TAL EFFECTOR-MEDIATED DNA MODIFICATION

CROSS-REFERENCE TO RELATED APPLICATIONS

This application claims benefit of priority from U.S. Provisional Application Serial No. 61/285,324, filed on December 10, 2009, U.S. Provisional Application Serial No. 61/352,108, filed on June 7, 2010, and U.S. Provisional Application Serial No. 61/366,685, filed on July 22, 2010, all of which are incorporated herein by reference in their entirety.

STATEMENT AS TO FEDERALLY SPONSORED RESEARCH

This invention was made with government support under grant nos. 0820831 and 0504304, awarded by the National Science Foundation. The government has certain rights in the invention.

TECHNICAL FIELD

The present invention relates to methods for gene targeting, and particularly to methods that include the use of transcription activator-like (TAL) effector sequences.

BACKGROUND

The ability to modify chromosomes through homologous recombination (gene targeting) has been a long sought goal of biologists. In plants, for example, gene targeting may help to discern the function of plant genes, opening up new possibilities for crop improvement. For example, with gene targeting it is possible to carry out the genetic surgery required to reorchestrate metabolic pathways to create high value crops, including seed with altered oil or carbohydrate profiles, food with enhanced nutritional qualities, or plants with increased resistance to disease and stress. In animals (e.g., mammals), gene targeting may be used for treatment of disease. For example, gene targeting may be used to engineer corrections in genes that are defective due to various types of mutations. Efficient methods for such gene targeting have been difficult to achieve.

SUMMARY

TAL effectors of plant pathogenic bacteria in the genus *Xanthomonas* play important roles in disease, or trigger defense, by binding host DNA and activating effector-specific host genes (*see*, e.g., Gu et al. (2005) *Nature* 435:1122; Yang et al. (2006) *Proc. Natl. Acad. Sci. USA* 103:10503; Kay et al. (2007) *Science* 318:648; Sugio et al. (2007) *Proc. Natl. Acad. Sci. USA* 104:10720; and Römer et al. (2007) *Science* 318:645). Specificity depends on an effector-variable number of imperfect, typically 34 amino acid repeats (Schornack et al. (2006) *J. Plant Physiol.* 163:256). Polymorphisms are present primarily at repeat positions 12 and 13, which are referred to herein as the repeat variable-diresidue (RVD).

The present invention is based in part on the fact that the RVDs of TAL effectors correspond to the nucleotides in their target sites in a direct, linear fashion, one RVD to one nucleotide, with some degeneracy and no apparent context dependence. This surprising finding represents a novel mechanism for protein-DNA recognition that enables target site prediction for new target specific TAL effector. As described herein, these proteins may be useful in research and biotechnology as targeted chimeric nucleases that can facilitate homologous recombination in genome engineering (e.g., to add or enhance traits useful for biofuels or biorenewables in plants). These proteins also may be useful as, for example, transcription factors, and especially for therapeutic applications requiring a very high level of specificity such as therapeutics against pathogens (e.g., viruses) as non limiting examples.

In one aspect, the present invention features a method for modifying the genetic material of a cell, comprising (a) providing a cell containing a target DNA sequence; and (b) introducing a transcription activator-like (TAL) effector -DNA modifying enzyme into the cell, the TAL effector-DNA modifying enzyme comprising (i) a DNA modifying enzyme domain that can modify double stranded DNA, and (ii) a TAL effector domain comprising a plurality of TAL effector repeat sequences that, in combination, bind to a specific nucleotide sequence in the target DNA sequence, such that the TAL effector-DNA modifying enzyme modifies the target DNA within or adjacent to the specific nucleotide sequence in the cell or progeny thereof. The method can further comprise

providing to the cell a nucleic acid comprising a sequence homologous to at least a portion of the target DNA sequence, such that homologous recombination occurs between the target DNA sequence and the nucleic acid. The cell can be a eukaryotic cell, a mammalian cell, a plant cell, or a prokaryotic cell. The target DNA can be chromosomal DNA. The introducing can comprise transfecting the cell with a vector encoding the TAL effector-DNA modifying enzyme, mechanically injecting the TAL effector-DNA modifying enzyme into the cell as a protein, delivering the TAL effector-DNA modifying enzyme into the cell as a protein by means of the bacterial type III secretion system, or introducing the TAL effector-DNA modifying enzyme into the cell as a protein by electroporation. The DNA modifying enzyme can be an endonuclease (e.g., a type II restriction endonuclease, such as *Fok*I).

The TAL effector domain that binds to a specific nucleotide sequence within the target DNA can comprise 10 or more DNA binding repeats, and preferably 15 or more DNA binding repeats. Each DNA binding repeat can include a repeat variable-diresidue (RVD) that determines recognition of a base pair in the target DNA sequence, wherein each DNA binding repeat is responsible for recognizing one base pair in the target DNA sequence, and wherein the RVD comprises one or more of: HD for recognizing C; NG for recognizing T; NI for recognizing A; NN for recognizing G or A; NS for recognizing A or C or G or T; N* for recognizing C or T, where * represents a gap in the second position of the RVD; HG for recognizing T; H* for recognizing T, where * represents a gap in the second position of the RVD; IG for recognizing T; NK for recognizing G; HA for recognizing C;ND for recognizing C; HI for recognizing C; HN for recognizing G; NA for recognizing G; SN for recognizing G or A; and YG for recognizing T. Each DNA binding repeat can comprise a RVD that determines recognition of a base pair in the target DNA sequence, wherein each DNA binding repeat is responsible for recognizing one base pair in the target DNA sequence, and wherein the RVD comprises one or more of: HA for recognizing C;ND for recognizing C; HI for recognizing C; HN for recognizing G; NA for recognizing G; SN for recognizing G or A; YG for recognizing T; and NK for recognizing G, and one or more of: HD for recognizing C; NG for recognizing T; NI for recognizing A; NN for recognizing G or A; NS for recognizing A or C or G or T; N* for recognizing C or T, wherein * represents a gap in the second position

of the RVD; HG for recognizing T; H* for recognizing T, wherein * represents a gap in the second position of the RVD; and IG for recognizing T.

In another aspect, the present invention features a method for generating a nucleic acid encoding a TAL effector specific for a selected nucleotide sequence, comprising: (1) linearizing a starter plasmid with *Psp*XI, the starter plasmid comprising a nucleotide sequence that encodes a first TAL effector DNA binding repeat domain having a repeat variable-diresidue (RVD) specific for the first nucleotide of the selected nucleotide sequence, wherein the first TAL effector DNA binding repeat domain has a unique *Psp*XI site at its 3' end; (2) ligating into the starter plasmid *Psp*XI site a DNA module encoding one or more TAL effector DNA binding repeat domains that have RVDs specific for the next nucleotide(s) of the selected nucleotide sequence, wherein the DNA module has *Xho*I sticky ends; and (3) repeating steps (1) and (2) until the nucleic acid encodes a TAL effector capable of binding to the selected nucleotide sequence. The method can further comprise, after the ligating, determining the orientation of the DNA module in the *Psp*XI site. The method can comprise repeating steps (1) and (2) from one to 30 times.

In another aspect, the present invention features a method for generating a nucleic acid encoding a transcription activator-like effector endonuclease (TALEN), comprising (a) identifying a first nucleotide sequence in the genome of a cell; and (b) synthesizing a nucleic acid encoding a TALEN that comprises (i) a plurality of DNA binding repeats that, in combination, bind to the first unique nucleotide sequence, and (ii) an endonuclease that generates a double-stranded cut at a position within or adjacent to the first nucleotide sequence, wherein each DNA binding repeat comprises a RVD that determines recognition of a base pair in the target DNA, wherein each DNA binding repeat is responsible for recognizing one base pair in the target DNA, and wherein the TALEN comprises one or more of the following RVDs: HD for recognizing C; NG for recognizing T; NI for recognizing A; NN for recognizing G or A; NS for recognizing A or C or G or T; N* for recognizing C or T; HG for recognizing T; H* for recognizing T; IG for recognizing T; NK for recognizing G; HA for recognizing C; ND for recognizing C; HI for recognizing C; HN for recognizing G; NA for recognizing G; SN for recognizing G or A; and YG for recognizing T.

The TALEN can comprises one or more of the following RVDs: HA for recognizing C; ND for recognizing C; HI for recognizing C; HN for recognizing G; NA for recognizing G; SN for recognizing G or A; YG for recognizing T; and NK for recognizing G, and one or more of: HD for recognizing C; NG for recognizing T; NI for recognizing A; NN for recognizing G or A; NS for recognizing A or C or G or T; N* for recognizing C or T; HG for recognizing T; H* for recognizing T; and IG for recognizing T.

The first nucleotide sequence can meet at least one of the following criteria: i) is a minimum of 15 bases long and is oriented from 5' to 3' with a T immediately preceding the site at the 5' end; ii) does not have a T in the first (5') position or an A in the second position; iii) ends in T at the last (3') position and does not have a G at the next to last position; and iv) has a base composition of 0-63% A, 11-63% C, 0-25% G, and 2-42% T.

The method can comprise identifying a first nucleotide sequence and a second nucleotide sequence in the genome of the cell, wherein the first and second nucleotide sequences meet at least one of the criteria set forth above and are separated by 15-18 bp. The endonuclease can generate a double-stranded cut between the first and second nucleotide sequences.

In another embodiment, the present invention features a TALEN comprising an endonuclease domain and a TAL effector DNA binding domain specific for a target DNA, wherein the DNA binding domain comprises a plurality of DNA binding repeats, each repeat comprising a RVD that determines recognition of a base pair in the target DNA, wherein each DNA binding repeat is responsible for recognizing one base pair in the target DNA, and wherein the TALEN comprises one or more of the following RVDs: HD for recognizing C; NG for recognizing T; NI for recognizing A; NN for recognizing G or A; NS for recognizing A or C or G or T; N* for recognizing C or T; HG for recognizing T; H* for recognizing T; IG for recognizing T; NK for recognizing G; HA for recognizing C; ND for recognizing C; HI for recognizing C; HN for recognizing G; NA for recognizing G; SN for recognizing G or A; and YG for recognizing T. The TALEN can comprise one or more of the following RVDs: HA for recognizing C; ND for recognizing C; HI for recognizing C; HN for recognizing G; NA for recognizing G; SN for recognizing G or A; YG for recognizing T; and NK for recognizing G, and one or more

of: HD for recognizing C; NG for recognizing T; NI for recognizing A; NN for recognizing G or A; NS for recognizing A or C or G or T; N* for recognizing C or T; HG for recognizing T; H* for recognizing T; and IG for recognizing T. The endonuclease domain can be from a type II restriction endonuclease (e.g., *Fok*I).

In still another aspect, the present invention features a TALEN comprising an endonuclease domain and a TAL effector domain, wherein the amino acid sequence of said TALEN is selected from the group consisting of SEQ ID NO:33 to SEQ ID NO:55, SEQ ID NO:72, and SEQ ID NO:73.

The present invention also features a method for generating an animal, comprising: providing a eukaryotic cell comprising a target DNA sequence into which it is desired to introduce a genetic modification; generating a double-stranded cut within the target DNA sequence with a TALEN comprising an endonuclease domain and a TAL effector domain that binds to the target DNA sequence; and generating an animal from the cell or progeny thereof in which a double-stranded cut has occurred. The method can further comprise introducing into the cell an exogenous nucleic acid comprising a sequence homologous to at least a portion of the target DNA, wherein the introducing is under conditions that permit homologous recombination to occur between the exogenous nucleic acid and the target DNA sequence in the cell or progeny thereof; and generating an animal from the cell or progeny thereof in which homologous recombination has occurred. The animal can be a mammal. The genetic modification can comprise a substitution, an insertion, or a deletion.

In yet another aspect, the present invention features a method for generating a plant, comprising providing a plant cell comprising a target DNA sequence into which it is desired to introduce a preselected genetic modification; generating a double-stranded cut within the target DNA sequence with a TALEN comprising an endonuclease domain and a TAL effector domain that binds to the target DNA sequence; and generating a plant from the cell or progeny thereof in which a double-stranded cut has occurred. The method can further comprise introducing into the plant cell an exogenous nucleic acid comprising a sequence homologous to at least a portion of the target DNA sequence, wherein the introducing is under conditions that permit homologous recombination to occur between the exogenous nucleic acid and the target DNA sequence in the cell or

progeny thereof; and generating a plant from the cell or progeny thereof in which homologous recombination has occurred.

In another aspect, the present invention features a method for targeted genetic recombination in a cell, comprising introducing into the cell a nucleic acid encoding a TAL effector endonuclease targeted to a selected DNA target sequence; inducing expression of the TAL effector endonuclease within the cell; and identifying a cell in which the selected DNA target sequence exhibits a mutation. The mutation can be selected from the group consisting of deletion of genetic material, insertion of genetic material, and both deletion and insertion of genetic material. The method can further comprise introducing donor DNA into the cell. The cell can be an insect cell, a plant cell, a fish cell, or a mammalian cell.

In another aspect, the present invention features a method for generating a TAL effector having enhanced targeting capacity for a target DNA, comprising generating a nucleic acid encoding a TAL effector that comprises DNA binding domain having a plurality of DNA binding repeats, wherein each repeat comprises a RVD that determines recognition of a base pair in the target DNA, wherein each DNA binding repeat is responsible for recognizing one base pair in the target DNA, wherein the generating comprises incorporating a nucleic acid encoding a variant 0th DNA binding repeat sequence with specificity for A, C, or G, thus eliminating the requirement for T at position -1 of the binding site.

In another aspect, the present invention features a method for generating a TAL effector having enhanced targeting capacity for a target DNA, comprising generating a nucleic acid encoding a TAL effector that comprises DNA binding domain having a plurality of DNA binding repeats, wherein each repeat comprises a RVD that determines recognition of a base pair in the target DNA, wherein each DNA binding repeat is responsible for recognizing one base pair in the target DNA, wherein the generating comprises incorporating one or more nucleic acids encoding TAL effector DNA binding domains that contain RVDs having enhanced specificity for G, and wherein said RVDs are selected from the group consisting of RN, R*, NG, NH, KN, K*, NA, NT, DN, D*, NL, NM, EN, E*, NV, NC, QN, Q*, NR, NP, HN, H*, NK, NY, SN, S*, ND, NW, TN,

T*, NE, NF, YN, Y*, and NQ, wherein * indicates a gap at the second position of the RVD.

The present invention also features a method for producing a polypeptide that selectively recognizes at least one base pair in a target DNA sequence, comprising synthesizing a polypeptide comprising a repeat domain, wherein the repeat domain comprises at least one repeat unit derived from a transcription activator-like (TAL) effector, wherein the repeat unit comprises a hypervariable region which determines recognition of a base pair in the target DNA sequence, wherein the repeat unit is responsible for the recognition of one base pair in the DNA sequence, and wherein the hypervariable region comprises a member selected from the group consisting of: (a) HD for recognition of C/G; (b) NI for recognition of A/T; (c) NG for recognition of T/A; (d) NS for recognition of C/G or A/T or T/A or G/C; (e) NN for recognition of G/C or A/T; (f) IG for recognition of T/A; (g) N for recognition of C/G; (h) HG for recognition of C/G or T/A; (i) H for recognition of T/A; and (j) NK for recognition of G/C. In addition, this invention features a polypeptide produced by the above method, and a DNA comprising a coding sequence for the polypeptide produced by the method. Also featured is an expression cassette comprising a promoter operably linked to the above-mentioned DNA, and a non-human host cell comprising the expression cassette. In another aspect, the present invention features a transformed, non-human organism comprising the expression cassette.

In still another aspect, the present invention features a method for selectively recognizing a base pair in a DNA sequence by a polypeptide, comprising constructing a polypeptide comprising a repeat domain, wherein the repeat domain comprises at least one repeat unit derived from a TAL effector, wherein the repeat unit comprises a hypervariable region which determines recognition of a base pair in the DNA sequence, wherein the repeat unit is responsible for the recognition of one base pair in the DNA sequence, and wherein the hypervariable region comprises a member selected from the group consisting of (a) HD for recognition of C/G; (b) NI for recognition of A/T; (c) NG for recognition of T/A; (d) NS for recognition of C/G or A/T or T/A or G/C; (e) NN for recognition of G/C or A/T; (f) IG for recognition of T/A; (g) N for recognition of C/G; (h)

HG for recognition of C/G or T/A; (i) H for recognition of T/A; and (j) NK for recognition of G/C .

The present invention also features a method of modulating expression of a target gene in a cell, wherein cells are provided which contain a polypeptide wherein the polypeptide comprises a repeat domain, wherein the repeat domain comprises at least one repeat unit derived from a TAL effector, wherein the repeat unit comprises a hypervariable region which determines recognition of a base pair in a DNA sequence, wherein the repeat unit is responsible for the recognition of one base pair in the DNA sequence, and wherein the hypervariable region comprises a member selected from the group consisting of (a) HD for recognition of C/G; (b) NI for recognition of A/T; (c) NG for recognition of T/A; (d) NS for recognition of C/G or A/T or T/A or G/C; (e) NN for recognition of G/C or A/T; (f) IG for recognition of T/A; (g) N for recognition of C/G; (h) HG for recognition of C/G or T/A; (i) H for recognition of T/A; and (j) NK for recognition of G/C .

In another aspect, the present invention features a polypeptide comprising a repeat domain, wherein the repeat domain comprises at least one repeat unit derived from a TAL effector, wherein the repeat unit comprises a hypervariable region which determines recognition of a base pair in a DNA sequence, wherein the repeat unit is responsible for the recognition of one base pair in the DNA sequence, and wherein the hypervariable region comprises a member selected from the group consisting of (a) HD for recognition of C/G; (b) NI for recognition of A/T; (c) NG for recognition of T/A; (d) NS for recognition of C/G or A/T or T/A or G/C; (e) NN for recognition of G/C or A/T; (f) IG for recognition of T/A; (g) N for recognition of C/G; (h) HG for recognition of C/G or T/A; (i) H for recognition of T/A; and (j) NK for recognition of G/C. The present invention also features a DNA comprising a coding sequence for the above-mentioned polypeptide.

In another aspect, the present invention features a DNA which is modified to include a base pair located in a target DNA sequence so that the base pair can be specifically recognized by a polypeptide comprising a repeat domain, wherein the repeat domain comprises at least one repeat unit derived from a TAL effector, wherein the repeat unit comprises a hypervariable region which determines recognition of a base pair in the DNA sequence, wherein the repeat unit is responsible for the recognition of one base pair

in the DNA sequence, and wherein, to receive a selective and determined recognition by the hypervariable region, the base pair is selected from the group consisting of (a) C/G for recognition by HD; (b) A/T for recognition by NI; (c) T/A for recognition by NG; (d) CT or A/T or T/A or G/C for recognition by NS; (e) G/C or A/T for recognition by NN; (f) T/A for recognition by IG; (g) C/G or T/A for recognition by N; (h) T/A for recognition by HG; (i) T/A for recognition by H; and (j) G/C for recognition by NK. Also featured are a vector comprising the above-mentioned DNA, a non-human host cell comprising the DNA, and a transformed, non-human organism comprising the DNA.

In yet another aspect, the present invention features a method for producing a DNA comprising a target DNA sequence that is selectively recognized by a polypeptide comprising a repeat domain, wherein the repeat domain comprises at least one repeat unit derived from a TAL effector, wherein the repeat unit comprises a hypervariable region which determines recognition of a base pair in the target DNA sequence, and wherein the repeat unit is responsible for the recognition of one base pair in the target DNA sequence, the method comprising synthesizing a DNA comprising a base pair that is capable of being recognized by the repeat unit, wherein the base pair is selected from the group consisting of (a) C/G for recognition by HD; (b) A/T for recognition by NI; (c) T/A for recognition by NG; (d) CT or A/T or T/A or G/C for recognition by NS; (e) G/C or A/T for recognition by NN; (f) T/A for recognition by IG; (g) C/G or T/A for recognition by N; (h) T/A for recognition by HG; (i) T/A for recognition by H; and (j) G/C for recognition by NK.

In another aspect, the present invention features a method for modifying the genetic material of a plant cell. The method can include (a) introducing into the plant cell (i) a first recombinant nucleic acid comprising a modified target nucleotide sequence, wherein the modified target nucleotide sequence comprises one or more modifications in nucleotide sequence with respect to a corresponding target nucleotide sequence present in the plant cell, and wherein the target nucleotide sequence further comprises a recognition site for a sequence-specific TAL effector endonuclease (TALEN); and (ii) a second recombinant nucleic acid comprising a nucleotide sequence encoding the sequence-specific transcription activator-like (TAL) effector endonuclease; (b) generating a plant containing the plant cell; (c) analyzing cells, seed, or tissue obtained from the plant, or

progeny thereof, for recombination at the target nucleotide sequence. The method can further include introducing into the plant cell (iii) a third recombinant nucleic acid comprising a nucleotide sequence encoding a selectable marker; and determining if the plant or progeny thereof expresses the selectable marker. The method can further include the step of screening the plant or progeny thereof for the absence of the selectable marker. The nucleotide sequence encoding the selectable marker may or may not be flanked on one or both sides by a sequence that is similar or identical to a sequence that is endogenous to the plant cell (e.g., a sequence at the site of cleavage for a second sequence-specific nuclease). The nucleotide sequence encoding the selectable marker may be flanked on both sides by recognition sites for a sequence-specific recombinase. The method can further include the step of out-crossing the plant, with or without the step of screening progeny of the out-cross for the absence of the selectable marker. The first and second recombinant nucleic acids can be simultaneously introduced into the plant cell. One or both of the recombinant nucleic acids can be linearized prior to the introducing step. The first and second recombinant nucleic acids may be present in the same construct.

In another aspect, the present invention features another method for modifying the genetic material of a cell. The method can include providing a primary cell containing chromosomal target DNA sequence in which it is desired to have homologous recombination occur; providing a TALEN comprising an endonuclease domain that can cleave double stranded DNA, and a TAL effector domain comprising a plurality of TAL effector repeat sequences that, in combination, bind to a specific nucleotide sequence within the target DNA in the cell; and contacting the target DNA sequence with the TALEN in the cell such that the TALEN cleaves both strands of a nucleotide sequence within or adjacent to the target DNA sequence in the cell. The method can further include providing a nucleic acid comprising a sequence homologous to at least a portion of the target DNA, such that homologous recombination occurs between the target DNA sequence and the nucleic acid. The target DNA sequence can be endogenous to the cell. The cell can be a plant cell, a mammalian cell, a fish cell, an insect cell or cell lines derived from these organisms for *in vitro* cultures or primary cells taken directly from living tissue and established for *in vitro* culture. The contacting can include transfecting

the cell with a vector comprising a TALEN coding sequence, and expressing the TALEN protein in the cell, mechanically injecting a TALEN protein into the cell, delivering a TAL effector endonuclease protein into the cell by means of the bacterial type III secretion system, or introducing a TALEN protein into the cell by electroporation. The endonuclease domain can be from a type II restriction endonuclease (e.g., *Fok*I). The TAL effector domain that binds to a specific nucleotide sequence within the target DNA can include 10 or more DNA binding repeats, more preferably 15 or more DNA binding repeats. The cell can be from any prokaryotic or eukaryotic organism.

In another aspect, the present invention features a method for designing a sequence specific TALEN capable of cleaving DNA at a specific location. The method can include identifying a first unique endogenous chromosomal nucleotide sequence adjacent to a second nucleotide sequence at which it is desired to introduce a double-stranded cut; and designing a sequence specific TALEN comprising (a) a plurality of DNA binding repeat domains that, in combination, bind to the first unique endogenous chromosomal nucleotide sequence, and (b) an endonuclease that generates a double-stranded cut at the second nucleotide sequence.

The present invention also features a TALEN comprising an endonuclease domain and a TAL effector DNA binding domain specific for a particular DNA sequence. The TALEN can further include a purification tag. The endonuclease domain can be from a type II restriction endonuclease (e.g., *Fok*I).

In another aspect, the present invention features a method for generating a genetically modified animal into which a desired nucleic acid has been introduced. The method can include providing a primary cell comprising an endogenous chromosomal target DNA sequence into which it is desired to introduce the nucleic acid; generating a double-stranded cut within the endogenous chromosomal target DNA sequence with a TALEN comprising an endonuclease domain and a TAL effector domain that binds to the endogenous chromosomal target DNA sequence; introducing an exogenous nucleic acid comprising a sequence homologous to at least a portion of the endogenous chromosomal target DNA into the primary cell under conditions that permit homologous recombination to occur between the exogenous nucleic acid and the endogenous chromosomal target DNA; and generating an animal from the primary cell in which homologous

recombination has occurred. The animal can be a mammal. The homologous sequence can be a nucleotide sequence selected from the group consisting of a nucleotide sequence that disrupts a gene after homologous recombination, a nucleotide sequence that replaces a gene after homologous recombination, a nucleotide sequence that introduces a point mutation into a gene after homologous recombination, and a nucleotide sequence that introduces a regulatory site after homologous recombination.

