CLAIMS

1. A method for generating a new mutation in an organism for non-therapeutic purpose, which comprises the following steps: sequentially generating two or more DNA breaks at a specific site in a genome of the organism and spontaneously repairing them respectively, wherein a later DNA break is generated based on a new sequence generated from a previous DNA break repair,

wherein the "sequentially generating two or more DNA breaks at a specific site" refers to that based on a new sequence formed by a previous DNA break repair event generated by ZFN or TALEN editing, a new ZFN or TALEN protein is designed to cut the site again; or based on a new sequence formed by a previous DNA break repair event generated by a CRISPR/Cas system, a new target RNA is designed to cut the site again.

2. The method according to claim 1, wherein the "DNA break" is achieved by delivering a nuclease with targeting property into a cell of the organism to contact with the specific site of genomic DNA.

3. The method according to claim 2, wherein the "nuclease with targeting property" is a ZFN, TALEN or CRISPR/Cas system.

4. The method according to any one of claims 1-3, wherein the "two or more DNA breaks" are generated by sequentially delivering different targeted nucleases into recipient cells of different generations, wherein a mutant cell that has completed the previous editing is used as a recipient to receive the delivery of the targeted nuclease for the later editing, thereby performing a second editing to generate a site-specific mutation; or

the "two or more DNA breaks" are generated by delivering different targeted nucleases for different targets into a same recipient cell; or

the "two or more DNA breaks" are generated when RNP complexes formed by a same CRISPR/Cas nuclease respectively with different gRNAs or sgRNAs sequentially cut corresponding target sequences; or

the "two or more DNA breaks" are generated when RNP complexes respectively formed by each of two or more CRISPR/Cas nucleases that recognize different PAM sequences with respective gRNA or sgRNA sequentially cut corresponding target sequences.

5. The method according to claim 4, wherein the targeted nuclease is any CRISPR/Cas nuclease capable of performing a genome editing.

6. The method according to claim 4, wherein the targeted nuclease is in a form of DNA; or

in a form of mRNA or protein instead of DNA.

7. The method according to claim 4, wherein the method for delivering targeted nuclease into a cell is selected from: 1) a PEG-mediated cell transfection method; 2) a liposome-mediated cell transfection method; 3) an electroporation transformation method; 4) a microinjection; 5) a gene gun bombardment; or 6) an *Agrobacterium*-mediated transformation method.

8. A method for lysing a target DNA for non-therapeutic purpose, comprising contacting the target DNA with a complex, the complex comprising:

(a) a Cas polypeptide; and

(b) at least two target RNAs, in which the first target RNA targets the DNA to cause a break at a specific site in the DNA, and the latter target RNA targets a sequence generated from a previous break repair event and generates a break again at said specific site.

9. The method according to claim 8, wherein the contacting comprises introducing the following into the cell: (a) the Cas polypeptide or a polynucleotide encoding the Cas polypeptide, and (b) the target RNA or a DNA polynucleotide encoding the target RNA.

10. A composition, comprising:

(a) a Cas polypeptide, or a polynucleotide encoding the Cas polypeptide; and

(b) at least two target RNAs, or DNA polynucleotides encoding the target RNAs, wherein a first RNA targets a DNA to cause a break at a specific site in the DNA, and a latter target RNA targets a sequence generated from a previous break repair event and generates a break again at said specific site.

11. A kit, comprising:

(a) a Cas polypeptide, or a nucleic acid comprising a nucleotide sequence encoding the Cas polypeptide; and

(b) at least two target RNAs, or nucleic acids comprising nucleotide sequences encoding the target RNAs, wherein a first RNA targets a DNA to cause a break at a specific site in the DNA, and the latter target RNA targets a sequence generated from a previous break repair event and generates a break again at said specific site;

wherein (a) and (b) are in a same or separate containers.

12. The kit according to claim 11, wherein the target RNA is a sgRNA or gRNA; or the target RNAs in (b) are in a same or separate containers.

