTTAACTGATGTTTTCACTTTTGACCAGGTAATCTTACCTTTGT
3601 3650
Zp15 WT Genomic Locus (970) ----------
Zp15 donor fragment (3450) TTTATTTTGGACTATCCCGACTCTCTTCTCAAGCATATGAATGACCAATT
147_5'_Border (2177) ----------
147_3'_Border (615) TTTATTTTGGACTATCCCGACTCTCTTCTCAAGCATATGAATGACCAATT
3651 3700
Zp15 WT Genomic Locus (970) GGTGTGGGCAGCCGAGCGCCATGTTCCAGCCGCTCCGGCAACAGTGCTGC
Zp15 donor fragment (3500) GGTGTGGGCAGCCGAGCGCCATGTTCCAGCCGCTCCGGCAACAGTGCTGC
147_5'_Border (2177) ----------
147_3'_Border (665) GGTGTGGGCAGCCGAGCGCCATGTTCCAGCCGCTCCGGCAACAGTGCTGC
3701 3750
Zp15 WT Genomic Locus (1020) CAGCAGCAGATGAGGATGATGGACGTGCAGTCCGTCGCGCAGCAGCTGCA
Zp15 donor fragment (3550) CAGCAGCAGATGAGGATGATGGACGTGCAGTCCGTCGCGCAGCAGCTGCA
147_5'_Border (2177) ----------
147_3'_Border (715) CAGCAGCAGATGAGGATGATGGACGTGCAGTCCGTCGCGCAGCAGCTGCA
3751 3800
Zp15 WT Genomic Locus (1070) GATGATGATGCAGCTTGAGCGTGCCGCTGCCGCCAGCAGCAGCCTGTACG
Zp15 donor fragment (3600) GATGATGATGCAGCTTGAGCGTGCCGCTGCCGCCAGCAGCAGCCTGTACG
147_5'_Border (2177) ----------
147_3'_Border (765) GATGATGATGCAGCTTGAGCGTGCCGCTGCCGCCAGCAGCAGCCTGTACG
3801 3850
Zp15 WT Genomic Locus (1120) AGCCAGCTCTGATGCAGCAGCAGCAGCAGCTGCTGGCAGCCCAGGGTCTC
Zp15 donor fragment (3650) AGCCAGCTCTGATGCAGCAGCAGCAGCAGCTGCTGGCAGCCCAGGGTCTC
147_5'_Border (2177) ----------
147_3'_Border (815) AGCCAGCTCTGATGCAGCAGCAGCAGCAGCTGCTGGCAGCCCAGGGTCTC
3851 3900
Zp15 WT Genomic Locus (1170) AACCCCATGGCCATGATGATGGCGCAGAACATGCCGGCCATGGGTGGACT
Zp15 donor fragment (3700) AACCCCATGGCCATGATGATGGCGCAGAACATGCCGGCCATGGGTGGACT
147_5'_Border (2177) ----------
147_3'_Border (865) AACCCCATGGCCATGATGATGGCGCAGAACATGCCGGCCATGGGTGGACT
3901 3950
Zp15 WT Genomic Locus (1220) CTACCAGTACCAGCTGCCCAGCTACCGCACCAACCCCTGTGGCGTCTCCG
Zp15 donor fragment (3750) CTACCAGTACCAGCTGCCCAGCTACCGCACCAACCCCTGTGGCGTCTCCG
147_5'_Border (2177) ----------
147_3'_Border (915) CTACCAGTACCAGCTGCCCAGCTACCGCACCAACCCCTGTGGCGTCTCCG
3951 4000
Zp15 WT Genomic Locus (1270) CTGCCATTCCGCCCTACTACTGATTCATGATATTTGGGAAATCTCCTCTA
Zp15 donor fragment (3800) CTGCCATTCCGCCCTACTACTGATTCATGATATTTGGGAAATCTCCTCTA
147_5'_Border (2177) ----------
147_3'_Border (965) CTGCCATTCCGCCCTACTACTGATTCATGATATTTGGGAAATCTCCTCTA
4001 4050
Zp15 WT Genomic Locus (1320) TCCATCTCTCTCTATCTATATATGTAATAATGCAGTAAGACGACACACAT
Zp15 donor fragment (3850) TCCATCTCTCTCTATCTATATATGTAATAATGCAGTAAGACGACACACAT
147_5'_Border (2177) ----------
147_3'_Border (1015) TCCATCTCTCTCTATCTATATATGTAATAATGCAGTAAGACGACACACAT
4051 4100
Zp15 WT Genomic Locus (1370) TATCATGTGTGGTATGACCAATAATATATGCATGGTCATAATAAAGTTTT
Zp15 donor fragment (3900) TATCATGTGTGGTATGACCAATAATATATGCATGGTCATAATAAAGTTTT
147_5'_Border (2177) ----------
147_3'_Border (1065) TATCATGTGTGGTATGACCAATAATATATGCATGGTCATAATAAAGTTTT
4101 4150
Zp15 WT Genomic Locus (1420) GGTTTTAATGAATCTATCGGCCGCTTGATGTCTATGATGGACAAATCAAA
Zp15 donor fragment (3950) GGTTTTAATGAATCTATCGGCCGCTTGATGTCTATGATGGACAAATCAAA