In still another aspect, the present invention features a method for generating a genetically modified plant in which a desired nucleic acid has been introduced. The method can include providing a plant cell comprising an endogenous target DNA sequence into which it is desired to introduce the nucleic acid; generating a double-stranded cut within the endogenous target DNA sequence with a TALEN comprising an endonuclease domain and a TAL effector domain that binds to the endogenous target nucleotide sequence; introducing an exogenous nucleic acid comprising a sequence homologous to at least a portion of the endogenous target DNA into the plant cell under conditions that permit homologous recombination to occur between the exogenous nucleic acid and the endogenous target DNA; and generating a plant from the plant cell in which homologous recombination has occurred.

In another aspect, the present invention features a method for targeted genetic recombination in a cell. The method can include introducing into the cell a nucleic acid molecule encoding a TALEN targeted to a selected DNA target sequence; inducing expression of the TALEN within the cell; and identifying a cell in which the selected DNA target sequence exhibits a mutation. The mutation can be selected from the group consisting of a deletion of genetic material, an insertion of genetic material, and both a deletion and an insertion of genetic material. The method can further include introducing donor DNA into the cell. The cell can be an insect cell, a plant cell, a fish cell, or a mammalian cell.

In yet another aspect, the present invention features a method for generating a nucleic acid encoding a sequence specific TALEN, comprising (1) selecting a starter plasmid comprising a nucleotide sequence that encodes a first TAL effector DNA binding repeat domain having a RVD specific for the first nucleotide of a selected nucleotide sequence, wherein the first TAL effector DNA binding repeat domain has a unique *PspX*I

site at its 3' end; (2) linearizing the starter plasmid with *PspX*I; (3) ligating into the *PspX*I site a DNA module encoding one or more TAL effector DNA binding repeat domains that have RVDs specific for the next nucleotide(s) of the selected nucleotide sequence, wherein the DNA module has *Xho*I sticky ends; and (4) repeating steps (2) and (3) until the nucleic acid encodes a TALEN capable of binding to the selected nucleotide sequence. In some cases, the method can further include, after the ligating in step (3), checking the orientation of the DNA module in the *PspX*I site.

Unless otherwise defined, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention pertains. Although methods and materials similar or equivalent to those described herein can be used to practice the invention, suitable methods and materials are described below. All publications, patent applications, patents, and other references mentioned herein are incorporated by reference in their entirety. In case of conflict, the present specification, including definitions, will control. In addition, the materials, methods, and examples are illustrative only and not intended to be limiting.

The details of one or more embodiments of the invention are set forth in the accompanying drawings and the description below. Other features, objects, and advantages of the invention will be apparent from the description and drawings, and from the claims.

DESCRIPTION OF DRAWINGS

FIGS. 1A-1D depict the TAL effector-DNA recognition cipher. FIG. 1A is a diagram of a generic TAL effector, showing the repeat region (open boxes) and a representative repeat sequence (SEQ ID NO:1) with the RVD underlined. FIG. 1B is a diagram showing best pattern matches (low entropy alignments) for various TAL effector RVDs and target gene promoter sequences (SEQ ID NOS:2-11). An asterisk indicates a deletion at residue 13. FIG. 1C is a diagram showing RVD-nucleotide associations in the alignments in B, plus ten more alignments obtained by scanning all rice promoters with 40 additional *X. oryzae* TAL effectors, retaining for each effector the best alignment for which the downstream gene was activated during infection. FIG. 1D is a diagram showing flanking nucleotide frequencies for the 20 TAL effector target sites. Positions

are relative to the 5' end of the target site; N, length of target site. Logos were generated using WebLogo.

FIGS. 2A and 2B provide evidence that *OsHen1* is activated by Tal1c of *Xanthomonas oryzae* pv. oryzicola strain BLS256. FIG. 2A is a picture of semi quantitative RT-PCR results, showing relative transcript abundance of *OsHen1*, with an actin gene for reference, in rice leaves 24 hours after inoculation with BLS256 marker exchange mutant M51, M51 carrying the empty cosmid vector (ev), M51 carrying cosmid pIJF92, which contains *tal1a*, *tal1b*, and *tal1c*, and the wild type (WT) strain. FIG. 2B is a schematic based on mapping of the single marker exchange mutation in M51 by rescue and end sequencing of a marker-containing *Xma*I fragment. The genome region, the coordinates of the rescued fragment, and the coordinates of the BLS256 genomic fragment contained in cosmid pIJF92 are shown.

FIG. 3 is a reference AvrBs3 amino acid sequence (SEQ ID NO:12).

FIG. 4 is a reference AvrBs3 nucleic acid sequence (SEQ ID NO:13).

FIG. 5 is a map of a TAL nuclease expression vector.

FIG. 6 is a map of a target reporter plasmid.

FIG. 7 is a diagram of the schematic architecture of TAL nucleases. The recognition sites of TAL DNA binding domain are represented as capital letters, while the spacer sequence is indicated in lowercase.

FIG. 8 is the amino acid sequence (SEQ ID NO:16) of the 17 and a half tandem repeats of the AvrBs3 recognition domain. Hypervariable amino acids at positions 12 and 13 are boxed.

FIG. 9 is a diagram showing a scheme for a yeast assay to test TAL effectiveness.

FIG. 10 is a graph plotting yeast assay results of AvrBs3 TAL nuclease.

FIG. 11 is a diagram showing a schematic representation of single, double, or triple AsvBs3 repeat modules and a cloning vector.

FIGS. 12A and 12B depict a single representative TAL effector repeat (FIG. 12A), as well as a representative truncated repeat (FIG. 12B) that is present at the end of the repeat region in most TAL effectors. Nucleotide and encoded amino acid sequences as shown. Ns represent nucleotides encoding the RVDs, which are indicated as "XX." Numbers are given for the amino acid positions. Sequences are taken from tal1c.

FIG. 13 is a schematic depicting the tal1c gene and the process by which the repeat region was reduced to a single, truncated repeat, resulting in pCS487, also shown. M, *Msc*I site; S, *Sph*I site.

FIG. 14 is a schematic depicting introduction of a translationally silent mutation at the end of the original truncated repeat in pCS487 to create a PspXI and XhoI site, yielding pCS489. Sequences of codons 18-21 in the original repeat (SEQ ID NO:21) and the mutated repeat (SEQ ID NO:23) are shown. The encoded amino acid sequence (SEQ ID NO:22) was not changed by the mutation. The mutated nucleotides are italicized.

FIG. 15 is a map of pCS488, which is a kanamycin resistant plasmid encoding only the N- and C-terminal portions of tal1c, without the repeat region, in the Gateway entry vector pENTR-D (Invitrogen, Carlsbad, CA).

FIG. 16 is a map of the single repeat starter plasmid designated pCS493, which encodes a repeat having the RVD NI. Three other plasmids, designated pCS494, pCS495, and pCS496, were identical except for the RVDs they encode (given at right).

FIG. 17A depicts nucleotide and encoded amino acid sequences for a single repeat module with the RVD NI. The 5' *Xho*I compatible cohesive end, the MscI site, and the 3' *PspXI*/*Xho*I compatible cohesive end are underlined. The RVD and the nucleotides encoding it are in bold type. Three other repeat modules were constructed that are identical to that shown except for the RVD encoding sequences, which encode HD, NI, and NG, respectively. FIG. 17B is a map of the single repeat module plasmid designated pCS502, which contains the repeat encoding sequence shown in FIG. 17A. Plasmids designated pCS503, pCS504, and pCS505 also were generated, and are identical to pCS502 except for the RVDs they encode (given at right).

FIG. 18A depicts nucleotide and encoded amino acid sequences for a single repeat module with RVD NI, in which nucleotide substitutions (italicized) prevent reconstitution of the *Xho*I site at the 5' end following ligation into a *PspXI*/*Xho*I site and destroy the internal MscI site. The RVD and its encoding nucleotides are in bold type. Three additional repeat modules were constructed that are identical to that shown except for the RVD encoding sequences, which encode HD, NI, and NG, respectively. FIG. 18B is a schematic of a three repeat module assembled by sequentially ligating additional repeat modules into a single repeat module plasmid. The MscI site in the first repeat and

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the *PspX*I site at the 3' end remain unique, and the entire module is flanked by two *Xho*I sites.

FIG. 19 is a list of the complete set of one-, two-, and three-repeat module plasmids.

FIG. 20 is a flow chart depicting the steps in a method that can be used to assemble any sequence of repeats into the tal1c "backbone" to generate a custom TAL effector gene.

FIGS. 21A and 21B are schematics depicting assembly of repeat modules in construction of TAL endonucleases that will target the nucleotide sequences shown. In FIG. 21A, repeat modules from plasmids designated pCS519, pCS524, pCS537, pCS551, pCS583, and pCS529 are sequentially added to the sequence in the starter plasmid designated pCS493, resulting in plasmids designated pMAT55, pMAT56, pMAT57, pMAT58, pMAT59, and pMAT60. In FIG. 21B, repeat modules from plasmids designated pCS530, pCS533, pCS522, and pCS541 are sequentially added to the sequence in the plasmid designated pMAT1, resulting in plasmids designated pMAT61, pMAT62, pMAT63, and pMAT64.

FIG. 22A is a schematic of a TAL effector protein. *BamH*I fragments (denoted by B's) were fused to the catalytic domain of the *Fok*I endonuclease to create TALENs. N, N-terminus; NLS, nuclear localization signal; B, *BamH*I site, AD, acidic activation domain. FIG. 22B is a graph plotting activity of TALENs constructed with TAL effectors AvrBs3 and PthXo1. Avr-FokI, AvrBs3 TALEN; Pth-FokI, PthXo1 TALEN, Avr-FokI and Pth-FokI, AvrBs3 and PthXo1 fusions to a catalytically inactive version of FokI (Bitinaite et al. (1998) *Proc. Natl. Acad. Sci. USA* 95:10570-10575); ZFN, zinc finger nuclease containing the Zif268 DNA binding domain (Porteus and Baltimore (2003) *Science* 300:763).

FIG. 23 is a reference PthXo1 amino acid sequence (SEQ ID NO:31).

FIG. 24 is a reference PthXo1 nucleic acid sequence (SEQ ID NO:32).

FIG. 25 is a diagram of the pFZ85 vector.

FIG. 26 shows the amino acid sequence of avrBs3_TALEN (SEQ ID NO:33).

FIG. 27 shows the amino acid sequence of pthXo1_TALEN (SEQ ID NO:34).

FIG. 28A is a graph plotting activity of AvrBs3 and PthXo1 TALENS on targets with different spacer lengths. ZFN, Zif268-derived zinc finger nuclease. FIG. 28B is a graph plotting activity of a heterodimeric TALEN. Activity in yeast containing PthXo1-FokI and AvrBs3-FokI expression vectors and a plasmid with a target consisting of recognition sites for each, in head to tail orientation separated by 15 bp is shown (Avr-FokI, Pth-FokI). Also shown for reference is activity of AvrBs3 (Avr-FokI) and PthXo1 (Pth-FokI) TALENS individually and Zif268 (ZFN) on their respective targets. As a negative control, a yeast culture with only the target site plasmid for Avr-FokI, Pth-FokI was assayed for LacZ activity (denoted as (-)).

FIG. 29A is a table showing the RVD sequences of individual custom TALENs and their respective DNA recognition sequences. FIG. 29B is a graph plotting the activity of custom TALENs. (-), negative control with target site plasmids only; ZFN, zinc finger nuclease positive control.

FIG. 30 is a depiction of the nucleotide and RVD frequencies at the termini of 20 target and TAL effector pairs.

FIG. 31 is a schematic of the Golden Gate cloning system [Engler et al. (2008) *PLoS One* 3:e3647; and Engler et al. (2009) *PLoS One* 4:e5553].

FIGS. 32A and 32B depict a set of 58 plasmids for assembly and cloning of custom TAL effector repeat encoding arrays using the Golden Gate cloning approach as described herein. Tet, tetracycline resistance gene, a marker for plasmid selection; spec, spectinomycin resistance gene, a marker for plasmid selection; amp, ampicillin resistance gene, a marker for plasmid selection.

FIG. 33 is a schematic of a method for assembly and cloning of custom TAL effector repeat encoding arrays by the Golden Gate approach using the set of plasmids shown in FIG. 32. For illustration purposes, assembly of an arbitrary repeat array is shown. spec, spectinomycin resistance gene, a marker for plasmid selection; amp, ampicillin resistance gene, a marker for plasmid selection.

FIGS. 34A-34U show the amino acid sequences of TALENs generated as described in Example 9 herein. FIG. 34A, telomerase-TALEN124; FIG. 34B, gridlock-TALEN105; FIG. 34C, adh1-TALEN58; FIG. 34D, adh1-TALEN63; FIG. 34E, adh1-TALEN68; FIG. 34F, adh1-TALEN73; FIG. 34G, adh1-TALEN89; FIG. 34H, gridlock-

TALEN106; FIG. 34I, adh1-TALEN64; FIG. 34J, adh1-TALEN69; FIG. 34K, adh1-TALEN74; FIG. 34L, tt4-TALEN90; FIG. 34M, telomerase-TALEN121; FIG. 34N, telomerase-TALEN126; FIG. 34O, gridlock-TALEN107; FIG. 34P, gridlock-TALEN117; FIG. 34Q, telomerase-TALEN131; FIG. 34R, telomerase-TALEN136; FIG. 34S, adh1-TALEN60; FIG. 34 T, tt4-TALEN85; FIG. 34U, gridlock-TALEN102.

FIG. 35 is a graph plotting TALEN activity as measured by the yeast assay using custom TALEN monomers of increasing length (9-, 10-, 12-, 13-, 15-, 16-, 17-, or 18 mers). The TALENs were targeted to *Arabidopsis* and zebrafish genes, as indicated.

FIG. 36A is a diagram showing two different DNA target sequences from the Arabidopsis *ADH1* gene that are targeted by two TALEN pairs. FIG. 36B is a graph plotting yeast assay data for functional TALEN pairs that target the Arabidopsis *ADH1* gene.

FIG. 37A is a schematic of a restriction endonuclease assay used to detect TALEN-induced mutations in *Arabidopsis* protoplasts. FIG. 37B shows the sequences of nine clones from undigested DNA in the restriction endonuclease assay. Six of the clones have mutations introduced by non-homologous end-joining (NHEJ).

FIG. 38A shows 0th repeat sequences of several phylogenetically distinct TAL effectors, AvrHah1 from *Xanthomonas gardneri*, AvrBs3 from *X. campestris* pv. vesicatoria, PthXo1 from *X. oryzae* pv. oryzae, PthA from *X. citri*, and Tal1c from *X. oryzae* pv. oryzicola. Polymorphic positions are boxed. FIG. 38B is a schematic showing the 0th and 1st repeats of PthXo1. The "0th" repeat immediately precedes the 1st repeat, shows 35% identity, and has a similar predicted secondary structure. The RVD of the 1st repeat and the candidate analogous residues of the 0th repeat are underlined. *, gap; H, helix; E, extended. The structure was predicted using JPred (Cole et al. (2008) *Nucl. Acids Res.* 36:W197-W201).

FIG. 39 shows a western blot of total protein isolated from human embryonic kidney 293T cells transfected with plasmids encoding V5-tagged TAL effector proteins AvrBs3, PthXo1, and Tal1c, as indicated, following immunodetection using a mouse-antiV5 antibody. Immunolabeled actin is shown as a control for equivalent loading in each lane.

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FIG. 40A shows the amino acid sequence of TALEN HPRT-3254-17, and FIG. 40B shows the amino acid sequence of TALEN HPRT-3286-20r.

FIG. 41A is a schematic showing the TALEN-targeted site in the human chromosomal HPRT gene. Binding sites for the HPRT-3254-17 and HPRT-3286-20r TALENs, the *Bpu*10I site in the spacer between those sites, and the primer sites for amplification of the region are indicated. Coordinates at the bottom give distance in base pairs from the first nucleotide of the coding sequence. FIG. 41B shows the results of *Bpu*10I digestion of products of PCR amplification of the region shown in FIG. 41A using genomic DNA isolated from TALEN-treated and untreated cells as templates. Genomic DNA was digested with *Bpu*10I prior to amplification. DNA fragments were separated by agarose gel electrophoresis and visualized using ethidium bromide.

DETAILED DESCRIPTION

The present patent application provides materials and methods related to sequence specific DNA recognition mediated by TAL effectors. As described herein, the primary amino acid sequences of TAL effectors dictate the nucleotide sequences to which they bind. The inventors have found that relationships between TAL effector amino acid sequences and their DNA target sequences are direct, enabling target site prediction for TAL effectors, and also allowing for TAL effector customization to bind to particular nucleotide sequences. Such prediction and customization can be harnessed for a variety of purposes. In one example, particular TAL effector sequences can be fused to endonuclease sequences, allowing for endonuclease targeting to specific DNA sequences, and subsequent cutting of the DNA at or near the targeted sequences. Cuts (i.e., double-stranded breaks) in DNA can dramatically increase the frequency of homologous recombination. Thus, in combination with DNA constructs that carry sequences having a high degree of sequence similarity to a particular target DNA sequence, TALENs can be used to facilitate site directed mutagenesis in complex genomes, that is, to knock out or alter gene function, or to add genes or other sequences with great precision and high efficiency.

Thus, included in the subject matter provided herein are, *inter alia*, materials and methods for making genetically modified organisms (including, without limitation,

plants, fungi, *Drosophila*, nematodes, zebrafish, mice, other mammals and humans). Such methods can include, for example, transfecting a cell with several recombinant nucleic acids. For example, a cell (e.g., a eukaryotic cell) can be transformed with a first recombinant nucleic acid construct containing a donor nucleotide sequence that includes alterations relative to a corresponding target nucleotide sequence found within the cell, and a second recombinant nucleic acid construct encoding a TAL-nuclease. In some embodiments, the cell also can be transformed with a third recombinant nucleic acid construct encoding a selectable marker. A nucleic acid sequence from the donor nucleic acid construct can become incorporated into the genome of the transformed cell as described herein. For example, plant cells produced using methods as described herein can be grown to produce plants having the altered donor nucleotide sequence incorporated into their genomes. Seeds from such plants can be used to produce plants having a phenotype such as, for example, an altered growth characteristic (e.g., increased resistance or tolerance to various biotic and abiotic stresses), altered appearance (e.g., altered color or height), or altered composition (e.g., increased or decreased levels of carbon, nitrogen, oil, protein, carbohydrate (e.g., sugar or starch), amino acid, fatty acid, or secondary metabolites) with respect to unmodified plants.

Polynucleotides and Polypeptides

Isolated nucleic acids and polypeptides are provided herein. The terms "nucleic acid" and "polynucleotide" are used interchangeably, and refer to both RNA and DNA, including cDNA, genomic DNA, synthetic (e.g., chemically synthesized) DNA, and DNA (or RNA) containing nucleic acid analogs. Polynucleotides can have any three-dimensional structure. A nucleic acid can be double-stranded or single-stranded (i.e., a sense strand or an antisense single strand). Non-limiting examples of polynucleotides include genes, gene fragments, exons, introns, messenger RNA (mRNA), transfer RNA, ribosomal RNA, ribozymes, cDNA, recombinant polynucleotides, branched polynucleotides, plasmids, vectors, isolated DNA of any sequence, isolated RNA of any sequence, nucleic acid probes, and primers, as well as nucleic acid analogs.

The polypeptides of the present invention (such as TAL effector-DNA modifying enzyme as non-limiting example) can be introduced in a cell by using a vector encoding

said polypeptides for example or as polypeptides *per se* by using delivery vectors associated or combined with any cellular permeabilization techniques such as sonoporation or electroporation or derivatives of these techniques.

As used herein, "isolated," when in reference to a nucleic acid, refers to a nucleic acid that is separated from other nucleic acids that are present in a genome, e.g., a plant genome, including nucleic acids that normally flank one or both sides of the nucleic acid in the genome. The term "isolated" as used herein with respect to nucleic acids also includes any non-naturally-occurring sequence, since such non-naturally-occurring sequences are not found in nature and do not have immediately contiguous sequences in a naturally-occurring genome.

An isolated nucleic acid can be, for example, a DNA molecule, provided one of the nucleic acid sequences normally found immediately flanking that DNA molecule in a naturally-occurring genome is removed or absent. Thus, an isolated nucleic acid includes, without limitation, a DNA molecule that exists as a separate molecule (e.g., a chemically synthesized nucleic acid, or a cDNA or genomic DNA fragment produced by PCR or restriction endonuclease treatment) independent of other sequences, as well as DNA that is incorporated into a vector, an autonomously replicating plasmid, a virus (e.g., a pararetrovirus, a retrovirus, lentivirus, adenovirus, or herpes virus), or the genomic DNA of a prokaryote or eukaryote. In addition, an isolated nucleic acid can include a recombinant nucleic acid such as a DNA molecule that is part of a hybrid or fusion nucleic acid. A nucleic acid existing among hundreds to millions of other nucleic acids within, for example, cDNA libraries or genomic libraries, or gel slices containing a genomic DNA restriction digest, is not to be considered an isolated nucleic acid.

A nucleic acid can be made by, for example, chemical synthesis or polymerase chain reaction (PCR). PCR refers to a procedure or technique in which target nucleic acids are amplified. PCR can be used to amplify specific sequences from DNA as well as RNA, including sequences from total genomic DNA or total cellular RNA. Various PCR methods are described, for example, in PCR Primer: A Laboratory Manual, Dieffenbach and Dveksler, eds., Cold Spring Harbor Laboratory Press, 1995. Generally, sequence information from the ends of the region of interest or beyond is employed to design oligonucleotide primers that are identical or similar in sequence to opposite strands of the

template to be amplified. Various PCR strategies also are available by which site-specific nucleotide sequence modifications can be introduced into a template nucleic acid.

Isolated nucleic acids also can be obtained by mutagenesis. For example, a donor nucleic acid sequence can be mutated using standard techniques, including oligonucleotide-directed mutagenesis and site-directed mutagenesis through PCR. See, Short Protocols in Molecular Biology, Chapter 8, Green Publishing Associates and John Wiley & Sons, edited by Ausubel *et al.*, 1992.

The term "polypeptide" as used herein refers to a compound of two or more subunit amino acids regardless of post-translational modification (e.g., phosphorylation or glycosylation). The subunits may be linked by peptide bonds or other bonds such as, for example, ester or ether bonds. The term "amino acid" refers to either natural and/or unnatural or synthetic amino acids, including D/L optical isomers.

By "isolated" or "purified" with respect to a polypeptide it is meant that the polypeptide is separated to some extent from the cellular components with which it is normally found in nature (e.g., other polypeptides, lipids, carbohydrates, and nucleic acids). An purified polypeptide can yield a single major band on a non-reducing polyacrylamide gel. A purified polypeptide can be at least about 75% pure (e.g., at least 80%, 85%, 90%, 95%, 97%, 98%, 99%, or 100% pure). Purified polypeptides can be obtained by, for example, extraction from a natural source, by chemical synthesis, or by recombinant production in a host cell or transgenic plant, and can be purified using, for example, affinity chromatography, immunoprecipitation, size exclusion chromatography, and ion exchange chromatography. The extent of purification can be measured using any appropriate method, including, without limitation, column chromatography, polyacrylamide gel electrophoresis, or high-performance liquid chromatography.

Recombinant Constructs

Recombinant nucleic acid constructs (e.g., vectors) also are provided herein. A "vector" is a replicon, such as a plasmid, phage, or cosmid, into which another DNA segment may be inserted so as to bring about the replication of the inserted segment. Generally, a vector is capable of replication when associated with the proper control

elements. Suitable vector backbones include, for example, those routinely used in the art such as plasmids, viruses, artificial chromosomes, BACs, YACs, or PACs. The term "vector" includes cloning and expression vectors, as well as viral vectors and integrating vectors. An "expression vector" is a vector that includes one or more expression control sequences, and an "expression control sequence" is a DNA sequence that controls and regulates the transcription and/or translation of another DNA sequence. Suitable expression vectors include, without limitation, plasmids and viral vectors derived from, for example, bacteriophage, baculoviruses, tobacco mosaic virus, herpes viruses, cytomegalovirus, retroviruses, vaccinia viruses, adenoviruses, and adeno-associated viruses. Numerous vectors and expression systems are commercially available from such corporations as Novagen (Madison, WI), Clontech (Palo Alto, CA), Stratagene (La Jolla, CA), and Invitrogen/Life Technologies (Carlsbad, CA).

The terms "regulatory region," "control element," and "expression control sequence" refer to nucleotide sequences that influence transcription or translation initiation and rate, and stability and/or mobility of the transcript or polypeptide product. Regulatory regions include, without limitation, promoter sequences, enhancer sequences, response elements, protein recognition sites, inducible elements, promoter control elements, protein binding sequences, 5' and 3' untranslated regions (UTRs), transcriptional start sites, termination sequences, polyadenylation sequences, introns, and other regulatory regions that can reside within coding sequences, such as secretory signals, Nuclear Localization Sequences (NLS) and protease cleavage sites.

