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(54) Title: I-CREI MEGANUCLEASE VARIANTS WITH MODIFIED SPECIFICITY, METHOD OF PREPARATION AND USES THEREOF

(57) Abstract: Method of preparing I-CreI meganuclease variants, with a modified specificity, i.e. able to cleave at least one homing site that is not cleaved by the wild-type I-CreI. I-CreI meganuclease variants obtainable by said method and their applications either for cleaving new DNA target or for genetic engineering and genome engineering for non-therapeutic purposes. Nucleic acids encoding said variants, expression cassettes comprising said nucleic acids, vectors comprising said expression cassettes, cells or organisms, plants or animals except humans, transformed by said vectors.

I-*CreI* MEGANUCLEASE VARIANTS WITH MODIFIED SPECIFICITY, METHOD OF PREPARATION AND USES THEREOF

The present invention relates to a method of preparing I-*CreI* meganuclease variants, with a modified specificity, *i.e.* able to cleave at least one homing site that is not cleaved by the wild-type I-*Cre*I. The invention relates also to the I-*CreI* meganuclease variants obtainable by said method and to their applications either for cleaving new DNA target or for genetic engineering and genome engineering for non-therapeutic purposes.

The invention also relates to nucleic acids encoding said variants, to expression cassettes comprising said nucleic acids, to vectors comprising said expression cassettes, to cells or organisms, plants or animals except humans, transformed by said vectors.

Meganucleases are sequence specific endonucleases recognizing large (>12bp; usually 14-40 bp) DNA cleavage sites (Thierry and Dujon, 1992). In the wild, meganucleases are essentially represented by homing endonucleases, generally encoded by mobile genetic elements such as inteins and class I introns (Belfort and Roberts, 1997; Chevalier and Stoddard, 2001). Homing refers to the mobilization of these elements, which relies on DNA double-strand break (DSB) repair, initiated by the endonuclease activity of the meganuclease. Early studies on the HO (Haber, 1998; Klar *et al.*, 1984; Kostriken *et al.*, 1983), I-*SceI* (Colleaux *et al.*, 1988; Jacquier and Dujon, 1985; Perrin *et al.*, 1993; Plessis *et al.*, 1992) and I-*TevI* (Bell-Pedersen *et al.*, 1990; Bell-Pedersen *et al.*, 1989; Bell-Pedersen *et al.*, 1991; Mueller *et al.*, 1996) proteins have illustrated the biology of the homing process. On another hand, these studies have also provided a paradigm for the study of DSB repair in living cells.

General asymmetry of homing endonuclease target sequences contrasts with the characteristic dyad symmetry of most restriction enzyme recognition sites. Several homing endonucleases encoded by introns ORF or inteins have been shown to promote the homing of their respective genetic elements into allelic intronless or inteinless sites. By making a site-specific double-strand break in the intronless or inteinless alleles, these nucleases create recombinogenic ends, which engage in a gene conversion process that duplicates the coding sequence and leads to the insertion of an intron or an intervening sequence at the DNA level.

Homing endonucleases fall into 4 separated families on the basis of pretty well conserved amino acids motifs [for review, see Chevalier and Stoddard (Nucleic Acids Research, 2001, 29, 3757-3774)]. One of them is the dodecapeptide family (dodecamer, DOD, D1-D2, LAGLIDADG, P1-P2). This is the largest family of proteins clustered by their most general conserved sequence motif: one or two copies (vast majority) of a twelve-residue sequence: the dodecapeptide. Homing endonucleases with one dodecapetide (D) are around 20 kDa in molecular mass and act as homodimers. Those with two copies (DD) range from 25 kDa (230 amino acids) to 50 kDa (HO, 545 amino acids) with 70 to 150 residues between each motif and act as monomer. Cleavage is inside the recognition site, leaving 4 nt staggered cut with 3'OH overhangs. Enzymes that contain a single copy of the LAGLIDADG motif, such as I-*Ceu*I and I-*Cre*I act as homodimers and recognize a nearly palindromic homing site.

The sequence and the structure of the homing endonuclease I-*CreI* (pdb accession code 1g9y) have been determined (Rochaix JD et al., NAR, 1985, 13, 975-984; Heath PJ et al., Nat. Struct. Biol., 1997, 4, 468-476; Wang et al., NAR, 1997, 25, 3767-3776; Jurica et al. Mol. Cell, 1998, 2, 469-476) and structural models using X-ray crystallography have been generated (Heath et al., 1997).

I-*CreI* comprises 163 amino acids (pdb accession code 1g9y); said endonuclease cuts as a dimer. The LAGLIDADG motif corresponds to residues 13 to 21; on either side of the LAGLIDADG α-helices, a four β-sheet (positions 21-29; 37-48; 66-70 and 73-78) provides a DNA binding interface that drives the interaction of the protein with the half-site of the target DNA sequence. The dimerization interface involves the two LAGLIDADG helix as well as other residues.

The homing site recognized and cleaved by I-*CreI* is 22-24 bp in length and is a degenerate palindrome (see figure 2 of Jurica MS et al, 1998 and SEQ ID NO:65). More precisely, said I-*CreI* homing site is a semi-palindromic 22 bp sequence, with 7 of 11 bp identical in each half-site (Seligman LM et al., NAR, 2002, 30, 3870-3879).

The endonuclease-DNA interface has also been described (see figure 4 of Jurica MS et al, 1998) and has led to a number of predictions about specific

protein-DNA contacts (Seligman LM et al., Genetics, 1997, 147, 1653-1664; Jurica MS et al., 1998; Chevalier B. et al., Biochemistry, 2004, 43, 14015-14026).

It emerges from said documents that:

- the residues G19, D20, Q47, R51, K98 and D137 are part of the endonucleolytic site of I-*Cre*I;

- homing site sequence must have at least 20 bp to achieve a maximal binding affinity of 0.2 nM;

- sequence-specific contacts are distributed across the entire length of the homing site;

- base-pair substitutions can be tolerated at many different homing site positions, without seriously disrupting homing site binding or cleavage;

- R51 and K98 are located in the enzyme active site and are candidates to act as Lewis acid or to activate a proton donor in the cleavage reaction; mutations in each of these residues have been observed to sharply reduce I-*Cre*I endonucleolytic activity (R51G, K98Q);

- five additional residues, which when mutated abolish I-*Cre*I endonuclease activity are located in or near the enzyme active site (R70A, L39R, L91R, D75G, Q47H).

These studies have paved the way for a general use of meganuclease for genome engineering. Homologous gene targeting is the most precise way to stably modify a chromosomal *locus* in living cells, but its low efficiency remains a major drawback. Since meganuclease-induced DSB stimulates homologous recombination up to 10 000-fold, meganucleases are today the best way to improve the efficiency of gene targeting in mammalian cells (Choulika *et al.*, 1995; Cohen-Tannoudji *et al.*, 1998; Donoho *et al.*, 1998; Elliott *et al.*, 1998; Rouet *et al.*, 1994), and to bring it to workable efficiencies in organisms such as plants (Puchta *et al.*, 1993; Puchta *et al.*, 1996) and insects (Rong and Golic, 2000; Rong and Golic, 2001; Rong *et al.*, 2002).

Meganucleases have been used to induce various kinds of homologous recombination events, such as direct repeat recombination in mammalian cells (Liang *et al.*, 1998), plants (Siebert and Puchta, 2002), insects (Rong *et al.*, 2002), and bacteria (Posfai *et al.*, 1999), or interchromosomal recombination (Moynahan and Jasin, 1997; Puchta, 1999; Richardson *et al.*, 1998).

However, this technology is still limited by the low number of potential natural target sites for meganucleases: although several hundreds of natural homing endonucleases have been identified (Belfort and Roberts, 1997; Chevalier and Stoddard, 2001), the probability to have a natural meganuclease cleaving a gene of interest is extremely low. The making of artificial meganucleases with dedicated specificities would bypass this limitation.

Artificial endonucleases with novel specificity have been made, based on the fusion of endonucleases domains to zinc-finger DNA binding domains (Bibikova *et al.*, 2003; Bibikova *et al.*, 2001; Bibikova *et al.*, 2002; Porteus and Baltimore, 2003).

Homing endonucleases have also been used as scaffolds to make novel endonucleases, either by fusion of different protein domains (Chevalier *et al.*, 2002; Epinat *et al.*, 2003), or by mutation of single specific amino acid residues (Seligman *et al.*, 2002; Sussman *et al.*, 2004) ; said thus obtained novel endonucleases have been proposed either for studying the cleavage mechanism of I-*Cre*I LAGLIDADG homing endonuclease (Chevalier B. et al., 2004) or for providing I-*Cre*I meganuclease variants with modified affinity to the DNA target of wild-type I-*Cre*I. Among the documents describing mutation of single specific amino acid residues, the followings may be cited:

- Chevalier B. et al. (Biochemistry, 2004, 43, 14015-14026) :

In view to study and characterize the role of bound divalent metal ions in the activity of the wild-type I-*CreI*, three active site residues were mutagenized: the only residue involved in direct metal ligation (D20), a second residue that contacts a metal-bound inner-shell water molecule (Q47) and a third residue that does not participate in metal binding but instead is found in a basic pocket near the scissile phosphate and nucleophilic water residue (K98).

The kinetic behavior and DNA binding properties of these mutants were assessed. More specifically, the following nine mutants were prepared: D20N, D20L, D20A; Q47N, Q47A, Q47M, Q47E; K98K and K98A.

Mutations at residue D20 demonstrate greater heterogeneity in their effect on DNA affinity (wild-type I-*CreI* target site) than those mutations at Q47.

- Seligman LM et al., 2002, describe mutations altering the cleavage specificity of I-*Cre*I. More specifically, they have studied the role of the nine amino acids of I-*Cre*I predicted to directly contact the DNA target (Q26, K28, N30, S32, Y33, Q38, Q44, R68 and R70). Among these nine amino acids, seven are thought to interact with nucleotides at symmetrical positions (S32, Y33, N30, Q38, R68, Q44 and R70). The mutants which have been designed and constructed have each of said nine amino acids and a tenth (T140) predicted to participate in a water-mediated interaction, converted to alanines.

As regards the mutations altering I-*CreI*, in view to determine the relative importance of these 10 residues in DNA recognition, alanine substitutions at these positions were constructed and examined in a *E. coli* based assay. The resulting I-*CreI* mutants fell into four distinct phenotypic classes in relation to the wild-type homing site.

It emerges from the results that:

- S32A and T140A contacts appear least important for homing site recognition,

- N30A, Q38A and Q44A displayed intermediate levels of activity in each assay,

- Q26A, R68A and Y33A are inactive,

- K28A and R70A are inactive and non-toxic.

As regards the mutations altering the seven symmetrical positions in the I-*CreI* homing site, it emerges from the obtained results that five of the seven symmetrical positions in each half-site appear to be essential for efficient site recognition *in vivo* by wild-type I-*Cre*I: 2/21, 3/20, 7/16, 8/15 and 9/14 (corresponding to positions –10/+10, -9/+9, -5/+5, -4/+4 and –3/+3 in SEQ ID NO:65). All mutants altered at these positions were resistant to cleavage by wild-type I-*Cre*I *in vivo*.

Seligman et al. have also studied the interaction between I-*Cre*I position 33 and homing site bases 2 and 21 or between I-C*re*I position 33 and homing site bases 1 and 22.

However, in all cases the I-*Cre*I mutants analysed, when displaying an increased affinity for a mutant homing site also displayed an activity for the wild-

type homing site (Table 1 and figure 3 of Seligman) even though said activity is decreased.

To sum up, Seligman et al. only show that:

- I-*Cre*I mutants at positions 30, 38, 44, 26, 68, 33, 28 and 70 have a modified behaviour in relation to the wild-type I-*CreI* homing site.

- homing site mutants altered at the positions 2/21, 3/20, 7/16, 8/15 and 9/14 are resistant to cleavage by wild-type I-*CreI in vivo*; however, i*n vitro* assay using *E. coli* appears to be more sensitive than the *in vivo* test and allows the detection of homing sites of wild-type I-*CreI* more effectively than the *in vivo* test; thus *in vitro* test shows that the DNA target of wild-type I-*CreI* may be the followings: gtc (recognized homing site in all the cited documents), gcc or gtt triplet at the positions -5 to -3, in reference to SEQ ID NO:65.

- altered specificity of I-*Cre*I derivative Y33 has been studied and is found to have increased affinities for I-*CreI* homing site mutated at positions 2/21.

However, it emerges from said document that it may however be problematic to extrapolate *in vivo* DNA recognition behaviour from *in vitro* studies.

- Sussman et al., 2004, report studies in which the homodimeric LAGLIDADG homing endonuclease I-*Cre*I is altered at individual side-chains corresponding to contact points to distinct base pairs in its target site (alteration in positions 6 and 10). The resulting enzymes constructs (Q26A, Q26C, Y66R, Q26C/Y66R) drive specific elimination of selected DNA targets *in vivo* and display shifted specificities of DNA binding and cleavage *in vitro*.

The overall result of the selection and characterization of enzyme point mutants against individual target site variants is both a shift and a broadening in binding specificity and in kinetics of substrate cleavage.

Each mutant displays a higher dissociation constant (lower affinity) against the original wild-type target site than does the wild-type enzyme, and each mutant displays a lower dissociation constant (higher affinity) against its novel target than does the wild-type enzyme.

The enzyme mutants display similar kinetics of substrate cleavage, with shifts and broadening in substrate preferences similar to those described for binding affinities.

- WO 2004/067736 describes a general method for producing a custom-made meganuclease able to cleave a targeted DNA sequence derived from an initial meganuclease. This general method comprises the steps of preparing a library of meganuclease variants and selecting the variants able to cleave the targeted DNA sequence. When the initial meganuclease is homing endonuclease I-*CreI*, a library lib2, wherein residues 44, 68 and 70 have been mutated was built and screened against a series of six targets close to the I-CreI natural target site; the screened mutants have altered binding profiles; however, they bind and/or cleave at least one of the same homing site than the wild-type I-*CreI*.

All the documents of the prior art describe I-*CreI* meganuclease variants with modified binding and/or cleavage properties than wild-type I-CreI. However, all of them cleave homing sites also cleaved by the wild-type I-*CreI*.

I-*Cre*I meganuclease variants which do not cleave wild-type I-*CreI* homing sites have not been described; however, there is a need for such novel I-*CreI* meganuclease variants with such a "modified specificity", i.e. meganuclease able to cleave at least one homing site other than the homing sites cleaved by the wild-type I-*CreI*.

Such variants would be of a particular interest for genetic and genome engineering.

Here the inventors found that one mutation of at least one of the amino acid residues in positions 44, 68 and 70 of I-*Cre*I is sufficient to obtain a I-*CreI* meganuclease variant able to cleave at least one homing site that is not cleaved by the wild-type meganuclease.

Therefore, the subject-matter of the present invention is a method of preparing a I-*CreI* meganuclease variant having at least a DNA target sequence (or homing site) which is different from the homing sites of the wild type I-*Cre*I meganuclease, said method comprising:

(a) replacing amino acids Q44, R68 and/or R70, in reference with I-*Cre*I pdb accession code 1g9y , with an amino acid selected in the group consisting of A, D, E, G, H, K, N, P, Q, R, S and T ;

(b) selecting the I-CreI meganuclease variants obtained in step (a) having at least one of the following R_3 triplet cleaving profile in reference to positions

–5 to –3 in a double-strand DNA target, said positions -5 to -3 corresponding to R_3 of the following formula I:

5'- R_1CAAA$R_2R_3R_4$R'$_4$R'$_3$R'$_2$TTTGR'$_1$ -3',

wherein:

R_1 is absent or present; and when present represents a nucleic acid fragment comprising 1 to 9 nucleotides corresponding either to a random nucleic acid sequence or to a fragment of a I-*CreI* meganuclease homing site situated from position -20 to -12 (from 5' to 3'), R_1 corresponding at least to position -12 of said homing site,

R_2 represents the nucleic acid doublet ac or ct and corresponds to positions -7 to -6 of said homing site,

R_3 represents a nucleic acid triplet corresponding to said positions -5 to -3, selected among g, t, c and a, except the following triplets : gtc, gcc, gtg, gtt and gct; therefore said nucleic acid triplet is preferably selected among the following triplets: ggg, gga, ggt, ggc, gag, gaa, gat, gac, gta, gcg, gca, tgg, tga, tgt, tgc, tag, taa, tat, tac, ttg, tta, ttt, ttc, tcg, tca, tct, tcc, agg, aga, agt, agc, aag, aaa, aat, aac, atg, ata, att, atc, acg, aca, act, acc, cgg, cga, cgt, cgc, cag, caa, cat, cac, ctg, cta, ctt, ctc, ccg, cca, cct and ccc and more preferably among the following triplets: ggg, ggt, ggc, gag, gat, gac, gta, gcg, gca, tag, taa, tat, tac, ttg, ttt, ttc, tcg, tct, tcc, agg, aag, aat, aac, att, atc, act, acc, cag, cat, cac, ctt, ctc, ccg, cct and ccc,

R_4 represents the nucleic acid doublet gt or tc and corresponds to positions –2 to –1 of said homing site,

R'$_1$ is absent or present; and when present represents a nucleic acid fragment comprising 1 to 9 nucleotides corresponding either to a random nucleic acid sequence or to a fragment of a I-CreI meganuclease homing site situated from position +12 to +20 (from 5' to 3'), R'$_1$ corresponding at least to position +12 of said homing site,

R'$_2$ represents the nucleic acid doublet ag or gt, and corresponds to positions +6 to +7 of said homing site,

R'$_3$ represents a nucleic acid triplet corresponding to said positions +3 to +5, selected among g, t, c, and a; R'$_3$ being different from gac, ggc, cac, aac, and agc, when R_3 and R'$_3$ are non-palindromic,

R'_4 represents the nucleic acid doublet gt or tc and corresponds to positions +1 to +2 of said homing site.

