

[Patent Literature]

[0003]

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[Summary]

[Technical Problem]

[0004]

Conventional genome engineering techniques fail to efficiently engineer two or more alleles at the same time by HDR. Furthermore, these techniques have a limitation in the size of a genomic region that can be engineered by HDR, and find difficulty in efficiently performing the large-scale engineering of the genome (e.g., 10 kbp).

[0005]

Accordingly, an object of the present disclosure is to provide a genome engineering method and a genome engineering kit which can efficiently engineer two or more alleles and are capable of engineering a relatively large region.

[Solution to Problem]

[0005a]

In one aspect, the present disclosure provides an *in vitro* method for preparing a cell in which two or more alleles in the chromosomal genome are engineered, comprising the steps of:

(a) introducing the following (i) and (ii) to a cell comprising two or more alleles to introduce a selective marker gene to each of the two or more alleles:

(i) a genome engineering system comprising a sequence-specific nucleic acid cleaving molecule that targets a target region in the two or more alleles in the chromosomal genome and cleaves the target region, or a polynucleotide encoding the sequence-specific nucleic acid cleaving molecule, and

(ii) two or more donor DNAs, each comprising at least one selective marker gene, each of which has an upstream homology arm having a nucleotide sequence homologous to an upstream nucleotide sequence of the target region and a downstream homology arm having a nucleotide sequence homologous to a downstream nucleotide sequence of the target region, and comprises the nucleotide sequence of the selective marker gene between the upstream homology arm and the downstream homology arm, the two or more donor DNAs comprising selective markers respectively having different selective marker genes which can be distinguished from each other, wherein the selective marker gene is different for each type of donor DNA comprising the selective marker, and the number of types of the donor DNAs comprising selective markers is equal to or more than the number of the alleles that are subject to genome engineering; and

(b) after the step (a), selecting a cell having the selective marker genes, which are respectively introduced in the two or more alleles by respective homologous recombination of the two or more alleles with the different types of donor DNAs comprising selective markers, and in which all the selective marker genes thus introduced are expressed (step for positive selection).

[0005b]

In another aspect, the present disclosure provides an *in vitro* method for engineering two or more alleles in the chromosomal genome, comprising the steps of:

(a) introducing the following (i) and (ii) to a cell comprising two or more alleles to introduce a selective marker gene to each of the two or more alleles:

(i) a genome engineering system comprising a sequence-specific nucleic acid cleaving molecule that targets a target region in the two or more alleles in the chromosomal genome and cleaves the target region, or a polynucleotide encoding the sequence-specific nucleic acid cleaving molecule, and

(ii) two or more donor DNAs, each comprising at least one selective marker gene, each of which has an upstream homology arm having a nucleotide sequence homologous to an upstream nucleotide sequence of the target region and a downstream homology arm having a nucleotide sequence homologous to a downstream nucleotide

sequence of the target region, and comprises the nucleotide sequence of the selective marker gene between the upstream homology arm and the downstream homology arm, the two or more donor DNAs comprising selective markers respectively having different selective marker genes which can be distinguished from each other, wherein the selective marker gene is different for each type of donor DNA comprising the selective marker, and the number of types of the donor DNAs comprising selective markers is equal to or more than the number of the alleles that are subject to genome engineering; and

(b) after the step (a), selecting a cell having the selective marker genes, which are respectively introduced in the two or more alleles by respective homologous recombination of the two or more alleles with the different types of donor DNAs comprising selective markers, and in which all the selective marker genes thus introduced are expressed(step for positive selection).

[0005c]

In yet another aspect, the present disclosure provides a genome engineering kit for engineering two or more alleles in the chromosomal genome, comprising the following (i) and (ii):

(i) a genome engineering system comprising a sequence-specific nucleic acid cleaving molecule that targets a target region in the chromosomal genome and cleaves the

target region, or a polynucleotide encoding the sequence-specific nucleic acid cleaving molecule, and

(ii) two or more donor DNAs, each comprising at least one selective marker gene, each of which has an upstream homology arm having a nucleotide sequence homologous to an upstream nucleotide sequence of the target region and a downstream homology arm having a nucleotide sequence homologous to a downstream nucleotide sequence of the target region, and comprises the nucleotide sequence of the selective marker gene between the upstream homology arm and the downstream homology arm, the two or more donor DNAs comprising selective markers respectively having different selective marker genes which can be distinguished from each other, wherein the selective marker gene is different for each type of donor DNA comprising the selective marker, and the number of types of the donor DNAs comprising selective markers is equal to or more than the number of the alleles that are subject to genome engineering.