As used herein, "operably linked" means incorporated into a genetic construct so that expression control sequences effectively control expression of a coding sequence of interest. A coding sequence is "operably linked" and "under the control" of expression control sequences in a cell when RNA polymerase is able to transcribe the coding sequence into RNA, which if an mRNA, then can be translated into the protein encoded by the coding sequence. Thus, a regulatory region can modulate, *e.g.*, regulate, facilitate or drive, transcription in the plant cell, plant, or plant tissue in which it is desired to express a modified target nucleic acid.

A promoter is an expression control sequence composed of a region of a DNA molecule, typically within 100 nucleotides upstream of the point at which transcription starts

(generally near the initiation site for RNA polymerase II). Promoters are involved in recognition and binding of RNA polymerase and other proteins to initiate and modulate transcription. To bring a coding sequence under the control of a promoter, it typically is necessary to position the translation initiation site of the translational reading frame of the polypeptide between one and about fifty nucleotides downstream of the promoter. A promoter can, however, be positioned as much as about 5,000 nucleotides upstream of the translation start site, or about 2,000 nucleotides upstream of the transcription start site. A promoter typically comprises at least a core (basal) promoter. A promoter also may include at least one control element such as an upstream element. Such elements include upstream activation regions (UARs) and, optionally, other DNA sequences that affect transcription of a polynucleotide such as a synthetic upstream element.

The choice of promoters to be included depends upon several factors, including, but not limited to, efficiency, selectability, inducibility, desired expression level, and cell or tissue specificity. For example, tissue-, organ- and cell-specific promoters that confer transcription only or predominantly in a particular tissue, organ, and cell type, respectively, can be used. In some embodiments, promoters specific to vegetative tissues such as the stem, parenchyma, ground meristem, vascular bundle, cambium, phloem, cortex, shoot apical meristem, lateral shoot meristem, root apical meristem, lateral root meristem, leaf primordium, leaf mesophyll, or leaf epidermis can be suitable regulatory regions. In some embodiments, promoters that are essentially specific to seeds ("seed-preferential promoters") can be useful. Seed-specific promoters can promote transcription of an operably linked nucleic acid in endosperm and cotyledon tissue during seed development. Alternatively, constitutive promoters can promote transcription of an operably linked nucleic acid in most or all tissues of a plant, throughout plant development. Other classes of promoters include, but are not limited to, inducible promoters, such as promoters that confer transcription in response to external stimuli such as chemical agents, developmental stimuli, or environmental stimuli.

A basal promoter is the minimal sequence necessary for assembly of a transcription complex required for transcription initiation. Basal promoters frequently include a "TATA box" element that may be located between about 15 and about 35 nucleotides upstream from the site of transcription initiation. Basal promoters also may include a "CCAAT box"

element (typically the sequence CCAAT) and/or a GGGCG sequence, which can be located between about 40 and about 200 nucleotides, typically about 60 to about 120 nucleotides, upstream from the transcription start site.

Non-limiting examples of promoters that can be included in the nucleic acid constructs provided herein include the cauliflower mosaic virus (CaMV) 35S transcription initiation region, the 1' or 2' promoters derived from T-DNA of *Agrobacterium tumefaciens*, promoters from a maize leaf-specific gene described by Busk ((1997) *Plant J.* 11:1285-1295), kn1-related genes from maize and other species, and transcription initiation regions from various plant genes such as the maize ubiquitin-1 promoter.

A 5' untranslated region (UTR) is transcribed, but is not translated, and lies between the start site of the transcript and the translation initiation codon and may include the +1 nucleotide. A 3' UTR can be positioned between the translation termination codon and the end of the transcript. UTRs can have particular functions such as increasing mRNA message stability or translation attenuation. Examples of 3' UTRs include, but are not limited to polyadenylation signals and transcription termination sequences. A polyadenylation region at the 3'-end of a coding region can also be operably linked to a coding sequence. The polyadenylation region can be derived from the natural gene, from various other plant genes, or from an *Agrobacterium* T-DNA.

The vectors provided herein also can include, for example, origins of replication, and/or scaffold attachment regions (SARs). In addition, an expression vector can include a tag sequence designed to facilitate manipulation or detection (e.g., purification or localization) of the expressed polypeptide. Tag sequences, such as green fluorescent protein (GFP), glutathione S-transferase (GST), polyhistidine, c-myc, hemagglutinin, or Flag™ tag (Kodak, New Haven, CT) sequences typically are expressed as a fusion with the encoded polypeptide. Such tags can be inserted anywhere within the polypeptide, including at either the carboxyl or amino terminus.

By " delivery vector" or " delivery vectors" is intended any delivery vector which can be used in the present invention to put into cell contact or deliver inside cells or subcellular compartments agents/chemicals and molecules (proteins or nucleic acids) needed in the present invention. It includes, but is not limited to liposomal delivery vectors, viral delivery vectors, drug delivery vectors, chemical carriers, polymeric

carriers, lipoplexes, polyplexes, dendrimers, microbubbles (ultrasound contrast agents), nanoparticles, emulsions or other appropriate transfer vectors. These delivery vectors allow delivery of molecules, chemicals, macromolecules (genes, proteins), or other vectors such as plasmids, peptides developed by Diatos. In these cases, delivery vectors are molecule carriers. By “delivery vector” or “delivery vectors” is also intended delivery methods to perform transfection.

- The terms "vector" or “vectors” refer to a nucleic acid molecule capable of transporting another nucleic acid to which it has been linked. A “vector” in the present invention includes, but is not limited to, a viral vector, a plasmid, a RNA vector or a linear or circular DNA or RNA molecule which may consists of a chromosomal, non chromosomal, semi-synthetic or synthetic nucleic acids. Preferred vectors are those capable of autonomous replication (episomal vector) and/or expression of nucleic acids to which they are linked (expression vectors). Large numbers of suitable vectors are known to those of skill in the art and commercially available.

Viral vectors include retrovirus, adenovirus, parvovirus (e. g. adenoassociated viruses), coronavirus, negative strand RNA viruses such as orthomyxovirus (e. g., influenza virus), rhabdovirus (e. g., rabies and vesicular stomatitis virus), paramyxovirus (e. g. measles and Sendai), positive strand RNA viruses such as picornavirus and alphavirus, and double-stranded DNA viruses including adenovirus, herpesvirus (e. g., Herpes Simplex virus types 1 and 2, Epstein-Barr virus, cytomegalovirus), and poxvirus (e. g., vaccinia, fowlpox and canarypox). Other viruses include Norwalk virus, togavirus, flavivirus, reoviruses, papovavirus, hepadnavirus, and hepatitis virus, for example. Examples of retroviruses include: avian leukosis-sarcoma, mammalian C-type, B-type viruses, D type viruses, HTLV-BLV group, lentivirus, spumavirus (Coffin, J. M., Retroviridae: The viruses and their replication, In Fundamental Virology, Third Edition, B. N. Fields, et al., Eds., Lippincott-Raven Publishers, Philadelphia, 1996).

- By “lentiviral vector” is meant HIV-Based lentivirus vectors that are very promising for gene delivery because of their relatively large packaging capacity, reduced immunogenicity and their ability to stably transduce with high efficiency a large range of different cell types. Lentiviral vectors are usually generated following transient transfection of three (packaging, envelope and transfer) or more plasmids into producer

cells. Like HIV, lentiviral vectors enter the target cell through the interaction of viral surface glycoproteins with receptors on the cell surface. On entry, the viral RNA undergoes reverse transcription, which is mediated by the viral reverse transcriptase complex. The product of reverse transcription is a double-stranded linear viral DNA, which is the substrate for viral integration in the DNA of infected cells. Said lentiviral vectors can be "non-integrative" or "integrative".

- By "integrative lentiviral vectors (or LV)", is meant such vectors as non limiting example, that are able to integrate the genome of a target cell.

- At the opposite by "non integrative lentiviral vectors (or NILV)" is meant efficient gene delivery vectors that do not integrate the genome of a target cell through the action of the virus integrase.

One type of preferred vector is an episome, i.e., a nucleic acid capable of extra-chromosomal replication. Preferred vectors are those capable of autonomous replication and/or expression of nucleic acids to which they are linked. Vectors capable of directing the expression of genes to which they are operatively linked are referred to herein as "expression vectors. A vector according to the present invention comprises, but is not limited to, a YAC (yeast artificial chromosome), a BAC (bacterial artificial), a baculovirus vector, a phage, a phagemid, a cosmid, a viral vector, a plasmid, a RNA vector or a linear or circular DNA or RNA molecule which may consist of chromosomal, non chromosomal, semi-synthetic or synthetic DNA. In general, expression vectors of utility in recombinant DNA techniques are often in the form of "plasmids" which refer generally to circular double stranded DNA loops which, in their vector form are not bound to the chromosome. Large numbers of suitable vectors are known to those of skill in the art. Vectors can comprise selectable markers, for example: neomycin phosphotransferase, histidinol dehydrogenase, dihydrofolate reductase, hygromycin phosphotransferase, herpes simplex virus thymidine kinase, adenosine deaminase, glutamine synthetase, and hypoxanthine-guanine phosphoribosyl transferase for eukaryotic cell culture; TRP1 for *S. cerevisiae*; tetracyclin, rifampicin or ampicillin resistance in *E. coli*. Preferably said vectors are expression vectors, wherein a sequence encoding a polypeptide of interest is placed under control of appropriate transcriptional and translational control elements to permit production or synthesis of said polypeptide.

Therefore, said polynucleotide is comprised in an expression cassette. More particularly, the vector comprises a replication origin, a promoter operatively linked to said encoding polynucleotide, a ribosome binding site, a RNA-splicing site (when genomic DNA is used), a polyadenylation site and a transcription termination site. It also can comprise an enhancer or silencer elements. Selection of the promoter will depend upon the cell in which the polypeptide is expressed. Suitable promoters include tissue specific and/or inducible promoters. Examples of inducible promoters are: eukaryotic metallothionine promoter which is induced by increased levels of heavy metals, prokaryotic lacZ promoter which is induced in response to isopropyl-β-D-thiogalacto-pyranoside (IPTG) and eukaryotic heat shock promoter which is induced by increased temperature. Examples of tissue specific promoters are skeletal muscle creatine kinase, prostate-specific antigen (PSA), α-antitrypsin protease, human surfactant (SP) A and B proteins, β-casein and acidic whey protein genes.

Inducible promoters may be induced by pathogens or stress, more preferably by stress like cold, heat, UV light, or high ionic concentrations (reviewed in Potenza et al. (2004) *In vitro Cell Dev Biol* 40:1-22). Inducible promoter may be induced by chemicals [reviewed in Moore et al. (2006); Padidam (2003); (Wang et al. (2003); and (Zuo and Chua (2000)].

Delivery vectors and vectors can be associated or combined with any cellular permeabilization techniques such as sonoporation or electroporation or derivatives of these techniques.

It will be understood that more than one regulatory region may be present in a recombinant polynucleotide, e.g., introns, enhancers, upstream activation regions, and inducible elements.

Recombinant nucleic acid constructs can include a polynucleotide sequence inserted into a vector suitable for transformation of cells (e.g., plant cells or animal cells). Recombinant vectors can be made using, for example, standard recombinant DNA techniques (*see*, e.g., Sambrook *et al.* (1989) Molecular Cloning: A Laboratory Manual, 2nd ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, NY).

A recombinant nucleic acid sequence as described herein can integrate into the genome of a cell via illegitimate (i.e., random, non-homologous, non site-specific)

recombination, or a recombinant nucleic acid sequence as described herein can be adapted to integrate into the genome of a cell via homologous recombination. Nucleic acid sequences adapted for integration via homologous recombination are flanked on both sides with sequences that are similar or identical to endogenous target nucleotide sequences, which facilitates integration of the recombinant nucleic acid at the particular site(s) in the genome containing the endogenous target nucleotide sequences. Nucleic acid sequences adapted for integration via homologous recombination also can include a recognition site for a sequence-specific nuclease. Alternatively, the recognition site for a sequence-specific nuclease can be located in the genome of the cell to be transformed. Donor nucleic acid sequences as described below typically are adapted for integration via homologous recombination.

In some embodiments, a nucleic acid encoding a selectable marker also can be adapted to integrate via homologous recombination, and thus can be flanked on both sides with sequences that are similar or identical to endogenous sequences within the plant genome (e.g., endogenous sequences at the site of cleavage for a sequence-specific nuclease). In some cases, nucleic acid containing coding sequence for a selectable marker also can include a recognition site for a sequence-specific nuclease. In these embodiments, the recognition site for the sequence-specific nuclease can be the same as or different from that contained within the donor nucleic acid sequence (i.e., can be recognized by the same nuclease as the donor nucleic acid sequence, or recognized by a different nuclease than the donor nucleic acid sequence).

In some cases, a recombinant nucleic acid sequence can be adapted to integrate into the genome of a cell via site-specific recombination. As used herein, "site-specific" recombination refers to recombination that occurs when a nucleic acid sequence is targeted to a particular site(s) within a genome not by homology between sequences in the recombinant nucleic acid and sequences in the genome, but rather by the action of recombinase enzymes that recognize specific nucleic acid sequences and catalyze the reciprocal exchange of DNA strands between these sites. Site-specific recombination thus refers to the enzyme-mediated cleavage and ligation of two defined nucleotide sequences. Any suitable site-specific recombination system can be used, including, for example, the Cre-lox system or the FLP-FRT system. In such embodiments, a nucleic

acid encoding a recombinase enzyme may be introduced into a cell in addition to a donor nucleotide sequence and a nuclease-encoding sequence, and in some cases, a selectable marker sequence. See, e.g., U.S. Patent No. 4,959,317.

Sequence-specific endonucleases

Sequence-specific nucleases and recombinant nucleic acids encoding the sequence-specific endonucleases are provided herein. The sequence-specific endonucleases can include TAL effector DNA binding domains and endonuclease domains. Thus, nucleic acids encoding such sequence-specific endonucleases can include a nucleotide sequence from a sequence-specific TAL effector linked to a nucleotide sequence from a nuclease.

TAL effectors are proteins of plant pathogenic bacteria that are injected by the pathogen into the plant cell, where they travel to the nucleus and function as transcription factors to turn on specific plant genes. The primary amino acid sequence of a TAL effector dictates the nucleotide sequence to which it binds. Thus, target sites can be predicted for TAL effectors, and TAL effectors also can be engineered and generated for the purpose of binding to particular nucleotide sequences, as described herein.

Fused to the TAL effector-encoding nucleic acid sequences are sequences encoding a nuclease or a portion of a nuclease, typically a nonspecific cleavage domain from a type II restriction endonuclease such as *Fok*I (Kim *et al.* (1996) *Proc. Natl. Acad. Sci. USA* 93:1156-1160). Other useful endonucleases may include, for example, *Hha*I, *Hind*III, *Not*I, *BbvC*I, *EcoR*I, *Bgl*I, and *Alw*I. The fact that some endonucleases (e.g., *Fok*I) only function as dimers can be capitalized upon to enhance the target specificity of the TAL effector. For example, in some cases each *Fok*I monomer can be fused to a TAL effector sequence that recognizes a different DNA target sequence, and only when the two recognition sites are in close proximity do the inactive monomers come together to create a functional enzyme. By requiring DNA binding to activate the nuclease, a highly site-specific restriction enzyme can be created.

A sequence-specific TALEN as provided herein can recognize a particular sequence within a preselected target nucleotide sequence present in a cell. Thus, in some embodiments, a target nucleotide sequence can be scanned for nuclease recognition sites,

and a particular nuclease can be selected based on the target sequence. In other cases, a TALEN can be engineered to target a particular cellular sequence. A nucleotide sequence encoding the desired TALEN can be inserted into any suitable expression vector, and can be linked to one or more expression control sequences. For example, a nuclease coding sequence can be operably linked to a promoter sequence that will lead to constitutive expression of the endonuclease in the species of plant to be transformed. Alternatively, an endonuclease coding sequence can be operably linked to a promoter sequence that will lead to conditional expression (e.g., expression under certain nutritional conditions). For example, a cauliflower mosaic virus 35S promoter can be used for constitutive expression. Other constitutive promoters include, without limitation, the nopaline synthase promoter, the ubiquitin promoter, and the actin promoter. In some embodiments, an artificial estrogen-induced promoter for can be used conditional expression, and high levels of transcription can be achieved when a plant is exposed to estrogen. Other conditional promoters that can be used include, for example, heat-inducible heat shock gene promoters, and light-regulated promoters such as that from the gene encoding the large subunit of ribulose bisphosphate carboxylase.

For purposes of therapy, the TAL effector-DNA modifying enzyme of the present invention and a pharmaceutically acceptable excipient are administered in a therapeutically effective amount. Such a combination is said to be administered in a "therapeutically effective amount" if the amount administered is physiologically significant. An agent is physiologically significant if its presence results in a detectable change in the physiology of the recipient. In the present context, an agent is physiologically significant if its presence results in a decrease in the severity of one or more symptoms of the targeted disease and in a genome correction of the lesion or abnormality. Vectors comprising targeting DNA and/or nucleic acid encoding a TAL effector-DNA modifying enzyme can be introduced into a cell by a variety of methods (e.g., injection, direct uptake, projectile bombardment, liposomes, electroporation). TAL effector-DNA modifying enzymes can be stably or transiently expressed into cells using expression vectors. Techniques of expression in eukaryotic cells are well known to those in the art. (See Current Protocols in Human Genetics: Chapter 12 “Vectors For Gene Therapy” and Chapter 13 “Delivery Systems for Gene Therapy”).

In one further aspect of the present invention, the TAL effector-DNA modifying enzyme is substantially non-immunogenic, i.e., engender little or no adverse immunological response. A variety of methods for ameliorating or eliminating deleterious immunological reactions of this sort can be used in accordance with the invention. In a preferred embodiment, the TAL effector-DNA modifying enzyme is substantially free of N-formyl methionine. Another way to avoid unwanted immunological reactions is to conjugate TAL effector-DNA modifying enzyme to polyethylene glycol ("PEG") or polypropylene glycol ("PPG") (preferably of 500 to 20,000 daltons average molecular weight (MW)). Conjugation with PEG or PPG, as described by Davis *et al.* (US 4,179,337) for example, can provide non-immunogenic, physiologically active, water soluble TAL effector-DNA modifying enzyme conjugates with anti-viral activity. Similar methods also using a polyethylene--polypropylene glycol copolymer are described in Saifer *et al.* (US 5,006,333).

Donor vectors

Also provided herein are recombinant nucleic acids including donor nucleotide sequences. A donor nucleotide sequence can include a variant sequence having one or more modifications (i.e., substitutions, deletions, or insertions) with respect to a preselected target nucleotide sequence found endogenously within the genome of a cell to be transformed (also referred to herein as a "modified target nucleotide sequence"). The variant sequence within the donor nucleic acid typically is flanked on both sides with sequences that are similar or identical to the endogenous target nucleotide sequence within the cell. The flanking sequences can have any suitable length, and typically are at least 50 nucleotides in length (e.g., at least 50 nucleotides, at least 75 nucleotides, at least 100 nucleotides, at least 200 nucleotides, at least 250 nucleotides, at least 300 nucleotides, at least 500 nucleotides, at least 750 nucleotides, at least 1000 nucleotides, from about 50 to about 5000 nucleotides, from about 100 to 2500 nucleotides, from about 100 to about 1000 nucleotides, from about 100 to 500 nucleotides, from about 200 to about 500 nucleotides, or from about 250 to 400 nucleotides). Thus, homologous recombination can occur between the recombinant donor nucleic acid construct and the endogenous target on both sides of the variant sequence, such that the resulting cell's

genome contains the variant sequence within the context of endogenous sequences from, for example, the same gene. A donor nucleotide sequence can be generated to target any suitable sequence within a genome. In a plant, for example, a donor nucleotide sequence can be targeted to a lipid biosynthetic gene, carbohydrate biosynthetic gene, seed storage protein gene, disease or pest resistance gene, stress tolerance gene, drought tolerance gene, or a gene that produces an anti-nutritional. In addition, the donor nucleotide sequence contains a recognition site for a sequence-specific nuclease, as described herein.

Selectable markers

Some of the methods provided herein include the use of a third recombinant nucleic acid encoding a selectable or screenable marker. A nucleotide sequence encoding a polypeptide that results in a selectable trait can be incorporated into an expression vector containing one or more expression control sequences. For example, an expression vector can include sequence encoding a selectable marker operably linked to a promoter sequence that will lead to constitutive expression in the plant cell to be transformed. Suitable selectable markers can include, without limitation, polypeptides conferring resistance to an antibiotic such as kanamycin, G418, bleomycin, ampicillin, or hygromycin, or an herbicide such as glufosinate, chlorosulfuron, or phosphinothricin.

In embodiments for use in plants, for example, a selectable marker can confer resistance to an herbicide that inhibits the growing point or meristem, such as an imidazolinone or a sulfonylurea. Exemplary polypeptides in this category code for mutant ALS and AHAS enzymes as described, for example, in U.S. Patent Nos. 5,767,366 and 5,928,937. U.S. Patent Nos. 4,761,373 and 5,013,659 are directed to plants resistant to various imidazolinone or sulfonamide herbicides. U.S. Patent No. 4,975,374 relates to plant cells and plants containing a gene encoding a mutant glutamine synthetase (GS) resistant to inhibition by herbicides that are known to inhibit GS, e.g., phosphinothricin and methionine sulfoximine. U.S. Patent No. 5,162,602 discloses plants resistant to inhibition by cyclohexanedione and aryloxyphenoxypropanoic acid herbicides. The resistance is conferred by an altered acetyl coenzyme A carboxylase (ACCase).

Polypeptides for resistance to glyphosate (sold under the trade name Roundup®) also are suitable for use in plants. See, for example, U.S. Patent Nos. 4,940,835 and 4,769,061. U.S. Patent No. 5,554,798 discloses transgenic glyphosate resistant maize plants, in which resistance is conferred by an altered 5-enolpyruvyl-3-phosphoshikimate (EPSP) synthase. Such polypeptides can confer resistance to glyphosate herbicidal compositions including, without limitation, glyphosate salts such as the trimethylsulphonium salt, the isopropylamine salt, the sodium salt, the potassium salt and the ammonium salt. See, e.g., U.S. Patent Nos. 6,451,735 and 6,451,732.

Polypeptides for resistance to phosphono compounds such as glufosinate ammonium or phosphinothricin, and pyridinoxy or phenoxy propionic acids and cyclohexones also are suitable. See, for example, European Publication No. 0 242 246, as well as U.S. Patent Nos. 5,879,903, 5,276,268, and 5,561,236.

Other herbicides include those that inhibit photosynthesis, such as triazine and benzonitrile (nitrilase). See, e.g., U.S. Patent No. 4,810,648. Other herbicides include 2,2-dichloropropionic acid, sethoxydim, haloxyfop, imidazolinone herbicides, sulfonylurea herbicides, triazolopyrimidine herbicides, s-triazine herbicides and bromoxynil. Also suitable are herbicides that confer resistance to a protox enzyme. See, e.g., U.S. Patent Publication No. 20010016956 and U.S. Patent No. 6,084,155.

In some embodiments, a recombinant nucleic acid encoding a selectable marker can be adapted to integrate into the genome of a cell (e.g., a plant cell or an animal cell) by site-specific recombination. For example, a sequence encoding a selectable marker can be flanked by recognition sequences for a recombinase such as, e.g., Cre or FLP. In other embodiments, a recombinant nucleic acid encoding a selectable marker can be adapted for integration into a plant genome by homologous recombination. In such nucleic acids, the sequence encoding the selectable marker can be flanked by sequences that are similar or identical to endogenous nucleotide sequences found within the genome of the plant cell into which the recombinant nucleic acid is to be introduced. At least one of the endogenous sequences can be at the cleavage site for a sequence-specific nuclease. The nucleic acid encoding the selectable marker also can contain a recognition site for a sequence-specific nuclease. The nuclease can be the same sequence-specific nuclease as that which is targeted to the donor nucleotide sequence, or a sequence-specific nuclease

that is different from that targeted to the donor nucleotide sequence. In still other embodiments, a recombinant nucleic acid encoding a selectable marker can be adapted for integration into the genome of a plant cell by illegitimate recombination. Such nucleic acids typically lack the flanking sequences and nuclease recognition sites that are contained within nucleic acids adapted for homologous or site-specific recombination as described herein.