Definitions

- Amino acid residues 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.

- In the present invention, unless otherwise mentioned, the residue numbers refer to the amino acid numbering of the I-*Cre*I sequence SWISSPROT P05725 or the pdb accession code 1g9y. According to this definition, a variant named "ADR" is I-*CreI* meganuclease in which amino acid residues Q44 and R68 have been replaced by alanine and aspartic acid, respectively, while R70 has not been replaced. Other mutations that do not alter the cleavage activity of the variant are not indicated and the nomenclature adopted here does not limit the mutations to the only three positions 44, 68 and 70.

- 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.

- As used herein, the term "wild-type I-*Cre*I" designates a I-*Cre*I meganuclase having the sequence SWISSPROT P05735 or pdb accession code 1g9y, and able to cleave the 24 bp double-strand polynucleotide sequence presented in figure 2B (positions -5 to -3: gtc) or double-strand polynucleotide sequences having at positions -5 to -3 the following other triplets: gtg, gtt, gct or gcc. Indeed, when using an *in vivo* assay in a yeast strain, the Inventors found that wild-type I-*Cre*I cleaves not only homing sites which palindromic sequence in positions –5 to –3 is gtc, gcc or gtt (Seligman et al., 2002), but also gtg and gct. It results that the variants of the invention are those able to cleave at least one homing site in which sequence in positions –5 to –3 differs from gtc, gct, gcc, gtt and gtg. Thus, wild-type I-*Cre*I is not only able to cleave homing site as described in figure 2B, *i.e.* with gtc in position –5 to –3 and gac

in position +3 to +5 (SEQ ID NO: 71), or a target with gcc in position –5 to –3 and ggc in position +3 to +5 (WO2004/067736), but is further able to cleave targets comprising in positions –5 to –3: gtg, gtt or gct (SEQ ID NO: 9, 11, and 15, respectively), said targets still having palindromic sequences at positions +/- 3 to 5.

- The term "modified specificity" relates to a I-*CreI* meganuclease variant able to cleave a homing site that is not cleaved, in the same conditions, by the wild-type I-*CreI*. A I-*Cre*I meganuclease variant with a modified specificity is able to cleave at least one target site that is not cleaved by wild-type I-*Cre*I.

- Heterodimeric form can be obtained for example by proceeding to the fusion of the two monomers. Resulting heterodimeric meganuclease can be able to cleave at least one target site that is not cleaved by the homodimeric form. Therefore a meganuclease variant is still part of the invention when used in a heteromeric form. The other monomer chosen for the formation of the heterodimeric meganuclease can be another variant monomer, but it can also be a wild-type monomer, for example a I-*Cre*I monomer or a I-*Dmo*I monomer.

- The terms "recognition site", "recognition sequence", "target", "target sequence", "DNA target", "homing recognition site", "homing site", "cleavage site" are indiscriminately used for designating a 14 to 40 bp double-stranded partially palindromic polynucleotide sequence that is recognized and cleaved by I-*Cre*I. These terms refer to a distinct DNA location, preferably a chromosomal location, at which a double stranded break (cleavage) is to be induced by the meganuclease; for example, the known homing recognition site of wild-type I-*Cre*I is represented by the sequence 5'-caaaacgtcgtgagacagtttg-3' (SEQ ID NO: 71), here only represented by one strand. This particular site is hereafter also named "I-*Cre*I natural target site".

- In the present application, when a sequence is given for illustrating a recognition or homing site, it is to be understood that it represents, from 5' to 3', only one strand of the double-stranded polynucleotide.

- The term "partially palindromic sequence", "partially symmetrical sequence", "degenerate palindrome", "pseudopalindromic sequence" are indiscriminately used for designating a palindromic sequence having a broken symmetry. For example the 22 bp sequence:

$c_{-11}a_{-10}a_{-9}a_{-8}a_{-7}c_{-6}g_{-5}t_{-4}c_{-3}g_{-2}t_{-1}g_{+1}a_{+2}g_{+3}a_{+4}c_{+5}a_{+6}g_{+7}t_{+8}t_{+9}t_{+10}g_{+11}$ (SEQ ID NO: 71) is a partially palindromic sequence in which symmetry is broken at base-pairs +/- 1, 2, 6 and 7. According to another formulation, nucleotide sequences of positions +/- 8 to 11 and +/- 3 to 5 are palindromic sequences. Symmetry axe is situated between the base-pairs in positions –1 and +1. Using another numbering, from the 5' extremity to the 3' extremity, palindromic sequences are in positions 1 to 4 and 19 to 22, and 7 to 9 and 14 to 16, symmetry is broken at base-pairs 5, 6, 10, 11, 12, 13, 17 and 18, and the symmetry axe is situated between the base-pairs in positions 11 and 12.

- The term "targeting DNA construct" corresponds to a DNA sequence comprising both the DNA target as defined hereabove and other DNA sequences allowing *in vivo* homologous recombination.

Thus, the inventors constructed a I-*Cre*I variants library, each of them presenting at least one mutation in the amino acid residues in positions 44, 68 and/or 70 (pdb code 1g9y), and each of them being able to cleave at least one target site not cleaved by a wild-type I-*Cre*I.

In this particular approach, the mutation consists of the replacement of at least one amino acid residue in position 44, 68, and/or 70 by another residue selected in the group comprising A, D, E, G, H, K, N, P, Q, R, S and T. Each mutated amino acid residue is changed independently from the other residues, and the selected amino acid residues can be the same or can be different from the other amino acid residues in position 44, 68 and/or 70. In this approach, the homing site, cleaved by the I-*Cre*I meganuclease variant according to the invention but not cleaved by wild-type I-*Cre*I, is the same as described above and illustrated in figure 2, except that the triplet sequence in positions –5 to –3 (corresponding to R_3 in formula I) and/or triplet sequence in positions +3 to +5 (corresponding to R_3' in formula I) differ from the triplet sequence in the same positions in the homing sites cleaved by the wild-type I-*Cre*I.

Unexpectedly, the I-*Cre*I meganuclease variants, obtainable by the method described above, i.e. with a "modified specificity" are able to cleave at least one target that differs from wild-type I-*Cre*I target in positions –5 to –3 and/or in positions +3 to +5. It must be noted that said DNA target is not necessarily palin-

dromic in positions +/- 3 to 5. I-*Cre*I is active in homodimeric form, but may be active in a heterodimeric form. Therefore I-*Cre*I variants according to the instant invention could be active not only in a homodimeric form, but also in a heterodimeric form, and in both cases, they could recognize a target with either palindromic or non palindromic sequence in position +/- 3 to 5, provided that the triplet in position –5 to –3 and/or +3 to +5 differs from gtc, gcc, gtg, gtt and gct, and from gac, ggc, cac, aac, and agc, respectively. Since each monomer of I-*Cre*I variant binds a half of the homing site, a variant able to cleave a plurality of targets could also cleave a target which sequence in position +/- 3 to 5 is not palindromic. Further, a variant could act both in a homodimeric form and in a heterodimeric form. I-*Cre*I variant could form a heterodimeric meganuclease, in which the other variant could be a wild-type I-*Cre*I monomer, another wild-type meganuclease monomer, such as I-*Dmo*I, another I-*Cre*I variant monomer, or a monomer of a variant from another meganuclease than I-*Cre*I.

According to an advantageous embodiment of said method, the I-*Cre*I meganuclease variant obtained in step (b) is selected from the group consisting of: A44/A68/A70, A44/A68/G70, A44/A68/H70, A44/A68/K70, A44/A68/N70, A44/A68/Q70, A44/A68/R70, A44/A68/S70, A44/A68/T70, A44/D68/H70, A44/D68/K70, A44/D68/R70, A44/G68/H70, A44/G68/K70, A44/G68/N70, A44/G68/P70, A44/G68/R70, A44/H68/A70, A44/H68/G70, A44/H68/H70, A44/H68/K70, A44/H68/N70, A44/H68/Q70, A44/H68/R70, A44/H68/S70, A44/H68/T70, A44/K68/A70, A44/K68/G70, A44/K68/H70, A44/K68/K70, A44/K68/N70, A44/K68/Q70, A44/K68/R70, A44/K68/S70, A44/K68/T70, A44/N68/A70, A44/N68/E70, A44/N68/G70, A44/N68/H70, A44/N68/K70, A44/N68/N70, A44/N68/Q70, A44/N68/R70, A44/N68/S70, A44/N68/T70, A44/Q68/A70, A44/Q68/D70, A44/Q68/G70, A44/Q68/H70, A44/Q68/N70, A44/Q68/R70, A44/Q68/S70, A44/R68/A70, A44/R68/D70, A44/R68/E70, A44/R68/G70, A44/R68/H70, A44/R68/K70, A44/R68/L70, A44/R68/N70, A44/R68/R70, A44/R68/S70, A44/R68/T70, A44/S68/A70, A44/S68/G70, A44/S68/K70, A44/S68/N70, A44/S68/Q70, A44/S68/R70, A44/S68/S70, A44/S68/T70, A44/T68/A70, A44/T68/G70, A44/T68/H70, A44/T68/K70, A44/T68/N70, A44/T68/Q70, A44/T68/R70, A44/T68/S70, A44/T68/T70, D44/D68/H70, D44/N68/S70, D44/R68/A70, D44/R68/K70, D44/R68/N70,

D44/R68/Q70, D44/R68/R70, D44/R68/S70, D44/R68/T70, E44/H68/H70,
E44/R68/A70, E44/R68/H70, E44/R68/N70, E44/R68/S70, E44/R68/T70,
E44/S68/T70, G44/H68/K70, G44/Q68/H70, G44/R68/Q70, G44/R68/R70,
G44/T68/D70, G44/T68/P70, G44/T68/R70, H44/A68/S70, H44/A68/T70,
H44/R68/A70, H44/R68/D70, H44/R68/E70, H44/R68/G70, H44/R68/N70,
H44/R68/R70, H44/R68/S70, H44/R68/T70, H44/S68/G70, H44/S68/S70,
H44/S68/T70, H44/T68/S70, H44/T68/T70, K44/A68/A70, K44/A68/D70,
K44/A68/E70, K44/A68/G70, K44/A68/H70, K44/A68/N70, K44/A68/Q70,
K44/A68/S70, K44/A68/T70, K44/D68/A70, K44/D68/T70, K44/E68/G70,
K44/E68/N70, K44/E68/S70, K44/G68/A70, K44/G68/G70, K44/G68/N70,
K44/G68/S70, K44/G68/T70, K44/H68/D70, K44/H68/E70, K44/H68/G70,
K44/H68/N70, K44/H68/S70, K44/H68/T70, K44/K68/A70, K44/K68/D70,
K44/K68/H70, K44/K68/T70, K44/N68/A70, K44/N68/D70, K44/N68/E70,
K44/N68/G70, K44/N68/H70, K44/N68/N70, K44/N68/Q70, K44/N68/S70,
K44/N68/T70, K44/P68/H70, K44/Q68/A70, K44/Q68/D70, K44/Q68/E70,
K44/Q68/S70, K44/Q68/T70, K44/R68/A70, K44/R68/D70, K44/R68/E70,
K44/R68/G70, K44/R68/H70, K44/R68/N70, K44/R68/Q70, K44/R68/S70,
K44/R68/T70, K44/S68/A70, K44/S68/D70, K44/S68/H70, K44/S68/N70,
K44/S68/S70, K44/S68/T70, K44/T68/A70, K44/T68/D70, K44/T68/E70,
K44/T68/G70, K44/T68/H70, K44/T68/N70, K44/T68/Q70, K44/T68/S70,
K44/T68/T70, N44/A68/H70, N44/A68/R70, N44/H68/N70, N44/H68/R70,
N44/K68/G70, N44/K68/H70, N44/K68/R70, N44/K68/S70, N44/N68/R70,
N44/P68/D70, N44/Q68/H70, N44/Q68/R70, N44/R68/A70, N44/R68/D70,
N44/R68/E70, N44/R68/G70, N44/R68/H70, N44/R68/K70, N44/R68/N70,
N44/R68/R70, N44/R68/S70, N44/R68/T70, N44/S68/G70, N44/S68/H70,
N44/S68/K70, N44/S68/R70, N44/T68/H70, N44/T68/K70, N44/T68/Q70,
N44/T68/R70, N44/T68/S70, P44/N68/D70, P44/T68/T70, Q44/A68/A70,
Q44/A68/H70, Q44/A68/R70, Q44/G68/K70, Q44/G68/R70, Q44/K68/G70,
Q44/N68/A70, Q44/N68/H70, Q44/N68/S70, Q44/P68/P70, Q44/Q68/G70,
Q44/R68/A70, Q44/R68/D70, Q44/R68/E70, Q44/R68/G70, Q44/R68/H70,
Q44/R68/N70, Q44/R68/Q70, Q44/R68/S70, Q44/S68/H70, Q44/S68/R70,
Q44/S68/S70, Q44/T68/A70, Q44/T68/G70, Q44/T68/H70, Q44/T68/R70,

R44/A68/G70, R44/A68/T70, R44/G68/T70, R44/H68/D70, R44/H68/T70, R44/N68/T70, R44/R68/A70, R44/R68/D70, R44/R68/E70, R44/R68/G70, R44/R68/N70, R44/R68/Q70, R44/R68/S70, R44/R68/T70, R44/S68/G70, R44/S68/N70, R44/S68/S70, R44/S68/T70, S44/D68/K70, S44/H68/R70, S44/R68/G70, S44/R68/N70, S44/R68/R70, S44/R68/S70, T44/A68/K70, T44/A68/R70, T44/H68/R70, T44/K68/R70, T44/N68/P70, T44/N68/R70, T44/Q68/K70, T44/Q68/R70, T44/R68/A70, T44/R68/D70, T44/R68/E70, T44/R68/G70, T44/R68/H70, T44/R68/K70, T44/R68/N70, T44/R68/Q70, T44/R68/R70, T44/R68/S70, T44/R68/T70, T44/S68/K70, T44/S68/R70, T44/T68/K70, and T44/T68/R70.

According to another advantageous embodiment of said method, the step (b) of selecting said I-*CreI* meganuclease variant is performed *in vivo* in yeast cells.

The subject-matter of the present invention is also the use of a I-*CreI* meganuclease variant as defined here above, i.e. obtainable by the method as described above, *in vitro* or *in vivo* for non-therapeutic purposes, for cleaving a double-strand nucleic acid target comprising at least a 20-24 bp partially palindromic sequence, wherein at least the sequence in positions +/- 8 to 11 is palindromic, and the nucleotide triplet in positions –5 to –3 and/or the nucleotide triplet in positions +3 to +5 differs from gtc, gcc, gtg, gtt, and gct, and from gac, ggc, cac, aac and agc, respectively. Formula I describes such a DNA target.

According to an advantageous embodiment of said use, said I-*CreI* meganuclease variant is selected from the group consisting of: A44/A68/A70, A44/A68/G70, A44/A68/H70, A44/A68/K70, A44/A68/N70, A44/A68/Q70, A44/A68/R70, A44/A68/S70, A44/A68/T70, A44/D68/H70, A44/D68/K70, A44/D68/R70, A44/G68/H70, A44/G68/K70, A44/G68/N70, A44/G68/P70, A44/G68/R70, A44/H68/A70, A44/H68/G70, A44/H68/H70, A44/H68/K70, A44/H68/N70, A44/H68/Q70, A44/H68/R70, A44/H68/S70, A44/H68/T70, A44/K68/A70, A44/K68/G70, A44/K68/H70, A44/K68/K70, A44/K68/N70, A44/K68/Q70, A44/K68/R70, A44/K68/S70, A44/K68/T70, A44/N68/A70, A44/N68/E70, A44/N68/G70, A44/N68/H70, A44/N68/K70, A44/N68/N70, A44/N68/Q70, A44/N68/R70, A44/N68/S70, A44/N68/T70, A44/Q68/A70,