13. A method for screening editing events independent of an exogenous transgenic marker for non-therapeutic purpose, comprising the following steps:

1) two or more DNA breaks are sequentially generated in sequence at a specific site of a first target gene of a recipient cell and spontaneously repaired respectively, wherein a later DNA break is generated based on a new sequence generated from a repair of a previous DNA break;

2) certain editing events are generated after the specific site of the first target gene is sequentially cut and repaired, which can confer a mutant cell with a resistance to a certain selection pressure to produce a phenotypic selectable trait, a corresponding selection pressure is applied to make a selection for the trait, and a cell, tissue, organ or complete organism that contains such editing events is isolated;

3) optionally, in addition to the first target gene, a targeted nuclease for at least one second target gene is used to edit another target site at the same time, and an editing event of the second target gene is enriched and screened synchronously through the screening of the selectable trait generated by mutations of the first target gene, and a cell, tissue, organ or complete organism that simultaneously contains the editing events of the first target gene and of the at least one second target gene is isolated;

wherein the feature that "two or more DNA breaks are sequentially generated in sequence at a specific site", refers to that based on a new sequence formed by a previous DNA break repair event generated by a CRISPR/Cas system, a new target RNA is designed to cut the site again.

14. The method according to claim 13, wherein the "first target gene" is a gene locus encoding at least one phenotypic selectable trait, wherein the at least one phenotypic selectable trait is a resistance/tolerance trait or a growth advantage trait.

15. The method according to claim 13 or 14, wherein the "specific site of a first target gene" refers to a site at which a certain type of mutation is generated after sequential cuttings and repairs, which is capable of conferring the recipient cell with a resistance to a certain selection pressure to produce at least one phenotypic selectable resistance/tolerance trait or growth advantage trait; wherein the "certain type of mutation" comprises substitution of single base, substitution of a plurality of bases, or insertion or deletion of an unspecified number of bases;

the "certain selection pressure" is an environmental pressure or a pressure resulted from an added compound.

16. The method according to any one of claims 13-15, wherein the "DNA break" is achieved

by delivering a nuclease with a targeting property into a cell of the organism to contact with the specific site of genomic DNA.

17. The method according to any one of claims 13-16 wherein the "two or more DNA breaks" are generated when RNP complexes formed by a same CRISPR/Cas nuclease respectively with different gRNAs or sgRNAs sequentially cut corresponding target sequences; or

the "two or more DNA breaks" are generated when RNP complexes respectively formed by two or more CRISPR/Cas nucleases that recognize different PAM sequences with respective gRNA or sgRNA, sequentially cut corresponding target sequences.

18. The method according to any one of claims 13-17, wherein the "targeted nuclease for at least one second target gene" and the CRISPR/Cas nuclease used for generating DNA break at a specific site of the first target gene are the same or different.

19. The method according to any one of claims 13-18, wherein the targeted nuclease is in a form of DNA; or

in a form of mRNA or protein instead of DNA.

20. A method for non-transgenic transient editing of an organism genome for non-therapeutic purpose, comprising the following steps:

1) a combination of at least two crRNA fragments or a combination of at least two sgRNA fragments is designed and synthesized for a specific site of a first target gene of a recipient cell, wherein the crRNA combination combined with tracrRNA or the sgRNA combination alone is capable of guiding a corresponding Cas protein to sequentially generate two or more DNA breaks at the specific site in the first target gene of the recipient cell and to spontaneously repair them respectively, wherein a later DNA break is generated based on a new sequence generated from a previous DNA break repair;

2) an appropriate amount of CRISPR/Cas protein or corresponding mRNA thereof is mixed with the combination of crRNA fragments and tracrRNA fragment or with the combination of sgRNA fragments alone as above designed and synthesized in advance that is capable of guiding a site-specific editing of the first target gene to generate endogenous selection markers, optionally, at least one of artificially synthesized crRNA and tracrRNA fragments or artificially synthesized sgRNA fragments targeting a second, third or more target genes is further added, and incubation is carried out in vitro to form an RNP complex;

3) the above RNP complex is delivered into the recipient cell and contacts with the specific

site of genomic DNA to achieve gene editing;

4) according to a phenotypic selectable trait generated by the site-specific editing of the first target gene by the RNP complex, a corresponding selection pressure is applied to make a selection for the trait, and a cell, tissue, organ or complete organism that contains the editing event is isolated, and optionally, a cell, tissue, organ or complete organism that simultaneously contains the editing events of the first target gene and of the at least one of a second, third or more target genes is isolated

wherein the feature that "to sequentially generate two or more DNA breaks at a specific site" refers to that based on a new sequence formed by a previous DNA break repair event generated by a CRISPR/Cas system, a new target RNA is designed to cut the site again.