Methods

One or more of the constructs provided herein can be used to transform cells and/or a DNA modifying enzyme can be introduced into cells, such that a genetically modified organism (e.g., a plant or an animal) is generated. Thus, genetically modified organisms and cells containing the nucleic acids and/or polypeptdes described herein also are provided. In some embodiments, a transformed cell has a recombinant nucleic acid construct integrated into its genome, i.e., can be stably transformed. Stably transformed cells typically retain the introduced nucleic acid sequence with each cell division. A construct can integrate in a homologous manner, such that a nucleotide sequence endogenous to the transformed cell is replaced by the construct, where the construct contains a sequence that corresponds to the endogenous sequence, but that contains one or more modifications with respect to the endogenous sequence. It is noted that while a plant or animal containing such a modified endogenous sequence may be termed a "genetically modified organism" (GMO) herein, the modified endogenous sequence is not considered a transgene. A construct also can integrate in an illegitimate manner, such that it integrates randomly into the genome of the transformed cell.

Alternatively, a cell can be transiently transformed, such that the construct is not integrated into its genome. For example, a plasmid vector containing a TALEN coding sequence can be introduced into a cell, such that the TALEN coding sequence is expressed but the vector is not stably integrated in the genome. Transiently transformed cells typically lose some or all of the introduced nucleic acid construct with each cell division, such that the introduced nucleic acid cannot be detected in daughter cells after sufficient number of cell divisions. Nevertheless, expression of the TALEN coding sequence is sufficient to achieve homologous recombination between a donor sequence

and an endogenous target sequence. Both transiently transformed and stably transformed cells can be useful in the methods described herein.

With particular respect to genetically modified plant cells, cells used in the methods described herein can constitute part or all of a whole plant. Such plants can be grown in a manner suitable for the species under consideration, either in a growth chamber, a greenhouse, or in a field. Genetically modified plants can be bred as desired for a particular purpose, e.g., to introduce a recombinant nucleic acid into other lines, to transfer a recombinant nucleic acid to other species or for further selection of other desirable traits. Alternatively, genetically modified plants can be propagated vegetatively for those species amenable to such techniques. Progeny includes descendants of a particular plant or plant line. Progeny of an instant plant include seeds formed on F_1, F_2, F_3, F_4, F_5, F_6 and subsequent generation plants, or seeds formed on BC_1, BC_2, BC_3, and subsequent generation plants, or seeds formed on F_1BC_1, F_1BC_2, F_1BC_3, and subsequent generation plants. Seeds produced by a genetically modified plant can be grown and then selfed (or outcrossed and selfed) to obtain seeds homozygous for the nucleic acid construct.

Genetically modified cells (e.g., plant cells or animal cells) can be grown in suspension culture, or tissue or organ culture, if desired. For the purposes of the methods provided herein, solid and/or liquid tissue culture techniques can be used. When using solid medium, cells can be placed directly onto the medium or can be placed onto a filter film that is then placed in contact with the medium. When using liquid medium, cells can be placed onto a floatation device, e.g., a porous membrane that contacts the liquid medium. Solid medium typically is made from liquid medium by adding agar. For example, a solid medium can be Murashige and Skoog (MS) medium containing agar and a suitable concentration of an auxin, e.g., 2,4-dichlorophenoxyacetic acid (2,4-D), and a suitable concentration of a cytokinin, e.g., kinetin.

A cell can be transformed with one recombinant nucleic acid construct or with a plurality (e.g., 2, 3, 4, or 5) of recombinant nucleic acid constructs. If multiple constructs are utilized, they can be transformed simultaneously or sequentially. Techniques for transforming a wide variety of species are known in the art. The polynucleotides and/or recombinant vectors described herein can be introduced into the genome of a host using

any of a number of known methods, including electroporation, microinjection, and biolistic methods. Alternatively, polynucleotides or vectors can be combined with suitable T-DNA flanking regions and introduced into a conventional *Agrobacterium tumefaciens* host vector. Such *Agrobacterium tumefaciens*-mediated transformation techniques, including disarming and use of binary vectors, are well known in the art. Other gene transfer and transformation techniques include protoplast transformation through calcium or PEG, electroporation-mediated uptake of naked DNA, liposome-mediated transfection, electroporation, viral vector-mediated transformation, and microprojectile bombardment (*see*, *e.g.*, U.S. Patents 5,538,880, 5,204,253, 5,591,616, and 6,329,571). If a plant cell or tissue culture is used as the recipient tissue for transformation, plants can be regenerated from transformed cultures using techniques known to those skilled in the art.

In some embodiments, a DNA modifying enzyme (e.g., a TALEN) can be directly introduced into a cell. For example, a polypeptide can be introduced into a cell by mechanical injection, by delivery via a bacterial type III secretion system, by electroporation, or by *Agrobacterium* mediated transfer. *See*, e.g., Vergunst et al. (2000) *Science* 290:979-982 for a discussion of the *Agrobacterium* VirB/D4 transport system, and its use to mediate transfer of a nucleoprotein T complex into plant cells.

With further respect to plants, the polynucleotides, vectors and polypeptides described herein can be introduced into a number of monocotyledonous and dicotyledonous plants and plant cell systems, including dicots such as safflower, alfalfa, soybean, coffee, amaranth, rapeseed (high erucic acid and canola), peanut or sunflower, as well as monocots such as oil palm, sugarcane, banana, sudangrass, corn, wheat, rye, barley, oat, rice, millet, or sorghum. Also suitable are gymnosperms such as fir and pine.

Thus, the methods described herein can be utilized with dicotyledonous plants belonging, for example, to the orders *Magniolales*, *Illiciales*, *Laurales*, *Piperales*, *Aristochiales*, *Nymphaeales, Ranunculales, Papeverales, Sarraceniaceae, Trochodendrales, Hamamelidales, Eucomiales, Leitneriales, Myricales, Fagales, Casuarinales, Caryophyllales, Batales, Polygonales, Plumbaginales, Dilleniales, Theales, Malvales, Urticales, Lecythidales, Violales, Salicales, Capparales, Ericales, Diapensales, Ebenales, Primulales, Rosales, Fabales, Podostemales, Haloragales,*

Myrtales, Cornales, Proteales, Santales, Rafflesiales, Celastrales, Euphorbiales, Rhamnales, Sapindales, Juglandales, Geraniales, Polygalales, Umbellales, Gentianales, Polemoniales, Lamiales, Plantaginales, Scrophulariales, Campanulales, Rubiales, Dipsacales, and *Asterales*. The methods described herein also can be utilized with monocotyledonous plants such as those belonging to the orders *Alismatales, Hydrocharitales, Najadales, Triuridales, Commelinales, Eriocaulales, Restionales, Poales, Juncales, Cyperales, Typhales, Bromeliales, Zingiberales, Arecales, Cyclanthales, Pandanales, Arales, Lilliales*, and *Orchidales*, or with plants belonging to *Gymnospermae*, e.g., *Pinales*, *Ginkgoales*, *Cycadales* and *Gnetales*.

The methods can be used over a broad range of plant species, including species from the dicot genera *Atropa, Alseodaphne*, *Anacardium, Arachis, Beilschmiedia*, *Brassica*, *Carthamus*, *Cocculus*, *Croton*, *Cucumis, Citrus*, *Citrullus*, *Capsicum*, *Catharanthus*, *Cocos*, *Coffea, Cucurbita*, *Daucus*, *Duguetia*, *Eschscholzia*, *Ficus*, *Fragaria*, *Glaucium*, *Glycine, Gossypium, Helianthus, Hevea, Hyoscyamus, Lactuca*, *Landolphia*, *Linum*, *Litsea, Lycopersicon*, *Lupinus*, *Manihot, Majorana, Malus, Medicago, Nicotiana, Olea, Parthenium*, *Papaver*, *Persea*, *Phaseolus*, *Pistacia*, *Pisum*, *Pyrus*, *Prunus*, *Raphanus*, *Ricinus*, *Senecio*, *Sinomenium*, *Stephania*, *Sinapis*, *Solanum*, *Theobroma*, *Trifolium*, *Trigonella, Vicia*, *Vinca*, *Vitis*, and *Vigna*; the monocot genera *Allium*, *Andropogon*, *Aragrostis*, *Asparagus, Avena*, *Cynodon*, *Elaeis, Festuca*, *Festulolium*, *Heterocallis*, *Hordeum*, *Lemna*, *Lolium, Musa, Oryza*, *Panicum*, *Pannesetum*, *Phleum*, *Poa*, *Secale*, *Sorghum*, *Triticum*, and *Zea*; or the gymnosperm genera *Abies*, *Cunninghamia*, *Picea*, *Pinus*, and *Pseudotsuga*.

A transformed cell, callus, tissue, or plant can be identified and isolated by selecting or screening the engineered cells for particular traits or activities, e.g., those encoded by marker genes or antibiotic resistance genes. Such screening and selection methodologies are well known to those having ordinary skill in the art. In addition, physical and biochemical methods can be used to identify transformants. These include Southern analysis or PCR amplification for detection of a polynucleotide; Northern blots, S1 RNase protection, primer-extension, or RT-PCR amplification for detecting RNA transcripts; enzymatic assays for detecting enzyme or ribozyme activity of polypeptides and polynucleotides; and protein gel electrophoresis, Western blots,

immunoprecipitation, and enzyme-linked immunoassays to detect polypeptides. Other techniques such as *in situ* hybridization, enzyme staining, and immunostaining also can be used to detect the presence or expression of polypeptides and/or polynucleotides. Methods for performing all of the referenced techniques are well known.

Polynucleotides that are stably incorporated into plant cells can be introduced into other plants using, for example, standard breeding techniques.

In the context of the present invention, "eukaryotic cells" refer to a fungal, yeast, plant or animal cell or a cell line derived from the organisms listed below and established for *in vitro* culture. More preferably, the fungus can be of the genus *Aspergillus*, *Penicillium*, *Acremonium*, *Trichoderma*, *Chrysoporium*, *Mortierella*, *Kluyveromyces* or *Pichia*. More preferably, the fungus can be of the species *Aspergillus niger*, *Aspergillus nidulans*, *Aspergillus oryzae*, *Aspergillus terreus*, *Penicillium chrysogenum*, *Penicillium citrinum*, *Acremonium chrysogenum*, *Trichoderma reesei*, *Mortierella alpine*, *Chrysosporium lucknowense*, *Kluyveromyces lactis*, *Pichia pastoris* or *Pichia ciferrii*.

In the present invention the plant can be of the genus *Arabidospis*, *Nicotiana*, *Solanum*, *Lactuca*, *Brassica*, *Oryza*, *Asparagus*, *Pisum*, *Medicago*, *Zea*, *Hordeum*, *Secale*, *Triticum*, *Capsicum*, *Cucumis*, *Cucurbita*, *Citrullis*, *Citrus*, or *Sorghum*. More preferably, the plant can be of the species *Arabidospis thaliana*, *Nicotiana tabaccum*, *Solanum lycopersicum*, *Solanum tuberosum*, *Solanum melongena*, *Solanum esculentum*, *Lactuca saliva*, *Brassica napus*, *Brassica oleracea*, *Brassica rapa*, *Oryza glaberrima*, *Oryza sativa*, *Asparagus officinalis*, *Pisum sativum*, *Medicago sativa*, *Zea mays*, *Hordeum vulgare*, *Secale cereal*, *Triticum aestivum*, *Triticum durum*, *Capsicum sativus*, *Cucurbita pepo*, *Citrullus lanatus*, *Cucumis melo*, *Citrus aurantifolia*, *Citrus maxima*, *Citrus medica*, or *Citrus reticulata*.

In the present invention the animal cell can be of the genus *Homo*, *Rattus*, *Mus*, *Sus*, *Bos*, *Danio*, *Canis*, *Felis*, *Equus*, *Salmo*, *Oncorhynchus*, *Gallus*, *Meleagris*, *Drosophila*, or *Caenorhabditis*; more preferably, the animal cell can be of the species *Homo sapiens*, *Rattus norvegicus*, *Mus musculus*, *Sus scrofa*, *Bos taurus*, *Danio rerio*, *Canis lupus*, *Felis catus*, *Equus caballus*, *Oncorhynchus mykiss*, *Gallus gallus*, or *Meleagris gallopavo*; the animal cell can be a fish cell from *Salmo salar*, Teleost fish or zebrafish species as non-limiting examples. The animal cell in the present invention can

also be an insect cell from *Drosophila melanogaster* as a non-limiting example; the animal cell can also be a worm cell from *Caenorhabditis elegans* as a non-limiting example.

In the present invention, the cell can be a plant cell, a mammalian cell, a fish cell, an insect cell or cell lines derived from these organisms for *in vitro* cultures or primary cells taken directly from living tissue and established for *in vitro* culture. As non limiting examples cell lines can be selected from the group consisting of CHO-K1 cells; HEK293 cells; Caco2 cells; U2-OS cells; NIH 3T3 cells; NSO cells; SP2 cells; CHO-S cells; DG44 cells; K-562 cells, U-937 cells; MRC5 cells; IMR90 cells; Jurkat cells; HepG2 cells; HeLa cells; HT-1080 cells; HCT-116 cells; Hu-h7 cells; Huvec cells; Molt 4 cells.

All these cell lines can be modified by the method of the present invention to provide cell line models to produce, express, quantify, detect, study a gene or a protein of interest; these models can also be used to screen biologically active molecules of interest in research and production in various fields such as chemical, biofuels, therapeutics and agronomy as non-limiting examples.

The present invention also provides methods for harnessing the sequence-specific DNA binding domains within TAL effectors to, for example, alter the genetic material within cells, to modulate gene expression, and to target pathogenic sequences in, e.g., anti-viral therapies. For example, in some embodiments, the present invention provides methods for modifying cellular genetic material. In some embodiments, the methods include introducing a polypeptide containing a TAL effector DNA binding domain, or a nucleic acid encoding such a polypeptide, into a cell. The TAL effector DNA binding domain can be fused to all or a portion of a DNA modifying enzyme (e.g., an endonuclease). In some embodiments, the methods include introducing two or more recombinant nucleic acids into a cell. A first recombinant nucleic acid contains a donor nucleotide sequence that includes one or more modifications (i.e., substitutions, deletions, or insertions) with respect to a corresponding, preselected target nucleotide sequence found in the cell. The donor nucleotide sequence can undergo homologous recombination with the endogenous target nucleotide sequence, such that the endogenous sequence or a portion thereof is replaced with the donor sequence or a portion thereof. The target nucleotide sequence typically includes a recognition site for a sequence-

specific TALEN. In some cases, a target nucleotide sequence can include recognition sites for two or more distinct TALENs (e.g., two opposed target sequences that are distinct, such that TALENs having distinct DNA sequence binding specificity can be used). In such cases, the specificity of DNA cleavage can be increased as compared to cases in which only one target sequence (or multiple copies of the same target sequence) is used.

A second recombinant nucleic acid contains a nucleotide sequence encoding a sequence specific TALEN that binds to the recognition site in the target nucleotide sequence. In some cases, the donor nucleotide sequence and the nucleotide sequence encoding the sequence-specific nuclease can be contained in the same nucleic acid construct. Alternatively, the donor nucleotide sequence and the TALEN coding sequence can be contained in separate constructs, or the TALEN polypeptide can be produced and introduced directly into a cell.

In some embodiments, a third recombinant nucleic acid containing a nucleotide sequence encoding a selectable marker also may be used. The second and third recombinant nucleic acids may undergo recombination with endogenous sequences and thus integrate into the genome of the cell. These recombination events can be illegitimate (i.e., random), or they can occur through homologous recombination or through site-specific recombination. The recombinant nucleic acids can be simultaneously or sequentially transformed into the cell, and can be linearized prior to transformation.

When the cell is a plant cell, the methods provided herein can further include steps such as generating a plant containing the transformed cell, generating progeny of the plant, selecting or screening for plants expressing the selectable marker (if included), generating progeny of the selected plants, and testing the plants (e.g., tissue, seed, precursor cells, or whole plants) or progeny of the plants for recombination at the target nucleotide sequence. In some cases, the methods can include out-crossing the selected plants to remove the selectable marker, and/or screening the selected or out-crossed plants for the absence of the sequence-specific nuclease.

In some embodiments, the present invention provides methods for modifying the genetic material of a cell, e.g., a prokaryotic cell, an animal cell, or a plant cell. The methods can include introducing into the cell a first recombinant nucleic acid containing

a modified target nucleotide sequence that includes one or more modifications in nucleotide sequence with respect to a corresponding target nucleotide sequence present in the cell, as well as a recognition site for a sequence-specific TALEN, and a second recombinant nucleic acid containing a nucleotide sequence encoding the sequence-specific TALEN. When the cell is a plant cell, a plant containing the cell can be generated, and cells, seed, or tissue obtained from the plant (or progeny thereof) can be analyzed for recombination at the target nucleotide sequence. The first and second recombinant nucleic acids can be simultaneously or serially transformed into the cell, and one or both may be linearized prior to transformation. In some cases, the first and second recombinant nucleic acids can be present in the same construct.

In some cases, the method also can include introducing into the cell a third recombinant nucleic acid containing a nucleotide sequence encoding a selectable marker, and determining whether the cell, an organism generated from the cell, or progeny thereof expresses the selectable marker. The method further can include screening the cell, the organism or progeny thereof for the absence of the selectable marker. The nucleotide sequence encoding the selectable marker may or may not be flanked on both sides by nucleotide sequences that are similar or identical to nucleotide sequences endogenous to the cell at the site of cleavage for a second sequence-specific nuclease, or by recognition sites for a sequence-specific recombinase. In some cases, the method also can include the step of out-crossing the organism. Progeny of the out-cross can be screened for the absence of the selectable marker.

The present invention also provides methods for modifying the genetic material of a cell (e.g., a plant cell or an animal cell), comprising providing a cell containing a target DNA sequence, e.g., a chromosomal, mitochondrial, or chloroplast sequence, in which it is desired to have homologous recombination occur, providing a TALEN that contains a DNA modifying enzyme domain (e.g., an endonuclease domain) and a TAL effector domain having a plurality of TAL effector repeats that, in combination, bind to a specific nucleotide sequence within the target DNA sequence, providing a nucleic acid containing a sequence homologous to at least a portion of the target DNA, and contacting the target DNA sequence in the cell with the TAL endonuclease such that both strands of a nucleotide sequence within or adjacent to the target DNA sequence in the cell are

cleaved. Such cleavage can enhance the frequency of homologous recombination at the target DNA sequence. The target DNA sequence can be endogenous to the cell. The methods can include introducing into the cell a vector containing a cDNA encoding the TAL endonuclease, and expressing a TAL endonuclease protein in the cell. In some cases, the TAL endonuclease protein itself can be introduced into the cell, for example, by mechanical injection, by delivery via a bacterial type III secretion system, by electroporation, or by *Agrobacterium* mediated transfer.

The methods described herein can be used in a variety of situations. In agriculture, for example, methods described herein are useful to facilitate homologous recombination at a target site can be used to remove a previously integrated transgene (e.g., a herbicide resistance transgene) from a plant line, variety, or hybrid. The methods described herein also can be used to modify an endogenous gene such that the enzyme encoded by the gene confers herbicide resistance, e.g., modification of an endogenous 5-enolpyruvyl shikimate-3-phosphate (EPSP) synthase gene such that the modified enzyme confers resistance to glyphosate herbicides. As another example, the methods described herein are useful to facilitate homologous recombination at regulatory regions for one or more endogenous genes in a plant or mammal metabolic pathway (e.g., fatty acid biosynthesis), such that expression of such genes is modified in a desired manner. The methods described herein are useful to facilitate homologous recombination in an animal (e.g., a rat or a mouse) in one or more endogenous genes of interest involved in, as non-limiting examples, metabolic and internal signaling pathways such as those encoding cell-surface markers, genes identified as being linked to a particular disease, and any genes known to be responsible for a particular phenotype of an animal cell.

The present invention also provides methods for designing sequence-specific TAL effectors capable of interacting with particular DNA sequences (e.g., TALENs capable of cleaving DNA at specific locations). The methods can include identifying a target nucleotide sequence (e.g., an endogenous chromosomal sequence, a mitochondrial DNA sequence, or a chloroplast DNA sequence) at which it is desired to have TAL effector binding (e.g., a sequence adjacent to a second nucleotide sequence at which it is desired to introduce a double-stranded cut), and designing a sequence specific TAL effector that contains a plurality of DNA binding repeats that, in combination, bind to the target

sequence. As described herein, TAL effectors include a number of imperfect repeats that determine the specificity with which they interact with DNA. Each repeat binds to a single base, depending on the particular di-amino acid sequence at residues 12 and 13 of the repeat. Thus, by engineering the repeats within a TAL effector (e.g., using standard techniques or the techniques described herein), particular DNA sites can be targeted. Such engineered TAL effectors can be used, for example, as transcription factors targeted to particular DNA sequences. A diagram of a generic TAL effector is shown in FIG. 1A, with the repeat region indicated by open boxes, and the RVD in the representative repeat sequence (SEQ ID NO:1) underlined.

Examples of RVDs and their corresponding target nucleotides are shown in Table 1A (*See*, also, PCT Publication No. WO2010/079430).

Table 1A

RVD	Nucleotide
HD	C
NG	T
NI	A
NN	G or A
NS	A or C or G
N*	C or T
HG	T
H*	T
IG	T

*Denotes a gap in the repeat sequence corresponding to a lack of an amino acid residue at the second position of the RVD.

Other RVDs and their corresponding target nucleotides are shown in Table 1B

Table 1B

RVD	Nucleotide
HA	C
ND	C
NK	G
HI	C
HN	G
NA	G
SN	G or A
YG	T

When it is desired to have sequence-specific DNA cleavage, for example, a sequence-specific TALEN can be designed to contain (a) a plurality of DNA binding repeat domains that, in combination, bind to the endogenous chromosomal nucleotide sequence, and (b) an endonuclease that generates a double-stranded cut at the second nucleotide sequence. Such sequence-specific DNA cleavage can be useful to enhance homologous recombination, as described herein. Other uses for TALENs include, for example, as therapeutics against viruses. TALENs can be engineered to target particular viral sequences, cleaving the viral DNA and reducing or abolishing virulence.

The materials and methods provided herein can be used to modify the sequence of a particular gene in a targeted manner. A gene may contain a plurality of sequences to which an engineered TAL effector could be targeted. As described herein, however, certain target sequences may be more effectively targeted. For example, as set forth in Example 9, sequences having particular characteristics may be more effectively targeted by TAL effectors. Thus, the methods provided herein can include identifying target sequences that meet particular criteria. These include sequences that: i) have a minimum length of 15 bases and an orientation from 5' to 3' with a T immediately preceding the site at the 5' end; ii) do not have a T in the first (5') position or an A in the second position;

iii) end in T at the last (3') position and do not have a G at the next to last position; and iv) have a base composition of 0-63% A, 11-63% C, 0-25% G, and 2-42% T.

Since TALENs as described herein generally work as dimers, some embodiments of the methods provided herein can include identifying a first genomic nucleotide sequence and a second genomic nucleotide sequence in a cell, wherein the first and second nucleotide sequences meet at least one of the criteria set forth above and are separated by 15-18 bp. In some cases, one TALEN polypeptide can bind to each nucleotide sequences, and the endonuclease contained in the TALEN can cleave within the 15-18 bp spacer.

The present invention also provides methods for generating genetically modified animals into which a desired nucleic acid has been introduced. Such methods can include obtaining a cell containing an endogenous chromosomal target DNA sequence into which it is desired to introduce the nucleic acid, introducing into the cell a TALEN to generate a double-stranded cut within the endogenous chromosomal target DNA sequence, introducing into the cell an exogenous nucleic acid containing a sequence homologous to at least a portion of the endogenous chromosomal target DNA, where the introduction is done under conditions that permit homologous recombination to occur between the exogenous nucleic acid and the endogenous chromosomal target DNA, and generating an animal from the primary cell in which homologous recombination has occurred. The homologous nucleic acid can include, e.g., a nucleotide sequence that disrupts a gene after homologous recombination, a nucleotide sequence that replaces a gene after homologous recombination, a nucleotide sequence that introduces a point mutation into a gene after homologous recombination, or a nucleotide sequence that introduces a regulatory site after homologous recombination.

The methods provided herein also can be used to generate genetically modified plants in which a desired nucleic acid has been introduced. Such methods can include obtaining a plant cell containing an endogenous target DNA sequence into which it is desired to introduce the nucleic acid, introducing a TALEN to generate a double-stranded cut within the endogenous target DNA sequence, introducing into the plant cell an exogenous nucleic acid containing a sequence homologous to at least a portion of the endogenous target DNA, where the introducing is under conditions that permit

homologous recombination to occur between the exogenous nucleic acid and the endogenous target DNA, and generating a plant from the plant cell in which homologous recombination has occurred.

The DNA in cells generated by the TALEN-facilitated homologous recombination methods provided herein is modified, as compared to cells that have not undergone such methods, and cells containing the modified DNA are referred to as "genetically modified." It is noted, however, that organisms containing such cells may not be considered GMO for regulatory purposes, since such a modification involves a homologous recombination and not random integration of a transgene. Thus, using the TALEN-facilitated methods described herein to generate genetic modifications may be advantageous in that, for example, standard regulatory procedures along with their associated time and cost may be avoided.