[0006]

As one example, the present disclosure includes the following examples.

[Document Name] Claims

[Claim 1]

An *in vitro* method for preparing a cell in which two or more alleles in the chromosomal genome are engineered, comprising the steps of:

(a) introducing the following (i) and (ii) to a cell comprising two or more alleles to introduce a selective marker gene to each of the two or more alleles:

(i) a genome engineering system comprising a sequence-specific nucleic acid cleaving molecule that targets a target region in the two or more alleles in the chromosomal genome and cleaves the target region, or a polynucleotide encoding the sequence-specific nucleic acid cleaving molecule, and

(ii) two or more donor DNAs, each comprising at least one selective marker gene, each of which has an upstream homology arm having a nucleotide sequence homologous to an upstream nucleotide sequence of the target region and a downstream homology arm having a nucleotide sequence homologous to a downstream nucleotide sequence of the target region, and comprises the nucleotide sequence of the selective marker gene between the upstream homology arm and the downstream homology arm, the two or more donor DNAs comprising selective markers respectively having different selective marker genes which can be distinguished from each other, wherein the selective marker gene is different for each type of

donor DNA comprising the selective marker, and the number of types of the donor DNAs comprising selective markers is equal to or more than the number of the alleles that are subject to genome engineering; and

(b) after the step (a), selecting a cell having the selective marker genes, which are respectively introduced in the two or more alleles by respective homologous recombination of the two or more alleles with the different types of donor DNAs comprising selective markers, and in which all the selective marker genes thus introduced are expressed(step for positive selection).

[Claim 2]

An *in vitro* method for engineering two or more alleles in the chromosomal genome, comprising the steps of:

(a) introducing the following (i) and (ii) to a cell comprising two or more alleles to introduce a selective marker gene to each of the two or more alleles:

(i) a genome engineering system comprising a sequence-specific nucleic acid cleaving molecule that targets a target region in the two or more alleles in the chromosomal genome and cleaves the target region, or a polynucleotide encoding the sequence-specific nucleic acid cleaving molecule, and

(ii) two or more donor DNAs, each comprising at least one selective marker gene, each of which has an upstream homology arm having a nucleotide sequence

homologous to an upstream nucleotide sequence of the target region and a downstream homology arm having a nucleotide sequence homologous to a downstream nucleotide sequence of the target region, and comprises the nucleotide sequence of the selective marker gene between the upstream homology arm and the downstream homology arm, the two or more donor DNAs comprising selective markers respectively having different selective marker genes which can be distinguished from each other, wherein the selective marker gene is different for each type of donor DNA comprising the selective marker, and the number of types of the donor DNAs comprising selective markers is equal to or more than the number of the alleles that are subject to genome engineering; and

(b) after the step (a), selecting a cell having the selective marker genes, which are respectively introduced in the two or more alleles by respective homologous recombination of the two or more alleles with the different types of donor DNAs comprising selective markers, and in which all the selective marker genes thus introduced are expressed(step for positive selection).

[Claim 3]

The method according to claim 1 or 2, wherein the target region has a length of 5 kbp or more, or 8 kbp or more.

[Claim 4]

The method according to any one of claims 1 to 3, wherein each of the two or more donor DNAs comprising selective markers has a selective marker gene for positive selection, a marker gene for negative selection, and a target sequence between the upstream homology arm and the downstream homology arm, wherein in the case of using the selective marker gene both for positive selection and for negative selection, another selective marker gene for negative selection is optionally absent,

the method further comprising the steps of:

(c) after the step (b), introducing the following (iii) and (iv) to the selected cell to introduce a donor DNA comprising a desired nucleotide sequence to each of the two or more alleles:

(iii) a genome engineering system comprising a sequence-specific nucleic acid cleaving molecule that targets the target sequence and cleaves the target sequence, or a polynucleotide encoding the sequence-specific nucleic acid cleaving molecule, and

(iv) a donor DNA comprising a desired nucleotide sequence, the donor DNA having an upstream homology arm having a nucleotide sequence homologous to an upstream nucleotide sequence of the target region and a downstream homology arm having a nucleotide sequence homologous to a downstream nucleotide sequence of the target region; and

(d) after the step (c), selecting a cell not expressing the marker gene for negative selection (step for negative selection).