A44/Q68/D70, A44/Q68/G70, A44/Q68/H70, A44/Q68/N70, A44/Q68/R70,
A44/Q68/S70, A44/R68/A70, A44/R68/D70, A44/R68/E70, A44/R68/G70,
A44/R68/H70, A44/R68/K70, A44/R68/L70, A44/R68/N70, A44/R68/R70,
A44/R68/S70, A44/R68/T70, A44/S68/A70, A44/S68/G70, A44/S68/K70,
A44/S68/N70, A44/S68/Q70, A44/S68/R70, A44/S68/S70, A44/S68/T70,
A44/T68/A70, A44/T68/G70, A44/T68/H70, A44/T68/K70, A44/T68/N70,
A44/T68/Q70, A44/T68/R70, A44/T68/S70, A44/T68/T70, D44/D68/H70,
D44/N68/S70, D44/R68/A70, D44/R68/K70, D44/R68/N70, D44/R68/Q70,
D44/R68/R70, D44/R68/S70, D44/R68/T70, E44/H68/H70, E44/R68/A70,
E44/R68/H70, E44/R68/N70, E44/R68/S70, E44/R68/T70, E44/S68/T70,
G44/H68/K70, G44/Q68/H70, G44/R68/Q70, G44/R68/R70, G44/T68/D70,
G44/T68/P70, G44/T68/R70, H44/A68/S70, H44/A68/T70, H44/R68/A70,
H44/R68/D70, H44/R68/E70, H44/R68/G70, H44/R68/N70, H44/R68/R70,
H44/R68/S70, H44/R68/T70, H44/S68/G70, H44/S68/S70, H44/S68/T70,
H44/T68/S70, H44/T68/T70, K44/A68/A70, K44/A68/D70, K44/A68/E70,
K44/A68/G70, K44/A68/H70, K44/A68/N70, K44/A68/Q70, K44/A68/S70,
K44/A68/T70, K44/D68/A70, K44/D68/T70, K44/E68/G70, K44/E68/N70,
K44/E68/S70, K44/G68/A70, K44/G68/G70, K44/G68/N70, K44/G68/S70,
K44/G68/T70, K44/H68/D70, K44/H68/E70, K44/H68/G70, K44/H68/N70,
K44/H68/S70, K44/H68/T70, K44/K68/A70, K44/K68/D70, K44/K68/H70,
K44/K68/T70, K44/N68/A70, K44/N68/D70, K44/N68/E70, K44/N68/G70,
K44/N68/H70, K44/N68/N70, K44/N68/Q70, K44/N68/S70, K44/N68/T70,
K44/P68/H70, K44/Q68/A70, K44/Q68/D70, K44/Q68/E70, K44/Q68/S70,
K44/Q68/T70, K44/R68/A70, K44/R68/D70, K44/R68/E70, K44/R68/G70,
K44/R68/H70, K44/R68/N70, K44/R68/Q70, K44/R68/S70, K44/R68/T70,
K44/S68/A70, K44/S68/D70, K44/S68/H70, K44/S68/N70, K44/S68/S70,
K44/S68/T70, K44/T68/A70, K44/T68/D70, K44/T68/E70, K44/T68/G70,
K44/T68/H70, K44/T68/N70, K44/T68/Q70, K44/T68/S70, K44/T68/T70,
N44/A68/H70, N44/A68/R70, N44/H68/N70, N44/H68/R70, N44/K68/G70,
N44/K68/H70, N44/K68/R70, N44/K68/S70, N44/N68/R70, N44/P68/D70,
N44/Q68/H70, N44/Q68/R70, N44/R68/A70, N44/R68/D70, N44/R68/E70,
N44/R68/G70, N44/R68/H70, N44/R68/K70, N44/R68/N70, N44/R68/R70,

N44/R68/S70, N44/R68/T70, N44/S68/G70, N44/S68/H70, N44/S68/K70, N44/S68/R70, N44/T68/H70, N44/T68/K70, N44/T68/Q70, N44/T68/R70, N44/T68/S70, P44/N68/D70, P44/T68/T70, Q44/A68/A70, Q44/A68/H70, Q44/A68/R70, Q44/G68/K70, Q44/G68/R70, Q44/K68/G70, Q44/N68/A70, Q44/N68/H70, Q44/N68/S70, Q44/P68/P70, Q44/Q68/G70, Q44/R68/A70, Q44/R68/D70, Q44/R68/E70, Q44/R68/G70, Q44/R68/H70, Q44/R68/N70, Q44/R68/Q70, Q44/R68/S70, Q44/S68/H70, Q44/S68/R70, Q44/S68/S70, Q44/T68/A70, Q44/T68/G70, Q44/T68/H70, Q44/T68/R70, R44/A68/G70, R44/A68/T70, R44/G68/T70, R44/H68/D70, R44/H68/T70, R44/N68/T70, R44/R68/A70, R44/R68/D70, R44/R68/E70, R44/R68/G70, R44/R68/N70, R44/R68/Q70, R44/R68/S70, R44/R68/T70, R44/S68/G70, R44/S68/N70, R44/S68/S70, R44/S68/T70, S44/D68/K70, S44/H68/R70, S44/R68/G70, S44/R68/N70, S44/R68/R70, S44/R68/S70, T44/A68/K70, T44/A68/R70, T44/H68/R70, T44/K68/R70, T44/N68/P70, T44/N68/R70, T44/Q68/K70, T44/Q68/R70, T44/R68/A70, T44/R68/D70, T44/R68/E70, T44/R68/G70, T44/R68/H70, T44/R68/K70, T44/R68/N70, T44/R68/Q70, T44/R68/R70, T44/R68/S70, T44/R68/T70, T44/S68/K70, T44/S68/R70, T44/T68/K70, and T44/T68/R70.

According to another advantageous embodiment of said use, the I-*Cre*I meganuclease variant is a homodimer.

According to another advantageous embodiment of said use, said I-*Cre*I meganuclease variant is a heterodimer.

According to said use:

- either the I-*Cre*I meganuclease variant is able to cleave a DNA target in which sequence in positions +/- 3 to 5 is palindromic,

- or, said I-*Cre*I meganuclease variant is able to cleave a DNA target in which sequence in positions +/- 3 to 5 is non-palindromic.

According to another advantageous embodiment of said use the cleaved nucleic acid target is a DNA target in which palindromic sequences in positions –11 to –8 and +8 to +11 are caaa and tttg, respectively.

According to another advantageous embodiment of said use, said I-*Cre*I meganuclease variant further comprises a mutation in position 75, preferably said mutation is D75N or D75V.

According to yet another advantageous embodiment of said use, said I-*Cre*I meganuclease variant has an alanine (A), an aspartic acid (D) or a threonine (T) in position 44, for cleaving a DNA target comprising nucleotide A in position -4, and/or T in position +4.

According to yet another advantageous embodiment of said use, said I-*Cre*I meganuclease variant has a lysine (K) or an arginine (R) in position 44, for cleaving a target comprising nucleotide C in position -4, and/or G in position +4.

The subject-matter of the present invention is also I-*CreI* meganuclease variants:

- Obtainable by the method of preparation as defined above;
- Having one mutation of at least one of the amino acid residues in positions 44, 68 and 70 of I-*Cre*I; said mutations being the only ones within the amino acids contacting directly the DNA target;
- Having at least a DNA target sequence (or homing site) which is different from the homing sites of the wild type I-CreI meganuclease and
- Being able to cleave at least one homing site that is not cleaved by the wild-type I-*CreI* meganuclease.

Thus, said I-*Cre*I meganuclease variant according to the invention, obtainable by the method as described above, has mainly a modified specificity, *i.e.* is able to cleave a DNA target that is not cleaved by wild-type I-*Cre*I.

Such novel I-*Cre*I meganucleases may be used either as very specific endonucleases in *in vitro* digestion, for restriction or mapping use, either *in vivo* or *ex vivo* as tools for genome engineering. In addition, each one can be used as a new scaffold for a second round of mutagenesis and selection/screening, for the purpose of making novel, second generation homing endonucleases.

The I-*Cre*I meganuclease variants according to the invention are mutated only at positions 44, 68 and/or 70 of the DNA binding domain. However, the instant invention also includes different proteins able to form heterodimers: heterodimerization of two different proteins from the above list result also in cleavage

of non palindromic sequences, made of two halves from the sites cleaved by the parental proteins alone. This can be obtained *in vitro* by adding the two different I-*Cre*I variants in the reaction buffer, and *in vivo* or *ex vivo* by coexpression. Another possibility is to build a single-chain molecule, as described by Epinat *et al.* (Epinat *et al.*, 2003). This single chain molecule would be the fusion of two different I-*Cre*I variants, and should also result in the cleavage of chimeric, non-palindromic sequences.

According to an advantageous embodiment of said I-*Cre*I meganuclease variant, the amino acid residue chosen for the replacement of the amino acid in positions 44, 68 and/or 70 is selected in the group comprising A, D, E, G, H, K, N, P, Q, R, S and T.

Said I-*Cre*I meganuclease variant is able to cleave at least one target, as defined above, that is not cleaved by the wild-type I-*Cre*I.

According to another advantageous embodiment, said I-*Cre*I meganuclease variant is selected in the group consisting of: A44/A68/A70, A44/A68/G70, A44/A68/H70, A44/A68/K70, A44/A68/N70, A44/A68/Q70, A44/A68/S70, A44/A68/T70, A44/D68/H70, A44/D68/K70, A44/D68/R70, A44/G68/H70, A44/G68/K70, A44/G68/N70, A44/G68/P70, A44/H68/A70, A44/H68/G70, A44/H68/H70, A44/H68/K70, A44/H68/N70, A44/H68/Q70, A44/H68/S70, A44/H68/T70, A44/K68/A70, A44/K68/G70, A44/K68/H70, A44/K68/N70, A44/K68/Q70, A44/K68/R70, A44/K68/S70, A44/K68/T70, A44/N68/A70, A44/N68/E70, A44/N68/G70, A44/N68/H70, A44/N68/K70, A44/N68/N70, A44/N68/Q70, A44/N68/R70, A44/N68/S70, A44/N68/T70, A44/Q68/A70, A44/Q68/D70, A44/Q68/G70, A44/Q68/H70, A44/Q68/N70, A44/Q68/S70, A44/R68/E70, A44/R68/K70, A44/R68/L70, A44/S68/A70, A44/S68/G70, A44/S68/N70, A44/S68/Q70, A44/S68/R70, A44/S68/S70, A44/S68/T70, A44/T68/A70, A44/T68/G70, A44/T68/H70, A44/T68/N70, A44/T68/Q70, A44/T68/S70, A44/T68/T70, D44/D68/H70, D44/N68/S70, D44/R68/A70, D44/R68/N70, D44/R68/Q70, D44/R68/R70, D44/R68/S70, D44/R68/T70, E44/H68/H70, E44/R68/A70, E44/R68/H70, E44/R68/N70, E44/R68/S70, E44/R68/T70, E44/S68/T70, G44/H68/K70, G44/Q68/H70, G44/R68/Q70, G44/T68/D70, G44/T68/P70, G44/T68/R70, H44/A68/S70,

H44/A68/T70, H44/R68/D70, H44/R68/E70, H44/R68/G70, H44/R68/N70, H44/R68/R70, H44/R68/S70, H44/S68/G70, H44/S68/S70, H44/S68/T70, H44/T68/S70, H44/T68/T70, K44/A68/A70, K44/A68/D70, K44/A68/E70, K44/A68/G70, K44/A68/H70, K44/A68/N70, K44/A68/Q70, K44/D68/A70, K44/D68/T70, K44/E68/G70, K44/E68/S70, K44/G68/A70, K44/G68/G70, K44/G68/N70, K44/G68/S70, K44/G68/T70, K44/H68/D70, K44/H68/E70, K44/H68/G70, K44/H68/N70, K44/H68/S70, K44/H68/T70, K44/K68/A70, K44/K68/D70, K44/K68/H70, K44/K68/T70, K44/N68/A70, K44/N68/D70, K44/N68/E70, K44/N68/G70, K44/N68/H70, K44/N68/N70, K44/N68/Q70, K44/N68/S70, K44/N68/T70, K44/P68/H70, K44/Q68/A70, K44/Q68/D70, K44/Q68/E70, K44/Q68/S70, K44/Q68/T70, K44/R68/A70, K44/R68/D70, K44/R68/E70, K44/R68/G70, K44/R68/H70, K44/R68/N70, K44/R68/S70, K44/S68/A70, K44/S68/D70, K44/S68/H70, K44/S68/N70, K44/S68/S70, K44/S68/T70, K44/T68/A70, K44/T68/D70, K44/T68/E70, K44/T68/G70, K44/T68/H70, K44/T68/N70, K44/T68/Q70, K44/T68/S70, K44/T68/T70, N44/A68/H70, N44/H68/N70, N44/H68/R70, N44/K68/G70, N44/K68/H70, N44/K68/R70, N44/K68/S70, N44/P68/D70, N44/Q68/H70, N44/R68/A70, N44/R68/D70, N44/R68/E70, N44/R68/K70, N44/S68/G70, N44/S68/H70, N44/S68/K70, N44/S68/R70, N44/T68/H70, N44/T68/K70, N44/T68/Q70, N44/T68/S70, P44/N68/D70, P44/T68/T70, Q44/G68/K70, Q44/G68/R70, Q44/K68/G70, Q44/N68/A70, Q44/N68/H70, Q44/N68/S70, Q44/P68/P70, Q44/Q68/G70, Q44/R68/D70, Q44/R68/E70, Q44/R68/G70, Q44/R68/Q70, Q44/S68/S70, Q44/T68/A70, Q44/T68/G70, Q44/T68/H70, R44/A68/G70, R44/A68/T70, R44/G68/T70, R44/H68/D70, R44/H68/T70, R44/N68/T70, R44/R68/A70, R44/R68/D70, R44/R68/E70, R44/R68/G70, R44/R68/Q70, R44/R68/S70, R44/R68/T70, R44/S68/G70, R44/S68/N70, R44/S68/S70, R44/S68/T70, S44/D68/K70, S44/R68/R70, S44/R68/S70, T44/A68/K70, T44/N68/P70, T44/N68/R70, T44/R68/E70, T44/R68/Q70, and T44/S68/K70.

According to yet another advantageous embodiment, the I-*Cre*I meganuclease variant has an alanine (A), an aspartic acid (D) or a threonine (T) in position 44 and cleaves a target comprising the nucleotide A in position -4, and/or T in position +4.

According to yet another advantageous embodiment, the I-*Cre*I meganuclease variant of the invention has a lysine (K) or an arginine (R) in position 44 and cleaves a target comprising c in position -4, and/or g in position +4.

As specified hereabove, in the frame of the definition of the I-*Cre*I meganuclease variant in the use application, said I-*Cre*I meganuclease variant may be a homodimer or a heterodimer. It may be able to cleave a palindromic or a non-palindromic DNA target. It may further comprise a mutation in position 75, as specified hereabove.

The subject-matter of the present invention is also a polynucleotide, characterized in that it encodes a I-*Cre*I meganuclease variant according to the invention.

Further, the subject-matter of the present invention is an expression cassette comprising said polynucleotide and regulation sequences such as a promoter, and an expression vector comprising said expression cassette.

The subject-matter of the present invention is also an expression vector, as described above, further comprising a targeting DNA construct.

According to an advantageous embodiment of said expression vector, said targeting DNA construct comprises a sequence sharing homologies with the region surrounding the cleavage site of the I-*Cre*I meganuclease variant of the invention.

According to another advantageous embodiment of said expression vector, said targeting DNA construct comprises:

a) sequences sharing homologies with the region surrounding the cleavage site of the I-*Cre*I meganuclease variant according to claim, and

b) sequences to be introduced flanked by sequence as in a).

The subject-matter of the present invention is also a cell, characterized in that it is modified by a polynucleotide as defined above or by a vector as defined above.

The subject-matter of the present invention is also a transgenic plant, characterized in that it comprises a polynucleotide as defined above, or a vector as defined above.

The subject-matter of the present invention is also a non-human transgenic mammal, characterized in that it comprises a polynucleotide as defined above or a vector as defined above.

The subject-matter of the present invention is further the use of a I-*Cre*I meganuclease variant, a polynucleotide, a vector, a cell, a transgenic plant, a non-human transgenic mammal, as defined above, for molecular biology, for *in vivo* or *in vitro* genetic engineering, and for *in vivo* or *in vitro* genome engineering, for non-therapeutic purposes.

Non therapeutic purposes include for example (i) gene targeting of specific loci in cell packaging lines for protein production, (ii) gene targeting of specific loci in crop plants, for strain improvements and metabolic engineering, (iii) targeted recombination for the removal of markers in genetically modified crop plants, (iv) targeted recombination for the removal of markers in genetically modified microorganism strains (for antibiotic production for example).

According to an advantageous embodiment of said use, it is for inducing a double-strand break in a site of interest comprising a DNA target sequence, thereby inducing a DNA recombination event, a DNA loss or cell death.

According to the invention, said double-strand break is for: repairing a specific sequence, modifying a specific sequence, restoring a functional gene in place of a mutated one, attenuating or activating an endogenous gene of interest, introducing a mutation into a site of interest, introducing an exogenous gene or a part thereof, inactivating or detecting an endogenous gene or a part thereof, translocating a chromosomal arm, or leaving the DNA unrepaired and degraded.

According to another advantageous embodiment of said use, said I-*Cre*I meganuclease variant, polynucleotide, vector, cell, transgenic plant or non-human transgenic mammal are associated with a targeting DNA construct as defined above.

The subject-matter of the present invention is also a method of genetic engineering, characterized in that it comprises a step of double-strand nucleic acid breaking in a site of interest located on a vector, comprising a DNA target of a I-*Cre*I meganuclease variant as defined hereabove, by contacting said vector with a I-*Cre*I meganuclease variant as defined above, thereby inducing a homologous recom-

bination with another vector presenting homology with the sequence surrounding the cleavage site of said I-*Cre*I meganuclease variant.

The subjet-matter of the present invention is also a method of genome engineering, characterized in that it comprises the following steps: 1) double-strand breaking a genomic locus comprising at least one recognition and cleavage site of a I-*Cre*I meganuclease variant as defined above, by contacting said cleavage site with said I-*Cre*I meganuclease variant; 2) maintaining said broken genomic locus under conditions appropriate for homologous recombination with a targeting DNA construct comprising the sequence to be introduced in said locus, flanked by sequences sharing homologies with the target locus.

The subjet-matter of the present invention is also a method of genome engineering, characterized in that it comprises the following steps: 1) double-strand breaking a genomic locus comprising at least one recognition and cleavage site of a I-*Cre*I meganuclease variant as defined above, by contacting said cleavage site with said I-*Cre*I meganuclease variant; 2) maintaining said broken genomic locus under conditions appropriate for homologous recombination with chromosomal DNA sharing homologies to regions surrounding the cleavage site.

The subject-matter of the present invention is also a composition characterized in that it comprises at least one I-*Cre*I meganuclease variant, a polynucleotide or a vector as defined above.

In a preferred embodiment of said composition, it comprises a targeting DNA construct comprising the sequence which repairs the site of interest flanked by sequences sharing homologies with the targeted locus.

The subject-matter of the present invention is also the use of at least one I-*Cre*I meganuclease variant, a polynucleotide or a vector, as defined above for the preparation of a medicament for preventing, improving or curing a genetic disease in an individual in need thereof, said medicament being administrated by any means to said individual.