21. The method according to claim 20, wherein the "first target gene" is a gene locus encoding at least one phenotypic selectable trait, wherein the at least one phenotypic selectable trait is a resistance/tolerance trait or a growth advantage trait.

22. The method according to claim 20 or 21, wherein the "specific site of the first target gene" refers to a site at which a certain type of mutation is generated after sequential cuttings and repairs, which is capable of conferring the recipient cell with a resistance to a certain selection pressure to produce at least one phenotypic selectable resistance/tolerance trait or growth advantage trait;

wherein the "certain type of mutation" comprises substitution of single base, substitution of a plurality of bases, or insertion or deletion of an unspecified number of bases, the "certain selection pressure" is an environmental pressure or a pressure e resulted from an added compound.

23. The method according to any one of claims 20-22, wherein the "two or more DNA breaks" are generated when RNP complexes formed by a same CRISPR/Cas nuclease respectively with different gRNAs or sgRNAs sequentially cut corresponding target sequences; or

the "two or more DNA breaks" are generated when RNP complexes respectively formed by each of two or more CRISPR/Cas nucleases that recognize different PAM sequences with respective gRNA or sgRNA, sequentially cut corresponding target sequences.

24. The method according to any one of claims 20-23, wherein the "at least one of artificially synthesized crRNA and tracrRNA fragments or artificially synthesized sgRNA fragments targeting a second, third or more target genes" shares the same Cas protein with the crRNA or sgRNA targeting the first target gene; or

the "at least one of artificially synthesized crRNA and tracrRNA fragments or artificially

synthesized sgRNA fragments targeting a second, third or more target genes" and the crRNA or sgRNA targeting the first target gene use Cas proteins that recognize different PAM sequences.

25. The method according to any one of claims 20-24, wherein when the organism is a plant, the recipient cell is replaced by a recipient plant cell or tissue; wherein the "recipient plant cell or tissue" is any cell or tissue that can serve as a recipient for transient expression and can be regenerated into a complete plant through tissue culture; wherein the cell is preferably a protoplast cell or a suspension cell; and the tissue is preferably a callus, immature embryo, mature embryo, leaf, shoot tip, young spike or hypocotyl.

26. The method according to claim 25, wherein the "first target gene" is at least one endogenous gene that encodes at least one phenotypic selectable trait selected from herbicide resistance/tolerance, wherein the herbicide resistance/tolerance is selected from the group consisting of resistance/tolerance to EPSPS inhibitor, resistance/tolerance to glutamine synthesis inhibitor, resistance/tolerance to ALS or AHAS inhibitor, resistance/tolerance to ACCase inhibitor, resistance/tolerance to carotenoid biosynthesis inhibitor, resistance/tolerance to cellulose inhibitor, resistance/tolerance to lipid synthesis inhibitor, resistance/tolerance to long-chain fatty acid inhibitor, resistance/tolerance to microtubule assembly inhibitor, resistance/tolerance to photosystem I electron shunting agent, resistance/tolerance to photosystem II inhibitor or resistance/tolerance to PPO inhibitor, and resistance/tolerance to synthetic growth hormone.