[Claim 5]

The method according to claim 4, wherein the region between the upstream homology arm and the downstream homology arm of the donor DNA comprising a desired nucleotide sequence has a length of 5 kbp or more, or wherein the region between the upstream homology arm and the downstream homology arm of the donor DNA comprising a desired nucleotide sequence has a length of 8 kbp or more.

[Claim 6]

A genome engineering kit for engineering two or more alleles in the chromosomal genome, comprising the following (i) and (ii):

(i) a genome engineering system comprising a sequence-specific nucleic acid cleaving molecule that targets a target region in the chromosomal genome and cleaves the target region, or a polynucleotide encoding the sequence-specific nucleic acid cleaving molecule, and
(ii) two or more donor DNAs, each comprising at least one selective marker gene, each of which has an upstream homology arm having a nucleotide sequence homologous to an upstream nucleotide sequence of the target region and a downstream homology arm having a nucleotide sequence homologous to a downstream nucleotide sequence of the

target region, and comprises the nucleotide sequence of the selective marker gene between the upstream homology arm and the downstream homology arm, the two or more donor DNAs comprising selective markers respectively having different selective marker genes which can be distinguished from each other, wherein the selective marker gene is different for each type of donor DNA comprising the selective marker, and the number of types of the donor DNAs comprising selective markers is equal to or more than the number of the alleles that are subject to genome engineering.

[Claim 7]

The kit according to claim 6, wherein the target region has a length of 5 kbp or more, or 8 kbp or more.

[Claim 8]

The kit according to any one of claims 6 to 7, further comprising a donor DNA comprising a desired nucleotide sequence, the donor DNA having an upstream homology arm having a nucleotide sequence homologous to an upstream nucleotide sequence of the target region and a downstream homology arm having a nucleotide sequence homologous to a downstream nucleotide sequence of the target region.

[Claim 9]

The kit according to claim 8, wherein the region between the upstream homology arm and the downstream homology arm of the donor DNA comprising a desired

nucleotide sequence has a length of 5 kbp or more, or 8 kbp or more.

[Claim 10]

The kit according to any one of claims 6 to 9, wherein the target region has a length of 5 kbp or more, and the region between the upstream homology arm and the downstream homology arm of the donor DNA comprising a desired nucleotide sequence has a length of 5 kbp or more, or wherein the target region has a length of 8 kbp or more, and the region between the upstream homology arm and the downstream homology arm of the donor DNA comprising a desired nucleotide sequence has a length of 8 kbp or more.

[Claim 11]

The method according to claim 4, wherein the donor DNA comprising a desired nucleotide sequence has no nucleotide sequence in the region between the upstream homology arm and the downstream homology arm of the donor DNA comprising a desired nucleotide sequence, and the upstream and downstream sequences of the target region are seamlessly linked, without insertion, substitution and deletion of a base, in the thus-engineered two or more alleles in the chromosomal genome.

[Claim 12]

The method according to claim 3, wherein a target sequence of site-specific recombinase is absent in the

thus-engineered two or more alleles in the chromosomal genome.

[Claim 13]

The method according to claim 4, wherein a target sequence of site-specific recombinase is absent in the thus-engineered two or more alleles in the chromosomal genome.

[Claim 14]

The method according to any one of claims 1 to 5 and 11 to 13, wherein in the step (b), single-cell cloning is not performed in a process up to the selection of the cell in which the two or more alleles are engineered.

[Claim 15]

The kit according to claim 8, wherein the donor DNA comprising a desired nucleotide sequence has no nucleotide sequence in the region between the upstream homology arm and the downstream homology arm of the donor DNA comprising a desired nucleotide sequence.