The subject-matter of the present invention is also the use of at least one I-*Cre*I meganuclease variant, a polynucleotide or a vector as defined above for the preparation of a medicament for preventing, improving or curing a disease caused by

an infectious agent that presents a DNA intermediate, in an individual in need thereof, said medicament being administrated by any means to said individual.

The subject-matter of the present invention is also the use of at least one I-*Cre*I meganuclease variant, a polynucleotide or a vector, as defined above, *in vitro*, for inhibiting the propagation, inactivating or deleting an infectious agent that presents a DNA intermediate, in biological derived products or products intended for biological uses or for disinfecting an object.

In a particular embodiment, said infectious agent is a virus.

The term "vector" refers to a nucleic acid molecule capable of transporting another nucleic acid to which it has been linked. 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 and commercially available, such as the following bacterial vectors: pQE7O, pQE6O, pQE-9 (Qiagen), pbs, pDIO, phagescript, psiXl74. pbluescript SK, pbsks, pNH8A, pNH16A, pNH18A, pNH46A (Stratagene); ptrc99a, pKK223-3, pKK233-3, pDR540, pRIT5 (Pharmacia); pWLNEO,pSV2CAT, pOG44, pXTI, pSG (Stratagene); pSVK3, pBPV, pMSG, pSVL (Pharmacia); pQE-30 (QlAexpress), pET (Novagen).

In addition to the preceding features, the invention further comprises other features which will emerge from the description which follows, which refers to examples illustrating the I-*Cre*I meganuclease variants and their uses according to the invention, as well as to the appended drawings in which:

Figure 1: principle of the screening assay. Yeast are transformed with the meganuclease expressing vector, marked with the LEU2 gene, and individually mated with yeast transformed with the reporter plasmid, marked by the TRP1 gene. In diploids (LEU2 TRP1), cleavage of the target site by the meganuclease induces homologous recombination between the two lacZ repeats, resulting in a functional beta-galactosidase gene, which can be monitored by X-Gal staining.

Figure 2: DNA targets.

A. Two palindromic targets derived from the natural I-*Cre*I target (here named C1234, SEQ ID NO: 65). The I-*Cre*I natural target contains two palindromes, boxed in grey: the -8 to -12 and +8 to +12 nucleotides on one hand, and the -5 to -3 and +3 to +5 nucleotide on another hand. Vertical dotted line, from which are numbered the nucleotide bases, represents the symmetry axe for the palindromic sequences. From the natural target can be derived two palindromic sequences, C1221 (SEQ ID NO: 12) and C4334 (SEQ ID NO:66). Both are cut by I-*Cre*I, *in vitro* and in yeast. Only one strand of each target site is shown.

B. I-*Cre*I natural DNA target and interaction with the Q44, R68 and R70 residues. Other amino acid residues interacting directly or indirectly with the DNA target are not shown. Arginine (R) residue in position 44 of a I-*Cre*I monomer directly interacts with guanine in position –5 of the target sequence, while glutamine (Q) residue of position 44 and Arginine (R) residue of position 70 directly interact with adenine in position +4 and guanine in position +3 of the complementary strand, respectively.

C. The 64 targets. The 64 targets are derived from the I-*Cre*I natural target site (here, also named C1221, SEQ ID NO: 12). They correspond to all the 24 bp palindromes resulting from substitutions at positions -5, -4, -3, +3, +4 and +5.

Figure 3: Nine examples of pattern. Nine meganucleases are tested 4 times against the 64 targets described in Figure 2C. The position of the different targets is indicated on the top, left panel. Meganucleases are identified by the amino acids in positions 44, 68 and 70 (ex: KSS is K44, S68, S70, or K44/S68/S70). Numeration of the amino acids is according to pdb code 1g9y. QRR corresponds to the wild type (Q44/R68/R70). The cleaved targets are indicated besides the panels.

Figure 4: cDNA sequence (SEQ ID NO: 69) used for obtaining the I-*Cre*I N75 scaffold protein (SEQ ID NO: 70). CDS is from base-pair 1 to base-pair 501 and the "STOP" codon TGA (not shown) follows the base-pair 501. In addition to the D75N mutation, the protein further contains mutations that do not alter its activity; in the protein sequence (SEQ ID NO:70), the two first N-terminal residues are methionine and alanine (MA), and the three C-terminal residues alanine, alanine and aspartic acid (AAD). These sequences allow having DNA coding sequences comprising the *NcoI* (ccatgg) and *EagI* (cggccg) restriction sites, which are used for cloning into various vectors.

Figure 5: pCLS0542 expression vector for meganucleases. The meganuclease expression vector is marked by LEU2. cDNAs encoding I-*Cre*I meganuclease variants are cloned into this vector digested with *NcoI* and *EagI*, in order to have the variant expression driven by the inducible Gal10 promoter.

Figure 6: pCLS0042 reporter vector. The reporter vector is marked by TRP1 and URA3. The LacZ tandem repeats share 800 bp of homology, and are separated by 1,3 kb of DNA. They are surrounded by ADH promoter and terminator sequences. Target sites are cloned into the *SmaI* site.

Figure 7: shows the results with 292 I-*Cre*I meganuclease variants with a "modified specificity". Proteins are defined by the amino acid present in positions 44, 68 and 70 (three first columns). Numeration of the amino acids is according to pdb accession code 1g9y. Targets are defined by nucleotides at positions -5 to -3 . For each protein, observed cleavage (1) or non observed cleavage (0) is shown for each one of the 64 targets.

Examples

The following examples are presented here only for illustrating the invention and not for limiting the scope thereof. Other variants, obtained from a cDNA, which sequence differs from SEQ ID NO: 69, and using appropriate primers, are still part of the invention.

Example 1: Experimental procedure

Construction of the library of the I-*Cre*I variants (Ulib2 library)

A combinatorial library was constructed by mutagenesis of the I-*Cre*I homing endonuclease replacing DNA binding residues. Three residues (Q44,

R68 and R70) capable of specific interactions with three bases in a single half-site within the DNA target (Jurica *et al.*, 1998) were selected. The combinatorial library was obtained by replacing the three corresponding codons with a unique degenerated vvk codon. vvk corresponds to 18 different codons coding for 12 different amino acids (A, D, E, G, H, K, N, P, Q, R, S and T), as a consequence of the degeneracy of the genetic code. Eventually, mutants in the protein library corresponded to independant combinations of any of the 12 amino acids encoded by the vvk codon at three residue positions. In consequence, the maximal (theoretical) diversity of the protein library was 12^3 or 1728. However, in terms of nucleic acids, the diversity is 18^3 or 5832.

First, residue D75, which is shielded from solvent by R68 and R70, was mutated to N in order to remove the likely energetic strain caused by replacements of those two basic residues in the library. Homodimers of mutant D75N (purified from *E. coli* cells wherein it was over-expressed using a pET expression vector) were shown to cleave the I-*Cre*I homing site.

D75N gene, i.e. a wild-type I-*Cre*I, which CDS is shown in figure 4A (SEQ ID NO: 69), was used as template for the PCR reaction using a forward primer defined by the sequence 5'-gtttaaacatcagctaagcttgacctttvvkgtgacttcaaaagacccag-3' (SEQ ID NO: 67), and a reverse primer, defined by the sequence 5'-gatgtagttggaaacggatccmbbatcmbbtacgtaaccaacgcc-3' (SEQ ID NO: 68). Such primers allow mutation of residues 44, 68 and 70 with a theoretical diversity of 12. The conditions of the PCR reaction are as follows: plasmid pET24-T45 containing the gene I-*Cre*I D75N was diluted at 1 ng/µl to be used as template for PCR. Degenerated oligonucleotides encoding the desired randomizations were used to amplify a PCR fragment in 50 µl PCR reactions. PCR products were pooled, EtOH precipitated and resuspended in 50 µl 10 mM Tris. PCR products were cloned by ligation into the D75N mutant gene, within a pET expression vector digested with specific restriction enzymes. Digestion of vector and insert DNA were conducted in two steps (single enzyme digestion) between which the DNA sample was extracted (using classic phenol:chloroform:isoamylalcohol-based methods) and EtOH-precipitated. 10 µg of digested vector DNA were used for ligation, with a 5:1 excess of insert DNA. *E coli* TG1 cells were transformed with the resulting vector by electroporation. To produce a

number of cell clones above the theoretical diversity of the library, $6x10^4$ clones were produced (35 times the diversity). Bacterial clones were scraped from plates and the corresponding plasmid vectors were extracted and purified.

The library was eventually recloned in the yeast pCLS0542 vector (Figure 5), by sub-cloning a *NcoI-EagI* DNA fragment containing the entire I-*Cre*I ORF of the Figure 4A (SEQ ID NO: 69) in which the stop codon TGA which follows the bp 501 is not shown into pCLS0542.

After ligation and transformation into bacteria, 70 000 bacterial clones were obtained (12 times the diversity). Bacteria were scraped and stored as a glycerol stock. In addition, an aliquot of this glycerol stock was used to inoculate a 200 ml culture and the library vector was extracted and purified from this culture for storage or potential subcloning.

Construction of the 64 target plasmids

The 64 palindromic targets are described in Figure 2C (positions –5 to –3 and +3 to +5) (SEQ ID NO: 1 to SEQ ID NO: 64).

64 couples of oligonucleotides were designed, corresponding to the two strands of the 64 DNA targets, with 12 pb of non palindromic extra sequence on each side, were annealed and cloned into a pGEM-T vector (Promega). Then, a *PvuII* restriction fragment was excised from each one of the 64 pGEM-T-derived vector, and cloned into pCLS0042 (Figure 6), resulting in 64 yeast reporter vectors. Steps of excision, digestion and ligation are performed using typical methods known by those skilled in the art. Insertion of the target sequence is made at the *SmaI* site of pCLS0042.

Yeast strains and transformation

The library of meganuclease expression variants and the A44/R68/L70 variant, were transformed into strain FYC2-6A: alpha, trp1Δ63, leu2Δ1, his3Δ200.

The target plasmids were transformed into yeast strain FYBL2-7B: a, ura3Δ851, trp1Δ63, leu2Δ1, lys2Δ202.

For transformation, a classical chemical/heat choc protocol can be used, and routinely gives 10^6 independent transformants per µg of DNA; transformants were selected on leucine drop-out synthetic medium (Gietz and Woods, 2002).

Screening

I-*Cre*I variant clones as well as yeast reporter strains were stocked in glycerol (20%) stock and replicated in novel microplates.

Each reporter strain was spotted 13 824 times on a nylon membrane, and on each one of this spot was spotted one out of the 13 824 yeast clones expressing a variant meganuclease. Membranes were laid on solid agarose YEPD rich medium, and incubated at 30°C for one night, to allow mating.

Then, membranes were laid on synthetic medium, lacking leucine and tryptophane, and with galactose (1%) as a carbon source, and incubated for five days at 37°C, to select for diploids, allow for meganuclease expression, reporter plasmid cleavage and recombination, and expression of beta-galactosidase. After 5 days, membranes were laid on solid agarose medium with 0.02% X-Gal in 0.5 M Sodium Phosphate buffer, pH 7.0, 0.1% SDS, 6% Dimethyl Formamide (DMF), 7 mM beta-mercaptoethanol, 1% agarose, and incubated at 37°C, to monitor beta-galactosidase activity. Positive clones were identified after two days of incubation, according to staining. For secondary screening, the same procedure was followed with the 292 selected positives, except that each mutant was tested 4 times on the same membrane (see figure 7).

Example 2: Identification of I-*Cre*I meganuclease variants with modified cleavage specificity.

A method for the identification of functional endonucleases in yeast *Saccharomyces cerevisiae* has previously been described (WO 04/067736) and is summarized in Figure 1.

Briefly, the meganucleases expressed from a replicative vector can be tested for their ability to cleave a DNA target in yeast cells, when this DNA target is placed between two direct repeats in another replicative vector. Efficient cleavage of the DNA target induces homologous recombination of direct repeats, resulting in the restoration of a functional beta-galactosidase marker, which can be monitored by X-Gal staining.

This method is used herein to screen a library of I-*Cre*I meganuclease variants with a collection of DNA targets, in order to identify novel I-*Cre*I-derived meganucleases with altered or modified specificities.

The library of I-*Cre*I meganuclease variants was made by mutagenesis of an I-*Cre*I scaffold which residue 75 was replaced with an Asparagine (N). Positions 44, 68 and 70 were randomized, and the regular amino acids (Q44, R68 and R70) replaced with one out of 12 amino acids (A, D, E, G, H, K, N, P, Q, R, S, or T, see Example 1). The resulting library has a complexity of 1728 in terms of protein (5832 in terms of nucleic acids, see Example 3) and was cloned in a yeast replicative expression vector carrying a LEU2 auxotrophic marker gene. This library was transformed into a leu2 mutant haploid yeast strain (FYC2-6A). 13 824 transformant (Leu^+) clones were individually picked in 96 wells microplates.

A series of 64 targets (SEQ ID NO: 1 to SEQ ID NO: 64) were derived from the I-*Cre*I natural target site (SEQ ID NO: 65). These targets are all palindromic in positions +/- 3 to 5 and +/- 8 to 11, and triplet sequence at positions –5 to –3 was randomised as shown in figure 2C. The 64 targets were cloned in the appropriate yeast reporter vector (see Example 1), and transformed into an haploid strain (FYBL2-7B), resulting in 64 tester strains.

In a primary screening experiment, the 13 824 yeast clones expressing I-*Cre*I meganuclease variants were mated with each one of the 64 tester strains, and diploids were tested for β-galactosidase activity. Altogether, 2 110 clones displayed cleavage activity with at least one out of the 64 targets. Meganuclease variant ORFs were amplified from each strain by PCR (see Example 1), and sequenced, and different meganuclease variants were identified among the 2110 clones. Consequently, each variant was tested in a second round of screening against the 64 targets, in quadriplate, and each cleavage profile was established, as shown on Figure 3.

Example 3: I-*Cre*I meganuclease variants with different cleavage profiles

Six examples of profiles, including wild-type I-*Cre*I, are shown on Figure 3, and results (only for variants with "modified specificity") are summarized in Figure 7. The "wild type" I-*Cre*I meganuclease (which residues in position 44, 68 and 70 are Q, R, and R, respectively, and which is named "Q44/R68/R70" or "QRR") cleaves a series of 5 targets, corresponding to the gtg, gtt, gtc, gct and gcc triplets in positions -5 to –3. A strong cleavage activity is observed with gtt, gtc and gcc, whereas gtg and gct are only faintly cut (Figure 3). Similar pattern is found with other

I-*Cre*I meganuclease variants, such as QKS and QRK. However, a lot of I-*Cre*I meganuclease variants display very different patterns. With a few I-*Cre*I meganuclease variants, cleavage of a unique sequence is observed. For example, I-*Cre*I meganuclease variants DRK, RAT and THR are active on the ggg, gct and gac targets, respectively, which were not cleaved by wild-type I-*Cre*I (Figure 3). QAT and QAN both cleave gtt, one of the targets cleaved by I-*Cre*I. NAR cleaves two different targets, gac and tac, both uncut by I-*Cre*I. Other I-*Cre*I meganuclease variants cleave efficiently a series of different targets, such as KSS (cleaves nct, ncc, ttt, ttc, ctt and ctc) and NRS (gag, gat, gac and gat).

Among the 64 targets that were tested, 25 are not cleaved by any of the 292 variants and it is notable that the nna sequence (except gta and gca) and the ngy sequence (except ggt and ggc) remain uncut.

Different groups of I-*Cre*I meganuclease variants emerge from these results, for example :

- a group comprising I-*Cre*I meganuclease variants that cleave more targets than QRR, such as GTP or NRK,
- a group comprising I-*Cre*I meganuclease variants that cleave less targets than QRR, such as TAR,
- a group comprising I-*Cre*I meganuclease variants that cleave only one target, which is not cut by the "wild-type QRR", such as ADH, ADK, AGH, AGK, AHK, AQD, HTT, DRA, DRK, DRR, DRT, GRQ, GTR, NAH, NHN, NKG, NKH, NSG, NTH, RAG, RAT, RGT, RNT, RRN, RSS, RST, SHR, THR, TKR, TRD, TSK or TSR,
- a group comprising I-*Cre*I meganuclease variants that cleave at least one target, and that do not cleave any target cleavable by the "wild-type QRR", such as AAA, ADH, DRK,
- a group comprising I-*Cre*I meganuclease variants only able to cleave two targets, such as NHR, TAR, etc.,
- a group comprising I-*Cre*I meganuclease variants only able to cleave two targets that are not cleaved by the "wild-type QRR", such as AAG, AAT, AHT, AKA, AKG, AKS, AKT, ANT, AQA, ATT, HRA, HSG, KKD, NHR, NKS, NRE, NSK, RRD, SRG, TTR,

- a group comprising variants that cleave at least the five targets also cleaved by the "wild-type QRR", such as ART, ERN, ERS, GTP, HTS, QKD, QRA, QRG, QRH, QRN, QRS, QSR, QTR or TRT,
- a group comprising I-*Cre*I meganuclease variants that cleave a target, which is not cleaved by any other I-*Cre*I meganuclease variant of the library, such as NRK (with ggt) or QRQ (with gca),
- a group comprising I-*Cre*I meganuclease variants that are able to cleave a large number of targets (8 or more), such as ARA, ARD, ARE, ARG, ARH, ARL, ARN, ARR, ARS, ART, ASR, DRN, ERA, ERN, ERS, ERT, EST, GTP, HRG, HRS, etc.,
- a group comprising I-*Cre*I meganuclease variants able to cleave at least one target with nna sequence in positions –5 to -3, such as ERA, ERT, QRA, QRQ, and QRS,
- a group comprising I-*Cre*I meganuclease variants able to cleave at least one target with gta and/or gca sequence in positions –5 to -3, such as ERA, ERT, QRA, QRQ, QRS,
- a group comprising I-*Cre*I meganuclease variants able to cleave at least one target with ngy sequence in positions –5 to -3, such as ARK, NRK, and TRK,
- a group comprising I-*Cre*I meganuclease variants able to cleave at least one target with ggt and/or ggc sequence in positions –5 to -3, such as ARK, NRK, TRK,
- a group comprising I-*Cre*I meganuclease variants able to cleave every target with nab sequences in positions –5 to -3, such as ARA, ARD, ARG, ARN, ARR, ARS, ART and ASR,
- a group comprising I-*Cre*I meganuclease variants able to cleave every target with ncy sequences in positions –5 to -3, such as KAA, KAG, KAN, KAS, KGS, KGT, KHS, KNS, KQS, KRE, KRG, KRN, KRQ, KRS, KRT, KSA, KSN, KSS, KST, KTG, KTN, KTS,
- a group comprising I-*Cre*I meganuclease variants able to cleave at least one target with nav sequence in positions –5 to -3, such as AAH, SRS,

- a group comprising I-*Cre*I meganuclease variants able to cleave at least one target with yyy sequence in positions –5 to -3, such as KHD, KNT, RRD,
- a group comprising I-*Cre*I meganuclease variants that differ from the "wild-type QRR" in only one amino acid residue, and that are able to cleave only one target, such as DRR,
- a group comprising I-*Cre*I meganuclease variants that differ from the "wild-type QRR" in only one amino acid residue, and that are able to cleave the five targets also cleaved by QRR, and that further cleave at least one more target, such as QRA, QRG, QRH, QRN, QRS, QSR, QTR.