147_5'_Border (2177)
147_3'_Border (1115) GGTTTTAATGAATCTATCGGCCGCTTGATGTCTATGATGGACAAATCAAA
4151 4200
Zp15 WT Genomic Locus (1470) GCTTCTCCTGTCAGGCATGTAAATATTTCAAAATCTCTATTCAGGCTCAA
Zp15 donor fragment (4000) ACTTCTCCTGTCAGGCATGTAAATATTTCAAAATCTCTATTCAGGCTCAA
147_5'_Border (2177)
147_3'_Border (1165) ACTTCTCCTGTCAGGCATGTAAATATTTCAAAATCTCTATTCAGGCTCAA
4201 4250
Zp15 WT Genomic Locus (1520) ATTCATAGCATATGGGTAGAGTAGTATGCTTGAGATTAGCAACTTTATAC
Zp15 donor fragment (4050) ATTCATAGCATATGGGTAGAGTAGTATGCTTGAGATTAGCAACTTTATAC
147_5'_Border (2177)
147_3'_Border (1215) ATTCATAGCATATGGGTAGAGTAGTATGCTTGAGATTAGCAACTTTATAC
4251 4300
Zp15 WT Genomic Locus (1570) TTGAGTATAGAGTATAAAACATAAAGTCATGTGTATTCTATTGGCTAGAT
Zp15 donor fragment (4100) TTGAGTATAGAGTATAAAACATAAAGTCATGTGTATTCTATTGGCTAGAT
147_5'_Border (2177)
147_3'_Border (1265) TTGAGTATAGAGTATAAAACATAAAGTCATGTGTATTCTATTGGCTAGAT
4301 4350
Zp15 WT Genomic Locus (1620) AAGTGTAAATGTGAGTTTAGAGGCAACAACCATGATTTGAATCCTAATTT
Zp15 donor fragment (4150) AAGTGTAAATGTGAGTTTAGAGGCAACAACCATGATTTGAATCCTAATTT
147_5'_Border (2177)
147_3'_Border (1315) AAGTGTAAATGTGAGTTTAGAGGCAACAACCATGATTTGAATCCTAATTT
4351 4400
Zp15 WT Genomic Locus (1670) ACACATAATTTTAGCGTTTTTTCCATTTAAAGGCGGGGCTTGACGAAGTT
Zp15 donor fragment (4200) ACACATAATTTTAGCGTTTTTTCCATTTAAAGGCGGGGCTTGACGAAGTT
147_5'_Border (2177)
147_3'_Border (1365) ACACATAATTTTAGCGTTTTTTCCATTTAAAGGCGGGGCTTGACGAAGTT
4401 4450
Zp15 WT Genomic Locus (1720) GGAAGCCGTGGAACTGCTGGGGCTTATCTTGACAACAAATCATTCCGGCA
Zp15 donor fragment (4250) GGAAGCCGTGGAACTGCTGGGGCTTATCTTGACAACAAATCATTCCGGCA
147_5'_Border (2177)
147_3'_Border (1415) GGAAGCCGTGGAACTGCTGGGGCTTATCTTGACAACAAATCATTCCGGCA
4451 4500
Zp15 WT Genomic Locus (1770) GGGACATCATTCTTAATAGATACTGAGGCCAATCCCTTGAACTTATTCAC
Zp15 donor fragment (4300) GAGACATCATTCTTAATAGATACTGAGGCCAATCCCTTGAACTTATTCAC
147_5'_Border (2177)
147_3'_Border (1465) GAGACATCATTCTTAATAGATACTGAGGCCAATCCCTTGAACTTATTCAC
4501 4550
Zp15 WT Genomic Locus (1820) GAGTAGTTTGATAACATTCTGTCACCCGAAAAGATTCTGTTAGATGGATG
Zp15 donor fragment (4350) GAGTAGTTTGATAACATTCTGTCACCCGAAAAGATTCTGTTAGATGGATG
147_5'_Border (2177)
147_3'_Border (1515) GAGTAGTTTGATAACATTCTGTCACCCGAAAAGATTCTGTTAGATGGATG
4551 4600
Zp15 WT Genomic Locus (1870) CAGCAACTAGGATCTGGTGATAACTAGCCCCGCTACCGGCGTGATTGGTT
Zp15 donor fragment (4400) CAGCAACTAGGATCTGGTGATAACTAGCCCCGCTACCGGCGTGATTGGTT
147_5'_Border (2177)
147_3'_Border (1565) CAGCAACTAGGATCTGGTGATAACTAGCCCCGCTACCGGCGTGATTGGTT
4601 4650
Zp15 WT Genomic Locus (1920) GTGGTGCCAACGGGAGCCATGCTCACGCTGGCCTGGACGATCCGGGAAGC
Zp15 donor fragment (4450) GTGGTGCCAACGGGAGCCATGCTCACGCTGGCCTGGACGATCCGGGAAGC
147_5'_Border (2177)
147_3'_Border (1615) GTGGTGCCAACGGGAGCCATGCTCACGCTGGCCTGGACGATCCGGGAAGC
4651 4700
Zp15 WT Genomic Locus (1970) CTCTCACTAGCATCTCCACGCGTGCAGGCGGAGGGTTGAAAAAATGCTTG
Zp15 donor fragment (4500) CTCTCACTAGCATCTCCACGCGTGCAGGCGGAGGGTTGAAAAAATGCTTG
147_5'_Border (2177)
147_3'_Border (1665) CTCTCACTAGCATCTCCACGCGTGCAGGCGGAGGGTTGAAAAAATGCTTG
4701 4750
Zp15 WT Genomic Locus (2020) GCCTGCTTCCGTGCATGCAGGCTACACCCGGATAGTGCAGGTAACCAATC
Zp15 donor fragment (4550) GCCTGCTTCCGTGCATGCAGGCTACACCCGGATAGTGCAGGTAACCAATC
147_5'_Border (2177)
147_3'_Border (1715) GCCTGCTTCCGTGCATGCAGGCTACACCCGGATAGTGCAGGTAACCAATC
4751 4800
Zp15 WT Genomic Locus (2070) GTATGCCCATTCACGGTCAATGCATACAACGAGCCTGAGTGTAGCTATCC
Zp15 donor fragment (4600) GTATGCCCATTCACGGTCAATGCATACAACGAGCCTGAGTGTAGCTATCC
147_5'_Border (2177)
147_3'_Border (1765) GTATGCCCATTCACGGTCAATGCATACAACGAGCCTGAGTGTAGCTATCC
4801 4850
Zp15 WT Genomic Locus (2120) GAGCAACCAATCACGTGGTACCTGACCTAAGTAATGACCAGCAAATAAAA

