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(54) **Titre : SYSTEMES DE RECOMBINAISON ET PROCEDES POUR RETIRER DES SEQUENCES D'ACIDE NUCLEIQUE DU
GENOME D'ORGANISMES EUCARYOTES**
(54) **Title: RECOMBINATION SYSTEMS AND A METHOD FOR REMOVING NUCLEIC ACID SEQUENCES FROM THE GENOME OF
EUKARYOTIC ORGANISMS**

(57) **Abrégé/Abstract:**

The invention relates to recombination systems and to a method for removing nucleic acid sequences from the chromosomal DNA of eukaryotic organisms. The invention also relates to transgenic organisms (preferably plants), containing said systems or produced by said method.



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(54) Title: RECOMBINATION SYSTEMS AND A METHOD FOR REMOVING NUCLEIC ACID SEQUENCES FROM THE GENOME OF EUKARYOTIC ORGANISMS

(54) Bezeichnung: REKOMBINATIONSSYSTEME UND VERFAHREN ZUM ENTFERNEN VON NUKLEINSÄURESEQUENZEN AUS DEM GENOM EUKARYOTISCHER ORGANISMEN

(57) Abstract: The invention relates to recombination systems and to a method for removing nucleic acid sequences from the chromosomal DNA of eukaryotic organisms. The invention also relates to transgenic organisms (preferably plants), containing said systems or produced by said method.

(57) Zusammenfassung: Die Erfindung betrifft Rekombinationssysteme und Verfahren zum Entfernen von Nukleinsäuresequenzen aus der chromosomalen DNA eukaryotischer Organismen, sowie transgene Organismen - bevorzugt Pflanzen - die diese Systeme enthalten bzw. mit diesen Verfahren hergestellt wurden.



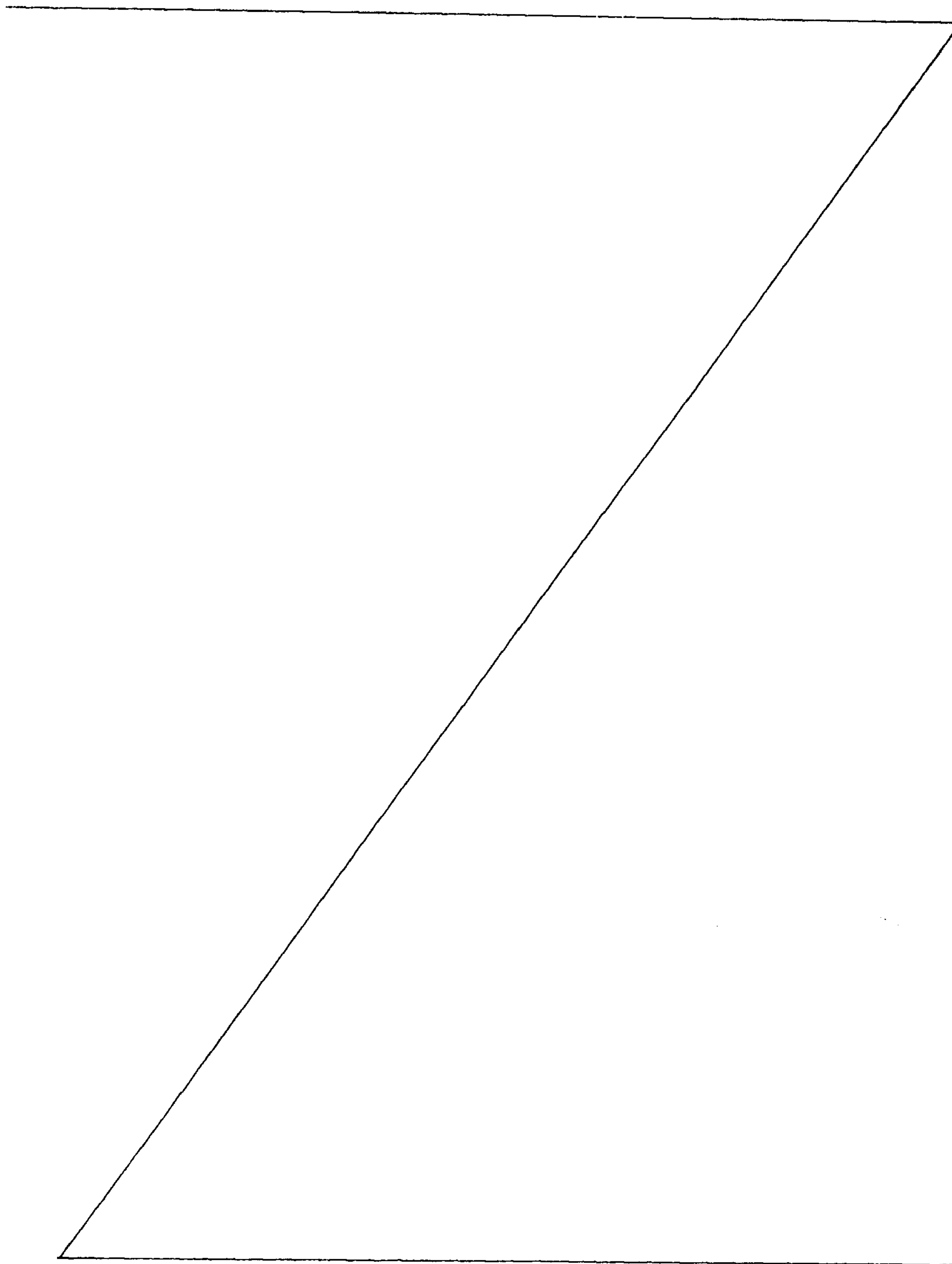
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RECOMBINATION SYSTEMS AND A METHOD FOR REMOVING NUCLEIC
ACID SEQUENCES FROM THE GENOME OF EUKARYOTIC ORGANISMS

- 5 The invention relates to recombination systems and methods for eliminating nucleic acid sequences from the genome of eukaryotic organisms, and to transgenic organisms – preferably plants – comprising these systems.
- 10 The purpose of biotechnological research into organisms consists in, inter alia, obtaining commercially utilizable information on the function of certain genes and gene products and the elucidation of biosynthetic pathways or disease mechanisms. The information obtained in this manner can be employed in a
15 multiplicity of ways. They serve for example for the production of novel medicaments, the development of alternative, biotechnological production methods or the generation of modified plants. An aim of biotechnological research into plants is the generation of plants with advantageous novel characteristics, for
20 example for increasing agricultural productivity, improving the quality in foodstuffs or for the production of certain chemicals or pharmaceuticals (Dunwell JM, J Exp Bot. 2000;51 Spec No:487-96).
- 25 In the generation of transgenic organisms, selection of the organisms which have been modified in the desired manner is required owing to the poor efficacy of the methods used (such as, for example, stable transformation or, in particular, homologous recombination). Transgenic plants can be generated by a series of
30 techniques (Review: Potrykus I. and Spangenberg G. ed. (1995) *Gene transfer to plants*. Springer, Berlin). In particular the gene transfer mediated by *Agrobacterium tumefaciens* and the bombardment of plant cells with the particle gun play an important role in this context. An important problem is the fact
35 that transgenic DNA, once stably introduced into an organism, can only be removed with difficulty. The genes for resistance to antibiotics or herbicides, which are used during the transformation procedure for selection purposes, remain in the transgenic plants, which contributes substantially to the lack of
40 acceptance of these "gene food" products among consumers.

1a

It has therefore been attempted for some time to develop techniques by means of which foreign DNA can be integrated into the plant genome at the specific sites or reexcised therefrom (Ow DW and Medberry SL (1995) Crit Rev in Plant Sci 14:239-261).



2

The skilled worker is familiar with a variety of systems for the site-directed removal of recombinantly introduced nucleic acid sequences. They are based on the use of sequence-specific recombinases and two recognition sequences of said recombinases 5 which flank the sequence to be removed. The effect of the recombinase on this construct brings about the excision of the flanked sequence, one of the recognition sequences remaining in the genome of the organism. Various sequence-specific recombination systems are described, such as the Cre/lox system 10 of the bacteriophage P1 (Dale EC and Ow DW (1991) Proc Natl Acad Sci USA 88:10558-10562; Russell SH et al. (1992) Mol Gene Genet 234: 49-59; Osborne BI et al. (1995) Plant J. 7, 687-701), the yeast FLP/FRT system (Kilby NJ et al. (1995) Plant J 8:637-652; Lyznik LA et al. (1996) Nucleic Acids Res 24:3784-3789), the Mu 15 phage Gin recombinase, the E. coli Pin recombinase or the R/RS system of the plasmid pSR1 (Onouchi H et al. (1995) Mol. Gen. Genet. 247:653-660.; Sugita K et al. (2000) Plant J. 22:461-469). Here, the recombinase (for example Cre or FLP) interacts specifically with its corresponding recombination sequences (34 20 bp lox sequence and 47 bp FRT sequence, respectively) in order to delete or invert the interposed sequences. Reports on successful applications of these systems in plants are limited. Thus, David Ow's group has demonstrated that a selection marker used for the transformation of plants which was flanked by two lox sequences 25 can be reexcised from the plant genome by the expression of Cre (Dale EC and Ow DW (1991) Proc Natl Acad Sci USA 88:10558-10562). A disadvantage of the sequence-specific recombination systems is the reversibility of the reaction, that is to say an equilibrium exists between excision and integration of the marker gene in 30 question. This frequently brings about the selection of mutations, i.e. as soon as a mutation blocks the further interaction of the lox recognition sequences with the enzyme, the (undesired) product is removed from the equilibrium and fixed. This not only applies to the Cre-lox system, but also to the 35 other sequence-specific recombinases (see above). A further disadvantage is the fact that one of the recognition sequences of the recombinase remains in the genome, which is thus modified. This may have effects on the characteristics of the organisms when, for example, the recognition sequence modifies or destroys 40 reading frames or genetic control elements such as promoters or enhancers. Furthermore, the recognition sequence which remains in the genome excludes a further use of the recombination system, for example for a second genetic modification, since interactions with the subsequently introduced recognition sequences cannot be 45 ruled out. Substantial chromosomal rearrangements or deletions may result.