These results do not limit the scope of the invention, since Figure 7 only shows results obtained with 292 variants (291 out of the 1728 (or 12^3) I-*Cre*I meganuclease variants obtainable in a complete library (see example 3).

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CLAIMS

1°) Method of preparing a I-*CreI* meganuclease variant having at least a target DNA sequence (or homing site) which is different from the homing sites of the wild type I-CreI meganuclease said method comprising:

(a) replacing amino acids Q44, R68 and/or R70, in reference with I-*Cre*I pdb accession code 1g9y , with an amino acid selected in the group consisting of A, D, E, G, H, K, N, P, Q, R, S and T;

(b) selecting the I-*Cre*I meganuclease variants obtained in step (a) having at least one of the following R_3 triplet cleaving profile in reference to positions –5 to –3 in a double-strand DNA target, said positions -5 to -3 corresponding to R_3 of the following formula I:

5'- $R_1CAAAR_2R_3R_4R'_4R'_3R'_2TTTGR'_1$ -3',

wherein:

R_1 is absent or present; and when present represents a nucleic acid fragment comprising 1 to 9 nucleotides corresponding either to a random nucleic acid sequence or to a fragment of a I-*CreI* meganuclease homing site situated from position -20 to -12 (from 5' to 3'), R_1 corresponding at least to position -12 of said homing site,

R_2 represents the nucleic acid doublet ac or ct and corresponds to positions -7 to -6 of said homing site,

R_3 represents a nucleic acid triplet corresponding to said positions -5 to -3, selected among g, t, c and a, except the following triplets : gtc, gcc, gtg, gtt and gct,

R_4 represents the nucleic acid doublet gt or tc and corresponds to positions –2 to -1 of said homing site,

R'_1 is absent or present; and when present represents a nucleic acid fragment comprising 1 to 9 nucleotides corresponding either to a random nucleic acid sequence or to a fragment of a I-CreI meganuclease homing site situated from position +12 to +20 (from 5' to 3'), R'_1 corresponding at least to position +12 of said homing site,

R'_2 represents the nucleic acid doublet ag or gt, and corresponds to positions +6 to +7 of said homing site,

R'_3 represents a nucleic acid triplet corresponding to said positions +3 to +5, selected among g, t, c, and a; R'_3 being different from gac, ggc, cac, aac, and agc, when R_3 and R'_3 are non-palindromic,

R'_4 represents the nucleic acid doublet gt or tc and corresponds to positions +1 to +2 of said homing site.

2°) Method according to claim 1, characterized in that said nucleic acid triplet R_3 is preferably selected among the following triplets: ggg, gga, ggt, ggc, gag, gaa, gat, gac, gta, gcg, gca, tgg, tga, tgt, tgc, tag, taa, tat, tac, ttg, tta, ttt, ttc, tcg, tca, tct, tcc, agg, aga, agt, agc, aag, aaa, aat, aac, atg, ata, att, atc, acg, aca, act, acc, cgg, cga, cgt, cgc, cag, caa, cat, cac, ctg, cta, ctt, ctc, ccg, cca, cct and ccc and more preferably among the following triplets: ggg, ggt, ggc, gag, gat, gac, gta, gcg, gca, tag, taa, tat, tac, ttg, ttt, ttc, tcg, tct, tcc, agg, aag, aat, aac, att, atc, act, acc, cag, cat, cac, ctt, ctc, ccg, cct and ccc.

3°) Method according to claim 1 or to claim 2, characterized in that the I-*Cre*I meganuclease variant obtained in step (b) is selected from the group consisting of: A44/A68/A70, A44/A68/G70, A44/A68/H70, A44/A68/K70, A44/A68/N70, A44/A68/Q70, A44/A68/R70, A44/A68/S70, A44/A68/T70, A44/D68/H70, A44/D68/K70, A44/D68/R70, A44/G68/H70, A44/G68/K70, A44/G68/N70, A44/G68/P70, A44/G68/R70, A44/H68/A70, A44/H68/G70, A44/H68/H70, A44/H68/K70, A44/H68/N70, A44/H68/Q70, A44/H68/R70, A44/H68/S70, A44/H68/T70, A44/K68/A70, A44/K68/G70, A44/K68/H70, A44/K68/K70, A44/K68/N70, A44/K68/Q70, A44/K68/R70, A44/K68/S70, A44/K68/T70, A44/N68/A70, A44/N68/E70, A44/N68/G70, A44/N68/H70, A44/N68/K70, A44/N68/N70, A44/N68/Q70, A44/N68/R70, A44/N68/S70, A44/N68/T70, A44/Q68/A70, A44/Q68/D70, A44/Q68/G70, A44/Q68/H70, A44/Q68/N70, A44/Q68/R70, A44/Q68/S70, A44/R68/A70, A44/R68/D70, A44/R68/E70, A44/R68/G70, A44/R68/H70, A44/R68/K70, A44/R68/L70, A44/R68/N70, A44/R68/R70, A44/R68/S70, A44/R68/T70, A44/S68/A70, A44/S68/G70, A44/S68/K70, A44/S68/N70, A44/S68/Q70, A44/S68/R70, A44/S68/S70, A44/S68/T70, A44/T68/A70, A44/T68/G70, A44/T68/H70, A44/T68/K70, A44/T68/N70, A44/T68/Q70, A44/T68/R70, A44/T68/S70, A44/T68/T70, D44/D68/H70, D44/N68/S70, D44/R68/A70, D44/R68/K70,

D44/R68/N70, D44/R68/Q70, D44/R68/R70, D44/R68/S70, D44/R68/T70,
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E44/R68/T70, E44/S68/T70, G44/H68/K70, G44/Q68/H70, G44/R68/Q70,
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Q44/T68/R70, R44/A68/G70, R44/A68/T70, R44/G68/T70, R44/H68/D70, R44/H68/T70, R44/N68/T70, R44/R68/A70, R44/R68/D70, R44/R68/E70, R44/R68/G70, R44/R68/N70, R44/R68/Q70, R44/R68/S70, R44/R68/T70, R44/S68/G70, R44/S68/N70, R44/S68/S70, R44/S68/T70, S44/D68/K70, S44/H68/R70, S44/R68/G70, S44/R68/N70, S44/R68/R70, S44/R68/S70, T44/A68/K70, T44/A68/R70, T44/H68/R70, T44/K68/R70, T44/N68/P70, T44/N68/R70, T44/Q68/K70, T44/Q68/R70, T44/R68/A70, T44/R68/D70, T44/R68/E70, T44/R68/G70, T44/R68/H70, T44/R68/K70, T44/R68/N70, T44/R68/Q70, T44/R68/R70, T44/R68/S70, T44/R68/T70, T44/S68/K70, T44/S68/R70, T44/T68/K70, and T44/T68/R70.

4°) Method according to any one of claims 1 to 3, characterized in that the step (b) of selecting said I-*CreI* meganuclease variant is performed *in vivo* in yeast cells.

5°) Use of a I-*CreI* meganuclease variant, obtainable by the method according to any one of claims 1 to 4, *in vitro* or *in vivo* for non-therapeutic purposes, for cleaving a double-strand nucleic acid target comprising at least a 20-24 bp partially palindromic sequence, wherein at least the sequence in positions +/- 8 to 11 is palindromic, and the nucleotide triplet in positions –5 to –3 and/or the nucleotide triplet in positions +3 to +5 differs from gtc, gcc, gtg, gtt, and gct, and from gac, ggc, cac, aac and agc, respectively.

6°) The use according to claim 5, characterized in that said I-*CreI* meganuclease variant is selected from the group consisting of: A44/A68/A70, A44/A68/G70, A44/A68/H70, A44/A68/K70, A44/A68/N70, A44/A68/Q70, A44/A68/R70, A44/A68/S70, A44/A68/T70, A44/D68/H70, A44/D68/K70, A44/D68/R70, A44/G68/H70, A44/G68/K70, A44/G68/N70, A44/G68/P70, A44/G68/R70, A44/H68/A70, A44/H68/G70, A44/H68/H70, A44/H68/K70, A44/H68/N70, A44/H68/Q70, A44/H68/R70, A44/H68/S70, A44/H68/T70, A44/K68/A70, A44/K68/G70, A44/K68/H70, A44/K68/K70, A44/K68/N70, A44/K68/Q70, A44/K68/R70, A44/K68/S70, A44/K68/T70, A44/N68/A70, A44/N68/E70, A44/N68/G70, A44/N68/H70, A44/N68/K70, A44/N68/N70, A44/N68/Q70, A44/N68/R70, A44/N68/S70, A44/N68/T70, A44/Q68/A70, A44/Q68/D70, A44/Q68/G70, A44/Q68/H70, A44/Q68/N70, A44/Q68/R70,

A44/Q68/S70, A44/R68/A70, A44/R68/D70, A44/R68/E70, A44/R68/G70,
A44/R68/H70, A44/R68/K70, A44/R68/L70, A44/R68/N70, A44/R68/R70,
A44/R68/S70, A44/R68/T70, A44/S68/A70, A44/S68/G70, A44/S68/K70,
A44/S68/N70, A44/S68/Q70, A44/S68/R70, A44/S68/S70, A44/S68/T70,
A44/T68/A70, A44/T68/G70, A44/T68/H70, A44/T68/K70, A44/T68/N70,
A44/T68/Q70, A44/T68/R70, A44/T68/S70, A44/T68/T70, D44/D68/H70,
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D44/R68/R70, D44/R68/S70, D44/R68/T70, E44/H68/H70, E44/R68/A70,
E44/R68/H70, E44/R68/N70, E44/R68/S70, E44/R68/T70, E44/S68/T70,
G44/H68/K70, G44/Q68/H70, G44/R68/Q70, G44/R68/R70, G44/T68/D70,
G44/T68/P70, G44/T68/R70, H44/A68/S70, H44/A68/T70, H44/R68/A70,
H44/R68/D70, H44/R68/E70, H44/R68/G70, H44/R68/N70, H44/R68/R70,
H44/R68/S70, H44/R68/T70, H44/S68/G70, H44/S68/S70, H44/S68/T70,
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K44/S68/T70, K44/T68/A70, K44/T68/D70, K44/T68/E70, K44/T68/G70,
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N44/K68/H70, N44/K68/R70, N44/K68/S70, N44/N68/R70, N44/P68/D70,
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N44/R68/S70, N44/R68/T70, N44/S68/G70, N44/S68/H70, N44/S68/K70,

N44/S68/R70, N44/T68/H70, N44/T68/K70, N44/T68/Q70, N44/T68/R70, N44/T68/S70, P44/N68/D70, P44/T68/T70, Q44/A68/A70, Q44/A68/H70, Q44/A68/R70, Q44/G68/K70, Q44/G68/R70, Q44/K68/G70, Q44/N68/A70, Q44/N68/H70, Q44/N68/S70, Q44/P68/P70, Q44/Q68/G70, Q44/R68/A70, Q44/R68/D70, Q44/R68/E70, Q44/R68/G70, Q44/R68/H70, Q44/R68/N70, Q44/R68/Q70, Q44/R68/S70, Q44/S68/H70, Q44/S68/R70, Q44/S68/S70, Q44/T68/A70, Q44/T68/G70, Q44/T68/H70, Q44/T68/R70, R44/A68/G70, R44/A68/T70, R44/G68/T70, R44/H68/D70, R44/H68/T70, R44/N68/T70, R44/R68/A70, R44/R68/D70, R44/R68/E70, R44/R68/G70, R44/R68/N70, R44/R68/Q70, R44/R68/S70, R44/R68/T70, R44/S68/G70, R44/S68/N70, R44/S68/S70, R44/S68/T70, S44/D68/K70, S44/H68/R70, S44/R68/G70, S44/R68/N70, S44/R68/R70, S44/R68/S70, T44/A68/K70, T44/A68/R70, T44/H68/R70, T44/K68/R70, T44/N68/P70, T44/N68/R70, T44/Q68/K70, T44/Q68/R70, T44/R68/A70, T44/R68/D70, T44/R68/E70, T44/R68/G70, T44/R68/H70, T44/R68/K70, T44/R68/N70, T44/R68/Q70, T44/R68/R70, T44/R68/S70, T44/R68/T70, T44/S68/K70, T44/S68/R70, T44/T68/K70.

7°) The use according to claim 5 or to claim 6, characterized in that said I-*Cre*I meganuclease variant is an homodimer.

8°) The use according to any ones of claims 5 and 6, characterized in that said I-*Cre*I meganuclease variant is an heterodimer.

9°) The use according to any one of claims 5 to 8, characterized in that sequence in positions +/- 3 to 5 of said DNA target is palindromic.

10°) The use, according to any one of claims 5 to 8, characterized in that sequence in positions +/- 3 to 5 of said DNA target is non-palindromic.

11°) The use according to any one of claims 5 to 10, characterized in that said palindromic sequences in positions –11 to –8 and +8 to +11 are caaa and tttg, respectively.

12°) The use according to anyone of claims 5 to 11, characterized in that said I-*Cre*I meganuclease variant further comprises a mutation in position 75.

13°) The use according to claim 12, characterized in that said mutation is D75N or D75V.

14°) The use according to anyone of claims 5 to 13, characterized in that said I-*Cre*I meganuclease variant has an alanine (A), an aspartic acid (D) or a threonine (T) in position 44 for cleaving a DNA target comprising nucleotide A in position -4, and/or T in position +4.

15°) The use according to anyone of claims 5 to 13, wherein said meganuclease variant has a lysine (K) or an arginine (R) in position 44 for cleaving a target comprising a nucleotide C in position -4, and/or G in position +4.

16°) A I-*CreI* meganuclease variant:

- Obtainable by the method of preparation according to claims 1 to 4;

- Having one mutation of at least one of the amino acid residues in positions 44, 68 and 70 of I-*Cre*I; said mutations being the only ones within the amino acids contacting directly the DNA target;

- Having at least a target DNA sequence (or homing site) which is different from the homing sites of the wild type I-CreI meganuclease and

- Being able to cleave at least one homing site that is not cleaved by the wild-type I-*CreI* meganuclease.

17°) The I-*CreI* meganuclease variant according to claim 16, characterized in that the amino acid residue chosen for the replacement of the amino acid in positions 44, 68 and/or 70 is selected in the group comprising A, D, E, G, H, K, N, P, Q, R, S and T.