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  Zp15 donor fragment  (4650) GAGCAAGCAATCACGTGGTACCTGACCTAAGTAATGACCAGCAAATAAAA
        147_5'_Border  (2177) ----------------------------------------------------
        147_3'_Border  (1815) GAGCAAGCAATCACGTGGTACCTGACCTAAGTAATGACCAGCAAATAAAA
                              4851                                          4900
Zp15 WT Genomic Locus  (2170) GTGTTGAGCAGCAAAATAGACAAGGTGGACGGTCCGCATCAGTATCGCGT
  Zp15 donor fragment  (4700) GTGTTGAGCAGCAAAATAGACAAGGTGGACGGTCCGCATCAGTATCG---
        147_5'_Border  (2177) ----------------------------------------------------
        147_3'_Border  (1865) GTGTTGAGCAGCAAAATAGACAAGGTGGACGGTCCGCATCAGTATCGCGT
                              4901                                          4950
Zp15 WT Genomic Locus  (2220) GCAGAGACAGTTAGGGTTCCGAGTTTCTTGTGACGGTTGTTAGGTAAATT
  Zp15 donor fragment  (4747) ----------------------------------------------------
        147_5'_Border  (2177) ----------------------------------------------------
        147_3'_Border  (1915) GCAGAGACAGTTAGGGTTCCGAGTTTCTTGTGACGGTTGTTAGGTAAATT
                              4951                                          5000
Zp15 WT Genomic Locus  (2270) CGCGCAATTAACTCGGGAGATTGGTTTGTAACGGGTCCAGACCCCTCCTC
  Zp15 donor fragment  (4747) ----------------------------------------------------
        147_5'_Border  (2177) ----------------------------------------------------
        147_3'_Border  (1965) CGCGCAATTAACTCGGGAGATTGGTTTGTAACGGGTCCAGACCCCTCCTC
                              5001                                          5050
Zp15 WT Genomic Locus  (2320) TATAAATATAAAGCAATACAGTTGATTGCGATAAACAATCGAACCTACAA
  Zp15 donor fragment  (4747) ----------------------------------------------------
        147_5'_Border  (2177) ----------------------------------------------------
        147_3'_Border  (2015) TATAAATATAAAGCAATACAGTTGATTGCGATAAACAATCGAACCTACAA
                              5051                                          5100
Zp15 WT Genomic Locus  (2370) TCAATAAAATTTGCATTTTATGTTGTACATTTAGGAGTCGCTCTAGTTTA
  Zp15 donor fragment  (4747) ----------------------------------------------------
        147_5'_Border  (2177) ----------------------------------------------------
        147_3'_Border  (2065) TCAATAAAATTTGCATTTTATGTTGTACATTTAGGAGTCGCTCTAGTTTA
                              5101                                          5150
Zp15 WT Genomic Locus  (2420) GTTCTAGTTTAACCTCTCAATCGCCCAAATTCTCTGTTTCTCTTCGGCTCT
  Zp15 donor fragment  (4747) ----------------------------------------------------
        147_5'_Border  (2177) ----------------------------------------------------
        147_3'_Border  (2115) GTTCTAGTTTAACCTCTCAATCGCCCAAATTCTCTGTTTCTCTTCGGCTCT
                              5151                                          5200
Zp15 WT Genomic Locus  (2470) ACATCGATTATAGGTGTCTAGGTCGGCCTGCCGACTATAGACAAAGCATA
  Zp15 donor fragment  (4747) ----------------------------------------------------
        147_5'_Border  (2177) ----------------------------------------------------
        147_3'_Border  (2165) ACATCGATTATAGGTGTC----------------------------------
                              5201
Zp15 WT Genomic Locus  (2520) GGACCT
  Zp15 donor fragment  (4747) ------
        147_5'_Border  (2177) ------
        147_3'_Border  (2183) ------
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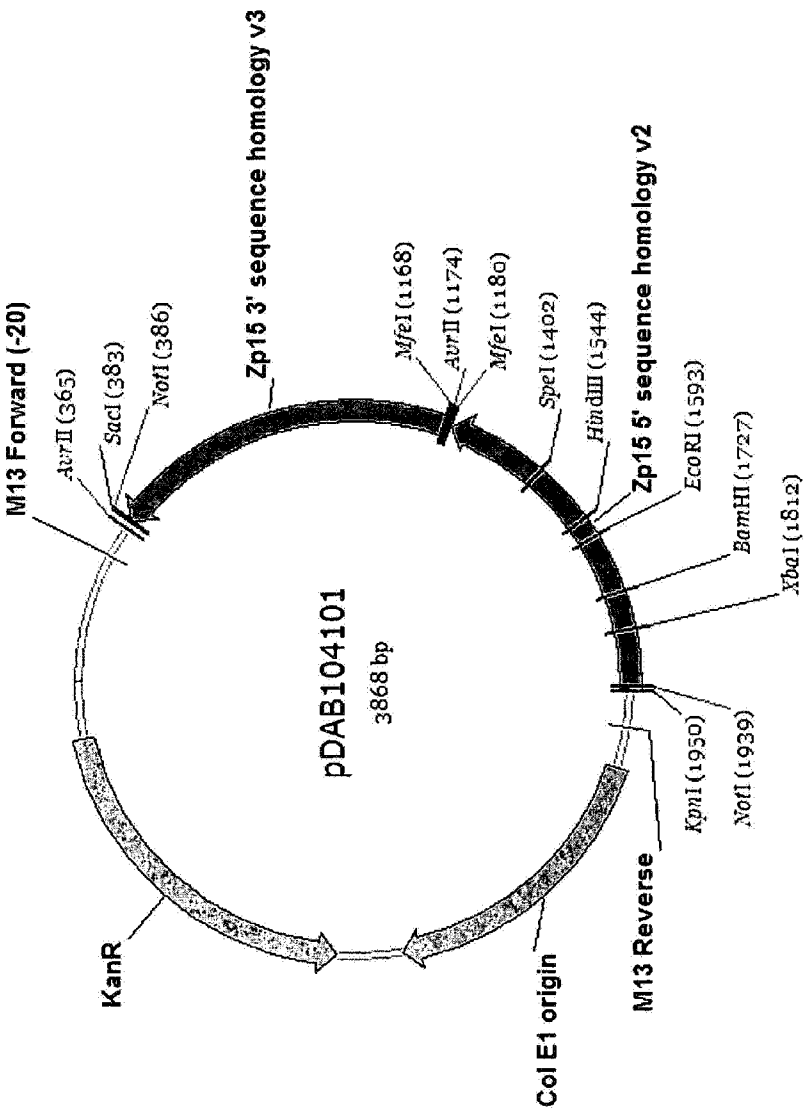
pDAB104101
3868 bp
M13 Forward (-20)
AvrII (365)
SacI (383)
NotI (386)
Zp15 3' sequence homology v3
MfeI (1168)
AvrII (1174)
MfeI (1180)
SpeI (1402)
HindIII (1544)
Zp15 5' sequence homology v2
EcoRI (1593)
BamHI (1727)
XbaI (1812)
KpnI (1950)
NotI (1939)
M13 Reverse
Col E1 origin
KanR