3

Zubko et al. describe a system for the deletion of nucleic acid sequences from the tobacco genome, where the sequence to be deleted is flanked by two 352 bp attP recognition sequences from the bacteriophage Lambda. Deletion of the flanked region takes place independently of the expression of helper proteins in two of eleven transgenic tobacco lines by spontaneous intrachromosomal recombination between the attP recognition regions. The disadvantages of this method are that recombination, or deletion, cannot be induced specifically at a particular point in time, but takes place spontaneously. The fact that the method worked only in a small number of lines suggests that the integration locus in the cases in question tends to be unstable (Puchta H (2000) Trends in Plant Sci 5:273-274).

On page 12 in the key to Figure 32, WO 96/14408 describes a method for eliminating a genetic locus in which in each case one recognition sequence of the homing restriction endonuclease I-SceI is inserted at the respective end of the sequence to be deleted. Treatment with the endonuclease leads to double-strand breaks at both ends of the sequence to be deleted. The free ends then join up by means of "recombination". The "recombination" cited here can only be an illegitimate recombination - as can also be seen from the Figure - (for example a non-homologous end-joining (NHEJ) event), since no homologous sequences exist at the two remaining ends of the genomic DNA. Illegitimate recombination, however, leads to unpredictable recombination events. This may have effects on the characteristics of the organisms if for example reading frames or genetic control elements such as promoters or enhancers are modified or destroyed thereby. The system requires two recognition sequences which flank the fragment to be deleted.

The generation of sequence-specific double-strand breaks with the aid of restriction enzymes in eukaryotic genomes such as yeast (Haber JE (1995) Bioassays 17:609-620), mammalian cells (Jasin M (1996) Trends Genet. 12:224-228) or plants (Puchta H (1999a) Methods Mol Biol 113:447-451) is described.

What is described is the induction of an intramolecular recombination on a plasmid DNA in *Xenopus* oocytes by sequence-specific cleavage with the endonuclease I-SceI (Segal DJ and Carroll D (1995) Proc Natl Acad Sci USA 92:806-810) or by synthetic, chimeric nucleases (Bibikova M et al. (2001) Mol Cell Biol 21(1):289-297). The aim is the site-directed recombination between two homologous sequences between which a suitable nuclease cleavage site is located. Both cases are extrachromosomal recombination events in which in each case only

4

part of the extra chromosomal plasmid DNA undergoes homologous recombination.

- Posfai et al. describe a method for exchanging genes in the
5 prokaryote *E. coli* (Posfai G et al. (1999) *Nucleic Acids Res*
27(22):4409-4415). Here, recombination between the endogenous and
the mutated gene results in the *E. coli* genome, induced by
cleavage with the restriction enzyme I-SceI. Aim and object was
the exchange of an endogenous gene for a mutated transgene.
10 Recombinations in *E. coli* proceed in a markedly simpler way and
with greater efficacy than in higher eukaryotes (for example
described by Kuzminov A (1999) *Microbiol Mol Biol Rev.*
63(4):751-813).
- 15 Dürrenberger et al. describe the induction of recombination in
chloroplasts of the single-celled green alga *Chlamydomonas*
reinhardtii using the I-SceI homing endonuclease (Dürrenberger F
et al. (1996) *Nucleic Acid Res* 24(17):3323-3331). Recombination
takes place between the endogenous 23S gene and an inserted 23S
20 cDNA which contains a I-SceI cleavage site. Double-strand breaks
are induced by mating the transgenic organism in question with an
organism expressing I-SceI. Recombinations in chloroplasts
proceed in a markedly simpler manner and with greater efficacy
than in the chromosomal DNA of higher eukaryotes. Thus, indeed,
25 homologous recombination appears to be the preferred, normal way
of DNA integration in plastids (chloroplastes) (described in:
Heifetz PB and Tuttle AM (2001) *Curr Opin Plant Biol*
4:157-161). It appears that plastids have a specific system which
enables them to undergo homologous recombination, as opposed to
30 the nucleus, and facilitates the site-directed introduction of
foreign DNA (Heifetz PB (2000) *Biochimie* 82:655-666).

The gene targeting technique, in which a site-directed
integration into the chromosomal DNA of the host organism is to
35 be achieved by means of homologous recombination works acceptably
well only in the case of prokaryotes and yeast. The generation of
corresponding transgenic organisms is possible in a few species
only (such as, for example, mice) and even then highly
complicated (see also Kanaar R Hoeijmakers JH (1997) *Genes Funct*
40 1(3):165-174). The existing, poor homologous recombination
efficacy (approx. $1:1 \times 10^6$) is compensated for in this case by the
use of complicated, sophisticated selection techniques which are
limited to the species in question (such as, for example, "ES"
cell technology). In other species – but above all in Higher
45 Plants – such technologies have not been established as yet
(Mengiste T and Paszkowski J (1999) *Biol Chem.* 380:749-758;
Vergunst AC and Hooykaas PJJ (1999) *Crit Rev Plant Sci* 18:1-31;

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Puchta H (1999) Methods Mol Biol 113:447-451; Hohn B and Puchta H (1999) Proc Natl Acad Sci USA 96:8321-8323). Attempts to achieve homologous recombination in plants resulted in random, nonhomologous "illegitimate" insertion events in most cases.

5 Here, the DNA introduced is integrated at one or more unpredictable sites in the plant genome. Integration takes place by means of illegitimate recombination (Roth DB and Wilson JH (1988) Illegitimate recombination in mammalian cells. In "Genetic recombination", R. Kucherlapati and G.R. Smith Edts., American
10 Society of Microbiology [sic], Washington, USA; pp.621-635) and not in sequence regions which are homologous to the transferred DNA (Puchta H and Hohn B (1996) Trends Plant Sci. 1:340-348). The problem of lacking efficacy in homologous recombination, which is serious predominantly in plants, is generally known to the
15 skilled worker. The causes are addressed by current research (Review article: Mengiste T and Paszkowski J (1999) Biological Chemistry 380(7-8):749-58). Increasing the efficacy of homologous recombination has long been a need in plant biotechnology which is hitherto unresolved.

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A further need which has long existed in biotechnological research and which is not addressed by any of the established systems is the provision of systems and methods which enables the site-directed elimination of nucleic acid sequences from the
25 chromosomal DNA of a eukaryotic organism and allow the repeated application to the same organism. For example, it is an aim of plant biotechnology further to improve by means of recombinant methods existing high-yielding varieties. In this context, it is particularly important to eliminate, after the transformation has
30 taken place, superfluous transgene sequences such as selection markers. In addition, methods for the predictable elimination of sequences, for example from the chromosomal DNA of an organism, would offer further applications in the field of genetic engineering which are of great interest scientifically and
35 economically.

It is an object of the present invention to develop systems and methods which enable the predictable elimination of defined nucleic acid sequences from the chromosomal DNA of a eukaryotic
40 organism and allow the repeated, successive application to the same organism.

We have found that this edition [sic] has been achieved in a surprising manner by providing the recombination system according
45 to the invention.

A first subject matter of the invention relates to a recombination system for eliminating a DNA sequence from the chromosomal DNA of a eukaryotic cell or organism, which comprises, in a eukaryotic cell or organism,

- I) a transgenic recombination construct inserted into the chromosomal DNA of a eukaryotic organism comprising a sequence consisting, in the 5'/3'-direction, of
- a1) a first homology sequence A and
 - b1) at least one recognition sequence for the site-directed induction of DNA double-strand breaks and
 - a2) a second homology sequence B, the homology sequences A and B having a sufficient length and sufficient homology in order to ensure homologous recombination,

together with

- II) an enzyme suitable for inducing DNA double-strand breaks at the recognition sequence (b1) for the site-directed induction of DNA double-strand breaks or a nucleic acid sequence encoding an enzyme suitable for inducing DNA double-strand breaks at the recognition sequence (b1).

20 As well, the object of the present invention is a recombination system which comprises, in a plant cell,

- I) a transgenic recombination construct inserted into the chromosomal DNA of the plant cell comprising a sequence consisting, in the 5'/3'-direction, of
- a1) a first homology sequence A and
 - b1) at least one recognition sequence for the site-directed induction of DNA double-strand breaks and

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a2) a second homology sequence B, the homology sequences A and B having a sufficient length and sufficient homology in order to ensure homologous recombination,

together with

II) an enzyme suitable for inducing DNA double-strand breaks at the recognition sequence (b) for the site-directed induction of DNA double-strand breaks or a nucleic acid sequence encoding an enzyme suitable for inducing DNA double-strand breaks at the recognition sequence (b),

10 wherein all recognition sequences for site-directed induction of DNA double-strand breaks are located between homology sequences A and B; and

wherein the homology sequences A and B have at least 20 base pairs and at least 70% homology that allows for homologous recombination to each other.