Other methods of targeted genetic recombination, as provided herein, can include introducing into a cell (e.g., a plant cell, insect cell, teleost fish cell, or animal cell) a nucleic acid molecule encoding a TALEN targeted to a selected DNA target sequence, inducing expression of the TALEN within the cell, and identifying a recombinant cell in which the selected DNA target sequence exhibits a mutation (e.g., a deletion of genetic material, an insertion of genetic material, or both a deletion and an insertion of genetic material). A donor DNA also can be introduced into the cell.

In some embodiments, a monomeric TALEN can be used. TALENs as described herein typically function as dimers across a bipartite recognition site with a spacer, such that two TAL effector domains are each fused to a catalytic domain of the *Fok*I restriction enzyme, the DNA recognition sites for each resulting TALEN are separated by a spacer sequence, and binding of each TALEN monomer to the recognition site allows *Fok*I to dimerize and create a double-strand break within the spacer (*see*, e.g., Moscou and Bogdanove (2009) *Science* 326:1501). Monomeric TALENs also can be constructed, however, such that single TAL effectors are fused to a nuclease that does not require dimerization to function. One such nuclease, for example, is a single-chain variant of *Fok*I in which the two monomers are expressed as a single polypeptide (Minczuk et al. (2008) *Nucleic Acids Res.* 36:3926-3938). Other naturally occurring or engineered monomeric nucleases also can serve this role. The DNA recognition domain used for a

monomeric TALEN can be derived from a naturally occurring TAL effector. Alternatively, the DNA recognition domain can be engineered to recognize a specific DNA target. Engineered single-chain TALENs may be easier to construct and deploy, as they require only one engineered DNA recognition domain.

In some embodiments, a dimeric DNA sequence-specific nuclease can be generated using two different DNA binding domains (e.g., one TAL effector binding domain and one binding domain from another type of molecule). As set forth above, the TALENs described herein typically function as dimers across a bipartite recognition site with a spacer. This nuclease architecture also can be used for target-specific nucleases generated from, for example, one TALEN monomer and one zinc finger nuclease monomer. In such cases, the DNA recognition sites for the TALEN and zinc finger nuclease monomers can be separated by a spacer of appropriate length. Binding of the two monomers can allow *Fok*I to dimerize and create a double-strand break within the spacer sequence. DNA binding domains other than zinc fingers, such as homeodomains, myb repeats or leucine zippers, also can be fused to *Fok*I and serve as a partner with a TALEN monomer to create a functional nuclease.

In some embodiments, a TAL effector can be used to target other protein domains (e.g., non-nuclease protein domains) to specific nucleotide sequences. For example, a TAL effector can be linked to a protein domain from, without limitation, a DNA interacting enzyme (e.g., a methylase, a topoisomerase, an integrase, a transposase, or a ligase), a transcription activators or repressor, or a protein that interacts with or modifies other proteins such as histones. Applications of such TAL effector fusions include, for example, creating or modifying epigenetic regulatory elements, making site-specific insertions, deletions, or repairs in DNA, controlling gene expression, and modifying chromatin structure.

In some embodiments, the spacer of the target sequence can be selected or varied to modulate TALEN specificity and activity. The results presented herein for TALENs that function as dimers across a bipartite recognition site with a spacer demonstrate that TALENs can function over a range of spacer lengths, and that the activity of TALENs varies with spacer length. *See*, e.g., Example 6 below. The flexibility in spacer length indicates that spacer length can be chosen to target particular sequences (e.g., in a

genome) with high specificity. Further, the variation in activity observed for different spacer lengths indicates that spacer length can be chosen to achieve a desired level of TALEN activity.

In some embodiments, TALEN activity can be modulated by varying the number and composition of repeats within the DNA binding domain(s). As described in Example 7 herein, for example, a PthXoI-based TALEN showed greater activity than an AvrBs3-based TALEN. PthXoI differs from AvrBs3 both in the number and RVD composition of its repeats. In addition, the naturally occurring DNA recognition sites for these proteins differ in their divergence from the respective recognition sequences predicted based on the TAL effector DNA cipher described by Moscou and Bogdanove (*supra*). Further, several custom TALENs of the same length (12 RVDs) but with differing RVD composition differed in their activity, and a 13 RVD custom TALEN had higher activity than a 12 RVD custom TALEN. Thus, not only can TALENs be engineered to recognize a DNA sequence of interest, but (1) the number of repeats can be varied to modulate activity, (2) different binding sites can be selected to achieve different levels of activity, and (3) the composition of RVDs and their fit to the target site (according to the cipher) can be varied to modulate TALEN activity.

When the TALEN is in a heterodimeric form, for instance with two different monomers including each a TAL effector domain and a FokI nuclease catalytic domain, the RVDs can be found in equivalent number in each of the two TAL effector domains, or each domain can display different numbers of RVDs. For instance, if a total of 22 RVDs is used to bind DNA in a particular heterodimeric TALEN, 11 repeats can be found in each of the two TAL effector domains; alternatively, 10 repeats can be found in one of the two TAL effector domains and 12 in the other. The present invention also encompasses TALEN with DNA modifying enzyme domain which functions as a monomer. In this case, all the RVDs can be found in a single TAL effector domain, which is fused to the monomeric enzyme. In this case, in order to have efficient binding, the number of RVDs must be equivalent to the total number of RVDs that would be found in an equivalent dimeric TALEN. For example, instead of having 10 repeats on two different TAL effector domains (as in the case for a dimeric TALEN), one would have 20 repeats in a single TAL effector domain (as in the case for a monomeric TALEN).

In a further aspect of the invention, the total number of repeats within the dimeric or monomeric TALEN is at least 14. In another further aspect of the invention, the total number of repeats within the dimeric or monomeric TALEN is at least 20. In another further aspect of the invention, the total number of repeats within the dimeric or monomeric TALEN is at least 24. In another further aspect of the invention, the total number of repeats within the dimeric or monomeric TALEN is at least 30.

This patent application also provides methods for generating TAL effector proteins having enhanced targeting capacity for a target DNA. The methods can include, for example, generating a nucleic acid encoding a TAL effector that has a DNA binding domain with a plurality of DNA binding repeats, each repeat containing a RVD that determines recognition of a base pair in the target DNA, where each DNA binding repeat is responsible for recognizing one base pair in the target DNA. As described in Example 12 below, relaxing the requirement for T at position -1 of the binding site may enhance the targeting capacity for engineered TAL effector proteins. Thus, generating a TAL effector encoding nucleic acid can include incorporating a nucleic acid encoding a variant 0th DNA binding repeat sequence with specificity for A, C, or G, thus eliminating the requirement for T at position -1 of the binding site.

In addition, methods are provided herein for generating TAL effectors having enhanced targeting capacity for a target DNA. Such methods can include generating a nucleic acid encoding a TAL effector that comprises DNA binding domain having a plurality of DNA binding repeats, each repeat containing a RVD that determines recognition of a base pair in the target DNA. As described in Example 12 below, the specificity of NN (the most common RVD that recognizes G) appears to be generally weak and can vary with context, but certain RVDs may have enhanced specificity for G. Thus, methods provided herein can include using alternate RVDs that may have more robust specificity for G. For example, one or more RVDs selected from the group consisting of RN, R*, NG, NH, KN, K*, NA, NT, DN, D*, NL, NM, EN, E*, NV, NC, QN, Q*, NR, NP, HN, H*, NK, NY, SN, S*, ND, NW, TN, T*, NE, NF, YN, Y*, and NQ can be used, where the asterisk indicates a gap at the second position of the RVD.

Articles of Manufacture

The present invention also provides articles of manufacture containing, for example, nucleic acid molecules encoding TALENs, TALEN polypeptides, compositions containing such nucleic acid molecules or polypeptides, or TAL endonuclease engineered cell lines. Such items can be used, for example, as research tools, or therapeutically.

In some embodiments, an article of manufacture can include seeds from plants generated using methods provided herein. The seeds can be conditioned using means known in the art and packaged using packaging material well known in the art to prepare an article of manufacture. A package of seed can have a label e.g., a tag or label secured to the packaging material, a label printed on the packaging material or a label inserted within the package. The label can indicate that the seeds contained within the package can produce a crop of genetically modified plants, and can described the traits that are altered by the genetic modification, relative to unmodified plants.

Other definitions

- Amino acid residues or subunits in a polypeptide sequence are designated herein according to the one-letter code, in which, for example, Q means Gln or Glutamine residue, R means Arg or Arginine residue and D means Asp or Aspartic acid residue.

- Amino acid substitution means the replacement of one amino acid residue with another, for instance the replacement of an Arginine residue with a Glutamine residue in a peptide sequence is an amino acid substitution.

- Nucleotides are designated as follows: one-letter code is used for designating the base of a nucleoside: a is adenine, t is thymine, c is cytosine, and g is guanine. For the degenerated nucleotides, r represents g or a (purine nucleotides), k represents g or t, s represents g or c, w represents a or t, m represents a or c, y represents t or c (pyrimidine nucleotides), d represents g, a or t, v represents g, a or c, b represents g, t or c, h represents a, t or c, and n represents g, a, t or c.

- The term "DNA modifying enzyme" refers to any protein which is capable of modifying the genetic material of a cell, whatever the level of DNA modification (cleavage, covalent interaction, water-mediated interaction...). DNA-interacting proteins (e.g., a methylase, a topoisomerase, an integrase, a transposase, or a ligase), transcription

activators or repressor, other proteins such as histones, and nucleases are intended to be included in the the meaning of "DNA modifying enzyme". When comprised in a TAL effector-DNA modifying enzyme the DNA modifying enzyme is refered as the DNA modifying enzyme domain.

- The term "nuclease" is intended to include exonucleases and endonucleases.

- The term "endonuclease" refers to any wild-type or variant enzyme capable of catalyzing the hydrolysis (cleavage) of bonds between nucleic acids within a DNA or RNA molecule, preferably a DNA molecule. Non-limiting examples of endonucleases include type II restriction endonucleases such as *FokI*, *Hha*I, *Hind*III, *Not*I, *BbvC*I, *EcoR*I, *Bgl*I, and *Alw*I. Endonucleases comprise also rare-cutting endonucleases when having typically a polynucleotide recognition site of about 12-45 base pairs (bp) in length, more preferably of 14-45 bp. Rare-cutting endonucleases significantly increase HR by inducing DNA double-strand breaks (DSBs) at a defined locus (Rouet, Smih et al. 1994; Rouet, Smih et al. 1994; Choulika, Perrin et al. 1995; Pingoud and Silva 2007). Rare-cutting endonucleases can for example be a homing endonuclease (Paques and Duchateau 2007), a chimeric Zinc-Finger nuclease (ZFN) resulting from the fusion of engineered zinc-finger domains with the catalytic domain of a restriction enzyme such as FokI (Porteus and Carroll 2005) or a chemical endonuclease (Eisenschmidt, Lanio et al. 2005 ; Arimondo, Thomas et al. 2006; Simon, Cannata et al. 2008). In chemical endonucleases, a chemical or peptidic cleaver is conjugated either to a polymer of nucleic acids or to another DNA recognizing a specific target sequence, thereby targeting the cleavage activity to a specific sequence. Chemical endonucleases also encompass synthetic nucleases like conjugates of orthophenanthroline, a DNA cleaving molecule, and triplex-forming oligonucleotides (TFOs), known to bind specific DNA sequences (Kalish and Glazer 2005). Such chemical endonucleases are comprised in the term "endonuclease" according to the present invention. Examples of such endonuclease include *I-Sce I, I-Chu I, I-Cre I, I-Csm I, PI-Sce I, PI-Tli I, PI-Mtu I, I-Ceu I, I-Sce II, I-Sce III, HO, PI-Civ I, PI-Ctr I, PI-Aae I, PI-Bsu I, PI-Dha I, PI-Dra I, PI-Mav I, PI-Mch I, PI-Mfu I, PI-Mfl I, PI-Mga I, PI-Mgo I, PI-Min I, PI-Mka I, PI-Mle I, PI-Mma I, PI-Msh I, PI-Msm I, PI-Mth I, PI-Mtu I, PI-Mxe I, PI-Npu I, PI-Pfu I, PI-Rma I, PI-Spb I, PI-Ssp I, PI-Fac I, PI-Mja I, PI-Pho I, PI-Tag I, PI-Thy I, PI-Tko I, PI-Tsp I, I-MsoI.*

The endonucleases according to the present invention can be part of a Transcription Activator-Like (TAL) effector endonuclease (TALEN).

- By "TALEN" is intended a protein comprising a Transcription Activator-like (TAL) effector binding domain and an endonuclease domain, the fusion of both domains resulting in a "monomeric TALEN". Some monomeric TALEN can be functional per se and others require dimerization with another monomeric TALEN. The dimerization can result in a homodimeric TALEN when both monomeric TALEN are identical or can result in a heterodimeric TALEN when monomeric TALEN are different. Two monomeric TALEN are different when, for example, their RVDs numbers are different, and / or when the content (i.e amino acid sequence) of at least one RVD is different.By "TAL effector-DNA modifying enzyme" is intended a protein comprising a Transcription Activator-Like effector binding domain and a DNA-modifying enzyme domain.

By "variant" is intended a "variant" protein, i.e. an protein that does not naturally exist in nature and that is obtained by genetic engineering or by random mutagenesis, i.e. an engineered protein. This variant protein can for example be obtained by substitution of at least one residue in the amino acid sequence of a wild-type, naturally-occurring, protein with a different amino acid. Said substitution(s) can for example be introduced by site-directed mutagenesis and/or by random mutagenesis.

By "cell" or "cells" is intended any prokaryotic or eukaryotic living cells, cell lines derived from these organisms for *in vitro* cultures, primary cells from animal or plant origin.

By "primary cell" or "primary cells" are intended cells taken directly from living tissue (i.e. biopsy material) and established for growth in vitro, that have undergone very few population doublings and are therefore more representative of the main functional components and characteristics of tissues from which they are derived from, in comparison to continuous tumorigenic or artificially immortalized cell lines. These cells thus represent a more valuable model to the *in vivo* state to which they refer.

- By "homologous" is intended a sequence with enough identity to another one to lead to homologous recombination between sequences, more particularly having at least 95 % identity, preferably 97 % identity and more preferably 99 %.

- "Identity" refers to sequence identity between two nucleic acid molecules or polypeptides. Identity can be determined by comparing a position in each sequence which may be aligned for purposes of comparison. When a position in the compared sequence is occupied by the same base, then the molecules are identical at that position. A degree of similarity or identity between nucleic acid or amino acid sequences is a function of the number of identical or matching nucleotides at positions shared by the nucleic acid sequences. Various alignment algorithms and/or programs may be used to calculate the identity between two sequences, including FASTA, or BLAST which are available as a part of the GCG sequence analysis package (University of Wisconsin, Madison, WI), and can be used with, e.g., default setting.

- by "mutation" is intended the substitution, deletion, insertion of one or more nucleotides/amino acids in a polynucleotide (cDNA, gene) or a polypeptide sequence. Said mutation can affect the coding sequence of a gene or its regulatory sequence. It may also affect the structure of the genomic sequence or the structure/stability of the encoded mRNA.

- By "gene" is meant the basic unit of heredity, consisting of a segment of DNA arranged in a linear manner along a chromosome, which codes for a specific protein or segment of protein. A gene typically includes a promoter, a 5' untranslated region, one or more coding sequences (exons), optionally introns, a 3' untranslated region. The gene may further comprise a terminator, enhancers and/or silencers.

- The term "gene of interest" refers to any nucleotide sequence encoding a known or putative gene product.

- As used herein, the term "locus" is the specific physical location of a DNA sequence (e.g. of a gene) on a chromosome. The term "locus" usually refers to the specific physical location of a target sequence on a chromosome.

- By "fusion protein" is intended the result of a well-known process in the art consisting in the joining of two or more genes which originally encode for separate proteins, the translation of said "fusion gene" resulting in a single polypeptide with functional properties derived from each of the original proteins.

- By "catalytic domain" is intended the protein domain or module of an enzyme containing the active site of said enzyme; by active site is intended the part of said

enzyme at which catalysis of the substrate occurs. Enzymes, but also their catalytic domains, are classified and named according to the reaction they catalyze. The Enzyme Commission number (EC number) is a numerical classification scheme for enzymes, based on the chemical reactions they catalyze (World Wide Web at chem.qmul.ac.uk/iubmb/enzyme/). In the scope of the present invention, any catalytic domain can be used as a partner and be fused to a TAL effector domain to generate a chimeric fusion protein resulting in a TAL effector-DNA modifying enzyme. Non-limiting examples of such catalytic domains can be those of MmeI, EsaSSII, CstMI, NucA, EndA Escherichia coli, NucM, EndA Streptococcus pneumonia, SNase Staphylococcus aureus, SNase Staphylococcus hyicus, SNase shigella flexneri, Bacillus subtilis yncB, EndodeoxyribonucleaseI Enterobacteria phage T7, EndoG bovine, ttSmr DNA mismatch repair protein mutS, cleavage domain of Metnase.

The practice of the present invention will employ, unless otherwise indicated, conventional techniques of cell biology, cell culture, molecular biology, transgenic biology, microbiology, recombinant DNA, and immunology, which are within the skill of the art. Such techniques are explained fully in the literature. *See*, for example, Current Protocols in Molecular Biology (Ausubel, 2000, Wiley and son Inc, Library of Congress, USA); Molecular Cloning: A Laboratory Manual, Third Edition, (Sambrook et al, 2001, Cold Spring Harbor, New York: Cold Spring Harbor Laboratory Press); Oligonucleotide Synthesis (M. J. Gait ed., 1984); U.S. Pat. No. 4,683,195; Nucleic Acid Hybridization (Harries and Higgins eds. 1984); Transcription and Translation (Hames and Higgins eds. 1984); Culture of Animal Cells (Freshney, Alan R. Liss, Inc., 1987); Immobilized Cells and Enzymes (IRL Press, 1986); Perbal, A Practical Guide to Molecular Cloning (1984); the series, Methods in Enzymology (Abelson and Simon, eds.-in-chief, Academic Press, Inc., New York), specifically, Vols.154 and 155 (Wu et al. eds.) and Vol. 185, "Gene Expression Technology" (Goeddel, ed.); Gene Transfer Vectors For Mammalian Cells (Miller and Calos eds., 1987, Cold Spring Harbor Laboratory); Immunochemical Methods in Cell and Molecular Biology (Mayer and Walker, eds., Academic Press, London, 1987); Handbook of Experimental Immunology, Vols. I-IV (Weir and Blackwell, eds., 1986); and Manipulating the Mouse Embryo, (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., 1986).

The above written description of the invention provides a manner and process of making and using it such that any person skilled in this art is enabled to make and use the same, this enablement being provided in particular for the subject matter of the appended claims, which make up a part of the original description.

As used above, the phrases "selected from the group consisting of," "chosen from," and the like include mixtures of the specified materials.

Where a numerical limit or range is stated herein, the endpoints are included. Also, all values and subranges within a numerical limit or range are specifically included as if explicitly written out.

The above description is presented to enable a person skilled in the art to make and use the invention, and is provided in the context of a particular application and its requirements. Various modifications to the preferred embodiments will be readily apparent to those skilled in the art, and the generic principles defined herein may be applied to other embodiments and applications without departing from the spirit and scope of the invention. Thus, this invention is not intended to be limited to the embodiments shown, but is to be accorded the widest scope consistent with the principles and features disclosed herein.

Having generally described this invention, a further understanding can be obtained by reference to certain specific examples, which are provided herein for purposes of illustration only, the invention being further described in the following examples, which do not limit the scope of the invention described in the claims unless otherwise specified.

EXAMPLES

Example 1 – A cipher governs TAL effector-DNA recognition

To determine whether there is a one-to-one, linear correspondence between RVDs and contiguous nucleotides in the TAL target site, the predicted promoter region (i.e., the 1,000 bp immediately preceding the annotated translational start site) of the known target gene for each of ten TAL effectors was scanned with the TAL effector RVD sequence for alignments that minimized entropy (randomness) in RVD-nucleotide associations. The following formula was used to quantify entropy, where R is the set of RVDs for the

effector, D is the set of four nucleotides (A, C, G, T), and $f_{i,j}$ represents the observed frequency with which the i^{th} RVD associates with the j^{th} nucleotide:

$$\sum_{i \in R} \sum_{j \in D} \max_{j}(f_i) - f_{i,j}$$

Multiple low entropy sites were present in each promoter. For effector AvrBs3, however, only one mapped to the 54 bp *upa20* promoter fragment identified previously as sufficient and necessary for activation, and it coincided with the UPA box common to genes directly activated by AvrBs3 (Kay et al., *supra*). Also, for effectors PthXo1 and AvrXa27, only one site each overlapped a polymorphism between the activated and non-activated alleles of their respective targets, *Os8N3* and *Xa27*. Across the alignments at these three sites, RVD-nucleotide associations were consistent, so the remaining alignments were selected based on those associations, resulting in exactly one site per TAL effector-target pair (FIG. 1B and Table 2). Each site is preceded by a T (FIG. 1D).

To assess the specificity conferred by the RVD-nucleotide associations, a weight matrix was first generated based on the frequencies of all RVD-nucleotide associations observed across the ten minimal entropy TAL effector-target site alignments (FIG. 1B). The weight matrix was then used to scan the promoter region, the 1,000 bp preceding the translational start, of each nonredundant gene model in rice, *Oryza sativa* spp. japonica cv. Nipponbare (Osa1, Release 6.0, rice.plantbiology.msu.edu) for best matches to the five TAL effectors of the rice pathogen *Xanthomonas oryzae* (AvrXa27, PthXo1, PthXo6, PthXo7, and Tal1c). For AvrXa27, the sequence upstream of *Xa27* (GenBank accession AY986492) was included. This upstream sequence is not present in Nipponbare. Observed association frequencies were weighted at 90% and the remaining 10% was distributed equally to frequencies of all possible associations. Alignments were ranked using a weight matrix score (y axis), taken as a negative log of the frequency score derived from the RVD-nucleotide association frequencies in FIG. 1B. Thus, the lower the score, the better the match. For PthXo1, PthXo6, PthXo7, and Tal1c, the experimentally identified target gene was the best or nearly best match. Better matches were not preceded by a T, were not represented on the microarray used to identify the target, or lacked introns and EST evidence. Scanning the reverse complement promoter sequences yielded no better scoring alignments than the forward sites for the known

targets. This result does not imply that TAL effectors bind to the positive strand, but indicates that they function in a forward orientation relative to the positive strand. The known target of the fifth effector, AvrXa27, is the disease resistance gene *Xa27* (Gu et al., *supra*). The poorer rank for this match (5,368) may reflect a calibrated, or recent and sub-optimal host adaptation. Better scoring sites likely comprise genes targeted by AvrXa27 for pathogenesis.

Using the weight matrix again, ten additional alignments were obtained by scanning all rice promoters with 40 additional *X. oryzae* TAL effectors and retaining the best alignments for which the downstream gene was activated during infection based on public microarray data (PLEXdb.org, accession OS3) (Table 3). As with the initial set, a T precedes each site, and no reverse-strand sites scored better. The RVD-nucleotide association frequencies of the total 20 alignments are shown in FIG. 1C. They constitute a strikingly simple cipher.

The RVD-nucleotide frequencies in the expanded set of 20 TAL effector nucleotide alignments were used to generate a new weight matrix, and a computational script was written in Python v2.5 (www.python.org). The script can be used to scan any collection of DNA sequences for matches to a particular TAL effector, with a user-definable weight factor for observed vs. unobserved RVD-nucleotide associations. *See* Moscou and Bogdanove (*supra*).

There is some degeneracy in the cipher. Strong associations may represent anchors that account for most of the binding affinity, with weak associations providing a measure of flexibility. Alternatively, neighbor effects may be involved. The latter possibility was investigated by determining the nucleotide association frequencies of every RVD conditioned on the RVD to either side and comparing them to the total observed frequencies – in other words, by sorting the RVD-nucleotide pairings according to the neighbor RVD to the left or right, and comparing the relative frequencies of each pair thus sorted with the overall frequency for that pair. The frequencies of the RVD-nucleotide associations sorted by neighbor did not deviate significantly from the total observed frequencies, suggesting that the associations are context independent.

Sequences flanking the 20 target sites revealed no conserved nucleotides except the T at -1, but they tend to be C-rich following the site and G-poor throughout (Fig. 1D).

With few exceptions, sites begin within 60 bp upstream of the annotated transcriptional start, and none are closer than 87 bp to the translational start (Table 2 and Table 3). Additional rules governing RVD/nucleotide associations are described in Examples 4 and 5.

Given these results, prediction of TAL effector targets in a genome and construction of targets *de novo* are now possible. The ability to predict sites will expedite identification of host genes important in disease. The ability to construct targets holds promise for designing durable resistance genes that are responsive to conserved or multiple TAL effectors. Customizing TAL effectors for arbitrary gene activation or targeting of fused proteins for DNA modification also is possible, as described herein.