Figure 7

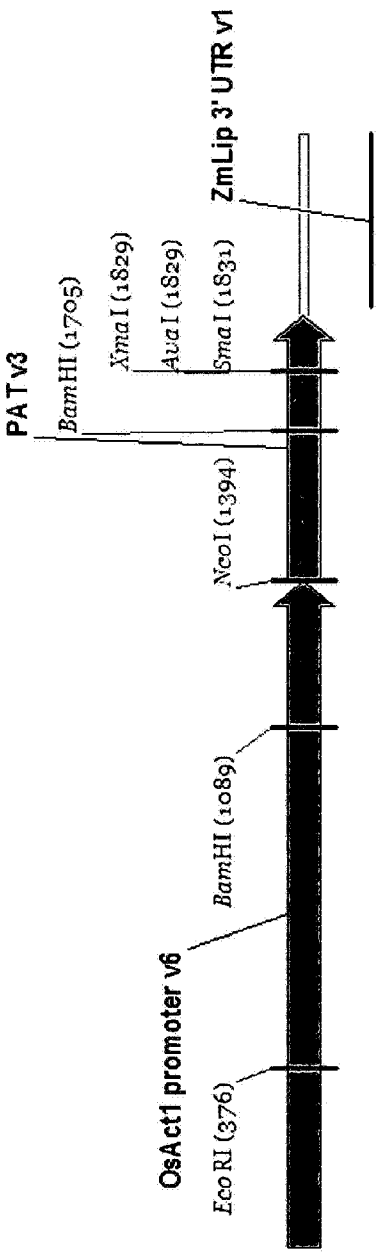
PAT Gene Cassette
2320 bp
OsAct1 promoter v6
Eco RI (376)
BamHI (1089)
NcoI (1394)
PAT v3
BamHI (1705)
XmaI (1829)
AvaI (1829)
SmaI (1831)
ZmLip 3' UTR v1