In addition, the object of the present invention is a method for removing a DNA sequence from the chromosomal DNA of a plant cell, which comprises combining, in the plant cell,

I) a transgenic recombination construct inserted into the chromosomal DNA of the plant cell comprising a sequence consisting, in the 5'/3'-direction, of

a1) a first homology sequence A and

20 b1) at least one recognition sequence for the site-directed induction of DNA double-strand breaks and

a2) a second homology sequence B, the homology sequences A and B having a sufficient length and sufficient homology in order to ensure homologous recombination,

together with

II) an enzyme suitable for inducing DNA double-strand breaks at the recognition sequence (b) for the site-directed induction of DNA double-strand breaks,

6b

and the induction of DNA double-strand breaks at the recognition sequence for the site-directed induction of DNA double-strand breaks and the homologous recombination taking place between the homology sequences A and B,

wherein all recognition sequences for site-directed introduction of DNA double-strand breaks are located between homology sequences A and B; and

wherein the homology sequences A and B have at least 20 base pairs and at least 70% homology that allows for homologous recombination to each other.

In addition, the object of the present invention is a recombination system which comprises, in a plant cell,

10 I) a transgenic recombination construct inserted into the chromosomal DNA of the plant cell comprising a sequence consisting, in the 5'/3'-direction, of

a1) a first homology sequence A;

b1) at least one recognition sequence (b) for the site-directed induction of DNA double-strand breaks; and

a2) a second homology sequence B, the homology sequences A and B having a sufficient length and sufficient homology in order to ensure homologous recombination,

together with

20 II) an enzyme suitable for inducing DNA double-strand breaks at the recognition sequence (b) for the site-directed induction of DNA double-strand breaks or a nucleic acid sequence encoding an enzyme suitable for inducing DNA double-strand breaks at the recognition sequence (b),

wherein all recognition sequences for site-directed induction of DNA double-strand breaks are located between homology sequences A and B;

wherein the homology sequences A and B have at least 20 base pairs and at least 70% homology that allow for homologous recombination to each other; and

wherein the enzyme suitable for inducing DNA double-strand breaks at the recognition sequence for the site-directed induction of DNA double-strand breaks is

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an homing endonuclease, a chimeric nuclease or a combination thereof.

In addition, the object of the present invention is a method for removing a DNA sequence from the chromosomal DNA of a plant cell, which comprises combining, in the plant cell,

I) a transgenic recombination construct inserted into the chromosomal DNA of the plant cell comprising a sequence consisting, in the 5'/3'-direction, of

a1) a first homology sequence A;

b1) at least one recognition sequence (b) for the site-directed induction of DNA double-strand breaks; and

a2) a second homology sequence B, the homology sequences A and B having a sufficient length and sufficient homology in order to ensure homologous recombination,

together with

II) an enzyme suitable for inducing DNA double-strand breaks at the recognition sequence (b) for the site-directed induction of DNA double-strand breaks,

and the induction of DNA double-strand breaks at the recognition sequence for the site-directed induction of DNA double-strand breaks and the homologous recombination taking place between the homology sequences A and B,

wherein all recognition sequences for site-directed introduction of DNA double-strand breaks are located between homology sequences A and B;

wherein the homology sequences A and B have at least 20 base pairs and at least 70% homology that allows for homologous recombination to each other; and

wherein the enzyme suitable for inducing DNA double-strand breaks at the recognition sequence for the site-directed induction of DNA double-strand breaks is an homing endonuclease, a chimeric nuclease or a combination thereof.

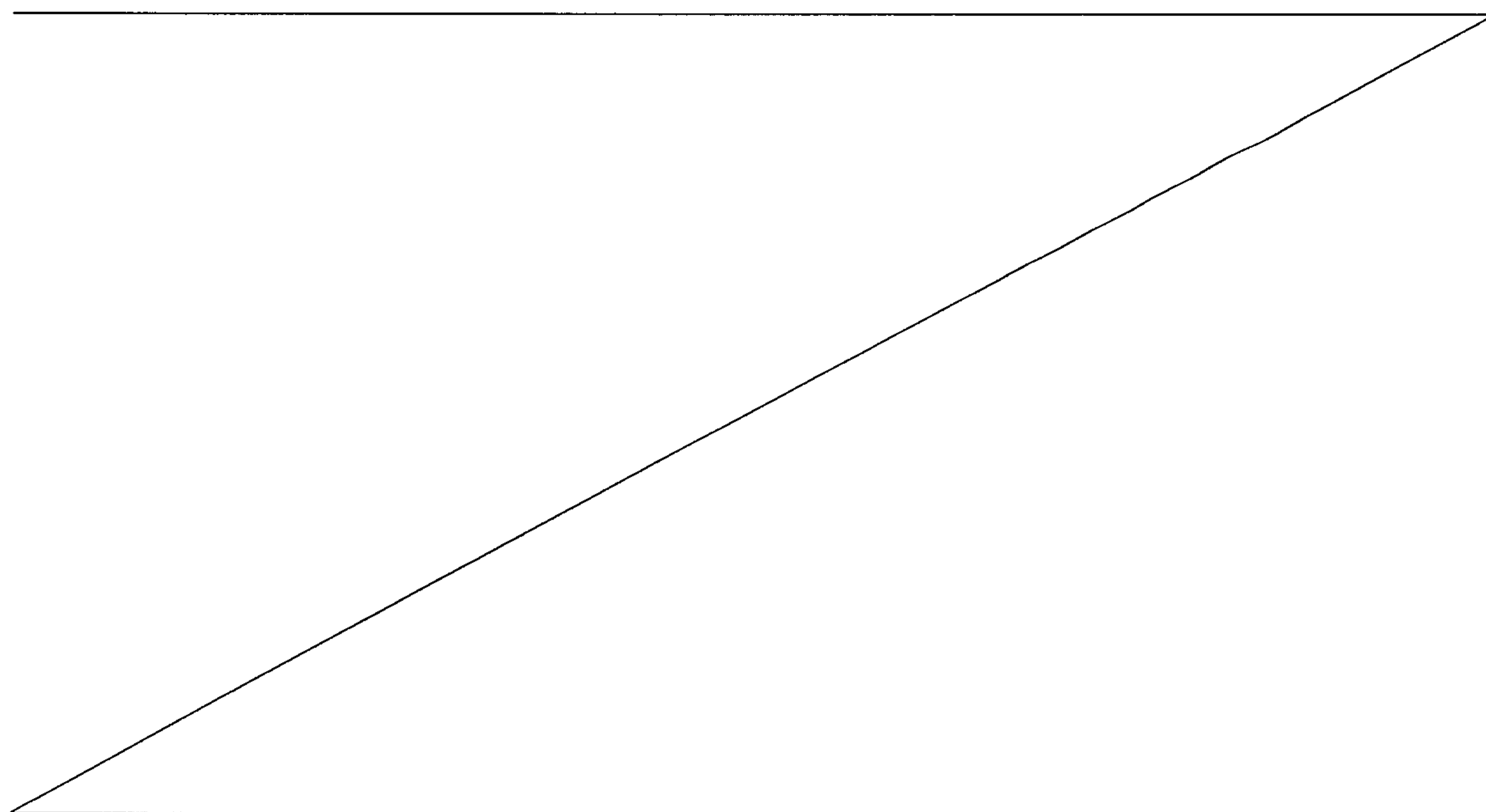
In addition, the object of the present invention is a plant cell comprising the recombination system as defined herein.

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In addition, the object of the present invention is the use of the plant cell as defined herein, for the production of a medicament.

A further subject matter of the invention relates to a method for eliminating a DNA sequence from the chromosomal DNA of a eukaryotic cell or organism, which comprises combining, in a eukaryotic cell or organism,

- I) a transgenic recombination construct inserted into the chromosomal DNA of a eukaryotic organism comprising a sequence consisting, in the 5'/3'-direction, of
 - a1) a first homology sequence A and
 - b1) at least one recognition sequence for the site-directed induction of DNA double-strand breaks and
 - a2) a second homology sequence B, the homology sequences A and B having a sufficient length and sufficient homology in order to ensure homologous recombination,together with



II) an enzyme suitable for inducing DNA double-strand breaks at the recognition sequence (b1) for the site-directed induction of DNA double-strand breaks,

5 and the induction of DNA double-strand breaks at the recognition sequence for the site-directed induction of DNA double-strand breaks and the homologous recombination taking place between the homology sequences A and B.

10 The invention enables sequences (for example selection markers such as genes for resistance to antibiotics or herbicides) to be deleted from the chromosomal DNA of an organism in an accurately predictable manner. In doing so, the sequence to be eliminated is flanked by recognition sequences for the site-directed induction
15 of DNA double-strand breaks (for example recognition sequences of rare-cleaving restriction enzymes) and combined with homologous sequences in the region of the cleavage sites. A double-strand break is induced by an enzyme suitable for inducing DNA
20 site-directed induction of DNA double-strand breaks, (for example a sequence-specific nuclease), which, in consequence, triggers the homologous recombination of homologous sequences located at the break, and thus the deletion of any nucleic acid sequences located between the sequences. The recognition sequence for the
25 site-directed induction of DNA double-strand breaks is likewise deleted, and the method can thus be used repeatedly for further controlled genetic modifications.