27. The method according to claim 26, wherein the "first target gene" is ALS, and the "specific site of gene" refers to site A122, P197, R198, D204, A205, D376, R377, W574, S653 or G654 in an *Arabidopsis* AtALS protein amino acid sequence, and amino acid sites in an ALS protein of another plant which correspond to the above-mentioned amino acid sites by using the AtALS amino acid sequence as reference standard; or

the crRNA or sgRNA targets a target sequence comprising a sequence encoding an AtALS protein amino acid sequence site selected from the group consisting of A122, P197, R198, D204, A205, D376, R377, W574, S653, G654 or any combination thereof, and a target sequence comprising a sequence encoding an amino acid site in an ALS protein of another plant which corresponds to the above-mentioned amino acid site, and any combination thereof, by using the AtALS amino acid sequence as reference standard; or

the "first target gene" is ACCase, and the "specific site of gene" refers to site I1781, E1874, N1878, W1999, W2027, I2041, D2078, C2088 or G2096 in an *Alopecurus myosuroides* AmACCase protein amino acid sequence, and amino acid sites in an ACCase protein of another monocotyledonous plant which correspond to the above-mentioned amino acid sites by using the

AmACCase amino acid sequence as reference standard; or

the crRNA or sgRNA targets a target sequence comprising a sequence encoding an AmACCase amino acid sequence site selected from the group consisting of I1781, E1874, N1878, W1999, W2027, I2041, D2078, C2088, G2096 or any combination thereof, and a target sequence comprising a sequence encoding an amino acid site in an ACCase protein of another monocotyledonous plant which corresponds to the above-mentioned amino acid sites, and any combination thereof, by using the AmACCase amino acid sequence as reference standard; or

the "first target gene" is HPPD, and the "specific site of gene" refers to site H141, L276, P277, N338, G342, R346, D370, P386, K418 or G419 in an *Oryza sativa* OsHPPD protein amino acid sequence, and amino acid sites in an HPPD protein of another plant which correspond to the above-mentioned amino acid sites by using the OsHPPD amino acid sequence as reference standard; or

the crRNA or sgRNA targets a target sequence comprising a sequence encoding an OsHPPD amino acid sequence site selected from the group consisting of H141, L276, P277, N338, G342, R346, D370, P386, K418, G419 or any combination thereof, and a target sequence comprising a sequence encoding an amino acid site in an HPPD protein of another plant which corresponds to the above-mentioned amino acid site, and any combination thereof, by using the OsHPPD amino acid sequence as reference standard; or

the "first target gene" is PPO, and the "specific site of gene" refers to site S128, V217, S223, V364, K373, L423, Y425 or W470 in an *Oryza sativa* OsPPO1 protein amino acid sequence, and amino acid sites in a PPO protein of another plant which correspond to the above-mentioned amino acid sites by using the amino acid sequence of OsPPO1 as reference standard; or

the crRNA or sgRNA targets a target sequence comprising a sequence encoding an OsPPO1 amino acid sequence site selected from the group consisting of S128, V217, S223, V364, K373, L423, Y425, W470 or any combination thereof, and a target sequence comprising a sequence encoding an amino acid site in a PPO protein of another plant which corresponds to the above-mentioned amino acid site, and any combination thereof, by using the OsPPO1 amino acid sequence as reference standard; or

the "first target gene" is TIR1, and the "specific site of gene" refers to site F93, F357, C413 or S448 in an *Oryza sativa* OsTIR1 protein amino acid sequence, and amino acid sites in a TIR1 protein of another plant which correspond to the above-mentioned amino acid sites by using the OsTIR1 amino acid sequence as reference standard; or

the crRNA or sgRNA targets a target sequence comprising a sequence encoding an OsTIR1

amino acid sequence site selected from the group consisting of F93, F357, C413, S448 or any combination thereof, and a target sequence comprising a sequence encoding an amino acid site in a TIR1 protein of another plant which corresponds to the above-mentioned amino acid site, and any combination thereof, by using the OsTIR1 amino acid sequence as reference standard.

28. A method for producing a plant with improved resistance or tolerance to herbicides, which comprises transforming or transfecting a plant cell with a recombinant expression vector comprising the nucleic acid obtained by the method according to any one of claims 20-27 and a promoter operably linked thereto or with an expression cassette comprising the nucleic acid obtained by the method according to any one of claims 20-27, and regenerating the transformed or transfected plant cell into a plant.

29. A method for controlling weeds in a plant cultivation site, wherein the plant produced by the method according to claim 28, the method comprises applying to the cultivation site one or more herbicides in an effective amount to control the weeds.