Figure 8

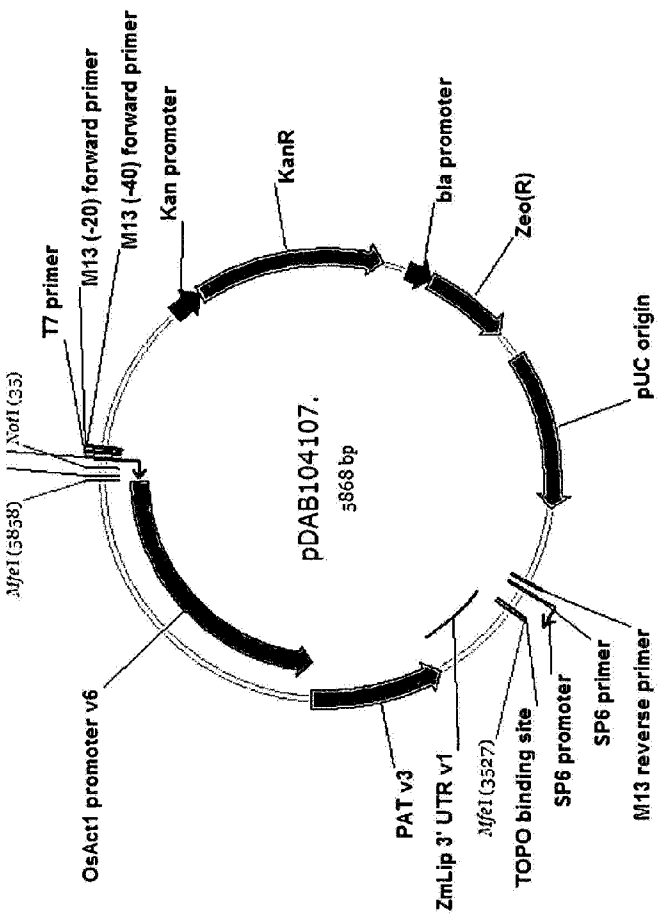
pDAB104107.
5868 bp
T7 primer
M13 (-20) forward primer
M13 (-40) forward primer
Kan promoter
KanR
bla promoter
Zeo(R)
pUC origin
M13 reverse primer
SP6 primer
SP6 promoter
TOPO binding site
MfeI (3527)
PAT v3
ZmLip 3' UTR v1
OsAct1 promoter v6
MfeI (5838)
NotI (33)

Figure 9

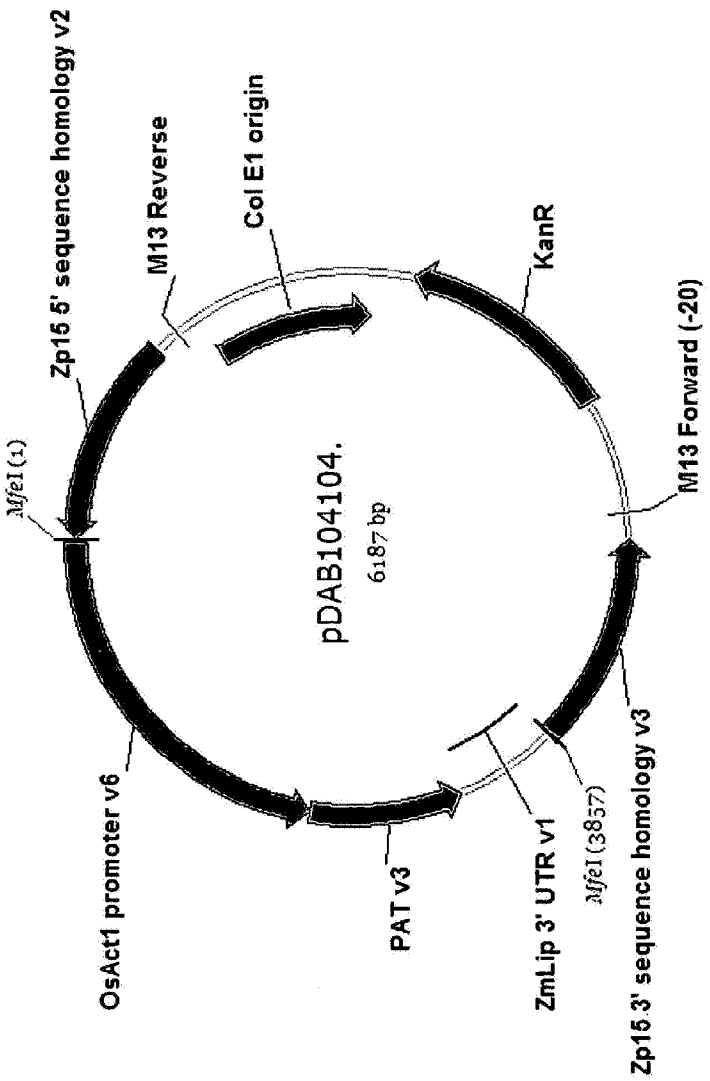
pDAB104104.
6187 bp
MfeI (1)
Zp15 5' sequence homology v2
M13 Reverse
Col E1 origin
KanR
M13 Forward (-20)
Zp15 3' sequence homology v3
MfeI (3857)
ZmLip 3' UTR v1
PAT v3
OsAct1 promoter v6