Surprisingly, this induced homologous recombination takes place
30 with high efficacy and precision, which is in contrast to previous experience in the field of homologous recombination, including in plants. The frequency can be compared with the parallel, nonhomologous events (for example non-homologous end-joining events) (cf. Example 5). This is a remarkable finding
35 which is in contrast to earlier observations, according to which the frequency of homologous recombination – above all in the case of plants – is secondary, almost negligible, in comparison with the “illegitimate” events.

40 The sequences which are deleted are those located between the homology sequences A and B. In contrast to systems such as, for example, the cre/lox or the FRT/FLP system, one is not bound to specific sequences when performing recombination. The skilled worker knows that any sequence can undergo homologous
45 recombination with another sequence provided that sufficient length and homology exist. Owing to the sequence-specific induction of the double-strand breaks, the homologous

8

recombination efficacy between the homology sequences A and B is increased considerably, indeed enabled in the first place in some cases.

5 With regard to the recombination construct, "transgene" refers to all those constructs which are the result of recombinant methods in which either

- 10
- a) at least one of the homology sequences A or B, or
 - b) at least one recognition sequence for the site directed induction of DNA double-strand breaks, or
 - c) (a) and (b)
- 15

are not located in their natural genetic environment (for example at their natural chromosomal locus) or have been modified by recombinant methods, it being possible for the modification to encompass, for example, substitutions, additions, deletions, 20 inversion [sic] or insertions of one or more nucleotide residues.

"Eukaryotic cell or organism" generally refers to any eukaryotic cell or organism and to cells, tissues, parts or propagation material (such as seeds or fruits) derived from these in which an 25 induction of double-strand breaks may take place at the recognition sequence for the site-directed induction of DNA double-strand breaks and the homologous recombination between the homology sequences A and B may take place while the recombination construct and the enzyme suitable for inducing DNA double-strand 30 breaks at the recognition sequence for the site-directed induction of DNA double-strand breaks are simultaneously present in one reaction space (for example in a cell or cell compartment). A particularly preferred embodiment encompasses compartments of a eukaryotic cell such as, for example, the 35 nucleus.

Cells or organisms which are especially preferably encompassed are those which constitute a multi-celled eukaryotic organism or are derived from the latter, and cells, tissues, parts or 40 propagation material (such as seeds or fruits) of same. Very especially preferably encompassed cells or organisms are those which constitute an animal or plant organism or are derived from the latter, and cells, tissues, parts or propagation material of same. Most preferably encompassed cells or organisms are those 45 which constitute a plant organism or are derived from the latter,

9

and cells, tissues, parts or propagation material of same. Preferred genera and species are detailed further below.

Referring to the homology sequences A and B, "sufficient length" preferably refers to sequences with a length of at least 20 base pairs, preferably at least 50 base pairs, especially preferably at least 100 base pairs, very especially preferably at least 250 base pairs, most preferably at least 500 base pairs.

10 Referring to the homology sequences A and B, "sufficient homology" preferably refers to sequences with at least 70%, preferably 80%, by preference at least 90%, especially preferably at least 95%, very especially preferably at least 99%, most preferably 100%, homology within these homology sequences over a length of of [sic] at least 20 base pairs, preferably at least 50 base pairs, especially preferably at least 100 base pairs, very especially preferably at least 250 base pairs, most preferably at least 500 base pairs.

20 Homology between two nucleic acid sequences is understood as meaning the identity of the nucleic acid sequence over in each case the entire sequence length which is calculated by alignment with the aid of the program algorithm GAP (Wisconsin Package Version 10.0, University of Wisconsin, Genetics Computer Group (GCG), Madison, USA), setting the following parameters:

Gap Weight: 12

Length Weight: 4

Average Match: 2,912

Average Mismatch:-2,003

30

In a preferred embodiment, only one recognition sequence for the site-directed induction of DNA double-strand breaks is located between the homology sequences A and B, so that the recombination construct employed in the recombination system or method according to the invention is constructed in the 5'/3'-direction as follows:

a1) a first homology sequence A and

40 b1) a recognition sequence for the site-directed induction of DNA double-strand breaks and

a2) a second homology sequence B, the homology sequences A and B having a sufficient length and sufficient homology in order to ensure homologous recombination.

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In a preferred embodiment, a further nucleic acid sequence is located between the homology sequences A and B, so that the recombination construct employed in the recombination system or method according to the invention is constructed as follows in 5 the 5'/3'-direction of

- a1) a first homology sequence A and
- 10 b1) a recognition sequence for the site-directed induction of DNA double-strand breaks and
- c) a further nucleic acid sequence and
- 15 a2) a second homology sequence B, the homology sequences A and B having a sufficient length and sufficient homology in order to ensure homologous recombination.

The recognition sequence for the site-directed induction of DNA double-strand breaks may also be located after or within the 20 further nucleic acid sequence.

In a further preferred embodiment, a second recognition sequence for the site-directed induction of double-strand breaks is present after the further nucleic acid sequence. This embodiment 25 is advantageous in particular in the case of homology sequences A and B which are further apart, or in the case of longer further nucleic acid sequences, since recombination efficacy is increased. In this embodiment, the recombination construct employed in the recombination system or method according to the 30 invention is constructed as follows in 5'/3' direction of

- a1) a first homology sequence A and
- 35 b1) a first recognition sequence for the site-directed induction of DNA double-strand breaks and
- c) a further nucleic acid sequence and
- 40 b2) a second recognition sequence for the site-directed induction of DNA double-strand breaks and
- a2) a second homology sequence B, the homology sequences A and B having a sufficient length and sufficient homology in order to ensure homologous recombination.

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11

Furthermore, other recognition sequences may also be present between the homology sequences A and B, in addition to the second recognition sequences for the site-directed induction of DNA double-strand breaks. The individual recognition sequences (for 5 example b1 or b2) for the site-directed induction of DNA double-strand breaks may be identical or different, i.e. they may act as recognition sequence for an individual enzyme for the site-directed induction of DNA double-strand breaks or else for a variety of enzymes. Here, the embodiment in which the recognition 10 sequences for the site-directed induction of DNA double-strand breaks act as recognition sequence for an individual enzyme for the site-directed induction of DNA double-strand breaks is preferred in this context.

15 The skilled worker is familiar with a variety of ways to obtain one of the recombination constructs according to the invention. They can be prepared by means of customary recombination and cloning techniques as are described, for example, in T. Maniatis, E.F. Fritsch and J. Sambrook, *Molecular Cloning: A Laboratory* 20 *Manual*, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY (1989), in T.J. Silhavy, M.L. Berman and L.W. Enquist, *Experiments with Gene Fusions*, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY (1984) and in Ausubel, F.M. et al., *Current Protocols in Molecular Biology*, Greene Publishing Assoc. 25 and Wiley Interscience (1987).

Preferably, the recombination construct according to the invention is generated by joining the abovementioned essential constituents of the recombination construct together in the 30 abovementioned sequence using the recombination and cloning techniques with which the skilled worker is familiar, and the result is then introduced into the chromosomal DNA of a host organism.

35 However, the skilled worker is aware that he may also obtain the recombination construct according to the invention in other ways. Thus, the host organism may already comprise one or more of the essential components of the recombination construct. The recombination construct according to the invention is then 40 generated by introducing one further, or more, essential components of the recombination construct in the correct position relative to the existing components in said organism. Thus, for example, the starting organism may already comprise one of the homology sequences A or B. If the organism already comprises a 45 homology sequence A, introducing a construct consisting of a recognition sequence for the site-directed induction of DNA double-strand breaks and a second homology sequence B after the

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homology sequence A gives rise to one of the recombination constructs according to the invention.

Furthermore, the skilled worker is familiar with various ways in which the recombination construct according to the invention may be introduced into the chromosomal DNA of a eukaryotic cell or organism. In this context, the insertion may be directed (i.e. taking place at a defined insertion site) or undirected (i.e. taking place randomly). Suitable techniques are known to the skilled worker and described by way of example hereinbelow.

"Enzyme suitable for inducing DNA double-strand breaks at the recognition sequence for the site-directed induction of DNA double-strand breaks" (hereinbelow "DSBI enzyme", which stands for "double strand-break inducing enzyme") generally refers to all those enzymes which are capable of generating double-strand breaks in double stranded DNA in a sequence-specific manner. The following may be mentioned by way of example, but not by limitation:

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1. Restriction endonucleases (type II), preferably homing endonucleases as described in detail hereinbelow.
2. Recombinases (such as, for example, Cre/lox; R-RS; FLP/FTR as described above)
3. Transposases, for example the P-element transposase (Kaufman PD and Rio DC (1992) Cell 69(1):27-39) or AcDs (Xiao YL and Peterson T (2000) Mol Gen Genet 263(1):22-29). In principle, all transposases or integrases are suitable as long as they have sequence specificity (Haren L et al. (1999) Annu Rev Microbiol. 1999;53:245-281; Beall EL, Rio DC (1997) Genes Dev. 11(16):2137-2151).
4. Chimeric nucleases as described in detail hereinbelow.
5. Enzymes which induce double-strand breaks in the immune system, such as the RAG1/RAG2 system (Agrawal A et al. (1998) Nature 394(6695):744-451).
6. Group II intron endonucleases. Modifications of the intron sequence allows group II introns to be directed to virtually any sequence in a double-stranded DNA, where group II introns can subsequently insert by means of a reverse splice mechanism (Mohr et al. (2000) Genes & Development 14:559-573; Guo et al. (2000) Science 289:452-457). During this reverse splice mechanism, a double-strand break is introduced into

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the target DNA, the excised intron RNA cleaving the sense strand while the protein portion of the group II intron endonuclease hydrolyses the antisense strand (Guo et al. (1997) EMBO J 16: 6835- 6848). If it is only desired to induce the double-strand break without achieving complete reverse splicing, as is the case in the present invention, it is possible to resort to, for example, group II intron endonucleases which lack the reverse transcriptase activity. While this does not prevent the generation of the double-strand break, the reverse splicing mechanism cannot proceed to completion.