Table 2

Predicted target site features for experimentally identified TAL effector-target pairs

TAL effector	Source	RVDs	Target gene	TcS	TATA box	TlS
AvrXa27[1]	*Xanthomonas oryzae* pv. oryzae PXO99^A	17	*Xa27* (rice)	27	-7	87
AvrBs3[2]	*X. campestris* pv. vesicatoria	18	*Bs3* (pepper)	59	1	123
AvrBs3[3]	*X. campestris* pv. vesicatoria	18	*UPA20* (pepper)	72	1	150
AvrBs3Δrep16[4,5]	Modified AvrBs3	14	*Bs3-E* (pepper)	85	1	136
AvrBs3Δrep109[4]	Modified AvrBs3	15	*Bs3* (pepper)	59	1	123
AvrHah1[6]	*X. gardneri*	14	*Bs3* (pepper)	59	1	121
PthXo1[7]	*X. oryzae* pv. oryzae PXO99^A	24	*Os8N3* (rice)	79	46	251
PthXo6[8]	*X. oryzae* pv. oryzae PXO99^A	23	*OsTFX1* (rice)	31	-780	136
PthXo7[8]	*X. oryzae* pv. oryzae PXO99^A	22	*OsTFIIAγ1* (rice)	333	44	469
Tal1c	*X. oryzae* pv. oryzicola BLS256	16	*OsHEN1* (rice)	10	-265	217

RVDs, repeat-variable diresidues; TcS, annotated transcriptional start site; TlS, translational start site. Locations are relative to the 5' end of the target site.

[1] Gu et al., *supra*
[2] Kay et al. (2007) *Science* 318:648
[3] Römer et al. (2007) *Science* 318:645
[4] Herbers et al. (1992) *Nature* 356:172
[5] Römer et al. (2009) *Plant Physiol.*
[6] Schornack et al. (2008) *New Phytologist* 179:546
[7] Yang et al. (2006) *Proc. Natl. Acad. Sci. USA* 103:10503
[8] Sugio et al. (2007) *Proc. Natl. Acad. Sci. USA*

Table 3

Xanthomonas oryzae TAL effector candidate targets in rice activated during infection.

Effector	Strain	RVDs	Rice locus	r	TcS	TATA box	TlS	*q*	Fold change
Tal1c	BLS256	16	OsHen1	1	10	-265	217	0.01	3.3
Tal2c	BLS256	27	Os03g03034	15	-16	-145	143	0.01	5.2
Tal2d	BLS256	16	Os04g49194	9	27	n.p.	102	3.9E-07	29.7
Tal3b	BLS256	18	Os05g27590	42	34	-1	104	3.4E-08	8.5
Tal4a	BLS256	26	Os03g37840	1	152	221	363	2.2E-04	2.6
Tal4b	BLS256	14	Os09g32100	72	68	n.p.	271	8.0E-03	3.6
Tal4c	BLS256	23	Os06g37080	18	31	n.p.	151	2.7E-10	17.1
Tal6	BLS256	20	Os07g47790	16	-15	-70	93	3.6E-02	21.6
PthXo1	PXO99^A	24	Os8N3	1	79	46	251	1.0E-08	84.2
PthXo6	PXO99^A	23	OsTFX1	2	31	-780	136	3.5E-03	2.8
PthXo7	PXO99^A	22	OsTFIIAγ1	7	333	44	469	1.6E-06	4.5
Tal9a	PXO99^A	20	OsHen1	1	44	-3	93	0.13	8.2
Tal7a/8a	PXO99^A	18	Os01g68740	2	32	-197	102	1.8E-01	1.7
Tal7b/8b	PXO99^A	20	Os01g40290	57	-2	-276	206	1.8E-01	1.7

RVDs, repeat-variable diresidues; r, rank out of 58,918 gene models scanned, based on the RVD weight matrix score; TcS, annotated transcriptional start site; n.p., not present; TlS, translational start site. Locations are relative to the 5' end of the target site. *q* values are for a comparison to mock across five time points up to 96 hours after inoculation, replicated four times; fold change given is at 96 hours (PLEXdb, accession OS3).

Example 2 – TALENs can function in yeast

Plasmid construction: The protein coding sequence of the TAL effector, AvrBs3, was obtained by digestion from a plasmid with *Bam*HI. A DNA fragment encoding principally the repeat domain was excised with *Sph*I. The amino acid sequence of AvrBs3 can be found under GENBANK Accession No. P14727 and SEQ ID NO:12 (FIG. 3), and the nucleic acid sequence under Accession No. X16130 and SEQ ID NO:13 (FIG. 4). In FIG. 4, the *Bam*HI and *Sph*I sites are in bold and underlined. The AvrBs3 *Bam*HI and *Sph*I fragments were cloned into the nuclease expression vector pDW1789_TAL (FIG. 5) adjacent to sequences encoding the *Fok*I nuclease domain. To clone the AvrBs3 target site into the target reporter plasmid, two complementary DNA oligos, containing two AvrBs3 recognition sites arranged in an inverted orientation with an 18 bp spacer sequence in between, were synthesized with *Bgl*II and *Spe*I overhangs at the 5' and 3' ends, respectively. Other reporter plasmids were made that had recognition sites with spacer lengths of 6, 9, 12 and 15 bp. The annealed DNA oligos were cloned into the reporter plasmid, pCP5 (FIG. 6), which was digested with *Bgl*II and *Spe*I.

Yeast assay: The target reporter plasmids were transformed into the yeast strain *YPH499* (a *MAT* a strain), and transformants were selected on synthetic complete medium lacking tryptophan (SC-W). The TALEN expression plasmids were transformed into *YPH*500 (a *MAT* α strain); and transformants were plated on SC medium lacking histidine (SC-H). Yeast colonies carrying the target reporter plasmid and colonies carrying the TALEN expression plasmid were cultured overnight at 30°C in liquid SC-W and SC-H media, respectively. The cultures were adjusted to the same OD_{600}, and 200 μl of each were mixed into 200 μl YPD medium. The mixture was incubated at 30°C for 4 hours to allow the two types of yeast strain to mate. The mixed culture was spun down and resuspended in 5 ml SC-W-H media at 30C overnight or until the OD_{600} reaches a range of 0.5-1. The cells were harvested and quantitative β-galactosidase assays were performed as described (Townsend et al. (2009) *Nature* 459:442-445).

Results: The TAL-*Fok*I fusion is a site-specific nuclease consisting of the TAL DNA recognition domain and the non-specific *Fok*I DNA cleavage domain. The TAL DNA recognition domain can be engineered to bind different DNA sequences. As described in Example 1 herein, the DNA recognition specificity for TAL effectors, a novel class of DNA binding domain, has been deciphered. In particular, the DNA binding domain of TAL effectors contain a various number of tandem, 34-amino acid repeats, which can recognize and bind to specific DNA sequences. Amino acid sequences of the repeats are conserved except for two adjacent highly variable residues at positions 12 and 13 of the repeats. These positions together specify individual nucleotides in the DNA binding site, one repeat to one nucleotide. The architecture of the TALENs is illustrated in FIG. 7. The TALENs function as dimers, with each monomer composed of engineered TAL DNA recognition repeats fused to a non-specific cleavage domain from the *Fok*I endonuclease. The DNA recognition repeats can be engineered to bind target DNA sequences within a genome of interest. TAL nuclease monomers bind to one of two DNA half-sites that are separated by a spacer sequence. This spacing allows the *Fok*I monomers to dimerize and create a double-strand DNA break (DSB) in the spacer sequence between the half-sites.

To explore the potential of the TAL effector DNA recognition domain, experiments were conducted to determine whether native TAL effectors can function as

nucleases when fused with the *Fok*I nuclease domain. The yeast-based assay was carried out by using a TAL nuclease expression construct and a target reporter construct. As illustrated in FIG. 5, the backbone of the nuclease expression construct contains a *Fok*I nuclease domain and an N-terminal nuclear localization signal (NLS) under control of the yeast TEF1 promoter. Several restriction sites are located between the *Fok*I nuclease domain and the NLS motif to facilitate cloning of various TAL effectors. The target reporter construct has a disrupted *lacZ* reporter gene with a 125 bp duplication of coding sequence as shown in FIG. 6. The duplication flanks a *URA3* gene and a target sequence (composed of two half sites and a spacer sequence) recognized by TAL DNA binding domains. If the TALEN binds and generates DNA double-strand breaks (DSBs) at the target site, such breaks, in yeast, are repaired predominantly by homologous recombination between the duplicated *lacZ* sequences through single strand annealing (Haber (1995) *Bioessays*17:609). Recombination results in reconstitution of a functional *lacZ* gene and loss of *URA3* (conferring 5-fluoroorotic acid resistance). Relative cleavage activity of TALENs was measured by determining *lacZ* enzyme activity.

In these studies, a native TAL effector, AvrBs3, which had a central nuclease repeat region as set forth in SEQ ID NO:16 (FIG. 8) was cloned into the nuclease expression vector, and the AvrBs3 target sites (two binding sites arranged in an inverted orientation) with an 18 bp spacer sequence were cloned into the target reporter vector. The yeast assay was performed using the scheme shown in FIG. 9 and described above. The results showed that the lacZ activity from yeast cells transformed with both the AvrBs3 nuclease plasmid and the target reporter plasmid was significantly higher (15.8-fold higher) than the control yeast cells that contained only the target reporter plasmid (FIG. 10). No activity was observed with nuclease fusions made with only the SphI fragment that encodes predominantly the repeat domain. This indicated that sequences other than the DNA binding domain are required for TALEN activity. Reporter plasmids with spacer lengths of 6 and 9 bp also failed to show activity, indicating that the space between the two binding sites is critical to allow FokI to dimerize. These data indicate that the AvrBs3 TAL nuclease can function as a site-specific nuclease that cleaves its cognate target sequence in yeast.

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Example 3 – Modular assembly of TAL effector repeats for customized TALENs

Complementary oligonucleotides corresponding to the 102 basepairs of each of four individual TAL effector repeats, each specifying a different nucleotide, are synthesized, annealed and cloned into a high copy bacterial cloning vector, individually and in combinations of 2 and 3 repeats in all permutations to yield 4 single, 16 double, and 64 triple repeat modules using standard restriction digestion and ligation techniques (e.g., as illustrated in FIG. 11). The desired TAL effector coding sequence is assembled by introducing the appropriate modules sequentially into a Gateway-ready high copy bacterial cloning vector containing a truncated form of the tal1c gene that lacks the central repeat region except for the characteristic final half repeat. For example, an 18 repeat TAL effector coding sequence can be assembled by sequentially introducing 5 triple modules and 1 double module into the truncated tal1c vector.

Example 4 – A system for modular assembly of TAL effector repeats

Plasmids and methods were developed for generating custom TAL effector-encoding genes. The functional specificity of TAL effectors is determined by the RVDs in the repeats, as described herein; other polymorphisms in the repeats and elsewhere in the proteins are rare and inconsequential with regard to functional specificity. Thus, custom TAL effector genes were generated by replacing the repeat region of an arbitrary TAL effector gene with repeats containing the desired RVDs. The repeat sequences outside the RVDs matched a consensus sequence (*see* below). DNA fragments encoding TAL effector repeats were sequentially assembled into modules encoding one, two, or three repeats, and the modules were cloned into a TAL effector gene from which the original repeats were removed. Each encoded repeat, with the exception of the last (half) repeat, had the sequence LTPDQVVAIASXXGGKQALETVQRLLPVLCQDHG (SEQ ID NO:18; FIG. 12A). The last (half) repeat had the sequence LTPDQVVAIASXXGGKQALES (SEQ ID NO:20; FIG. 12B). In both sequences, "XX" indicates the location of the RVD. The RVDs used in the modular repeats were NI, HD, NN, and NG, which specify binding to A, C, G, and T, respectively. In the experiments described below, the *tal1c* gene of *Xanthomonas oryzae* pv. oryzicola strain BLS256,

with its repeats removed, was used as the "backbone" for building custom TAL effector genes.

The method described herein included five components: (1) generation of single repeat starter plasmids; (2) generation of single repeat module plasmids; (3) generation of multiple repeat modules; (4) generation of a complete set of one-, two-, and three-repeat module plasmids; and (5) assembly of custom TAL effector coding sequences.

To generate single repeat starter plasmids, the *tal1c* gene was digested with *Msc*I and religated to remove the entire repeat region except for the first part of the first repeat and the last part of the last, truncated repeat, resulting in the plasmid designated pCS487 (FIG. 13). The resulting gene encoded the RVD NI and, like most TAL effector genes, contained two *Sph*I sites that flanked the repeat region. The gene contained no *Xho*I site.

Next, a translationally silent mutation was introduced into pCS487 to create a unique *Psp*XI site, which encompasses a unique *Xho*I site centered on codons 19 and 20. The mutation is depicted in FIG. 14, which shows the original and altered nucleotide sequences for codons 18-21 (SEQ ID NO:21 and SEQ ID NO:23, respectively), both of which encode the amino acid sequence ALES (SEQ ID NO:22). The resulting plasmid was designated pCS489.

By further mutagenesis, three additional constructs were generated with the RVDs HD, NN, and NG, to create the plasmids designated pCS490, pCS491, and pCS492, respectively. The *Sph*I fragment encompassing the modified repeat region was transferred from pCS489, pCS490, pCS491, and pCS492 to the kanamycin resistant plasmid designated pCS488 (FIG. 15), which encoded only the N- and C-terminal portions of *tal1c*, without the repeat region, in the Gateway entry vector pENTR-D (Invitrogen, Carlsbad, CA). This transfer resulted in the single repeat starter plasmids designated pCS493 (FIG. 16), pCS494, pCS495, and pCS496, respectively. The *Psp*XI/*Xho*I site in the truncated repeat remained unique in these plasmids. The TAL effector gene in pCS488 and each of its derivatives was preceded by Shine-Dalgarno and Kozak sequences for efficient translation in prokaryotes and eukaryotes, respectively.

Single repeat module plasmids were then constructed. One plasmid was generated for each of the four chosen RVDs (NI, HD, NN, and NG). Each plasmid had a 5' compatible cohesive end that reconstituted a *Xho*I but not a *Psp*XI site when ligated

into a *Psp*XI site, and a 3' compatible cohesive end that reconstituted both a *Xho*I and a *Psp*XI site. The plasmids were generated by cloning annealed synthetic, complementary oligonucleotides with overhangs (FIG. 17A) into the *Psp*XI/*Xho*I site of pBluescript SK-, resulting in plasmids designated pCS502 (FIG. 17B), pCS503, pCS504, and pCS505, respectively. Each plasmid allowed for introduction of additional repeats at the 3' end of the single repeat module at the unique reconstituted *Psp*XI site, or for excision of the repeat module using the reconstituted *Xho*I sites.

Additional single repeat modules, one each for NI, HD, NN, and NG, were generated. Each had a 5' compatible cohesive end that did not reconstitute a *Psp*XI or *Xho*I site when ligated into a *Psp*XI site, a 3' compatible cohesive end that reconstituted both the *Xho*I and a *Psp*XI site, and a translationally silent nucleotide substitution that destroyed the internal *Msc*I site (FIG. 18A). These modules were generated by annealing synthetic, complementary oligonucleotides with overhangs. Ligating any of these additional single repeat modules into the unique *Psp*XI/*Xho*I site of a single repeat module plasmid (pCS502, pCS503, pCS504, or pCS505) resulted in no new *Xho*I site at the 5' junction, but restoration of the unique 3' *Psp*XI/*Xho*I site, so the resulting plasmids could be linearized for introduction of more additional repeats by cutting with *Psp*XI. Reiteration of this process resulted in modules containing multiple repeats (FIG. 18B). Further, each entire multiple repeat module could be excised using *Xho*I. Because the *Msc*I site was destroyed in the additional single repeat modules, the *Msc*I site in the initial repeat remained unique, and was useful to check orientation upon subsequent subcloning of the multiple repeat module.

Additional single repeat modules were cloned iteratively into the single repeat module plasmids to generate, along with the single repeat module plasmids, a complete set of all possible one-, two-, and three-repeat modules, for a total of 84 plasmids designated pCS502 through pCS585 (FIG. 19). Modules containing more than three repeats (e.g., four, five, six, seven, eight, nine, ten, or more than ten repeats) are generated in the same manner.

A method was then devised to assemble any sequence of repeats into the *tal1c* "backbone" to generate a custom TAL effector gene. The method included the following steps, which also are depicted in FIG. 20:

(1) Choose a single repeat starter plasmid with the first desired repeat (pCS493, pCS494, pCS495, or pCS495, encoding RVD NI, HD, NN, or NG, respectively);

(2) linearize the plasmid with *Psp*XI;

(3) isolate the module for the next repeat(s) from the appropriate module plasmid (pCS502 through pCS585) using *Xho*I;

(4) ligate;

(5) check orientation by digestion with *Msc*I and confirm sequence from the 3' end using a vector based primer; and

(6) repeat steps 2-5 until all repeats are assembled.

Example 5 – Library of plasmids for modular assembly of TALENs

Assembly of TALEN repeats as described herein (e.g., using the steps depicted in FIG. 20) results in numerous intermediate plasmids containing increasing numbers of repeats. Each of these plasmids is stored such that a library of plasmids for modular assembly of TALENs (pMATs) is generated. For example, FIGS. 21A and 21B depict the assembly of repeat modules in construction of TAL endonucleases that will target the nucleotide sequences shown. In FIG. 21A, repeat modules from plasmids designated pCS519, pCS524, pCS537, pCS551, pCS583, and pCS529 are sequentially added to the sequence in the starter plasmid designated pCS493, resulting in plasmids designated pMAT55, pMAT56, pMAT57, pMAT58, pMAT59, and pMAT60. In FIG. 21B, repeat modules from plasmids designated pCS530, pCS533, pCS522, and pCS541 are sequentially added to the sequence in the plasmid designated pMAT1, resulting in plasmids designated pMAT61, pMAT62, pMAT63, and pMAT64.

Example 6 – Generation and testing of customized TALENs

The TAL DNA recognition domain was used to create TALENs that recognize and cleave particular DNA targets (FIG. 22A), using the system described in Examples 4 and 5. To assess TALEN function, a yeast assay was adapted in which LacZ activity serves as an indicator of DNA cleavage (Townsend et al., *supra*). In this assay, a target plasmid and a TALEN expression plasmid are brought together in the same cell by mating. The target plasmid has a *lacZ* reporter gene with a 125-bp duplication of coding

sequence. The duplication flanks a target site recognized by a given TALEN. When a double-strand DNA break occurs at the target site, it is repaired through single-strand annealing between the duplicated sequences, which creates a functional *lacZ* gene whose expression can be measured using standard β-galactosidase assays that provide a quantifiable readout (FIG. 22A). This assay has been demonstrated to be a good predictor of the ability of a ZFN to create chromosomal mutations by NHEJ or to stimulate homologous recombination for gene editing in higher eukaryotes (Townsend et al., *supra*; and Zhang et al. (2010) *Proc. Natl. Acad. Sci. USA* 107:12028-12033).

Two well characterized TAL effectors were used – AvrBs3 from the pepper pathogen *Xanthomonas campestris* pv. vesicatoria and PthXo1 from the rice pathogen *X. oryzae* pv. oryzae (Bonas et al. (1989) *Mol. Gen. Genet.* 218:127-136; and Yang et al. (2006) *Proc. Natl. Acad. Sci. USA* 103:10503-10508). The amino acid sequence of AvrBs3 can be found under GENBANK Accession No. P14727 and SEQ ID NO:12 (FIG. 3), and the nucleic acid sequence under Accession No. X16130 and SEQ ID NO:13 (FIG. 4). The amino acid sequence of PthXo1 can be found under GENBANK Accession No. ACD58243 and SEQ ID NO:31 (FIG. 23), and the nucleic acid sequence under Accession No. CP000967, gene ID 6305128, and SEQ ID NO:32 (FIG. 24). The amino acid sequence of PthXo1 under GENBANK Accession No. ACD58243 is truncated at the N-terminus due to a misannotation of the start codon. The complete sequence is presented in FIG. 23.

The repeat domains of both AvrBs3 and PthXo1 are encoded entirely within a conserved *Sph*I fragment (FIGS. 4 and 24). Both TAL effector-encoding genes also have a *BamH*I restriction fragment that encompasses the coding sequence for the repeat domain and 287 amino acids prior and 231 amino acids after (FIGS. 4 and 24; *see*, also, FIG. 22A). Absent from the *BamH*I fragment is the TAL effector transcriptional activation domain. Both the *Sph*I fragments and the *Bam*HI fragments were fused to a DNA fragment encoding *Fok*I that is present in the nuclease expression vector pFZ85 (FIG. 25). The fusion proteins between *Fok*I nuclease and the *Bam*HI fragments encoded by AvrBs3 and PthXo1 are given in FIGS. 26 and 27; SEQ ID NOS:33 and 34.

The *Fok*I monomers must dimerize in order to cleave, but the appropriate spacer length between the two DNA recognition sites was unclear. For ZFNs, in which the zinc

finger array is separated from *Fok*I by a 4-7 amino acid linker, the typical spacer between the two recognition sites is 5-7 bp (Handel et al. (2009) *Mol. Ther.* 17:104-111). Since, for example, 235 amino acids separate the repeat domain from *Fok*I in the *Bam*HI TALEN constructs used herein, a variety of spacer lengths for both the *Bam*HI and *Sph*I constructs (6, 9, 12, 15, and 18 bp) were used. As a positive control, a well-characterized zinc finger nuclease with a DNA binding domain derived from the mouse transcription factor Zif268 (Porteus and Baltimore (2003) *Science* 300:763) was used. As negative controls, the TAL effector domains were fused to a catalytically inactive *Fok*I variant or tested against non-cognate DNA targets.

Haploid cell types containing either TALEN expression or target plasmid in 200 μl of overnight culture were mated in YPD medium at 30°C. After 4 hours, the YPD medium was replaced with 5 ml of selective medium and incubated overnight at 30°C. Mated cultures were lysed, ONPG substrate added, and absorbance read at 415 nm using a 96-well plate reader (Townsend et al., *supra*). β-galactosidase levels were calculated as a function of substrate cleavage velocity. The results obtained with target reporter constructs that had a 15 bp spacer separating the two recognition sites are shown in FIG. 22B. All nuclease expression constructs derived from the *Sph*I fragment, which encoded principally the repeat array, failed to show activity, indicating that amino acid sequences in addition to those in the repeat array are required for function (FIG. 22B). Robust activity, however, was observed for both the AvrBs3 and the PthXo1 TALENs derived from the *Bam*HI fragment (FIG. 22B). The activity of the PthXo1 TALEN approximated that of the ZFN positive control. The activity required the functional *Fok*I domain and was specific for the DNA target recognized by a given TALEN.

Experiments also were conducted to test various distances between the TAL effector binding sites (11 length variants between 12 and 30 bp), in order to identify spacer lengths that enable *Fok*I to dimerize most efficiently (FIG. 28A). Both enzymes showed two spacer length optima – one at 15 bp and the other at either 21 bp (AvrBs3) or 24 bp (PthXo1). For PthXo1, activity was observed for all tested spacer lengths 13 bp and longer. Some spacer lengths for AvrBs3 showed no activity, however, suggesting that spacer length is critical for certain TALENs.

The above experiments tested activity of homodimeric TALENs, which bind two

identical recognition sequences placed in opposition on either side of the spacer. Since such palindromic sites are unlikely to occur naturally in genomic targets, experiments were conducted to test whether TALENs could function as heterodimers. AvrBs3 and PthXo1 recognition sites were placed in head to tail orientation on either side of a 15 bp spacer. Activity of AvrBs3 and PthXo1 TALENS individually and Zif268 on their respective targets was measured as controls. As a negative control, a yeast culture with only the target site plasmid for the heterodimeric site was assayed for LacZ activity. The resulting activity of the heterodimeric TALEN approximated an average of the activities observed with the two homodimeric enzymes (FIG. 28B).

To test whether repeat domains can be assembled to target TALENs to arbitrary chromosomal sequences, two genes were chosen that were previously targeted for mutagenesis with ZFNs – *ADH1* from Arabidopsis and *gridlock* from zebrafish (Foley et al. (2009) *PLoS One* 4:e4348; and Zhang et al., *supra*). A search was conducted for 12-13 bp sequences in the coding regions that were preceded by a 5' T and with a nucleotide composition similar to that of TAL effector binding sites identified by Moscou and Bogdanove (*supra*). In *ADH1* and *gridlock*, such sites occurred on average every 7-9 bp. Four 12 bp sites were selected in *ADH1* (at positions 360, 408, 928, and 975 of the chromosomal gene sequence) and one 13 bp site in *gridlock* (at position 2356 of the chromosomal gene sequence; FIG. 29A). TAL effector repeat domains were constructed to recognize these targets, using the most abundant RVDs from native TAL effectors (NI for A, HD for C, NN for G, and NG for T). To construct custom TALENs, repeats with these RVDs were synthesized individually and assembled into modules of one, two, or three repeats as described in Examples 4 and 5. These modules were ligated sequentially into a derivative of the *tal1c* gene (Moscou and Bogdanove, *supra*) from which the original repeats had been removed, and *BamH*I fragments from these engineered TAL effectors were fused to sequences encoding the catalytic domain of *Fok*I in pFZ85 (FIG. 25). Five custom TALENs targeted to *ADH1* from Arabidopsis and the zebrafish *gridlock* gene were created.