Figure 10

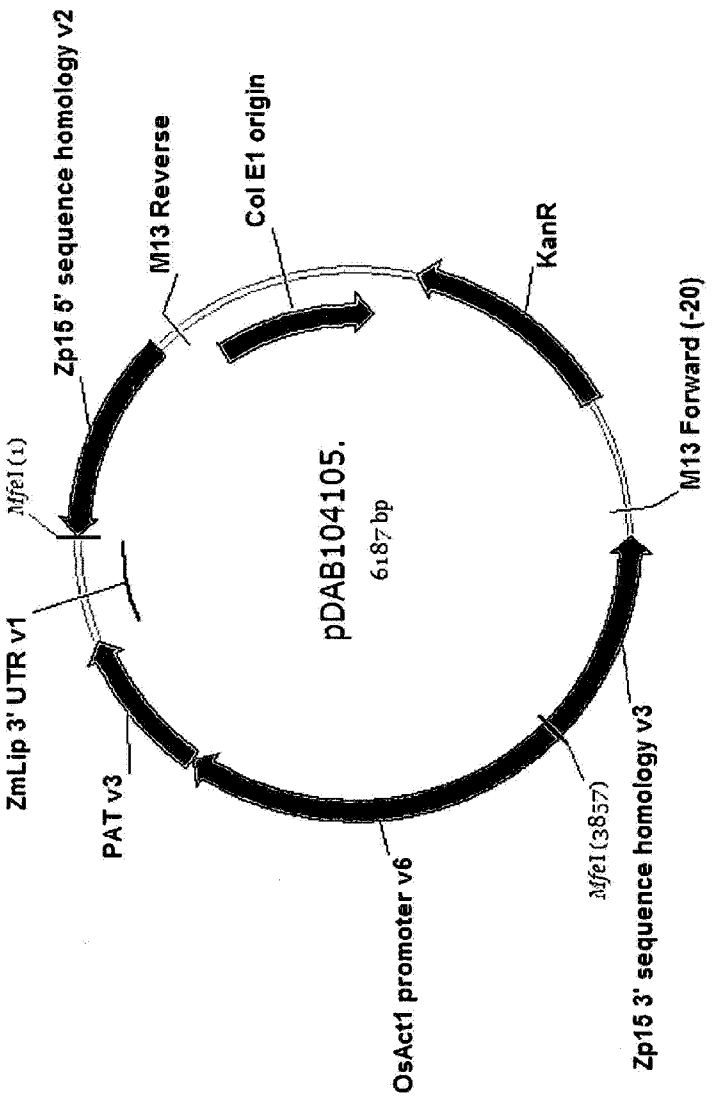
pDAB104105.
6187 bp
Zp15 5' sequence homology v2
M13 Reverse
Col E1 origin
KanR
M13 Forward (-20)
Zp15 3' sequence homology v3
MfeI (3857)
OsAct1 promoter v6
PAT v3
ZmLip 3' UTR v1
MfeI (1)

Figure 11

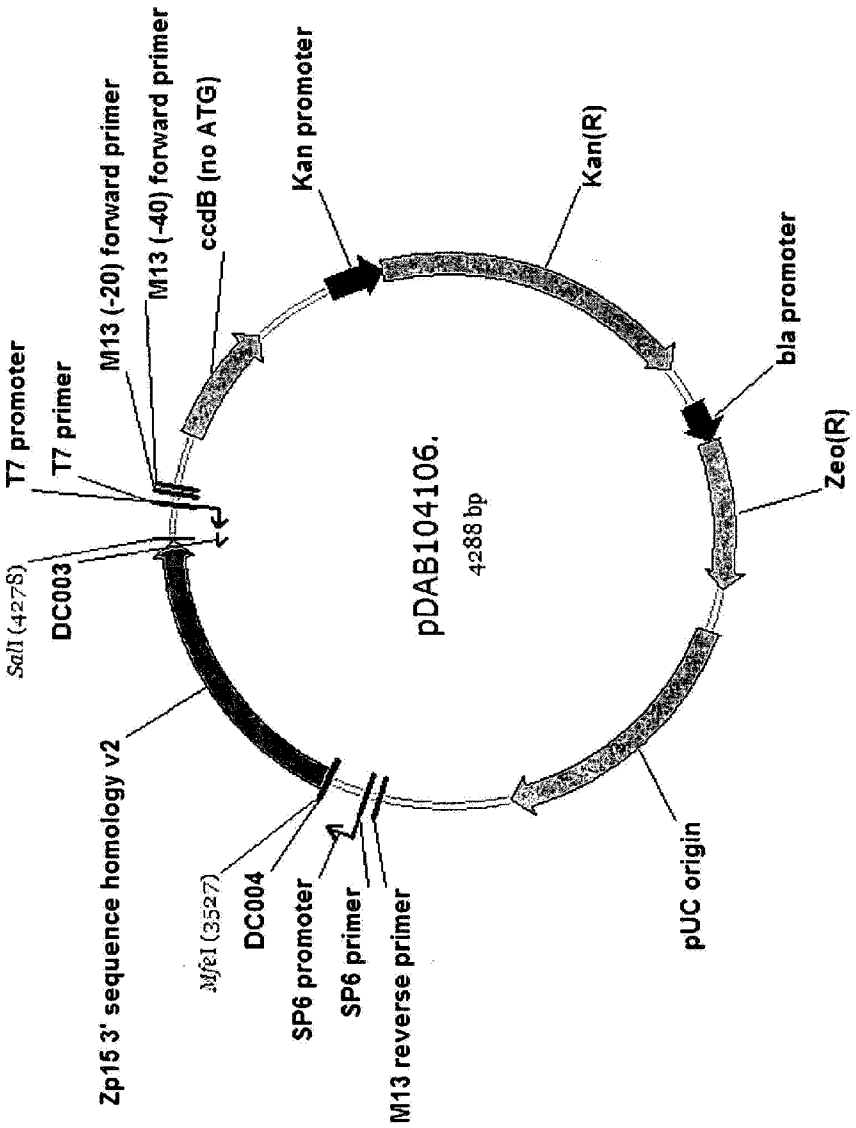
pDAB104106.
4288 bp
T7 promoter
T7 primer
M13 (-20) forward primer
M13 (-40) forward primer
ccdB (no ATG)
Kan promoter
Kan(R)
bla promoter
Zeo(R)
pUC origin
M13 reverse primer
SP6 primer
SP6 promoter
DC004
MfeI (3527)
Zp15 3' sequence homology v2
DC003
SalI (4278)