Suitable enzymes are not only natural enzymes, but also synthetic enzymes.

Preferred enzymes are all those DSBI enzymes whose recognition sequence is known and which can either be obtained in the form of their proteins (for example by purification) or expressed using their nucleic acid sequence.

Especially preferred are restriction endonucleases (restriction enzymes) which have no or only a few recognition sequences – besides the recognition sequences present in the transgenic recombination construct – in the chromosomal DNA sequence of a particular eukaryotic organism. This avoids further double-strand breaks at undesired loci in the genome.

This is why homing endonucleases are very especially preferred (Review: (Belfort M and Roberts RJ (1997) Nucleic Acids Res 25: 3379-3388; Jasin M (1996) Trends Genet. 12:224-228; Internet: <http://rebase.neb.com/rebase/rebase.homing.html>). Owing to their long recognition sequences, they have no, or only a few, further recognition sequences in the chromosomal DNA of eukaryotic organisms in most cases.

The sequences encoding for such homing endonucleases can be isolated for example from the chloroplast genome of *Chlamydomonas* (Turmel M et al. (1993) J Mol Biol 232: 446-467). They are small (18 to 26 kD) and their open reading frame (ORF) has a "coding usage" which is suitable directly for nuclear expression in eukaryotes (Monnat RJ Jr et al. (1999) Biochem Biophys Res Com 255:88-93). Homing endonucleases which are very especially preferably isolated are the homing endonucleases I-SceI (W096/14408), I-SceII (Sarguiel B et al. (1990) Nucleic Acids Res 18:5659-5665), I-SceIII (Sarguiel B et al. (1991) Mol Gen Genet. 255:340-341), I-CeuI (Marshall (1991) Gene 104:241-245), I-CreI (Wang J et al. (1997) Nucleic Acids Res 25: 3767-3776), I-ChuI

14

(Cote V et al.(1993) Gene 129:69-76), I-TevI (Chu et al. (1990) Proc Natl Acad Sci USA 87:3574-3578; Bell-Pedersen et al. (1990) Nucleic Acids Res18:3763-3770), I-TevII (Bell-Pedersen et al. (1990) Nucleic Acids Res18:3763-3770), I-TevIII (Eddy et al. 5 (1991) Genes Dev. 5:1032-1041), Endo SceI (Kawasaki et al. (1991) J Biol Chem 266:5342-5347), I-CpaI (Turmel M et al. (1995a) Nucleic Acids Res 23:2519-2525) and I-CpaII (Turmel M et al. (1995b) Mol. Biol. Evol. 12, 533-545).

- 10 Further homing endonucleases are detailed in the abovementioned Internet website, and examples which may be mentioned are homing endonucleases such as F-SceI, F-SceII, F-SuvI, F-TevI, F-TevII, I-AmaI, I-AniI, I-CeuI, I-CeuAIIP, I-ChuI, I-CmoeI, I-CpaI, I-CpaII, I-CreI, I-CrepsbIP, I-CrepsbIIP, I-CrepsbIIIIP, 15 I-CrepsbIVP, I-CsmI, I-CvuI, I-CvuAIP, I-DdiI, I-DdiII, I-DirI, I-DmoI, I-HmuI, I-HmuII, I-HspNIP, I-LlaI, I-MsoI, I-NaaI, I-NanI, I-NclIP, I-NgrIP, I-NitI, I-NjaI, I-Nsp236IP, I-PakI, I-PboIP, I-PcuIP, I-PcuAI, I-PcuVI, I-PgrIP, I-PobIP, I-PorI, I-PorIIP, I-PpbIP, I-PpoI, I-SPBetaIP, I-ScaI, I-SceI, I-SceII, 20 I-SceIII, I-SceIV, I-SceV, I-SceVI, I-SceVII, I-SexIP, I-SneIP, I-SpomCP, I-SpomIP, I-SpomIIP, I-SquIP, I-Ssp6803I, I-SthPhiJP, I-SthPhiST3P, I-SthPhiS3bP, I-TdeIP, I-TevI, I-TevII, I-TevIII, I-UarAP, I-UarHGPA1P, I-UarHGPA13P, I-VinIP, I-ZbiIP, PI-MtuI, PI-MtuHIP, PI-MtuHIIP, PI-PfuI, PI-PfuII, PI-PkoI, PI-PkoII, 25 PI-PspI, PI-Rma43812IP, PI-SPBetaIP, PI-SceI, PI-TfuI, PI-TfuII, PI-ThyI, PI-TliI, PI-TliII.

Preferred in this context are the homing endonucleases whose gene sequences are already known, such as, for example,

- 30 F-SceI, I-CeuI, I-ChuI, I-DmoI, I-CpaI, I-CpaII, I-CreI, I-CsmI, F-TevI, F-TevII, I-TevI, I-TevII, I-AniI, I-CvuI, I-DdiI, I-HmuI, I-HmuII, I-LlaI, I-NanI, I-MsoI, I-NitI, I-NjaI, I-PakI, I-PorI, I-PpoI, I-ScaI, I-Ssp6803I, PI-PkoI, PI-PkoII, PI-PspI, PI-TfuI, PI-TliI.

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Very especially preferred are commercially available homing endonucleases such as I-CeuI, I-SceI, I-DmoI, I-PpoI, PI-PspI or PI-SceI.

- 40 The enzymes can be isolated from their organisms of origin in the manner with which the skilled worker is familiar, and/or their coding nucleic acid sequence can be cloned. The sequences of various enzymes are deposited in GenBank.

- 45 Very especially preferred are the homing endonucleases I-SceI, I-CpaI, I-CpaII, I-CreI and I-ChuI. Most preferred are the homing endonucleases as shown in SEQ ID NO: 2, 4, 6, 8 or 10.

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Synthetic DSBI enzymes which may be mentioned by way of example are chimeric nucleases which are composed of an unspecific nuclease domain and a sequence-specific DNA binding domain consisting of zinc fingers (Bibikova M et al. (2001) Mol Cell Biol. 21:289-297). These DNA-binding zinc finger domains can be adapted to suit any DNA sequence. Suitable methods for preparing suitable zinc finger domains are described and known to the skilled worker (Beerli RR et al., Proc Natl Acad Sci U S A. 2000; 97 (4):1495-1500; Beerli RR, et al., J Biol Chem 2000; 275(42):32617-32627; Segal DJ and Barbas CF 3rd., Curr Opin Chem Biol 2000; 4(1):34-39; Kang JS and Kim JS, J Biol Chem 2000; 275(12):8742-8748; Beerli RR et al., Proc Natl Acad Sci USA 1998; 95(25):14628-14633; Kim JS et al., Proc Natl Acad Sci USA 1997; 94(8):3616-3620; Klug A, J Mol Biol 1999; 293(2):215-218; Tsai SY et al., Adv Drug Deliv Rev 1998;30(1-3):23-31; Mapp AK et al., Proc Natl Acad Sci USA 2000; 97(8):3930-3935; Sharrocks AD et al., Int J Biochem Cell Biol 1997; 29(12):1371-1387; Zhang L et al., J Biol Chem 2000; 275(43):33850-33860).

The DSBI enzyme is preferably expressed as a fusion protein with a nuclear localization sequence (NLS). This NLS sequence enables facilitated transport into the nucleus and increases the efficacy of the recombination system. A variety of NLS sequences are known to the skilled worker and described, inter alia, by Jicks GR and Raikhel NV (1995) Annu. Rev. Cell Biol. 11:155-188. Preferred for plant organisms is, for example, the NLS sequence of the SV40 large antigen. Very especially preferred are the following NLS sequences:

NLS1: N-Pro-Lys-Thr-Lys-Arg-Lys-Val-C (SEQ ID NO: 29)

NLS2: N-Pro-Lys-Lys-Lys-Arg-Lys-Val-C (SEQ ID NO: 30)

The homing endonucleases as shown in SEQ ID NO: 4, 6, 8 or 10 used in the use examples are fusion proteins of the native nucleases and the NLS2 nuclear localization sequence.

Owing to the small size of many DSBI enzymes (such as, for example, the homing endonucleases), an NLS sequence is not necessarily required. These enzymes are capable of passing through the nuclear pores even without any aid. This is confirmed by the efficacy of the homing endonuclease as shown in SEQ ID NO: 2 which has been used and which encompasses no nuclear localization sequence.