18°) The I-*CreI* meganuclease variant according to claim 16 or claim 17, characterized in that said I-*Cre*I meganuclease variant is selected in the group consisting of: A44/A68/A70, A44/A68/G70, A44/A68/H70, A44/A68/K70, A44/A68/N70, A44/A68/Q70, A44/A68/S70, A44/A68/T70, A44/D68/H70, A44/D68/K70, A44/D68/R70, A44/G68/H70, A44/G68/K70, A44/G68/N70, A44/G68/P70, A44/H68/A70, A44/H68/G70, A44/H68/H70, A44/H68/K70, A44/H68/N70, A44/H68/Q70, A44/H68/S70, A44/H68/T70, A44/K68/A70, A44/K68/G70, A44/K68/H70, A44/K68/N70, A44/K68/Q70, A44/K68/R70, A44/K68/S70, A44/K68/T70, A44/N68/A70, A44/N68/E70, A44/N68/G70, A44/N68/H70, A44/N68/K70, A44/N68/N70, A44/N68/Q70, A44/N68/R70, A44/N68/S70, A44/N68/T70, A44/Q68/A70, A44/Q68/D70, A44/Q68/G70,

A44/Q68/H70, A44/Q68/N70, A44/Q68/S70, A44/R68/E70, A44/R68/K70, A44/R68/L70, A44/S68/A70, A44/S68/G70, A44/S68/N70, A44/S68/Q70, A44/S68/R70, A44/S68/S70, A44/S68/T70, A44/T68/A70, A44/T68/G70, A44/T68/H70, A44/T68/N70, A44/T68/Q70, A44/T68/S70, A44/T68/T70, D44/D68/H70, D44/N68/S70, D44/R68/A70, D44/R68/N70, D44/R68/Q70, D44/R68/R70, D44/R68/S70, D44/R68/T70, E44/H68/H70, E44/R68/A70, E44/R68/H70, E44/R68/N70, E44/R68/S70, E44/R68/T70, E44/S68/T70, G44/H68/K70, G44/Q68/H70, G44/R68/Q70, G44/T68/D70, G44/T68/P70, G44/T68/R70, H44/A68/S70, H44/A68/T70, H44/R68/D70, H44/R68/E70, H44/R68/G70, H44/R68/N70, H44/R68/R70, H44/R68/S70, H44/S68/G70, H44/S68/S70, H44/S68/T70, H44/T68/S70, H44/T68/T70, K44/A68/A70, K44/A68/D70, K44/A68/E70, K44/A68/G70, K44/A68/H70, K44/A68/N70, K44/A68/Q70, K44/D68/A70, K44/D68/T70, K44/E68/G70, K44/E68/S70, K44/G68/A70, K44/G68/G70, K44/G68/N70, K44/G68/S70, K44/G68/T70, K44/H68/D70, K44/H68/E70, K44/H68/G70, K44/H68/N70, K44/H68/S70, K44/H68/T70, K44/K68/A70, K44/K68/D70, K44/K68/H70, K44/K68/T70, K44/N68/A70, K44/N68/D70, K44/N68/E70, K44/N68/G70, K44/N68/H70, K44/N68/N70, K44/N68/Q70, K44/N68/S70, K44/N68/T70, K44/P68/H70, K44/Q68/A70, K44/Q68/D70, K44/Q68/E70, K44/Q68/S70, K44/Q68/T70, K44/R68/A70, K44/R68/D70, K44/R68/E70, K44/R68/G70, K44/R68/H70, K44/R68/N70, K44/R68/S70, K44/S68/A70, K44/S68/D70, K44/S68/H70, K44/S68/N70, K44/S68/S70, K44/S68/T70, K44/T68/A70, K44/T68/D70, K44/T68/E70, K44/T68/G70, K44/T68/H70, K44/T68/N70, K44/T68/Q70, K44/T68/S70, K44/T68/T70, N44/A68/H70, N44/H68/N70, N44/H68/R70, N44/K68/G70, N44/K68/H70, N44/K68/R70, N44/K68/S70, N44/P68/D70, N44/Q68/H70, N44/R68/A70, N44/R68/D70, N44/R68/E70, N44/R68/K70, N44/S68/G70, N44/S68/H70, N44/S68/K70, N44/S68/R70, N44/T68/H70, N44/T68/K70, N44/T68/Q70, N44/T68/S70, P44/N68/D70, P44/T68/T70, Q44/G68/K70, Q44/G68/R70, Q44/K68/G70, Q44/N68/A70, Q44/N68/H70, Q44/N68/S70, Q44/P68/P70, Q44/Q68/G70, Q44/R68/D70, Q44/R68/E70, Q44/R68/G70, Q44/R68/Q70, Q44/S68/S70, Q44/T68/A70, Q44/T68/G70, Q44/T68/H70, R44/A68/G70, R44/A68/T70, R44/G68/T70, R44/H68/D70,

R44/H68/T70, R44/N68/T70, R44/R68/A70, R44/R68/D70, R44/R68/E70, R44/R68/G70, R44/R68/Q70, R44/R68/S70, R44/R68/T70, R44/S68/G70, R44/S68/N70, R44/S68/S70, R44/S68/T70, S44/D68/K70, S44/R68/R70, S44/R68/S70, T44/A68/K70, T44/N68/P70, T44/N68/R70, T44/R68/E70, T44/R68/Q70, and T44/S68/K70.

19°) The I-*CreI* meganuclease variant according to anyone of claims 16 to 18, characterized in that said I-*Cre*I meganuclease variant has an alanine (A), an aspartic acid (D) or a threonine (T) in position 44 and cleaves a target comprising the nucleotide A in position -4, and/or T in position +4.

20°) The I-*CreI* meganuclease variant according to anyone of claims 16 to 18, characterized in that said I-*Cre*I meganuclease variant of the invention has a lysine (K) or an arginine (R) in position 44 and cleaves a target comprising c in position -4, and/or g in position +4.

21°) A polynucleotide, characterized in that it encodes a I-*Cre*I meganuclease variant according to anyone of claims 16 to 20.

22°) An expression cassette, comprising a polynucleotide according to claim 21 and regulation sequences.

23°) An expression vector, characterized in that it comprises an expression cassette according to claim 22.

24°) An expression vector, according to claim 23, characterized in that it further comprises a targeting DNA construct.

25°) An expression vector, according to claim 24, characterized in that said targeting DNA construct comprises a sequence sharing homologies with the region surrounding the cleavage site of the I-*Cre*I meganuclease variant according to anyone of claims 16 to 20.

26°) The expression vector, according to claim 24 characterized in that said targeting DNA construct comprises :

a) sequences sharing homologies with the region surrounding the cleavage site of the variant according to claims 16 to 20, and

b) sequences to be introduced flanked by sequence as in a).

27°) A cell, characterized in that it is modified by a polynucleotide according to claim 21 or by a vector according to any one of claims 23 to 26.

28°) A transgenic plant, characterized in that it comprises a polynucleotide according to claim 21, or a vector according to any one of claims 23 to 26.

29°) A non-human transgenic mammal, characterized in that it comprises a polynucleotide according to claim 21 or a vector according to any one of claims 23 to 26.

30°) Use of a I-*Cre*I meganuclease variant, according to any one of claims 16 to 20, a polynucleotide according to claim 21, a vector according to any one of claims 23 to 26, a cell according to claim 27, a transgenic plant according to claim 28, a non-human transgenic mammal according to claim 29, for molecular biology, for *in vivo* or *in vitro* genetic engineering, and for *in vivo* or *in vitro* genome engineering.

31°) The use, according to claim 30, for inducing a double-strand nucleic acid break in a site of interest comprising a DNA target sequence, thereby inducing a DNA recombination event, a DNA loss or cell death.

32°) The use, according to claim 30 or to claim 31, characterized in that said double-strand nucleic acid break is for: repairing a specific sequence, modifying a specific sequence, restoring a functional gene in place of a mutated one, attenuating or activating an endogenous gene of interest, introducing a mutation into a site of interest, introducing an exogenous gene or a part thereof, inactivating or detecting an endogenous gene or a part thereof, translocating a chromosomal arm, or leaving the DNA unrepaired and degraded.

33°) The use according to any one of claims 30 to 32, characterized in that said I-*Cre*I meganuclease variant, polynucleotide, vector, cell, transgenic plant or non-human transgenic mammal are associated with a targeting DNA construct as defined in claim 26.

34°) A method of genetic engineering, characterized in that it comprises a step of double-strand nucleic acid breaking in a site of interest located on a vector, comprising a DNA target of a I-*Cre*I meganuclease variant according to claims 16 to 20, by contacting said vector with a I-*Cre*I meganuclease variant as defined above, thereby inducing a homologous recombination with another vector presenting homology with the sequence surrounding the cleavage site of said I-*Cre*I meganuclease variant.

35°) A method of genome engineering, characterized in that it comprises the following steps: 1) double-strand breaking a genomic locus comprising at least one recognition and cleavage site of a I-*Cre*I meganuclease variant according to claims 16 to 20, by contacting said cleavage site with said I-*Cre*I meganuclease variant; 2) maintaining said broken genomic locus under conditions appropriate for homologous recombination with a targeting DNA construct comprising the sequence to be introduced in said locus, flanked by sequences sharing homologies with the target locus.

36°) A method of genome engineering, characterized in that it comprises the following steps: 1) double-strand breaking a genomic locus comprising at least one recognition and cleavage site of a I-*Cre*I meganuclease variant according to claims 16 to 20, by contacting said cleavage site with said I-*Cre*I meganuclease variant; 2) maintaining said broken genomic locus under conditions appropriate for homologous recombination with chromosomal DNA sharing homologies to regions surrounding the cleavage site.

37°) A composition characterized in that it comprises at least one I-*Cre*I meganuclease variant according to claims 16 to 20, a polynucleotide according to claim 21, or a vector according to any one of claims 23 to 26.

38°) The composition according to claim 37, characterized in that said composition further comprises a targeting DNA construct comprising the sequence which repairs the site of interest flanked by sequences sharing homologies with the targeted locus.

39°) Use of at least one I-*Cre*I meganuclease variant according to claims 16 to 20, a polynucleotide according to claim 21, or a vector according to any one of claims 23 to 26 for the preparation of a medicament for preventing, improving or curing a genetic disease in an individual in need thereof, said medicament being administrated by any means to said individual.

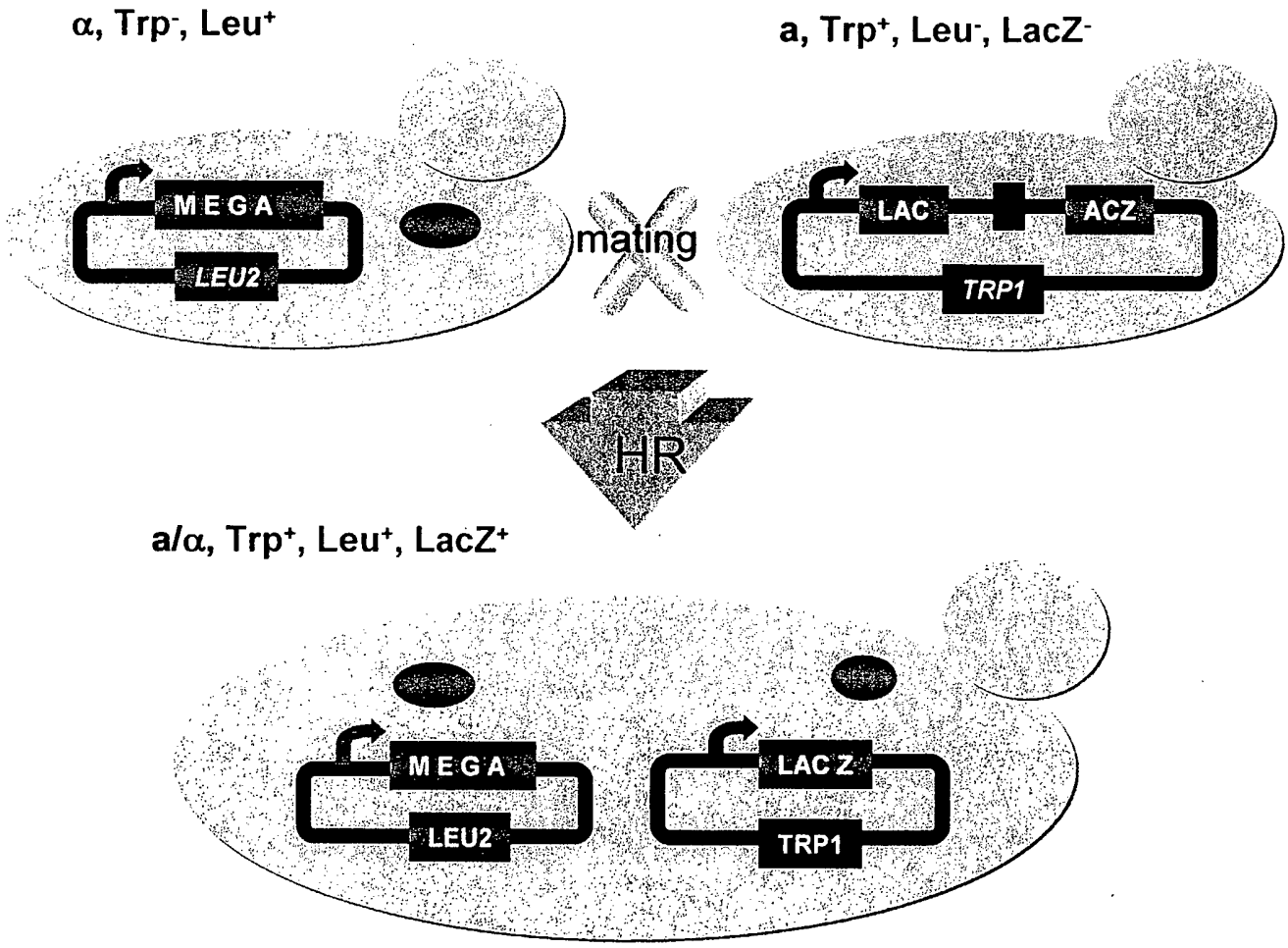
40°) Use of at least one I-*Cre*I meganuclease variant according to claims 16 to 20, a polynucleotide according to claim 21, or a vector according to any one of claims 23 to 26, for the preparation of a medicament for preventing, improving or curing a disease caused by an infectious agent that presents a DNA intermediate, in

an individual in need thereof, said medicament being administrated by any means to said individual.

41°) Use of at least one I-*Cre*I meganuclease variant according to claims 16 to 20, a polynucleotide according to claim 21, or a vector according to any one of claims 23 to 26, *in vitro*, for inhibiting the propagation, inactivating or deleting an infectious agent that presents a DNA intermediate, in biological derived products or products intended for biological uses or for disinfecting an object.

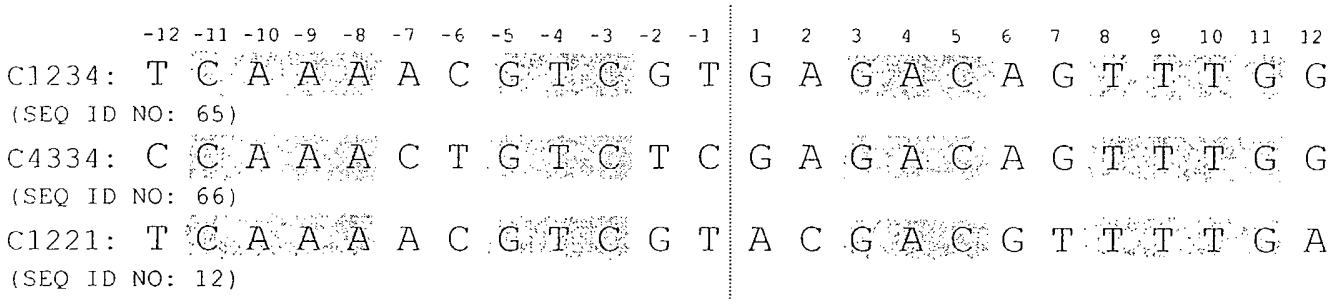
42°) The use according to any one of claim 39 to 41, characterized in that said infectious agent is a virus.

Figure 1



Selection of diploides, X-Gal test

Figure 2A


-12 -11 -10 -9 -8 -7 -6 -5 -4 -3 -2 -1 1 2 3 4 5 6 7 8 9 10 11 12
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(SEQ ID NO: 65)
C4334: C C A A A C T G T C T C G A G A C A G T T T G G
(SEQ ID NO: 66)
C1221: T C A A A A C G T C G T A C G A C G T T T T G A
(SEQ ID NO: 12)

Figure 2B

(SEQ ID NO: 65)

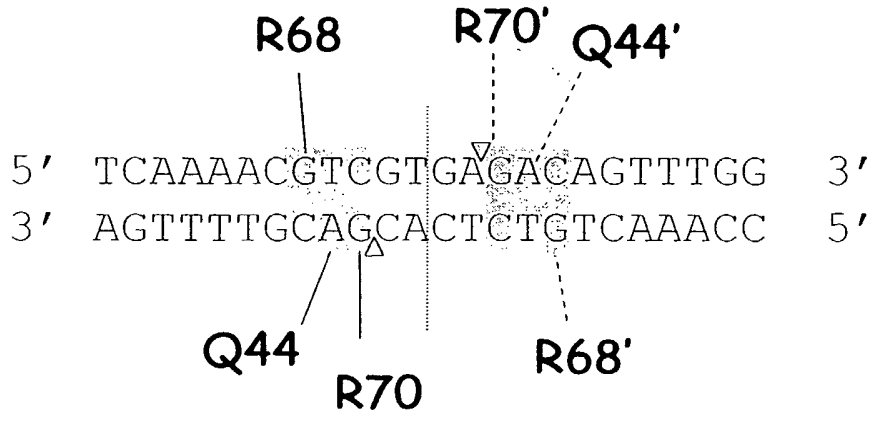

Figure 2C

	-12	-11	-10	-9	-8	-7	-6	-5	-4	-3	-2	1	1	2	3	4	5	6	7	8	9	10	11	12
SEQ ID NO: 1:	T	C	A	A	A	A	C	G	G	G	G	T	A	C	C	C	C	G	T	T	T	T	G	A
SEQ ID NO: 2:	T	C	A	A	A	A	C	G	G	A	G	T	A	C	T	C	C	G	T	T	T	T	G	A
SEQ ID NO: 3:	T	C	A	A	A	A	C	G	G	T	G	T	A	C	A	C	C	G	T	T	T	T	G	A
SEQ ID NO: 4:	T	C	A	A	A	A	C	G	G	C	G	T	A	C	G	C	C	G	T	T	T	T	G	A
SEQ ID NO: 5:	T	C	A	A	A	A	C	G	A	G	G	T	A	C	C	T	C	G	T	T	T	T	G	A
SEQ ID NO: 6:	T	C	A	A	A	A	C	G	A	A	G	T	A	C	T	T	C	G	T	T	T	T	G	A
SEQ ID NO: 7:	T	C	A	A	A	A	C	G	A	T	G	T	A	C	A	T	C	G	T	T	T	T	G	A
SEQ ID NO: 8:	T	C	A	A	A	A	C	G	A	C	G	T	A	C	G	T	C	G	T	T	T	T	G	A
SEQ ID NO: 9:	T	C	A	A	A	A	C	G	T	G	G	T	A	C	C	A	C	G	T	T	T	T	G	A
SEQ ID NO: 10:	T	C	A	A	A	A	C	G	T	A	G	T	A	C	T	A	C	G	T	T	T	T	G	A
SEQ ID NO: 11:	T	C	A	A	A	A	C	G	T	T	G	T	A	C	A	A	C	G	T	T	T	T	G	A
SEQ ID NO: 12:	T	C	A	A	A	A	C	G	T	C	G	T	A	C	G	A	C	G	T	T	T	T	G	A
SEQ ID NO: 13:	T	C	A	A	A	A	C	G	C	G	G	T	A	C	C	G	C	G	T	T	T	T	G	A
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SEQ ID NO: 15:	T	C	A	A	A	A	C	G	C	T	G	T	A	C	A	G	C	G	T	T	T	T	G	A
SEQ ID NO: 16:	T	C	A	A	A	A	C	G	C	C	G	T	A	C	G	G	C	G	T	T	T	T	G	A
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Figure 2C (continuated)