Figure 12

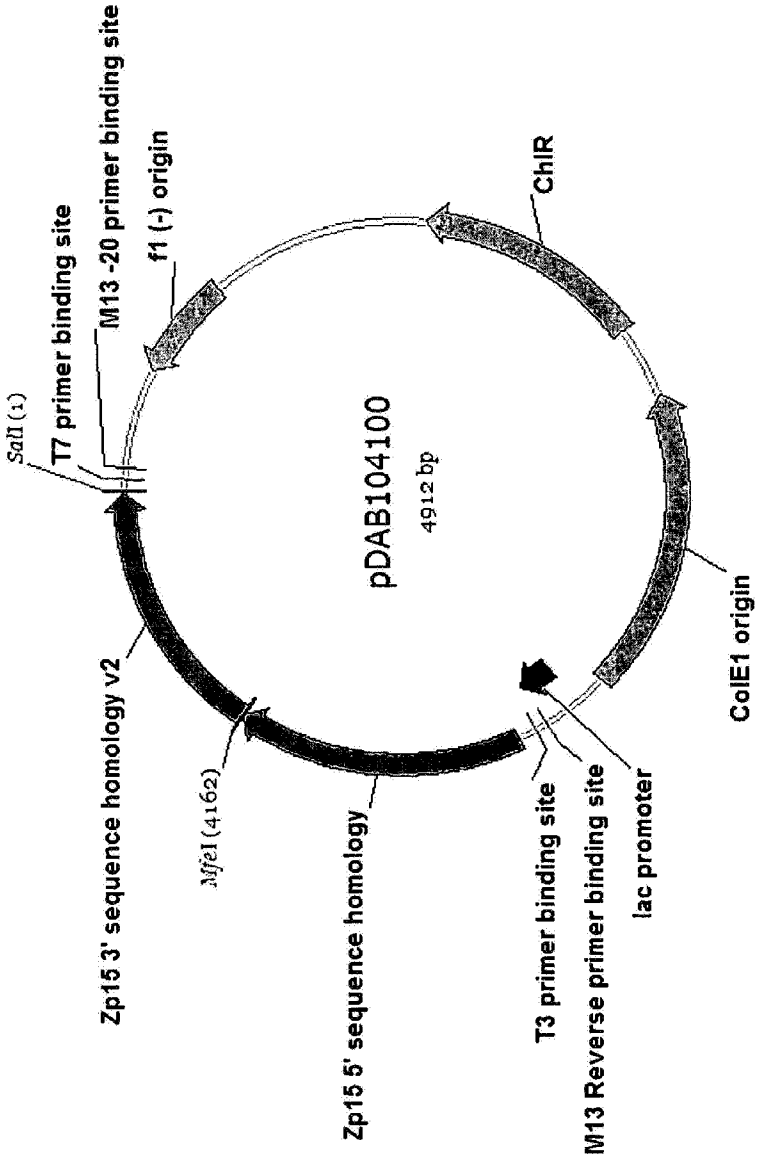
SalI (1)
T7 primer binding site
M13 -20 primer binding site
f1 (-) origin
ChlR
pDAB104100
4912 bp
Zp15 3' sequence homology v2
MfeI (4162)
Zp15 5' sequence homology
T3 primer binding site
M13 Reverse primer binding site
lac promoter
ColE1 origin