16

In a further preferred embodiment, the activity of the DSBI enzyme can be induced. Suitable methods have been described for sequence-specific recombinases (Angrand PO et al. (1998) Nucl. Acids Res. 26(13):3263-3269; Logie C and Stewart AF (1995) Proc Natl Acad Sci USA 92(13):5940-5944; Imai T et al. (2001) Proc Natl Acad Sci USA 98(1):224-228). These methods employ fusion proteins of the DSBI enzyme and the ligand binding domain for steroid hormone receptor (for example the human androgen receptor, or mutated variants of the human estrogen receptor as described therein). Induction may be effected with ligands such as, for example, estradiol, dexamethasone, 4-hydroxytamoxifen or raloxifen.

Some DSBI enzymes are active as dimers (homo- or heterodimers; I-CreI forms a homodimer; I-SecIV forms a heterodimer) (Wernette CM (1998) Biochemical & Biophysical Research Communications 248(1):127-333). Dimerization can be designed as an inducible feature, for example by exchanging the natural dimerization domains for the binding domain of a low-molecular-weight ligand. Addition of a dimeric ligand then brings about dimerization of the fusion protein. Corresponding inducible dimerization methods, and the preparation of the dimeric ligands, have been described (Amara JF et al. (1997) Proc Natl Acad Sci USA 94(20): 10618-1623; Muthuswamy SK et al. (1999) Mol Cell Biol 19(10):6845-685; Schultz LW and Clardy J (1998) Bioorg Med Chem Lett. 8(1):1-6; Keenan T et al. (1998) Bioorg Med Chem. 6(8):1309-1335).

"Recognition sequence for the site-directed induction of DNA double-strand breaks" generally refers to those sequences which, under the conditions in the eukaryotic cell or organism used in each case, enable the recognition and cleavage by the DSBI enzyme. The recognition sequences for the respective DSBI enzymes detailed are mentioned in Table 1 hereinbelow by way of example, but not by limitation.

Table 1: Recognition sequences and organisms of origin of DSBI enzymes ("^" indicates the cleavage site of the DSBI enzyme within a recognition sequence).

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	DSBI enzyme	Organism of origin	Recognition sequence
5	CRE	Bacteriophage P1	5'-AACTCTCATCGCTTCGGATAACTTCCTGTTATCCGAAACAT ATCACTCACTTTGGTGATTTACCGTAACTGTCTATGATTAATG -3'
	FLP	Saccharomyces cerevisiae	5'-GAAGTTCCTATTCCGAAGTTCCTATTCTCTAGAAAGTATAG GAACTTC-3'
10	R	pSR1 Plasmids	5'-CGAGATCATATCACTGTGGACGTTGATGAAAGAATACGTTA TTCTTTCATCAAATCGT
	P-Element Transposase	Drosophila	5'-CTAGATGAAATAACATAAGGTGG
15	I-AniI	Aspergillus nidulans	5'-TTGAGGAGGTT^TCTCTGTAAATAANNNNNNNNNNNNNNNNN 3'-AACTCCTCAAAGAGACATTTATTNNNNNNNNNNNNNNNNNN^
	I-DdiI	Dictyostelium discoideumAX3	5'-TTTTTTGGTCATCCAGAAGTATAT 3'-AAAAAACCAG^TAGGTCTTCATATA
	I-CvuI	Chlorella vulgaris	5'-CTGGGTTCAAACGTCGTGA^GACAGTTTGG 3'-GACCCAAGTTTGCAG^CACTCTGTCAAACC
20	I-CsmI	Chlamydomonas smithii	5'-GTACTAGCATGGGGTCAAATGTCTTTCTGG
	I-CmoEI	Chlamydomonas moewusii	5'-TCGTAGCAGCT^CACGGTT 3'-AGCATCG^TCGAGTGCCAA
	I-CreI	Chlamydomonas reinhardtii	5'-CTGGGTTCAAACGTCGTGA^GACAGTTTGG 3'-GACCCAAGTTTGCAG^CACTCTGTCAAACC
25	I-ChuI	Chlamydomonas humicola	5'-GAAGGTTTGGCACCTCG^ATGTCGGCTCATC 3'-CTTCAAACCGTG^GAGCTACAGCCGAGTAG
	I-CpaI	Chlamydomonas pallidostigmatica	5'-CGATCCTAAGGTAGCGAA^ATTCA 3'-GCTAGGATTCCATC^GCTTTAAGT
30	I-CpaII	Chlamydomonas pallidostigmatica	5'-CCCGGCTAACTC^TGTGCCAG 3'-GGGCCGAT^TGAGACACGGTC
	I-CeuI	Chlamydomonas eugametos	5'-CGTAACTATAACGGTCCTAA^GGTAGCGAA 3'-GCATTGATATTGCCAG^GATTCCATCGCTT
35	I-DmoI	Desulfurococcus mobilis	5'-ATGCCTTGCCGGGTAA^GTTCCGGCGCGCAT 3'-TACGGAACGGCC^CATTCAAGGCCGCGCGTA
	I-SceI	S.cerevisiae	5'-AGTTACGCTAGGGATAA^CAGGGTAATATAG 3'-TCAATGCGATCCC^TATTGTCCATTATATC 5'-TAGGGATAA^CAGGGTAAT 3'-ATCCC^TATTGTCCATTA ("Core"-Sequence)
40	I-SceII	S.cerevisiae	5'-TTTTGATTCTTTGGTCACCC^TGAAGTATA 3'-AAAATAAGAAACCAG^TGGGACTTCATAT
	I-SceIII	S.cerevisiae	5'-ATTGGAGGTTTTGGTAAC^TATTTATTACC 3'-TAACCTCAAACC^ATTGATAAATAATGG
	I-SceIV	S.cerevisiae	5'-TCTTTTCTCTTGATTA^GCCCTAATCTACG 3'-AGAAAAGAGAAC^TAATCGGGATTAGATGC
45	I-SceV	S.cerevisiae	5'-AATAATTTTCT^TCTTAGTAATGCC 3'-TTATTAAAGAAGAATCATTA^CGG
	I-SceVI	S.cerevisiae	5'-GTTATTTAATG^TTTTAGTAGTTGG 3'-CAATAAATTACAAAATCATCA^ACC

	DSBI enzyme	Organism of origin	Recognition sequence
	I-SceVII	<i>S.cerevisiae</i>	5'-TGTCACATTGAGGTGCACTAGTTATTAC
5	PI-SceI	<i>S.cerevisiae</i>	5'-ATCTATGTCGGGTGC^GGAGAAAGAGGTAAT 3'-TAGATACAGCC^CACGCCTCTTTCTCCATTA
	F-SceI	<i>S.cerevisiae</i>	5'-GATGCTGTAGGC^ATAGGCTTGGTT 3'-CTACGACA^TCCGTATCCGAACCAA
	F-SceII	<i>S.cerevisiae</i>	5'-CTTCCGCAACA^GTAAAATT 3'-GAAAGGCG^TTGTCATTTTAA
10	I-HmuI	<i>Bacillus subtilis</i> bacteriophage SPO1	5'-AGTAATGAGCCTAACGCTCAGCAA 3'-TCATTACTCGGATTGC^GAGTCGTT
15	I-HmuII	<i>Bacillus subtilis</i> bacteriophage SP82	5'-AGTAATGAGCCTAACGCTCAACAANNNNNNNNNNNNNNNNNNNNNN NNNNNNNNNNNNNNNNNNNNNNNNNNNN
	I-LlaI	<i>Lactococcus lactis</i>	5'-CACATCCATAAC^CATATCATTTTT 3'-GTGTAGGTATTGGTATAGTAA^AAA
	I-MsoI	<i>Monomastix</i> species	5'-CTGGGTTCAAACGTCGTGA^GACAGTTTGG 3'-GACCCAAGTTTTGCAG^CACTCTGTCAAACC
20	I-NanI	<i>Naegleria andersoni</i>	5'-AAGTCTGGTGCCA^GCACCCGC 3'-TTCAGACC^ACGGTCGTGGGCG
	I-NitI	<i>Naegleria italica</i>	5'-AAGTCTGGTGCCA^GCACCCGC 3'-TTCAGACC^ACGGTCGTGGGCG
	I-NjaI	<i>Naegleria jamiesoni</i>	5'-AAGTCTGGTGCCA^GCACCCGC 3'-TTCAGACC^ACGGTCGTGGGCG
25	I-PakI	<i>Pseudonoclonium akinetum</i>	5'-CTGGGTTCAAACGTCGTGA^GACAGTTTGG 3'-GACCCAAGTTTTGCAG^CACTCTGTCAAACC
	I-PorI	<i>Pyrobaculum organotrophum</i>	5'-GCGAGCCCGTAAGGGT^GTGTACGGG 3'-CGCTCGGGCATT^CCCACACATGCCC
30	I-PpoI	<i>Physarum polycephalum</i>	5'-TAACTATGACTCTCTTAA^GGTAGCCAAAT 3'-ATTGATACTGAGAG^AATTCCATCGGTTTA
	I-ScaI	<i>Saccharomyces capensis</i>	5'-TGTCACATTGAGGTGCACT^AGTTATTAC 3'-ACAGTGTAACCTCCAC^GTGATCAATAATG
	I-Ssp6803I	<i>Synechocystis</i> species	5'-GTCGGGCT^CATAACCCGAA 3'-CAGCCCGAGTA^TTGGGCTT
35	PI-PfuI	<i>Pyrococcus furiosus</i> Vc1	5'-GAAGATGGGAGGAGGG^ACCGGACTCAACTT 3'-CTTCTACCCTCC^TCCCTGGCCTGAGTTGAA
	PI-PfuII	<i>Pyrococcus furiosus</i> Vc1	5'-ACGAATCCATGTGGAGA^AGAGCCTCTATA 3'-TGCTTAGGTACAC^CTCTTCTCGGAGATAT
40	PI-PkoI	<i>Pyrococcus kodakaraensis</i> KOD1	5'-GATTTTAGAT^CCCTGTACC 3'-CTAAAA^TCTAGGGACATGG
	PI-PkoII	<i>Pyrococcus kodakaraensis</i> KOD1	5'-CAGTACTACG^GTTAC 3'-GTCATG^ATGCCAATG
45	PI-PspI	<i>Pyrococcus</i> sp.	5'-AAAATCCTGGCAAACAGCTATTAT^GGGTAT 3'-TTTTAGGACCGTTTGTGCGAT^AATACCCATA
	PI-TfuI	<i>Thermococcus fumicolans</i> ST557	5'-TAGATTTTAGGT^CGCTATATCCTTCC 3'-ATCTAAAA^TCCAGCGATATAGGAAGG