The resulting custom TALENs were tested in the yeast assay as homodimeric TALENs (that is, the identical DNA binding site was duplicated in inverse orientation on either side of a 16-18 bp spacer), although it is noted that heterodimeric TALENs would

need to be constructed to direct cleavage at naturally occurring DNA targets. Spacer lengths were chosen based on the distance closest to 15 bp from the 3' end of the next neighboring (and opposing) candidate site. Sixteen bp spacers were used for *ADH1*-360-12, *ADH1*-408-12r, and 18 bp spacers for *ADH1*-928-12, *ADH1*-975-12r, and *gridlock*-2356-13r. The yeast assay was performed as described above.

Robust nuclease activity was observed for the *ADH1*-360-12 and *gridlock*-2356-13r TALEN (FIG. 29B). The *ADH1*-928-12 TALEN had modest activity that was nonetheless significantly above the negative controls. For each TALEN that gave positive results, nuclease activity was specific to the cognate target. These results indicate that novel, functional TALENs can be created by assembly of customized repeat domains.

Example 7 – Naturally occurring target and TAL effector pairs show overall and positional bias in nucleotide and RVD composition

The 20 paired targets and TAL effectors analyzed by Moscou and Bogdanove (*supra*) were evaluated for overall composition bias and for positional effects on nucleotide or RVD frequencies. It was observed that sites (on the positive strand) were generally A- and C-rich, and G-poor. The average percent A was 31 ± 16% (1 standard deviation). The average percent C was 37 ± 13%. The average percent G was 9 ± 8%, and the average percent T was 22 ± 10%. Since the alignments vary in length, the analysis of positional effects was restricted to the five positions on each end. Strikingly, bias in the target sequences was apparent for A and against T at positions 1 and 3, and for T at position N and possibly 2. G was particularly rare at position N-1. This bias was reflected by matching RVDs in the effectors, with NI being most common at positions 1 and 3, no NG at position 1, nearly always NG at position N, and rarely NN at position N-1 (FIG. 30).

Example 8 – Method and reagents for rapid assembly and cloning of custom TAL effector repeat arrays

The Golden Gate cloning method [Engler et al. (2008), *supra*; and Engler et al. (2009), *supra*] employs the ability of Type IIS restriction endonucleases (e.g. *Bsa*I) to cut

outside their recognition sites to create custom overhangs for ordered ligation of multiple DNA fragments simultaneously. Using this method, several DNA fragments can be fused into an array in a specific order and cloned into a desired destination vector in a single reaction (FIG. 31).

A method and reagents for assembling custom TAL effector repeat encoding arrays were developed based on the Golden Gate system. When *Bsa*I sites are positioned on either side of a TAL effector repeat coding sequence, cleavage releases a repeat fragment flanked by 4-bp overhangs. Because the cleavage site is not sequence-specific, by staggering, repeat clones can be released with ordered, complementary overhangs (sticky ends), enabling the ordered assembly of multi-repeat arrays.

A library of 58 plasmids (FIGS. 32A and 32B) was generated to allow the simultaneous assembly of up to 10 repeat units into "subarrays," followed by simultaneous assembly of one, two, or three of these subarrays together with a final truncated repeat into a complete, custom array. Ten staggered sets of four fragments, each fragment in a set encoding a repeat module with a different one of the four most common RVDs, HD, NG, NI, and NN, were synthesized and cloned into a vector carrying the tetracycline resistance gene, for a total of 40 plasmids. Four more fragments that encoded the terminal truncated TAL effector repeat of 20 amino acids, each fragment encoding a different one of the four most common RVDs, were synthesized and cloned into a different vector carrying the spectinomycin resistance gene to yield four more plasmids, designated as "last repeat plasmids," FIG. 32A). All fragments in the staggered sets are flanked by *Bsa*I sites in the vector so that cleavage with *Bsa*I releases the fragments with different sticky ends that allow for assembly in the appropriate order; that is, i.e. the overhang at the 3' end of a fragment for repeat module 1 is complementary only to the overhang at the 5' end of the fragment for repeat module 2, the overhang at the 3' end of repeat module 2 is complementary only to the overhang at the 5' end of repeat module 3, and so on. The fragments in the last repeat plasmids are flanked by sites for a different Type IIS restriction endonuclease, *Esp*3I. Fourteen additional plasmids, described following, were constructed as destination vectors to receive assembled subarrays.

The first destination vector, plasmid pFUS_A was constructed to receive the first subarray of 10 repeats to be assembled into a final array of 21 or fewer repeats (counting the final, truncated repeat). pFUS_A was constructed such that cleavage by *Bsa*I creates an overhang on one side complementary to the overhang at the 5' end of the first repeat module and an overhang at the other side complementary to the overhang at the 3' end of the l0th repeat module. To receive a second subarray of 10 or fewer repeats to be assembled into such a final array, destination vector plasmids pFUS_B1, pFUS_B2, pFUS_B3, pFUS_B4, pFUS_B5, pFUS_B6, pFUS_B7, pFUS_B8, pFUS_B9, and pFUS_B10 were constructed that when cleaved by *Bsa*I have overhangs respectively complementary to the overhang at the 5' end of the first repeat module and the 3' end of the repeat module for the corresponding numbered position (e.g., the pFUS_B6 overhang for the 3' end of the subarray matches the overhang of the four repeat module fragments for position 6). Arrays cloned in pFUS_A and the pFUS_B series of plasmids are flanked by *Esp*3I sites in the vector and when released by digestion with *Esp*3I the arrays have unique complementary overhangs that allow for them to be ligated in order along with a final truncated repeat fragment into destination vector pTAL, which encodes a TALEN missing the repeat region. pTAL was constructed so that cleavage with *Esp*3I allows insertion of the repeat array at the correct location and in the correct orientation by virtue of an overhang at one end that is complementary to the overhang at the 5' end of the first ten repeat subarray and an overhang at the other end complementary to the overhang at the 3' end of the final truncated repeat fragment (FIG. 33).

The final two destination vector plasmids, pFUS_A30A and pFUS_A30B were constructed to receive the first and second ten repeat subarrays to be assembled into a final array of 22-31 repeats . pFUS_A30A and pFUS_A30B were constructed such that digestion with *Esp*3I releases the arrays with the appropriate complementary overhangs such that the arrays can be ligated in order along with a third array from a pFUS_B vector and a final truncated repeat fragment from a last repeat plasmid, released similarly by digestion with *Esp*3I, into pTAL (FIG. 32).

All destination vectors have the LacZ gene cloned in between the Type IIS restriction endonuclease sites, allowing for blue-white screening for recombinants.

Except for pTAL, which carries a gene for ampicillin resistance, all the destination vectors carry a gene for spectinomycin resistance.

To rapidly construct a custom TAL effector repeat array using these reagents, the following method was established. In the first step, the appropriate individual RVD module plasmids for the necessary subarrays of ten or fewer repeats are mixed together with the appropriate destination vector in one tube. T4 DNA ligase and *Bsa*I endonuclease are added and the reaction is incubated in a PCR machine for 10 cycles of 5 minutes at 37°C and 10 minutes at 16°C, the respective optimal temperatures for the two enzymes. The reaction mixture is then treated with the PLASMID-SAFE™ nuclease to hydrolyze all linear dsDNA fragments in order to prevent cloning of shorter, incomplete arrays by *in vivo* recombination, and then the mixture is used to transform chemically competent *E. coli* cells. The resulting recombinant plasmids are isolated and the correct constructs confirmed. Then, in the second step, the confirmed plasmids from the first step are mixed together with the appropriate last repeat plasmid and pTAL, and the digestion and ligation reaction cycle carried out as in the first step. Finally, the reaction products are introduced into *E. coli*, and the full length, final array construct is isolated and confirmed. The protocol can be completed by one person within a week's time.

Expression constructs for TALENS 85, 102 and 117 in Table 4A, as well as TALENS HPRT-3254-17 and HPRT-3286-20r, described in Example 14 below, were made using the method and reagents described in this example.

Repeat arrays cloned in pTAL are subcloned readily into other TAL effector gene contexts using the conserved *Sph*I restriction endonuclease sites that flank the repeat region.

Example 9 – Custom TALEN data show initial support for "rules" and a correlation between RVD number and activity

Example 6 describes experiments conducted to engineer the TALEN DNA binding domain so that it can recognize unique DNA sequences. As described, these custom TALENs recognized sites in the Arabidopsis *ADH1* and zebrafish *gridlock* genes. Additional custom TAL effector DNA binding domains were engineered to recognize not only sites in these genes, but also in the *TT4* gene from Arabidopsis, and *telomerase* from

zebrafish (Foley et al., *supra*; and Zhang et al., *supra*). These custom TALENs were made using the methods described in Examples 3, 4 and 8. In engineering the custom TALENs, the observed compositional and positional biases were adopted as design principles or "rules." First, a search was conducted for sequences in the coding regions that were preceded by a 5' T and at least 15 bp in length, and that had a nucleotide composition consistent with the averages noted above. Specifically, only those sites with 0-63% A, 11-63% C, 0-25% G, and 2-42% T were selected. Such sites occurred on average every 7-9 bp. Sites were then selected that conformed to the observed positional biases described above. From this set, two pairs of binding sites in each gene were identified that were 15-19 bp in length and separated by 15-18 bp, so that binding of the engineered TALENs would allow *Fok*I to dimerize. The modular assembly methods (Examples 3 and 4) generated partial length constructs.

In total, 21 intermediate and full length TALENs designed to target 16 nucleotide sequences, each with an array of nine repeats or longer. The amino acid sequences of these TALENs are provided in FIGS. 34A-34U (SEQ ID NOS:35-55). These 21 TALENs were tested for their ability to cleave DNA using the yeast assay described in Examples 2 and 6. Activity data are shown in FIG. 35 and summarized in Table 4A.

Some of the intermediate, partial length TALENs correspond to targets that break the rules for nucleotide composition and terminal T. Table 4A shows length, conformity to these two rules, and activity relative to that of ZFN268 for each TALEN. The results reveal a general trend that increasing the length of the RVD array increases activity of the resulting TALEN. This suggests that there is a minimal number of RVDs that are needed before a DNA target can be recognized *in vivo*. Further, conformity to the rules appears to be important. Of the six TALENs showing no detectable activity, two violated the target composition rule, two did not end in NG, and another broke both rules (one obeyed both rules). Three of the eight TALENs with activity less than 25% of ZFN268 violated one of the rules, and one of four TALENs with activity 25-50% of ZFN268 did not have an RVD sequence ending in NG. It is noted that TALENs with activity 50% or greater than that of ZFN268 obeyed all the rules, and for TALENs of the same length, rule breakers generally had less activity than obedient arrays. Consistent with the overall trend regarding length, even for intermediates that broke no rules, the corresponding full

length TALENs had higher activity (Table 4A and FIG. 35). Variation in spacer length due to TALEN length differences on the same target may have contributed to this observation, but some range of spacer lengths is tolerated (Christian et al., *supra*).

Some complexities in the data were apparent. For example, activity varied among obedient TALENs of the same length, some short arrays had moderately high activity, and some long arrays that were obedient had little or no activity (Table 4B). Nonetheless, the results provided support for the conclusions that 1) generally a greater number of repeats results in greater activity, and 2) conformity to composition and positional bias rules is important for activity. Therefore, the following design principles were derived.

- TAL effector binding sites are designed to be a minimum of 15 bases long and oriented from 5' to 3' with a T immediately preceding the site at the 5' end.
- A site may not have a T in the first (5') position or an A in the second position.
- A site must end in T (3'), and may not have a G at the next to last position.
- The base composition of the site must fall within specified ranges (average ± two standard deviations): A 0-63%, C 11-63%, G 0-25%, and T 2-42%.

Table 4A

Activity, conformity to rules, and length of TALENs tested in the yeast assay.

Gene	TALEN	Names from Christian et al. (*supra*)	RVDs	Activity	% GATC	Ends in NG	RVD sequence[1]
telomerase	124		9	-	N	Y	HD NN NN NG NG NG NN HD NG
gridlock	105		10	+	N	N	NI HD HD HD HD NG HD NG HD HD
ADH1	58	ADH1-360-12	12	++	Y	N	NI NG HD NI NI NN NI NG NG HD NG HD
ADH1	63	ADH1-408-12r	12	-	Y	N	HD HD HD NI NN NI NI NN NG NI NI NI
ADH1	68	ADH1-928-12	12	+	Y	Y	HD HD NN NN NI NG NN HD NG HD HD NG
ADH1	73	ADH1-975-12r	12	-	N	N	NI NN NI HD NI NI NI HD HD NI HD NI
TT4	89		12	-	Y	N	NN NN HD NI HD NG NN HD NG NI NI HD
gridlock	106	gridlock-2356-13r	13	++	Y	Y	NI HD HD HD HD NG HD NG HD HD NN HD NG
ADH1	64		15	+	Y	Y	HD HD HD NI NN NI NI NN NG NI NI NI HD NI NG
ADH1	69		15	+++	Y	Y	ND ND NN NN NI NG NN HD NG HD HD NG HD NG NG
ADH1	74		15	++	Y	Y	NI NN NI HD NI NI NI HD HD NI HD NI NI HD NG
TT4	90		15	-	Y	Y	NN NN HD NI HD NG NN HD NG NI NI HD HD HD NG
telomerase	121		15	+	Y	Y	HD NG NG NN NG HD HD NN HD NI NG NN NI NG NG
telomerase	126		15	-	N	Y	HD NN NN NG NG NG NN HD NG NI NG HD NN NG NG
gridlock	107		16	++++	Y	Y	NI HD HD HD HD NG HD NG HD HD NN HD NG NG HD NG
gridlock	117		16	++	Y	Y	HD HD HD NN NN NI NI NN HD HD NN NI HD NN HD NG
telomerase	131		16	+	Y	Y	NI NG NG HD HD HD HD NI HD NN NI NN HD NG HD NG
telomerase	136		17	+	N	Y	NI NN NI HD NI NN NN NI NI NN NG NN NN NI NN HD NG
ADH1	60		18	+++++	Y	Y	NI NG HD NI NI NN NI HG NG HD NG HD NG NG HD NI HD NG
TT4	85		18	+	Y	Y	NI HD NG HD HD NN HD HD NG NN NI NI NN HD NI HD NI NG
gridlock	102		18	+	Y	N	NN NN HD NG HD NI HD HD NG NI HD NI NI HD NN NI HD NI

[1] Target sequences tested consist of inverted repeats of the corresponding nucleotide sequence, where HD, NG, NI, and NN correspond to C, T, A, and G, respectively, separated by a spacer sequence of 16-18 bp.

Table 4B

Excerpt of Table 4A, sorted by activity level

RVDs	Activity	% GATC	Ends in NG
9	-	n	y
12	-	y	n
12	-	n	n
12	-	y	n
15	-	y	y
15	-	n	y
10	+	n	n
12	+	y	y
15	+	y	y
15	+	y	y
16	+	y	y
17	+	n	y
18	+	y	y
18	+	y	n
12	++	y	n
13	++	y	y
15	++	y	y
16	++	y	y
15	+++	y	y
16	++++	y	y
18	+++++	y	y

Example 10 – Heterodimeric TALEN pairs cleave their intended naturally occurring target sequences in the yeast assay

The data in Examples 2, 6 and 9 demonstrate that custom TALENs can be engineered to recognize novel target DNA sequences. The yeast activity data for the custom TALENs was gathered using individual TALEN monomers that recognized a homodimeric target site. That is, the target sequence of the TALEN was duplicated in inverse orientation on either side of a 15-18 bp spacer. Cleavage of endogenous chromosomal sequences, however, generally would require that two different custom TALENs recognize two different sequences on either side of a spacer. As described in Example 6, this ability was demonstrated for the AvrBs3 and PthXo1 TALENS together

using a corresponding chimeric target site in the yeast assay. We tested whether two different custom TALENs could recognize and cleave a naturally occurring DNA sequence. Using the yeast assay described in Example 2, custom TALENs designed to cleave two different target sequences in the Arabidopsis ADH1 gene were assayed for activity on these targets. The DNA sequences of the target sites and the corresponding TALENs are shown in FIG. 36A. The amino acid sequences of the TALENs are provided in FIG. 34. The beta-galactosidase activity obtained in the yeast assay is plotted in the graph shown in FIG. 36B. The activity of the TALENs on their naturally occurring target sequence was significantly above the negative controls, indicating that TALENs can be engineered to recognize and cleave endogenous target DNA sequences.

Example 11 – TALENs cleave native genes in Arabidopsis and introduce mutations by imprecise non-homologous end-joining

One of the active TALEN pairs designed to recognize a target sequence in the Arabidopsis *ADH1* gene was tested to determine whether it can bind, cleave and mutate chromosomal DNA. Each of the individual *ADH1* TALENs comprising this pair (pTALENs 69 and 74) was cloned into the plant expression vector pFZ14, which places the TALENs under the control of the constitutive 35S promoter (Zhang et al., *supra*). The resulting constructs were then introduced into Arabidopsis protoplasts by electroporation. After 48 hours, genomic DNA was isolated and digested with Tth111I. A Tth111I cleavage site is located in the spacer sequence between the two TALEN recognition sites (FIG. 37A). Cleavage of the chromosomal DNA by the TALEN would be expected to introduce mutations by imprecise non-homologous end-joining (NHEJ), which would result in failure to cleave by Tth111I. A 375 bp fragment encompassing the TALEN recognition site was then PCR amplified. The PCR product was digested again with Tth111I to remove most of the remaining genomic DNA that was not modified by TALEN-mediated NHEJ. The digestion products were then run on an agarose gel. An uncleaved PCR product was observed, and such uncleaved PCR products are diagnostic of nuclease activity (in this case TALEN activity) at the endogenous target sequence (Zhang et al., *supra*). The uncut DNA was cloned and analyzed by DNA sequencing. The sequencing of nine independent clones revealed that six carried mutations introduced

by NHEJ (FIG. 37B). Thus, TALENS cleave endogenous chromosomal loci and introduce DNA double strand breaks and mutations.

Example 12 – Enhancing targeting capacity

At the core of the TAL effector DNA cipher, the four most common RVDs each have apparent one-to-one specificity for the four nucleotides, based on association frequencies. This is markedly so for HD, NG, and NI, but less so for NN (FIG. 1C). NN associates most frequently with G, but almost as commonly with A, and sometimes with C or T. For a randomly assembled TAL effector with NN at four locations in a 13 RVD sequence, having G at all corresponding positions in an artificial target gave the best activity (Boch et al. (2009) *Science* 326:1509-1512). A reduced but did not abolish activity, and C and T eliminated detectable activity. A drastic loss of activity was observed when C, T, or A was substituted for G at just the first position in the binding site for the 24 RVD effector PthXo1, which is an NN (Romer et al. (2010) *New Phytol.* 187:1048-1057). This was in contrast, however, to the observation that the much shorter AvrHah1 (14 RVDs) begins with an NN that aligns with A, and the 23 RVD effector PthXo6 has three NNs in a row at positions 4–6 that each align with A, yet both of these proteins are highly active (*see*, Schornack et al. (2008) *New Phytol.* 179:546-556; and Romer et al., *supra*). Thus the specificity of NN for G appears to be generally weak and can vary with context.

The observed invariance of the thymine immediately preceding TAL effector target sites is a requirement for several effectors [Boch et al., *supra*; Romer et al., *supra*; and Romer et al. (2009) *Plant Physiol.* 150:1697-1712]. The amino acid sequence immediately preceding the repeat region in TAL effectors, which is highly conserved (FIG. 38A), shares significant similarity with the repeat, both in amino acid sequence and in predicted secondary structure (FIG. 38B and Bodganove et al. (2010) *Curr. Opin. Plant Biol.* 13:394-401). It was hypothesized that this sequence, termed the "0^{th}" repeat, is the basis for the requirement for T at position -1 of the binding site, and that residues in the RVD-analogous position (FIG. 38B) specify the nucleotide.

Based on these findings, it was hypothesized that by incorporating repeats with high specificity for G, and by relaxing the requirement for T at -1, targeting capacity for

engineered TAL effector proteins can be enhanced. Experiments were initiated to test novel and rare RVDs for more robust specificity for G than NN displays, and to replace the RVD-analogous residues of the 0^{th} repeat with common RVDs.

Novel and rare RVDs for robust specificity for G: The modules disclosed above (see, e.g., Example 4) used four particular RVDs (NI, HD, NN, and NG) to specify binding to the four nucleotide bases (A, C, G, and T, respectively). Repeats containing other RVDs also may be useful, and may have increased specificity and/or affinity for the four bases as compared to NI, HD, NN, and NG. Toward improving specificity for G, several repeats encoding novel and rare RVDs were constructed. The rare RVDs NK, HN, and NA associated with G, suggesting that N may be important as one or the other of the residues (FIG. 1C). Thus, a broad set of derivatives encoding repeats having the RVDs shown in Table 5 were constructed. The left column lists RVDs having a polar amino acid (R, K, D, E, Q, H, S, T, or Y) at position 12 and N at position 13. The right columns list combinations of N in the first position with any of 17 other amino acids (G, L, V, R, K, D, E, Q, H, T, M, C, P, Y, W, or F) in the second position of the RVD. To account for the possibility of greater specificity without N, repeats also were made with a polar amino acid (R, K, D, E, Q, H, S, T, or Y) at position 12 and a gap (*) at position 13 (middle column).

Novel artificial RVDs are tested for function in a quantitative reporter gene based assay for transcriptional activation activity of TAL effectors, such as a GUS or dual luciferase reporter based, Agrobacterium-mediated transient expression assay in *Nicotiana benthamiana*, or in the lacZ reporter based TALEN assay in *Saccharomyces cerevisiae*, described above (*see*, e.g., Example 2) Repeat modules containing RVDs to be tested are incorporated into a TAL effector or TALEN with measurable and sub-saturation levels of activity, and the resulting proteins are tested for differences in activity on a set of DNA targets with integrated permutations of all four nucleotides at corresponding positions. In particular, beginning with the PthXo1 variant(s) minimally active in the *in planta* and yeast assays and responsive to mismatches at three added repeats, TALENs containing each of the novel and rare repeats (in homomeric threes) are tested *in vivo* against targets with G at each of the corresponding positions. For any that

show increased activity, the assays are repeated with targets permutated to the other nucleotides at those positions, to ascertain specificity.

Table 5. RVDs to be tested[a]

Polar+N	Polar*	N+all	
RN	R*	NG	NH
KN	K*	NA	NT
DN	D*	NL	NM
EN	E*	NV	NC
QN	Q*	NR	NP
HN	H*	NK	NY
SN	S*	ND	NW
TN	T*	NE	NF
YN	Y*	NQ	

[a]N*, NG, and NS nt association frequencies are known. An asterisk represents a gap corresponding to the 2nd position in the RVD (i.e., the 13th position of the consensus repeat sequence).

Common RVD substitutions for the RVD-analogous position of the 0th repeat to relax specificity of T at position -1: Secondary structure predictions and alignment of the 0th repeat and repeat consensus sequences suggested that positions occupied by KR* (asterisk denotes a gap) in the 0th repeat were analogous to the RVD and were therefore the residues that specify the T at -1. Variants of PthXo1 with substitutions of HD, NG, NI, and NN for KR and separately for R* were constructed in the Tal1c "backbone" construct described above. Activities of these variants are compared to the wild type effector in the *in planta* and yeast assays using targets with corresponding nucleotides at position -1, namely, C, T, A, and G, respectively. Additional variants of PthXo1 are constructed that have S, the residue at position 11 of the consensus repeat sequence, substituted for the K at position 11 of the 0th repeat. And other variants are constructed that have this substitution combined with a substitution of K, the residue at position 16 of the consensus repeat sequence, for the V at position 15 of the 0th repeat (Table 6). A proximal TATA box for TAL effector activity may be included. In addition, PthXo1 is

useful for this experiment because unlike AvrBs3, for which the T at -1 appears to be part of a TATA box, the TATA box closest to the PthXo1 binding site is 46 bp downstream and would not be perturbed by modifications at -1.

If the above modifications do not result in enhanced targeting for G or increased ability to target sequences preceded by nucleotides other than T, then a more comprehensive set of artificial RVDs are tested for G specificity, and substitutions other than the common RVDs are tested for the 0th repeat.