Figure 13

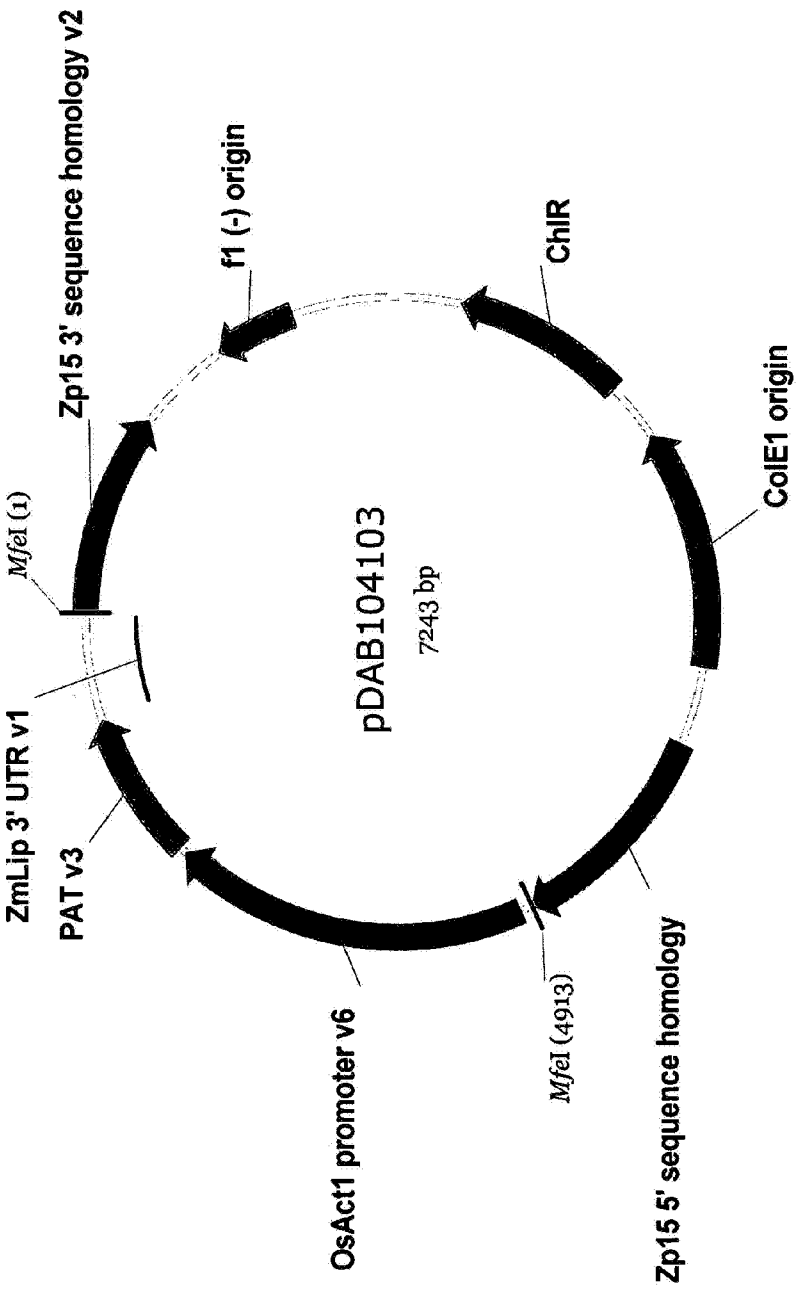
pDAB104103
7243 bp
Zp15 3' sequence homology v2
f1 (-) origin
ChlR
ColE1 origin
Zp15 5' sequence homology
MfeI (4913)
OsAct1 promoter v6
PAT v3
ZmLip 3' UTR v1
MfeI (1)

Figure 14

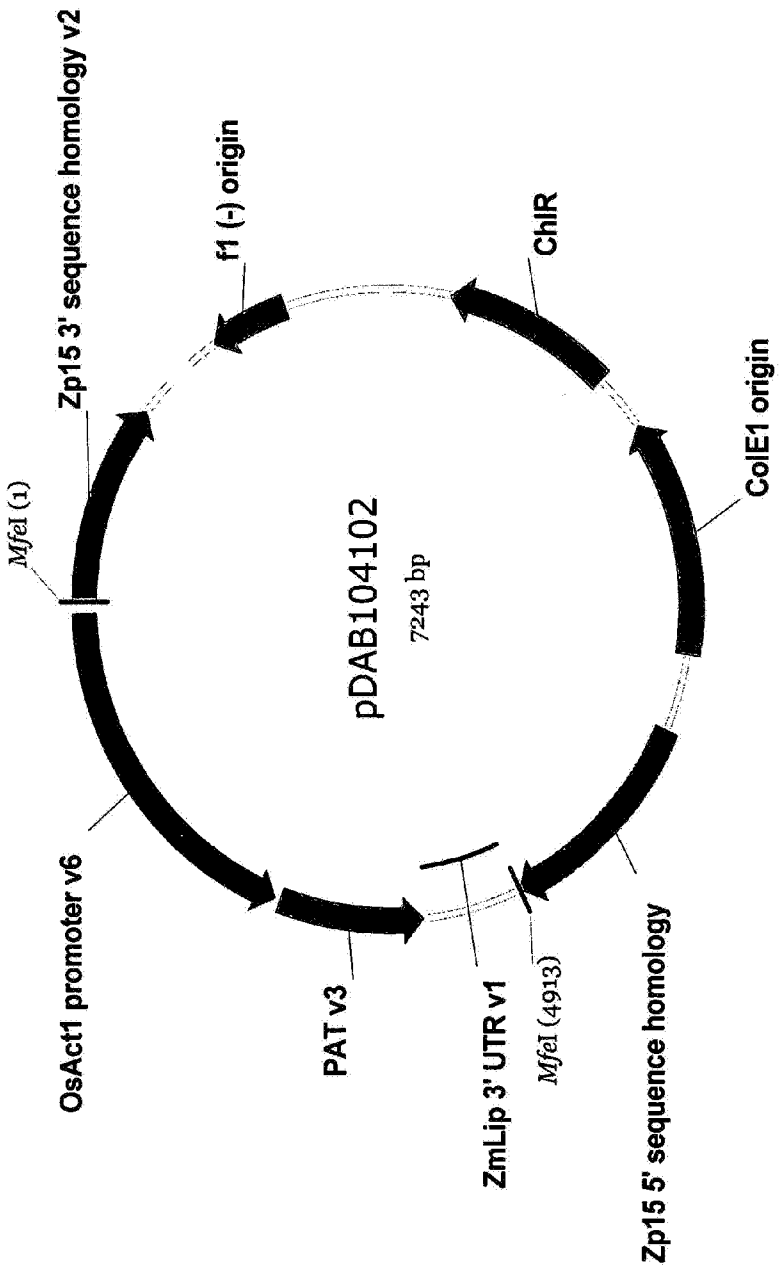
pDAB104102
7243 bp
Zp15 3' sequence homology v2
f1 (-) origin
ChlR
ColE1 origin
Zp15 5' sequence homology
ZmLip 3' UTR v1
PAT v3
OsAct1 promoter v6
MfeI (1)
MfeI (4913)

Figure 15