	DSBI enzyme	Organism of origin	Recognition sequence
5	PI-TfuII	Thermococcus fumicolans ST557	5'-TAYGCNGAYACN^GACGGYTTYT 3'-ATRCGNCT^RTGNCTGCCRAARA
	PI-ThyI	Thermococcus hydrothermalis	5'-TAYGCNGAYACN^GACGGYTTYT 3'-ATRCGNCT^RTGNCTGCCRAARA
	PI-TliI	Thermococcus litoralis	5'-TAYGCNGAYACNGACGG^YTTYT 3'-ATRCGNCTRTGNC^TGCCRAARA
10	PI-TliIII	Thermococcus litoralis	5'-AAATTGCTTGCAAACAGCTATTACGGCTAT
	I-TevI	Bacteriophage T4	5'-AGTGGTATCAAC^GCTCAGTAGATG 3'-TCACCATAGT^TCCGAGTCATCTAC
15	I-TevII	Bacteriophage T4	5'-GCTTATGAGTATGAAGTGAACACGT^TATTC 3'-CGAATACTCATACTTCACTTGTG^CAATAAG
	F-TevI	Bacteriophage T4	5'-GAAACACAAGA^AATGTTTAGTAAANNNNNNNNNNNNN 3'-CTTTGTGTTCTTTACAAATCATTNNNNNNNNNNNNNNN^
	F-TevII	Bacteriophage T4	5'-TTTAATCCTCGCTTC^AGATATGGCAACTG 3'-AAATTAGGAGCGA^AGTCTATACCGTTGAC

20 Also encompassed are minor deviations (degenerations) of the recognition sequence which still enable recognition and cleavage by the DSBI enzyme in question. Such deviations - also in connection with different framework conditions such as, for example, calcium or magnesium concentration - have been described
25 (Argast GM et al. (1998) J Mol Biol 280: 345-353). Also encompassed are core sequences of these recognition sequences. It is known that the inner portions of the recognition sequences suffice for an induced double-strand break and that the outer ones are not absolutely relevant, but can codetermine the
30 cleavage efficacy. Thus, for example, an 18bp core sequence can be defined for I-SceI.

Recombination construct and DSBI enzyme can be combined to give one of the recombination systems or methods according to the
35 invention in various ways with which the skilled worker is familiar. Thus, the recombination constructs and the DSBI enzyme can be combined in an organism, a cell, a cell compartment or a tissue for example as follows:

40 1.) Organisms which have the recombination cassette inserted into the chromosomal DNA are generated in the customary manner. For example, such plants can be generated preferably by agrobacteria-mediated transformation. The primary transformants which contain the recombination cassette are
45 employed for the transformation with an expression cassette which ensures expression of the DSBI enzyme or grown in a suitable manner until homozygous, when they act as the host

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- organism (for example host plant) for the transformation with an expression cassette which ensures expression of the DSBI enzyme. Starting from these host plants, it is possible, for example, to initiate, establish and use for transformation purposes in-vitro cultures such as, for example, callus cultures or embryogenic cultures. Transformation with the expression cassette for the DSBI enzyme can be in each case stable or transient.
- 5
- 10 2.) Organisms known as master organisms, which bear and express the corresponding gene for the DSBI enzyme (or an expression cassette which ensures the expression of the DSBI enzyme) are generated in the customary manner. For example, such master plants can be generated preferably by agrobacterium-mediated transformation. The primary transformants which express the DSBI enzyme are employed for the transformation with the recombination construct or grown in a suitable manner until homozygous, which is when they act as master organism or host organism (for example master plant) into which the recombination constructs are introduced. Starting from these master plants, it is possible, for example, to initiate, establish and use for transformation purposes in-vitro cultures such as, for example, callus cultures or embryogenic cultures.
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- 20
- 25 3.) The gene encoding the DSBI enzyme (or an expression cassette which ensures the expression of the DSBI enzyme) can be integrated into a vector which already bears the recombination cassette and thus introduced into plant cells simultaneously with the target gene. It is preferred to insert the gene encoding the DSBI enzyme between the homology sequences and thus to delete it from the chromosomal DNA after it has fulfilled its function. Very especially preferably, expression of the DSBI enzyme is inducible in such a case (for example under the control of one of the inducible promoters described hereinbelow), in a development-dependent fashion using a development-dependent promoter, or else DSBI enzymes are employed whose activity is inducible in order to avoid cleaving the recombination construct immediately after the transformation and prior to its insertion into the genome.
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- 40
- 45 4.) Relying on the co-transformation technique, the expression cassette which ensures the expression of the DSBI enzyme can be transformed into the cells simultaneously with the recombination construct, but on a separate vector. Co-transformation can be in each case stable or transient. In

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- such a case, expression of the DSBI enzyme is preferably inducible (for example under the control of one of the inducible promoters described hereinbelow), in a development-dependent fashion using a development-dependent promotor, or else DSBI enzymes are employed whose activity is inducible in order to avoid cleaving the recombination construct immediately after the transformation and prior to its insertion into the genome.
- 5
- 10 5.) Organisms, for example plants or else animals, expressing the DSBI enzyme may also act as parent individuals. In the progeny from the hybridization between organisms expressing the DSBI enzyme on the one hand and organisms bearing the recombination construct on the other hand, the desired
- 15 double-strand breaks and recombination between the homology sequences are observed, with the possible deletion of the sequences located between the homology sequences.
- 6.) Expression of the DSBI enzyme is also conceivable in a transient transformation approach in which the possibilities
- 20 2 to 4 can be exploited.
- 7.) The DSBI enzyme can also be introduced into cells comprising or bearing the transgenic recombination construct directly,
- 25 for example via microinjection, particle bombardment (biolistic method), polyethylene glycol transfection or liposome-mediated transfection. This embodiment is advantageous since no DSBI-enzyme-encoding sequences can remain in the genome. Such a method has been described for
- 30 example by Segal DJ et al. (1995) Proc Natl Acad Sci USA 92:806-810.
- 8.) The DSBI enzyme may also be generated by introducing the DSBI-enzyme-encoding, in-vitro-generated mRNA into cells (for
- 35 example via microinjection, particle bombardment (biolistic method) or liposome-mediated transfection). This embodiment is advantageous since no DSBI-enzyme-encoding sequences can remain in the genome.
- 40 9.) The DSBI enzyme can be introduced into plant cells as a fusion protein with the VirE2 or VirF protein of an agrobacterium. Such methods have been described for example for Cre recombinase (Vergunst AC et al. (2000) Science. 290: 979-982). If the expression cassette for the fusion protein
- 45 is located outside the border sequences, it is not inserted

22

into the plant genome. This embodiment is advantageous since no DSBI-enzyme-encoding sequences can remain in the genome.

The recombination system or method according to the invention can
5 be realized in intact organisms or else in parts, cells or propagation material derived therefrom, especially preferably in intact plants or else in any plant tissue or plant in-vitro cultures including callus. An in-vitro application using, for example, wheat germ extract or reticulocyte extract can also be
10 envisaged.

As described above, the DSBI enzyme can be generated using an expression cassette which comprises the DNA encoding a DSBI enzyme and is introduced into a eukaryotic cell or organism. In
15 this context, the expression cassette for the DSBI enzyme preferably comprises a nucleic acid sequence encoding a DSBI enzyme as shown in SEQ ID NO: 2, 4, 6, 8 or 10 or a functional equivalent of same which is capable of generating DNA
20 double-strand breaks in double-stranded DNA using the essentially identical recognition sequence. Essentially identical recognition sequences refer to those recognition sequences which, while deviating from the recognition sequence identified as optimal for the enzyme in question, still permit cleavage by this enzyme. Very especially preferably, the expression cassettes for the DSBI
25 enzyme comprise a nucleic acid sequence as shown in SEQ ID NO: 1, 3, 5, 7 or 9.

Expression cassette – for example when referring to the expression cassette for the DSBI enzyme – means those
30 constructions in which the DNA to be expressed is linked operably to at least one genetic control element that [sic] enables or regulates its expression (i.e. transcription and [lacuna] or translation). Here, expression may be for example stable or transient, constitutive or inducible. For introducing it, the
35 skilled worker may resort to various direct methods (for example transfection, particle bombardment, microinjection) or indirect methods (for example infection with agrobacteria, infection with viruses), all of which are detailed further below.