Table 6. 0^{th} repeat constructs to be made and tested for specificity for targets with A, C, G, or T at the -1 position

Native 0^{th} repeat sequence (specifies T at -1)	**Substitution specifying T**	**Substitution specifying A**	**Substitution specifying C**	**Substitution specifying G**
…KIA*__KR__GGV…(74)[†]	…KIA*__NG__GGV…(75)	…KIA*__NI__GGV…(76)	…KIA*__HD__GGV…(77)	…KIA*__NN__GGV…(78)
…KIA*__KR__GGV…(79)	…KIA__S__NGGGV…(80)	…KIA__S__NIGGV…(81)	…KIA__S__HDGGV…(82)	…KIA__S__NNGGV…(83)
…KIAK__R__*GGV…(84)	…KIAK__NG__GGV…(85)	…KIAK__NI__GGV…(86)	…KIAK__HD__GGV…(87)	…KIAK__NN__GGV…(88)
…KIA*__KR__GGV…(89)	…KIA__S__NGGG__K__…(90)	…KIA__S__NIGG__K__…(91)	…KIA__S__HDGG__K__…(92)	…KIA__S__NNGG__K__…(93)

Candidate and substituted RVDs are bold. Other substitutions or modifications are underlined. Asterisks denote a gap relative to the consensus repeat sequence.

[†] SEQ ID NO:

Example 13 - Novel predicted nucleotide specific RVDs

It was observed that when the RVDs listed in Tables 1A and 1B were grouped by the second amino acid residue in the RVD (i.e., the 13th in the overall repeat), there was a near perfect correlation of that amino acid with the nucleotide(s) specified by the RVD, irrespective of the amino acid at the first position of the RVD (Table 7). Thus, RVDs ending in a gap (denoted by an asterisk) specify C or T, or T; RVDs ending in D specify C; RVDs ending in G specify T; and RVDs ending in N specify G or A, or G. It also was observed that amino acids at position 1 of the RVD were either H, I, N, S, or Y. These observations suggested that RVD specificity is determined by the residue in the second position, independent of whether the residue at the first position is H, I, N, S, or Y. Therefore, specificities were predicted for several novel (i.e., yet unobserved) RVDs that combine residues observed at the second position with residues H, I, S, N, or Y at the first position. Thus, I*, S*, and Y* were predicted to specify C or T, or T; ID, SD, and YD were predicted to specify C; SG was predicted to specify T: and IN and YN were predicted to specify G or A, or G. Also, although there was only one instance of K at the second position, based on the observed specificity of NK, it was predicted that HK, IK, SK, and YK specify G.

These novel RVDs are tested and compared to existing RVDs for function and specificity in quantitative TAL effector and TALEN activity assays as described in Examples 2 and 11.

Table 7[1]

RVDs grouped and ordered by their second residue

1st residue	2nd residue	Nucleotide
N	*	C or T
H	*	T
H	A	C
N	A	G
H	D	C
N	D	C
H	G	T
I	G	T
N	G	T
Y	G	T
N	I	A
H	I	C
N	K	G
H	N	G
S	N	G or A
N	N	G or A[1]
N	S	A or C or G[1]

[1] An asterisk denotes a gap. RVD groups with like specificities are boxed in thick lines.

Example 14 – Custom TALENs cleave endogenous targets in animal cells and introduce mutations by imprecise non-homologous end-joining

To test whether TALENs could be used for targeted mutagenesis in animal cells, first, expression of TAL effectors AvrBs3, PthXo1, and Tal1c was tested in human embryonic kidney (HEK) 293T cells. The stop codon was removed from the AvrBs3, PthXo1,and Tal1c encoding genes and the genes were subcloned into mammalian expression vector pcDNA3.2/V5-DEST (Invitrogen, Carlsbad, CA) in frame with the downstream sequence in that vector that encodes the V5 epitope for protein immunodetection. pcDNA3.2/V5-DEST places the TAL effector gene under the control of the constitutive human cytomegalovirus (CMV) promoter. HEK 293T cells were transfected using Lipofectamine 2000 (Invitrogen) with the resulting plasmids individually, and after 24 hours, total proteins were isolated from each transfected batch

of cells and subjected to polyacrylamide gel electrophoresis, western blotting and immunolabeling using a mouse anti-V5 antibody. The labeled proteins were detected with a goat anti-mouse antibody-horse radish peroxidase conjugate using the SuperSignal Weat Pico Chemiluminescent kit (ThermoScientific, Inc.). Equivalent loading was confirmed by immunolabeling and detection of actin. Each TAL effector protein was detectably expressed with no apparent degradation (FIG. 39).

Next, a pair of TALENs were designed as described in Example 9 to target a sequence in the endogenous human *HPRT* gene, and named HPRT-3254-17 and HPRT-3286-20r (FIG. 40A and FIG. 40B). Plasmids pTALEN141 encoding HPRT-3254-17 and plasmid pTALEN142 encoding HPRT-3286-20r were constructed using the Golden Gate cloning-based method and reagents described in Example 8. The TALEN genes were then subcloned into the mammalian expression vector pCDNA3.1(-) (Invitrogen, Inc.), which places them under control of the constitutive CMV promoter, yielding plasmids pTALEN141M and pTALEN 142M. HEK 293T cells were then transfected with both pTALEN141M and pTALEN142M together and separately with pCDNA3.1(-) as a negative control. After 72 hours, genomic DNA was isolated and digested with restriction endonuclease *Bpu*10I. A *Bpu*10I site exists within the spacer that separates the HPRT-3254-17 and HPRT-3286-20r binding sites in *HPRT* (FIG 41A). Following *Bpu*10I digestion, PCR was used to amplify a 244 bp fragment spanning the TALEN-targeted site from both the TALEN-treated and the control samples. The expected fragment was amplified from both samples, indicating that *Bpu*10I digestion of the genomic DNA had been incomplete. Subsequent digestion of the PCR products with *Bpu*10I, however, resulted in complete cleavage of the product amplified from the control sample, but incomplete cleavage of the product from the TALEN treated sample (FIG. 41B). The presence of cleavage-resistant PCR product in the TALEN-treated sample provides evidence that the endogenous *Bpu*10I site was mutated *in vivo* as a result of imperfect repair by non-homologous end joining of a TALEN-mediated double strand break at the intended target in *HPRT*. Thus, TALENs can be used for targeted mutagenesis in mammalian cells.

OTHER EMBODIMENTS

It is to be understood that while the invention has been described in conjunction with the detailed description thereof, the foregoing description is intended to illustrate and not limit the scope of the invention, which is defined by the scope of the appended claims. Other aspects, advantages, and modifications are within the scope of the following claims.

WHAT IS CLAIMED IS:

1. A method for modifying the genetic material of a cell, comprising:

(a) providing a cell containing a target DNA sequence; and

(b) introducing a transcription activator-like (TAL) effector -DNA modifying enzyme into the cell, the TAL effector-DNA modifying enzyme comprising:

(i) a DNA modifying enzyme domain that can modify double stranded DNA, and

(ii) a TAL effector domain comprising a plurality of TAL effector repeat sequences that, in combination, bind to a specific nucleotide sequence in the target DNA sequence,

such that the TAL effector-DNA modifying enzyme modifies the target DNA within or adjacent to the specific nucleotide sequence in the cell or progeny thereof.

2. The method of claim 1, further comprising providing to the cell a nucleic acid comprising a sequence homologous to at least a portion of the target DNA sequence, such that homologous recombination occurs between the target DNA sequence and the nucleic acid.

3. The method of claim 1, wherein the cell is a eukaryotic cell.

4. The method of claim 1, wherein the cell is a mammalian cell.

5. The method of claim 1, wherein the cell is a plant cell.

6. The method of claim 1, wherein the cell is a prokaryotic cell.

7. The method of claim 1, wherein the target DNA is chromosomal DNA.

8. The method of claim 1, wherein the introducing comprises transfecting the cell with a vector encoding the TAL effector-DNA modifying enzyme.

9. The method of claim 1, wherein the introducing comprises mechanically injecting the TAL effector-DNA modifying enzyme into the cell as a protein.

10. The method of claim 1, wherein the introducing comprises delivering the TAL effector-DNA modifying enzyme into the cell as a protein by means of the bacterial type III secretion system.

11. The method of claim 1, wherein the introducing comprises introducing the TAL effector-DNA modifying enzyme into the cell as a protein by electroporation.

12. The method of claim 1, wherein the DNA modifying enzyme domain is an endonuclease.

13. The method of claim 12, wherein the endonuclease is a type II restriction endonuclease.

14. The method of claim 13, wherein the type II restriction endonuclease is *Fok*I.

15. The method of claim 1, wherein the TAL effector domain that binds to a specific nucleotide sequence within the target DNA comprises 10 or more DNA binding repeats, more preferably 15 or more DNA binding repeats.

16. The method of claim 15, wherein each DNA binding repeat comprises a repeat variable-diresidue (RVD) that determines recognition of a base pair in the target DNA sequence, wherein each DNA binding repeat is responsible for recognizing one base pair in the target DNA sequence, and wherein the RVD comprises one or more of:

HD for recognizing C;

NG for recognizing T;

NI for recognizing A;

NN for recognizing G or A;

NS for recognizing A or C or G or T;

N* for recognizing C or T, wherein * represents a gap in the second position of the RVD;

HG for recognizing T;

H* for recognizing T, wherein * represents a gap in the second position of the RVD;

IG for recognizing T;

NK for recognizing G;

HA for recognizing C;

ND for recognizing C;

HI for recognizing C;

HN for recognizing G;

NA for recognizing G;

SN for recognizing G or A; and

YG for recognizing T.

17. The method of claim 16, wherein each DNA binding repeat comprises a RVD that determines recognition of a base pair in the target DNA sequence, wherein each DNA binding repeat is responsible for recognizing one base pair in the target DNA sequence, and wherein the RVD comprises one or more of:

HA for recognizing C;

ND for recognizing C;

HI for recognizing C;

HN for recognizing G;

NA for recognizing G;

SN for recognizing G or A;

YG for recognizing T; and

NK for recognizing G,

and one or more of:

HD for recognizing C;

NG for recognizing T;

NI for recognizing A;

NN for recognizing G or A;

NS for recognizing A or C or G or T;

N* for recognizing C or T, wherein * represents a gap in the second position of the RVD;

HG for recognizing T;

H* for recognizing T, wherein * represents a gap in the second position of the

RVD; and

IG for recognizing T.

18. A method for generating a nucleic acid encoding a TAL effector specific for a selected nucleotide sequence, comprising:

(1) linearizing a starter plasmid with *Psp*XI, the starter plasmid comprising a nucleotide sequence that encodes a first TAL effector DNA binding repeat domain having a repeat variable-diresidue (RVD) specific for the first nucleotide of the selected nucleotide sequence, wherein the first TAL effector DNA binding repeat domain has a unique *Psp*XI site at its 3' end;

(2) ligating into the starter plasmid *Psp*XI site a DNA module encoding one or more TAL effector DNA binding repeat domains that have RVDs specific for the next nucleotide(s) of the selected nucleotide sequence, wherein the DNA module has *Xho*I sticky ends; and

(3) repeating steps (1) and (2) until the nucleic acid encodes a TAL effector capable of binding to the selected nucleotide sequence.

19. The method of claim 18, further comprising, after the ligating, determining the orientation of the DNA module in the *Psp*XI site.

20. The method of claim 18, comprising repeating steps (1) and (2) from one to 30 times.

21. A method for generating a nucleic acid encoding a transcription activator-like effector endonuclease (TALEN), comprising:

(a) identifying a first unique nucleotide sequence in the genome of a cell; and

(b) synthesizing a nucleic acid encoding a TALEN that comprises (i) a plurality of DNA binding repeats that, in combination, bind to the first unique nucleotide sequence, and (ii) an endonuclease that generates a double-stranded break at a second nucleotide sequence within or adjacent to the first nucleotide sequence, wherein each DNA binding repeat comprises a RVD that determines recognition of a base pair in the target DNA, wherein each DNA binding repeat is responsible for recognizing one base pair in the target DNA, and wherein the TALEN comprises one or more of the following RVDs:

HD for recognizing C;

NG for recognizing T;

NI for recognizing A;

NN for recognizing G or A;

NS for recognizing A or C or G or T;

N* for recognizing C or T;

HG for recognizing T;

H* for recognizing T;

IG for recognizing T;

NK for recognizing G;

HA for recognizing C;

ND for recognizing C;

HI for recognizing C;

HN for recognizing G;

NA for recognizing G;

SN for recognizing G or A; and

YG for recognizing T.

22. The method of claim 21, wherein the TALEN comprises one or more of the following RVDs:

HA for recognizing C;

ND for recognizing C;

HI for recognizing C;

HN for recognizing G;

NA for recognizing G;

SN for recognizing G or A;

YG for recognizing T;and

NK for recognizing G,

and one or more of:

HD for recognizing C;

NG for recognizing T;

NI for recognizing A;

NN for recognizing G or A;

NS for recognizing A or C or G or T;

N* for recognizing C or T;

HG for recognizing T;

H* for recognizing T; and

IG for recognizing T.

23. The method of claim 21, wherein the first nucleotide sequence meets at least one of the following criteria:

i) is a minimum of 15 bases long and is oriented from 5' to 3' with a T immediately preceding the site at the 5' end;

ii) does not have a T in the first (5') position or an A in the second position;

iii) ends in T at the last (3') position and does not have a G at the next to last position; and

iv) has a base composition of 0-63% A, 11-63% C, 0-25% G, and 2-42% T.

24. The method of claim 23, comprising identifying a first nucleotide sequence and a second nucleotide sequence in the genome of the cell, wherein the first and second nucleotide sequences meet at least one of the criteria set forth in claim 23, and are separated by 15-18 bp.

25. The method of claim 24, wherein the endonuclease generates a double-stranded break between the first and second nucleotide sequences.

26. A TALEN comprising an endonuclease domain and a TAL effector DNA binding domain specific for a target DNA, wherein the DNA binding domain comprises a plurality of DNA binding repeats, each repeat comprising a RVD that determines recognition of a base pair in the target DNA, wherein each DNA binding repeat is responsible for recognizing one base pair in the target DNA, and wherein the TALEN comprises one or more of the following RVDs:

HD for recognizing C;

NG for recognizing T;

NI for recognizing A;

NN for recognizing G or A;

NS for recognizing A or C or G or T;

N* for recognizing C or T;

HG for recognizing T;

H* for recognizing T;

IG for recognizing T;

NK for recognizing G;

HA for recognizing C;

ND for recognizing C;

HI for recognizing C;

HN for recognizing G;

NA for recognizing G;

SN for recognizing G or A; and

YG for recognizing T.

27. The TALEN of claim 26, wherein the TALEN comprises one or more of the following RVDs:

HA for recognizing C;

ND for recognizing C;

HI for recognizing C;

HN for recognizing G;

NA for recognizing G;

SN for recognizing G or A;

YG for recognizing T; and

NK for recognizing G,

and at least one or more of :

HD for recognizing C;

NG for recognizing T;

NI for recognizing A;

NN for recognizing G or A;

NS for recognizing A or C or G or T;

N* for recognizing C or T;

HG for recognizing T;

H* for recognizing T; and

IG for recognizing T.

28. The TALEN of claim 26, wherein the endonuclease domain is from a type II restriction endonuclease.

29. The TALEN of claim 28, wherein the type II restriction endonuclease is *Fok*I.

30. A TALEN comprising an endonuclease domain and a TAL effector domain, wherein the amino acid sequence of said TALEN is selected from the group consisting of SEQ ID NO:33 to SEQ ID NO:55, SEQ ID NO:72, and SEQ ID NO:73.

31. A method for generating an animal, comprising:

providing a eukaryotic cell comprising a target DNA sequence into which it is desired to introduce a genetic modification;

generating a double-stranded break within the target DNA sequence with a TALEN comprising an endonuclease domain and a TAL effector domain that binds to the target DNA sequence; and

generating an animal from the cell or progeny thereof in which a double-stranded break has occurred.

32. The method of claim 31, further comprising:

introducing into the cell an exogenous nucleic acid comprising a sequence homologous to at least a portion of the target DNA, wherein the introducing is under conditions that permit homologous recombination to occur between the exogenous nucleic acid and the target DNA sequence in the cell or progeny thereof; and

generating an animal from the cell or progeny thereof in which homologous recombination has occurred.

33. The method of claim 31, wherein the animal is a mammal.

34. The method of claim 31, wherein the genetic modification comprises a substitution, an insertion, or a deletion.

35. A method for generating a plant, comprising:

providing a plant cell comprising a target DNA sequence into which it is desired to introduce a preselected genetic modification;

generating a double-stranded break within the target DNA sequence with a TALEN comprising an endonuclease domain and a TAL effector domain that binds to the target DNA sequence; and

generating a plant from the cell or progeny thereof in which a double-stranded break has occurred.

36. The method of claim 35, further comprising:

introducing into the plant cell an exogenous nucleic acid comprising a sequence homologous to at least a portion of the target DNA sequence, wherein the introducing is under conditions that permit homologous recombination to occur between the exogenous nucleic acid and the target DNA sequence in the cell or progeny thereof; and

generating a plant from the cell or progeny thereof in which homologous recombination has occurred.

37. A method for targeted genetic recombination in a cell, comprising:

introducing into the cell a nucleic acid encoding a TAL effector endonuclease targeted to a selected DNA target sequence;

inducing expression of the TAL effector endonuclease within the cell; and

identifying a cell in which the selected DNA target sequence exhibits a mutation.

38. The method of claim 37, wherein the mutation is selected from the group consisting of deletion of genetic material, insertion of genetic material, and both deletion and insertion of genetic material.

39. The method of claim 37, further comprising introducing donor DNA into the cell.

40. The method of claim 37, wherein the cell is an insect cell, a plant cell, a fish cell, or a mammalian cell.

41. A method for generating a TAL effector having enhanced targeting capacity for a target DNA, said method comprising generating a nucleic acid encoding a TAL effector that comprises DNA binding domain having a plurality of DNA binding repeats, wherein each repeat comprises a RVD that determines recognition of a base pair in the target DNA, wherein each DNA binding repeat is responsible for recognizing one base pair in the target DNA,
wherein said generating comprises incorporating a nucleic acid encoding a variant 0th DNA binding repeat sequence with specificity for A, C, or G, thus eliminating the requirement for T at position -1 of the binding site.

42. A method for generating a TAL effector having enhanced targeting capacity for a target DNA, said method comprising generating a nucleic acid encoding a TAL effector that comprises DNA binding domain having a plurality of DNA binding repeats, wherein each repeat comprises a RVD that determines recognition of a base pair in the target DNA, wherein each DNA binding repeat is responsible for recognizing one base pair in the target DNA,
wherein said generating comprises incorporating one or more nucleic acids encoding TAL effector DNA binding domains that contain RVDs having enhanced specificity for G, and wherein said RVDs are selected from the group consisting of RN, R*, NG, NH, KN, K*, NA, NT, DN, D*, NL, NM, EN, E*, NV, NC, QN, Q*, NR, NP, HN, H*, NK, NY, SN, S*, ND, NW, TN, T*, NE, NF, YN, Y*, and NQ, wherein * indicates a gap at the second position of the RVD.

43. A method for producing a polypeptide that selectively recognizes at least one base pair in a target DNA sequence, the method comprising synthesizing a polypeptide comprising a repeat domain, wherein the repeat domain comprises at least one repeat unit derived from a transcription activator-like (TAL) effector, wherein the repeat unit comprises a hypervariable region which determines recognition of a base pair in the target DNA sequence, wherein the repeat unit is responsible for the recognition of one base pair in the DNA sequence, and wherein the hypervariable region comprises a member selected from the group consisting of:

(a) HD for recognition of C/G;

(b) NI for recognition of A/T;

(c) NG for recognition of T/A;

(d) NS for recognition of C/G or A/T or T/A or G/C;

(e) NN for recognition of G/C or A/T;

(f) IG for recognition of T/A;

(g) N for recognition of C/G;

(h) HG for recognition of C/G or T/A;

(i) H for recognition of T/A; and

(j) NK for recognition of G/C.

44. A polypeptide produced by the method of claim 43.

45. A DNA comprising a coding sequence for the polypeptide produced by the method of claim 43.

46. An expression cassette comprising a promoter operably linked to the DNA of claim 45.

47. A non-human host cell comprising the expression cassette of claim 46.

48. A transformed, non-human organism comprising the expression cassette of claim 46.

49. A method for selectively recognizing a base pair in a DNA sequence by a polypeptide, the method comprising constructing a polypeptide comprising a repeat domain, wherein the repeat domain comprises at least one repeat unit derived from a TAL effector, wherein the repeat unit comprises a hypervariable region which determines recognition of a base pair in the DNA sequence, wherein the repeat unit is responsible for the recognition of one base pair in the DNA sequence, and wherein the hypervariable region comprises a member selected from the group consisting of:

(a) HD for recognition of C/G;

(b) NI for recognition of A/T;

(c) NG for recognition of T/A;

(d) NS for recognition of C/G or A/T or T/A or G/C;

(e) NN for recognition of G/C or A/T;

(f) IG for recognition of T/A;

(g) N for recognition of C/G;

(h) HG for recognition of C/G or T/A;

(i) H for recognition of T/A; and

(j) NK for recognition of G/C .

50. A method of modulating expression of a target gene in a cell, wherein cells are provided which contain a polypeptide wherein the polypeptide comprises a repeat domain, wherein the repeat domain comprises at least one repeat unit derived from a TAL effector, wherein the repeat unit comprises a hypervariable region which determines recognition of a base pair in a DNA sequence, wherein the repeat unit is responsible for the recognition of one base pair in the DNA sequence, and wherein the hypervariable region comprises a member selected from the group consisting of:

(a) HD for recognition of C/G;

(b) NI for recognition of A/T;

(c) NG for recognition of T/A;

(d) NS for recognition of C/G or A/T or T/A or G/C;

(e) NN for recognition of G/C or A/T;

(f) IG for recognition of T/A;

(g) N for recognition of C/G;

(h) HG for recognition of C/G or T/A;

(i) H for recognition of T/A; and

(j) NK for recognition of G/C .

51. A polypeptide comprising a repeat domain, wherein the repeat domain comprises at least one repeat unit derived from a TAL effector, wherein the repeat unit comprises a hypervariable region which determines recognition of a base pair in a DNA sequence, wherein the repeat unit is responsible for the recognition of one base pair in the DNA sequence, and wherein the hypervariable region comprises a member selected from the group consisting of:

(a) HD for recognition of C/G;

(b) NI for recognition of A/T;

(c) NG for recognition of T/A;

(d) NS for recognition of C/G or A/T or T/A or G/C;

(e) NN for recognition of G/C or A/T;

(f) IG for recognition of T/A;

(g) N for recognition of C/G;

(h) HG for recognition of C/G or T/A;

(i) H for recognition of T/A; and

(j) NK for recognition of G/C .

52. A DNA comprising a coding sequence for the polypeptide of claim 51.

53. A DNA which is modified to include a base pair located in a target DNA sequence so that the base pair can be specifically recognized by a polypeptide comprising a repeat domain, wherein the repeat domain comprises at least one repeat unit derived from a TAL effector, wherein the repeat unit comprises a hypervariable region which determines recognition of a base pair in the DNA sequence, wherein the repeat unit is responsible for the recognition of one base pair in the DNA sequence, and wherein, to receive a selective and determined recognition by the hypervariable region, the base pair is selected from the group consisting of:

(a) C/G for recognition by HD;

(b) A/T for recognition by NI;

(c) T/A for recognition by NG;

(d) CT or A/T or T/A or G/C for recognition by NS;

(e) G/C or A/T for recognition by NN;

(f) T/A for recognition by IG;

(g) C/G or T/A for recognition by N;

(h) T/A for recognition by HG;

(i) T/A for recognition by H; and

(j) G/C for recognition by NK.

54. A vector comprising the DNA of claim 53.

55. A non-human host cell comprising the DNA of claim 53.

56. A transformed, non-human organism comprising the DNA of claim 53.

57. A method for producing a DNA comprising a target DNA sequence that is selectively recognized by a polypeptide comprising a repeat domain, wherein the repeat domain comprises at least one repeat unit derived from a TAL effector, wherein the repeat unit comprises a hypervariable region which determines recognition of a base pair in the target DNA sequence, and wherein the repeat unit is responsible for the recognition of one base pair in the target DNA sequence, the method comprising synthesizing a DNA comprising a base pair that is capable of being recognized by the repeat unit, wherein the base pair is selected from the group consisting of:

(a) C/G for recognition by HD;

(b) A/T for recognition by NI;

(c) T/A for recognition by NG;

(d) CT or A/T or T/A or G/C for recognition by NS;

(e) G/C or A/T for recognition by NN;

(f) T/A for recognition by IG;

(g) C/G or T/A for recognition by N;

(h) T/A for recognition by HG;

(i) T/A for recognition by H; and

(j) G/C for recognition by NK.