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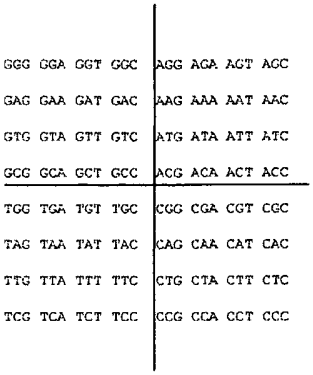
Figure 3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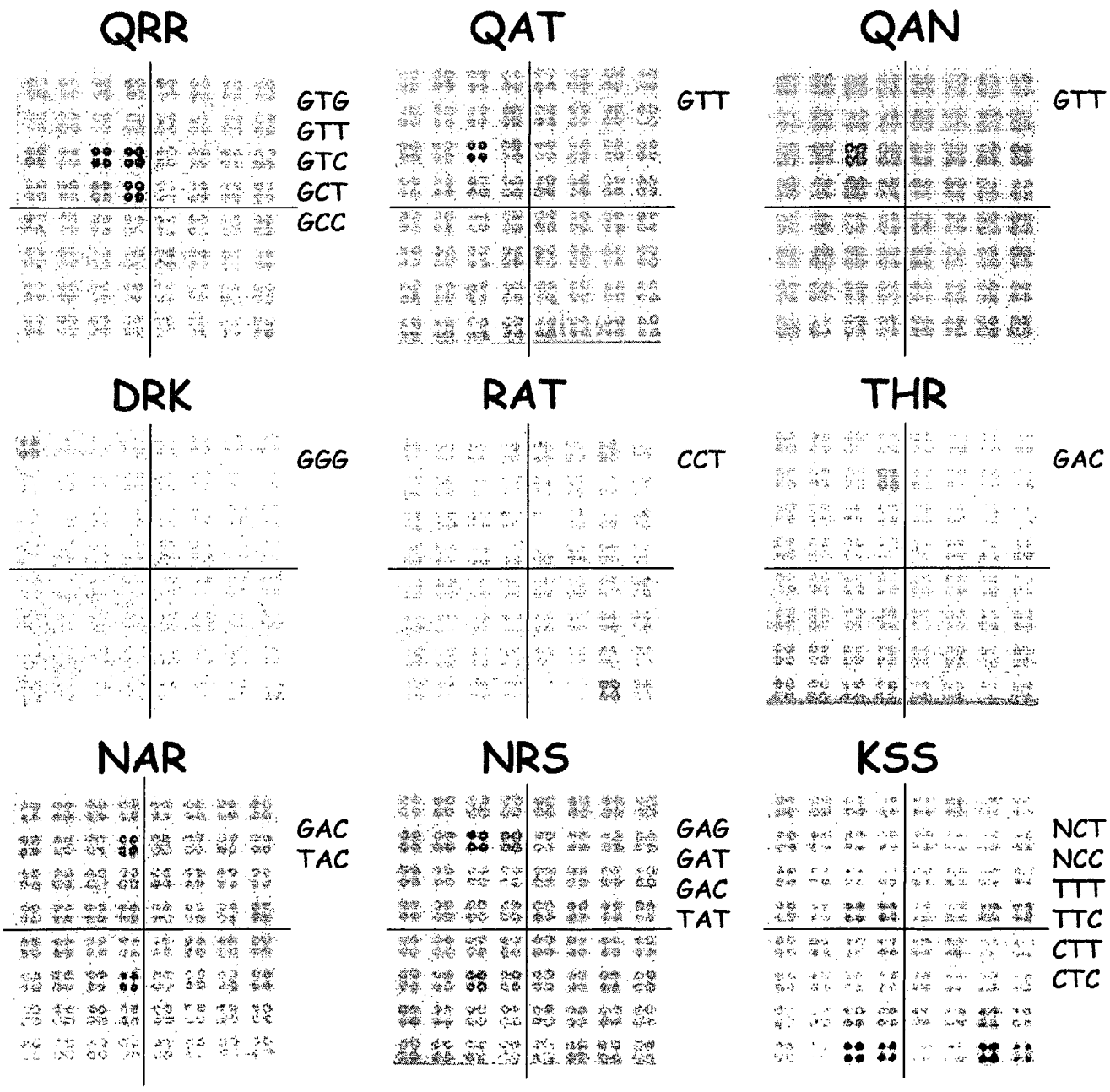
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CTT
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Figure 4A

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201  CGTACGTGAT CGCGGATCCG TTTCCAACTA CATCTTAAGC GAAATCAAGC
251  CGCTGCACAA CTTCCTGACT CAACTGCAGC CGTTTCTGAA ACTGAAACAG
301  AAACAGGCAA ACCTGGTTCT GAAAATTATC GAACAGCTGC CGTCTGCAAA
351  AGAATCCCCG GACAAATTCC TGGAAGTTTG TACCTGGGTG GATCAGATTG
401  CAGCTCTGAA CGATTCTAAG ACGCGTAAAA CCACTTCTGA AACCGTTCGT
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Figure 4B

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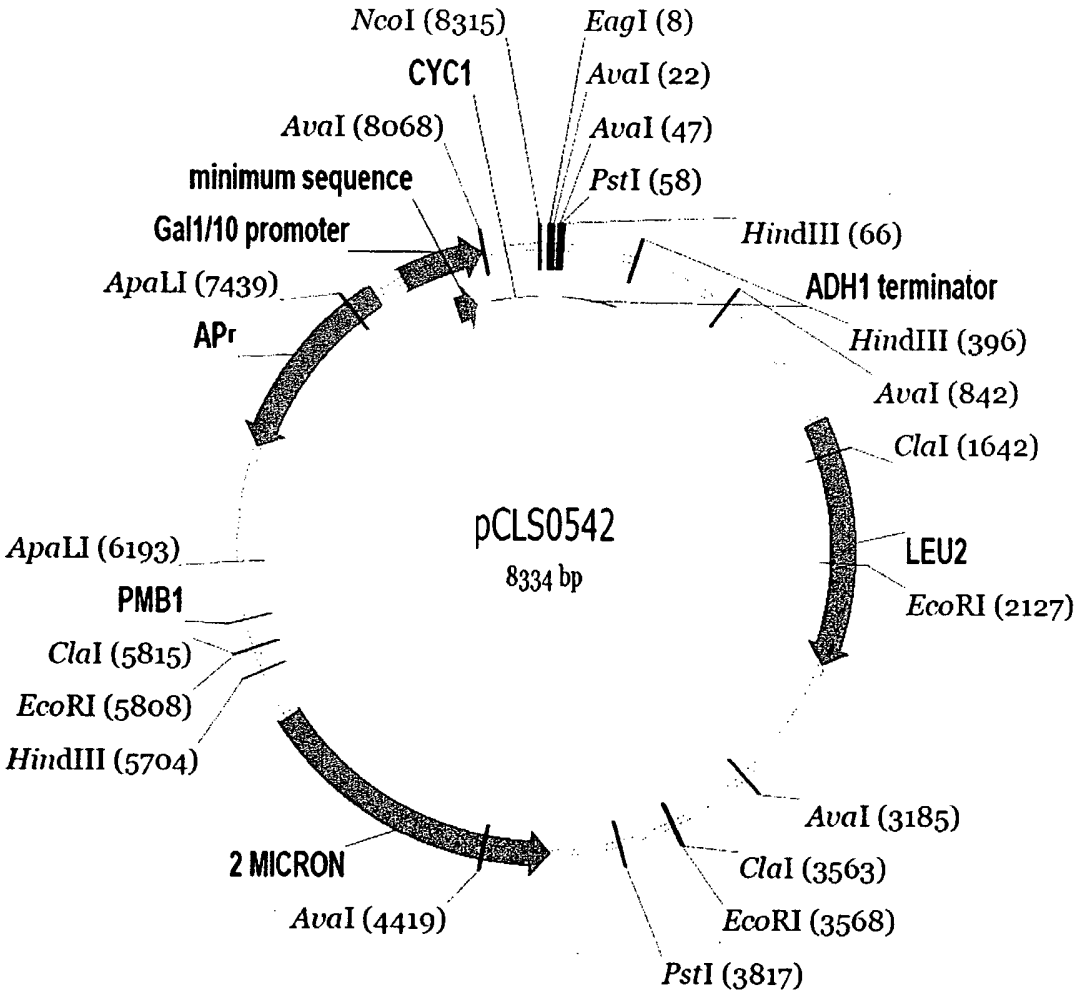
V = A or G or C

M = A or C

B = G or C or T

K = G or T

Figure 5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HindIII (66)
ApaLI (7439)
ADH1 terminator
APr
HindIII (396)
AvaI (842)
ClaI (1642)
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EcoRI (2127)
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2 MICRON
EcoRI (3568)
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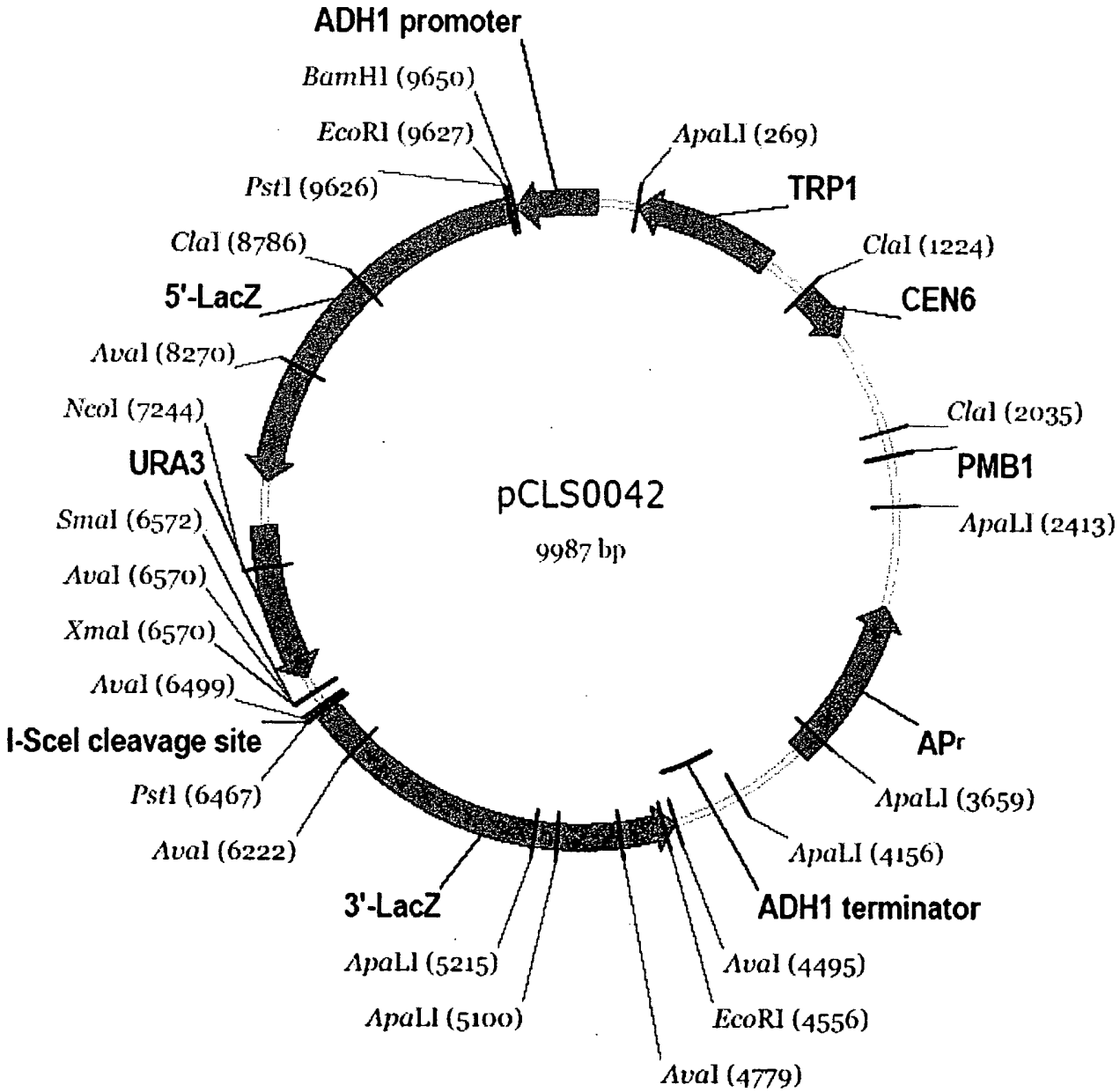
Figure 6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AvaI (8270)
NcoI (7244)
URA3
SmaI (6572)
AvaI (6570)
XmaI (6570)
AvaI (6499)
I-SceI cleavage site
PstI (6467)
AvaI (6222)
3'-LacZ
ApaLI (5215)
ApaLI (5100)
AvaI (4779)
EcoRI (4556)
AvaI (4495)
ADH1 terminator
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APr
ApaLI (3659)
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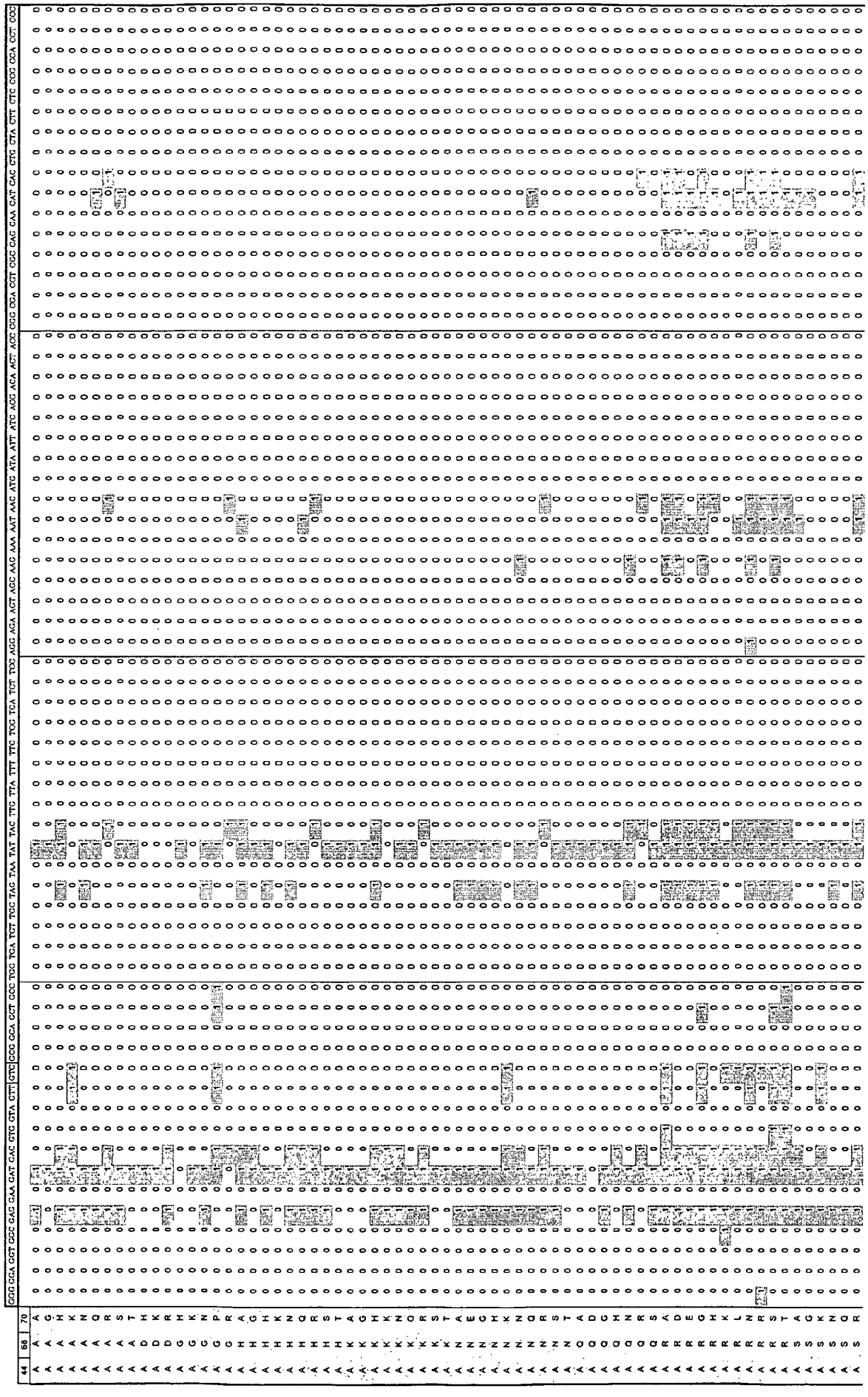

FIGURE 7.1

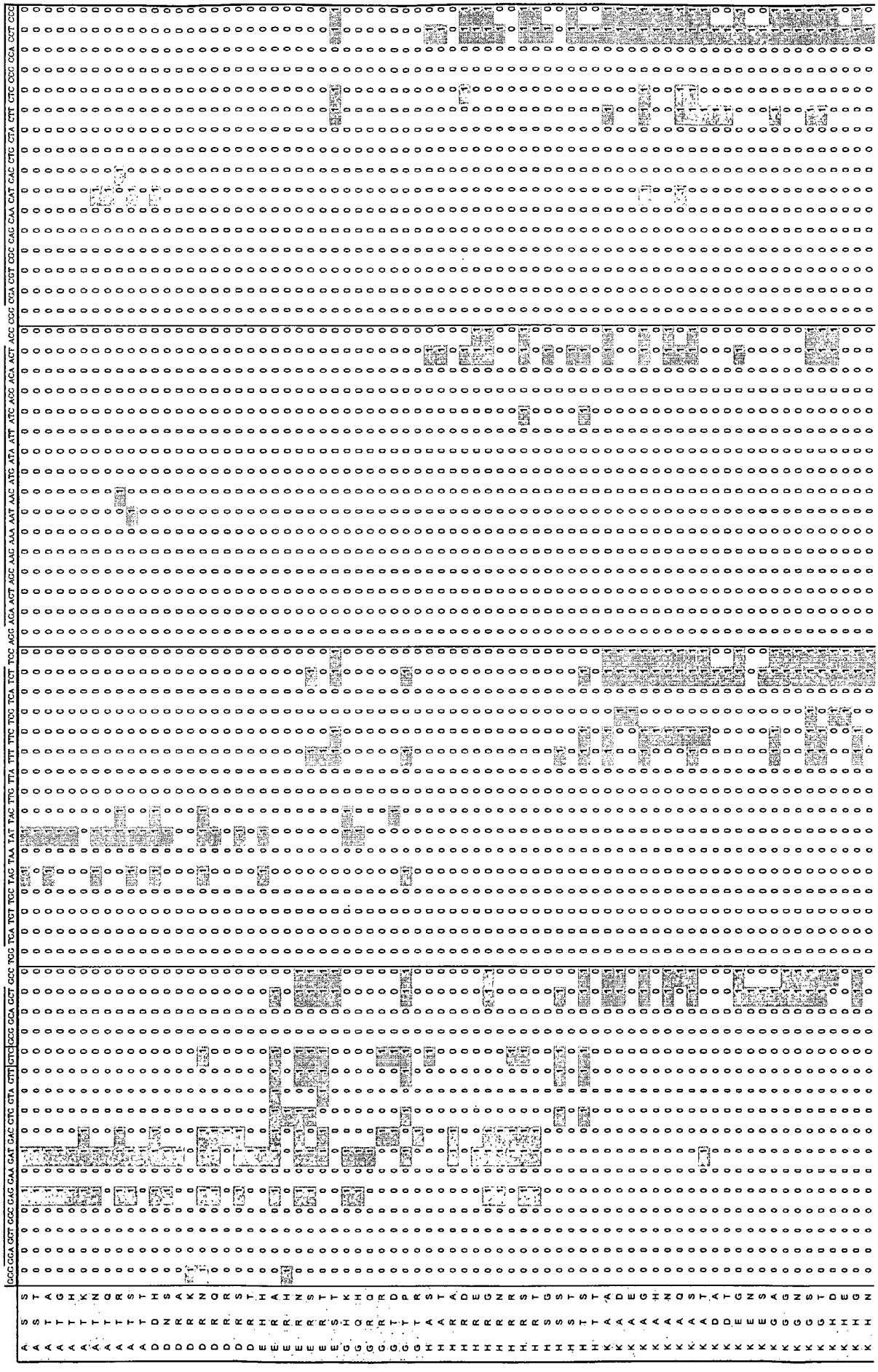

FIGURE 7.2

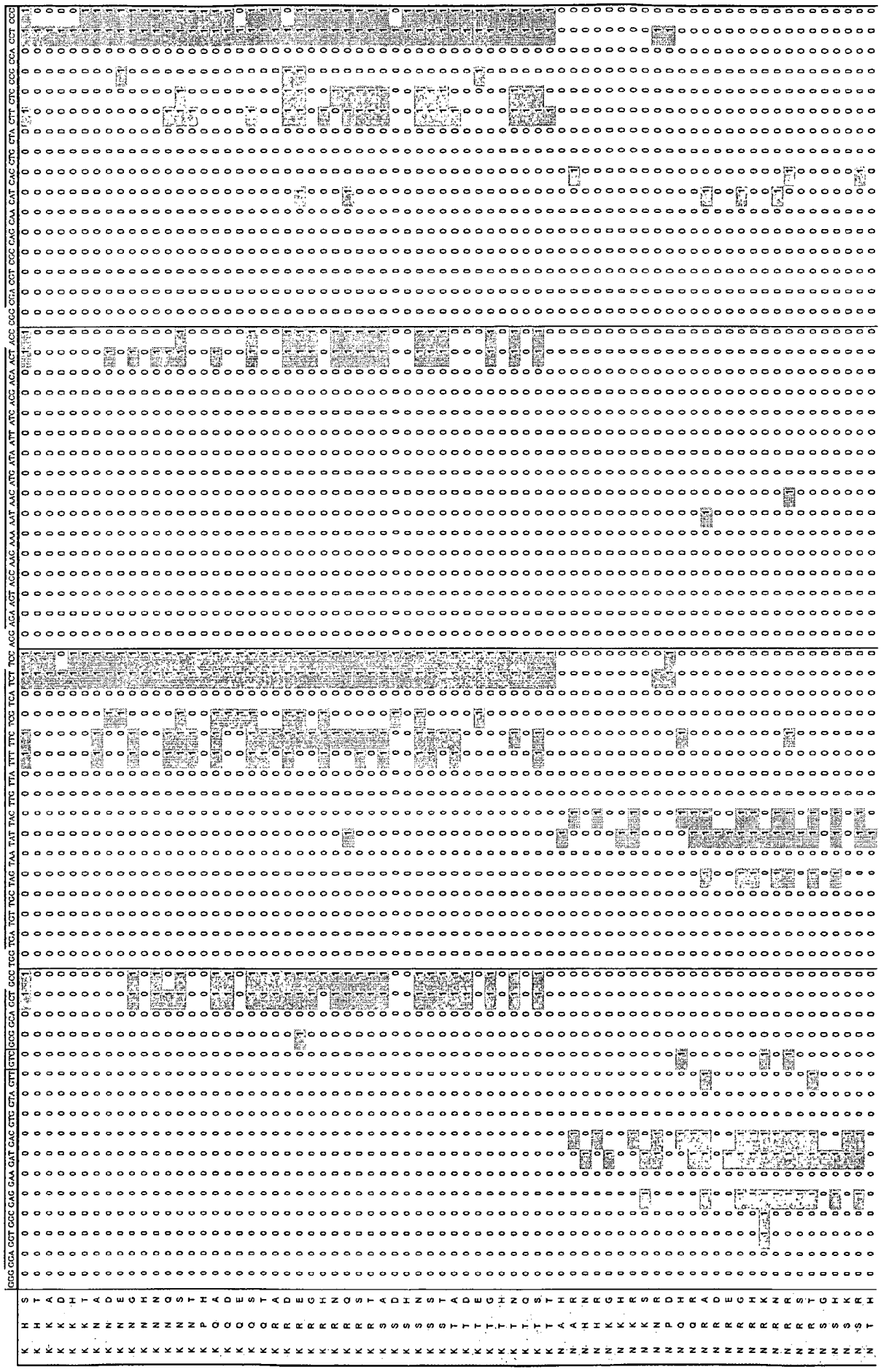

FIGURE 7.3

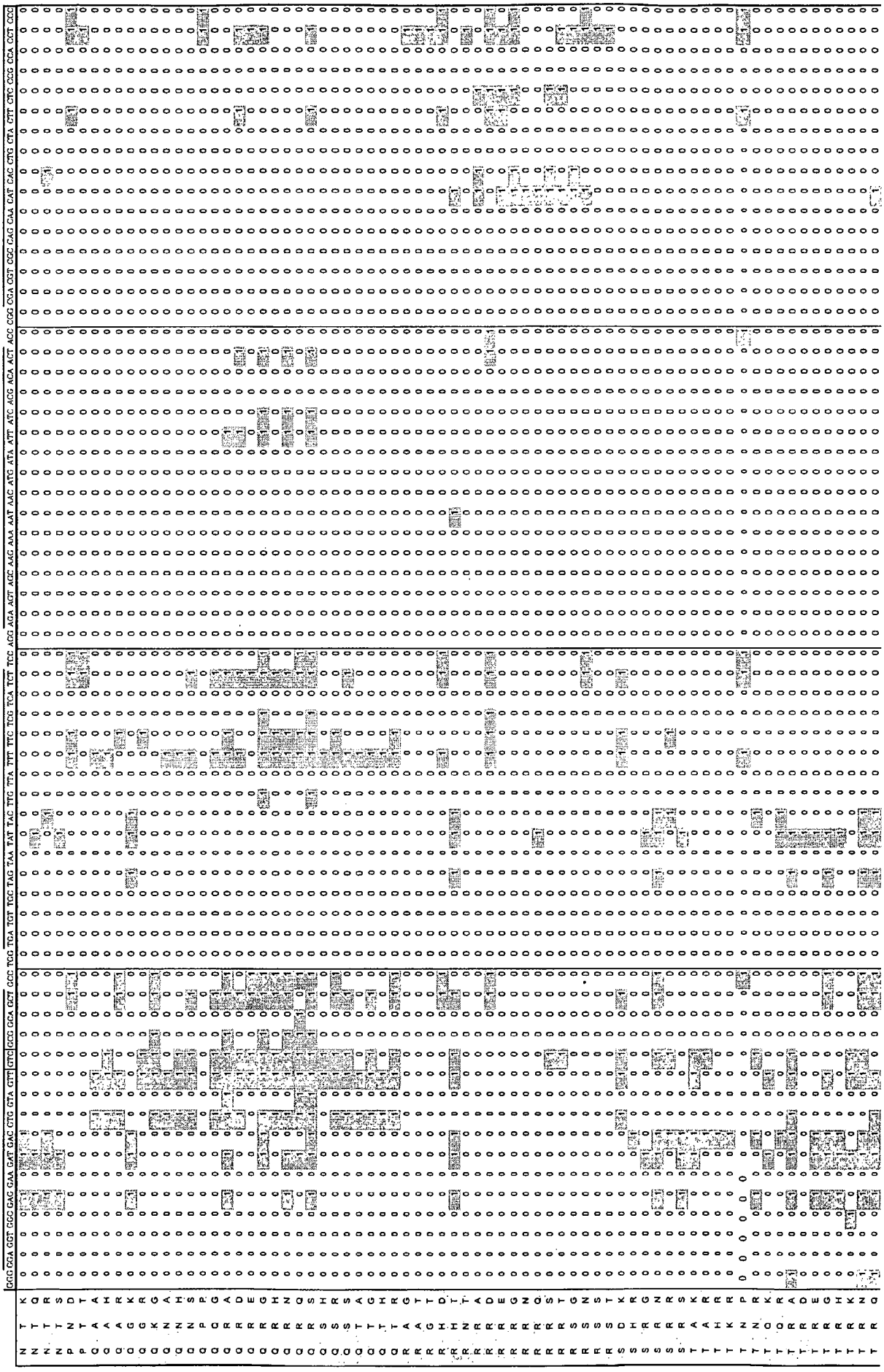

FIGURE 7.4

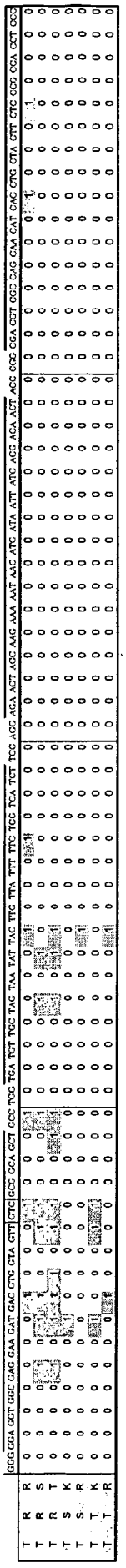

FIGURE 7.5

F1546-5PCT Listage.ST25
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Pâques, Frédéric

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ttt gtg gac ggt gac ggt agc atc atc gct cag att aaa cca aac cag       96
Phe Val Asp Gly Asp Gly Ser Ile Ile Ala Gln Ile Lys Pro Asn Gln
            20                  25                  30

tct tat aag ttt aaa cat cag cta agc ttg acc ttt cag gtg act caa      144
Ser Tyr Lys Phe Lys His Gln Leu Ser Leu Thr Phe Gln Val Thr Gln
        35                  40                  45

aag acc cag cgc cgt tgg ttt ctg gac aaa cta gtg gat gaa att ggc      192
Lys Thr Gln Arg Arg Trp Phe Leu Asp Lys Leu Val Asp Glu Ile Gly
    50                  55                  60

gtt ggt tac gta cgt gat cgc gga tcc gtt tcc aac tac atc tta agc      240
Val Gly Tyr Val Arg Asp Arg Gly Ser Val Ser Asn Tyr Ile Leu Ser
65                  70                  75                  80

gaa atc aag ccg ctg cac aac ttc ctg act caa ctg cag ccg ttt ctg      288
Glu Ile Lys Pro Leu His Asn Phe Leu Thr Gln Leu Gln Pro Phe Leu
                85                  90                  95

aaa ctg aaa cag aaa cag gca aac ctg gtt ctg aaa att atc gaa cag      336
Lys Leu Lys Gln Lys Gln Ala Asn Leu Val Leu Lys Ile Ile Glu Gln
            100                 105                 110

ctg ccg tct gca aaa gaa tcc ccg gac aaa ttc ctg gaa gtt tgt acc      384
Leu Pro Ser Ala Lys Glu Ser Pro Asp Lys Phe Leu Glu Val Cys Thr
        115                 120                 125

tgg gtg gat cag att gca gct ctg aac gat tct aag acg cgt aaa acc      432
Trp Val Asp Gln Ile Ala Ala Leu Asn Asp Ser Lys Thr Arg Lys Thr
    130                 135                 140

act tct gaa acc gtt cgt gct gtg ctg gac agc ctg agc gag aag aag      480
Thr Ser Glu Thr Val Arg Ala Val Leu Asp Ser Leu Ser Glu Lys Lys
145                 150                 155                 160

aaa tcc tcc ccg gcg gcc gac                                         501
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<212> PRT

<213> Chlamydomonas reinhardtii

<400> 70

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Phe Val Asp Gly Asp Gly Ser Ile Ile Ala Gln Ile Lys Pro Asn Gln
            20                  25                  30

Ser Tyr Lys Phe Lys His Gln Leu Ser Leu Thr Phe Gln Val Thr Gln
        35                  40                  45

Lys Thr Gln Arg Arg Trp Phe Leu Asp Lys Leu Val Asp Glu Ile Gly
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Val Gly Tyr Val Arg Asp Arg Gly Ser Val Ser Asn Tyr Ile Leu Ser
65 70 75 80

Glu Ile Lys Pro Leu His Asn Phe Leu Thr Gln Leu Gln Pro Phe Leu
85 90 95

Lys Leu Lys Gln Lys Gln Ala Asn Leu Val Leu Lys Ile Ile Glu Gln
100 105 110

Leu Pro Ser Ala Lys Glu Ser Pro Asp Lys Phe Leu Glu Val Cys Thr
115 120 125

Trp Val Asp Gln Ile Ala Ala Leu Asn Asp Ser Lys Thr Arg Lys Thr
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Thr Ser Glu Thr Val Arg Ala Val Leu Asp Ser Leu Ser Glu Lys Lys
145 150 155 160

Lys Ser Ser Pro Ala Ala Asp
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<223> I-CreI homing site

<400> 71
caaaacgtcg tgagacagtt tg 22

INTERNATIONAL SEARCH REPORT

Interi al Application No
PCT/IB2005/000981

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12N9/22 C12N15/55 C12N15/10 C12N15/64 C12N15/79 C12N15/90

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, EMBASE, BIOSIS, FSTA, WPI Data, PAJ

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 2004/067736 A (CELLECTIS; ARNOULD, SYLVAIN; BRUNEAU, SYLVIA; CABANIOLS, JEAN-PIERRE;) 12 August 2004 (2004-08-12) cited in the application page 74 - page 86	16-19, 21-42
A	SUSSMAN D ET AL: "Isolation and Characterization of New Homing Endonuclease Specificities at Individual Target Site Positions" JOURNAL OF MOLECULAR BIOLOGY, LONDON, GB, vol. 342, no. 1, 3 September 2004 (2004-09-03), pages 31-41, XP004844889 ISSN: 0022-2836 cited in the application	

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[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
31 October 2005	10/11/2005

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Piret, B

INTERNATIONAL SEARCH REPORT

Interi al Application No
PCT/IB2005/000981

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	SELIGMAN L M ET AL: "Mutations altering the cleavage specificity of a homing endonuclease" NUCLEIC ACIDS RESEARCH, OXFORD UNIVERSITY PRESS, SURREY, GB, vol. 30, no. 17, 1 September 2002 (2002-09-01), pages 3870-3879, XP002282592 ISSN: 0305-1048 cited in the application	

INTERNATIONAL SEARCH REPORT

Information on patent family members

Inter ial Application No
PCT/IB2005/000981

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 2004067736 A	12-08-2004	AU 2004208030 A1	12-08-2004
		AU 2004208031 A1	12-08-2004
		WO 2004067753 A2	12-08-2004