40 Operable linkage is generally understood as meaning an arrangement in which a genetic control sequence is capable of exerting its function with regard to a nucleic acid sequence, for example while encoding a DSBI enzyme. Function, in this context, may mean for example control of the expression, i.e.
45 transcription and/or translation, of the nucleic acid sequence, for example one encoding a DSBI enzyme. Control, in this context, encompasses for example initiating, increasing, governing or

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suppressing the expression, i.e. transcription and, if appropriate, translation. Controlling, in turn, may be, for example, tissue- and [lacuna] or time-specific. It may also be inducible, for example by certain chemicals, stress, pathogens
5 and the like.

Operable linkage is understood as meaning for example the sequential arrangement of a promotor, of the nucleic acid sequence to be expressed - for example one encoding a DSBI enzyme
10 - and, if appropriate, further regulatory elements such as, for example, a terminator, in such a way that each of the regulatory elements can fulfil its function when the nucleic acid sequence - for example one encoding a DSBI enzyme - is expressed.

15 This does not necessarily require a direct linkage in the chemical sense. Genetic control sequences such as, for example, enhancer sequences are also capable of exerting their function on the target sequence from positions located at a distance or indeed other DNA molecules. Preferred arrangements are those in
20 which the nucleic acid sequence to be expressed - for example one encoding a DSBI enzyme - is positioned after a sequence acting as promotor so that the two sequences are linked covalently to one another. The distance between the promotor sequence and the nucleic acid sequence - for example one encoding a DSBI enzyme -
25 is preferably less than 200 base pairs, especially preferably less than 100 base pairs, very especially preferably less than 50 base pairs.

The skilled worker is familiar with a variety of ways in order to
30 obtain such an expression cassette. For example, it is preferably prepared by directly fusing a nucleic acid sequence which acts as promotor with a nucleotide sequence to be expressed - for example one encoding a DSBI enzyme. Operable linkage can be achieved by means of customary recombination and cloning techniques as are
35 described, for example, in T. Maniatis, E.F. Fritsch and J. Sambrook, Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY (1989), in T.J. Silhavy, M.L. Berman and L.W. Enquist, Experiments with Gene Fusions, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
40 (1984) and in Ausubel, F.M. et al., Current Protocols in Molecular Biology, Greene Publishing Assoc. and Wiley Interscience (1987).

However, an expression cassette may also be constructed in such a
45 way that the nucleic acid sequence to be expressed (for example one encoding a DSBI enzyme) is brought under the control of an endogenous genetic control element, for example a promotor, for

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example by means of homologous recombination or else by random insertion. Such constructs are likewise understood as being expression cassettes for the purposes of the invention.

5 The skilled worker furthermore knows that nucleic acid molecules may also be expressed using artificial transcription factors of the zinc finger protein type (Beerli RR et al. (2000) Proc Natl Acad Sci USA 97(4):1495-500). These factors can be adapted to suit any sequence region and enable expression independently of
10 certain promotor sequences.

The term "genetic control sequences" is to be understood in the broad sense and refers to all those sequences which affect the coming into existence, or the function, of the expression
15 cassette according to the invention. For example, genetic control sequences ensure transcription and, if appropriate, translation in prokaryotic or eukaryotic organisms. Preferably, the expression cassettes according to the invention encompass 5'-upstream of the respective nucleic acid sequence to be
20 expressed a promotor and 3'-downstream a terminator sequence as additional genetic control sequence, and, if appropriate, further customary regulatory elements, in each case in operable linkage with the nucleic acid sequence to be expressed.

25 Genetic control sequences are described, for example, in "Goeddel; Gene Expression Technology: Methods in Enzymology 185, Academic Press, San Diego, CA (1990)" or "Gruber and Crosby, in: Methods in Plant Molecular Biology and Biotechnolgy, CRC Press, Boca Raton, Florida, eds.:Glick and Thompson, Chapter 7, 89-108"
30 and the references cited therein.

Examples of such control sequences are sequences to which inductors or repressors bind and thus regulate the expression of the nucleic acid. The natural regulation of the sequences before
35 the actual structural genes may still be present in addition to these novel control sequences or instead of these sequences and, if appropriate, may have been genetically modified in such a way that the natural regulation has been switched off and gene expression increased. However, the expression cassette may also
40 be simpler in construction, that is to say no additional regulatory signals are inserted before the abovementioned genes, and the natural promotor together with its regulation is not removed. Instead, the natural control sequence is mutated in such a way that regulation no longer takes place and gene expression
45 is increased. These modified promotors may also be placed on their own before the natural genes for increasing the activity.

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A variety of control sequences are suitable, depending on the host organism or starting organism described in greater detail hereinbelow, which, owing to the introduction of the expression cassettes or vectors, becomes a genetically modified, or
5 transgenic, organism.

Advantageous control sequences for the expression cassettes or vectors according to the invention are present for example in promoters such as *cos*, *tac*, *trp*, *tet*, *phoA*, *tat*, *lpp*, *lac*, *lacIq*,
10 T7, T5, T3, *gal*, *trc*, *ara*, SP6, λ -PR or in the λ -PL promoter, which are advantageously used in Gram-negative bacteria.

Further advantageous control sequences are present for example in the Gram-positive promoters *amy* and SPO2, in the yeast or fungal
15 promoters ADC1, MFa, AC, P-60, CYC1, GAPDH, TEF, rp28, ADH or in the plant promoters CaMV/35S (Franck et al. (1980) Cell 21:285-294), PRP1 (Martini N et al. (1993) Mol Gen Genet. 236(2-3):179-186), SSU, OCS, LEB4, USP, STLS1, B33, NOS; FBPaseP (WO 98/18940) or in the ubiquitin or phaseolin promoters.

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Vectors which are suitable for expression in vertebrates, preferably in mammals, are vectors like the TK promoter, the RSV 3' LTR promoter, the CMV promoter or the SV40 early or late promoter. The skilled worker is familiar with other promoters.
25 Inducible promoters suitable for use in vertebrates, preferably in mammals, encompass for example the Tet promoter/repressor, which is inducible or repressible by tetracyclin or derivatives, the dexamethasone-inducible MMTV-LTR promoter, the Drosophila minimal heat shock promoter, which is inducible by ecdysone or
30 the analog ponasterone A (for example within the pVgRXR expression system; Invitrogen, Inc.).

A preferred promoter is, in principle, any promoter which is capable of controlling the expression of genes, in particular
35 foreign genes, in plants. Preferred promoters are those which enable constitutive expression in plants (Benfey et al. (1989) EMBO J. 8:2195-2202). A promoter which is preferably used is, in particular, a plant promoter or a promoter derived from a plant virus. Especially preferred is the promoter of the cauliflower
40 mosaic virus 35S transcript (Franck et al. (1980) Cell 21:285-294; Odell et al. (1985) Nature 313:810-812; Shewmaker et al. (1985) Virology 140:281-288; Gardner et al. 1986, Plant Mol. Biol. 6, 221-228) or the 19S CaMV promoter (US 5,352,605 and WO 84/02913). It is known that this promoter comprises a variety
45 of recognition sequences for transcriptional effectors which, in their totality, bring about permanent and constitutive expression of the gene introduced (Benfey et al. (1989) EMBO J 8:2195-2202).

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A further suitable constitutive promoter is the Rubisco small subunit (SSU) promoter (US 4,962,028). A further example of a suitable promoter is the leguminB promoter (GenBank Acc.-No.: X03677). Further preferred constitutive promoters are, for
5 example, the Agrobacterium nopaline synthase promoter, the TR dual promoter, the agrobacterium OCS (octopine synthase) promoter, the ubiquitin promoter (Holtorf S et al. (1995) Plant Mol Biol 29:637-649), the promoters of the vacuolar ATPase subunits, or the promoter of a wheat prolin-rich protein (WO
10 91/13991).

The Expression cassettes may also comprise an inducible, preferably chemically inducible, promoter (Aoyama T and Chua NH (1997) Plant J 11:605-612; Caddick MX et al. (1998) Nat.
15 Biotechnol 16:177-180; Review: Gatz, Annu Rev Plant Physiol Plant Mol Biol 1997, 48:89-108), by means of which the expression of the exogenous gene in the plant can be controlled at a specific point in time. Such promoters, such as, for example, the PRP1 promoter (Ward et al., Plant. Mol. Biol. 22 (1993), 361-366), a
20 salicylic-acid-inducible promoter (WO 95/19443), a benzenesulfonamide-inducible promoter (EP-A-0388186), a tetracyclin-inducible promoter (Gatz et al., (1992) Plant J. 2, 397-404), an abscisic acid-inducible promoter (EP-A 335528), a salicylic acid-inducible promoter (WO 95/19443) or an ethanol-
25 (Salter MG et al. (1998) Plant J. 16:127-132) or cyclohexanone-inducible (WO 93/21334) promoter may likewise be used.

In an especially preferred embodiment, the DSBI-enzyme-encoding
30 nucleic acid, in particular, is expressed under the control of an inducible promoter. This leads to a controlled, governable expression and deletion - for example in plants -, and any problems caused by a constitutive expression of a DSBI enzyme are avoided.

35 Other preferred promoters are promoters induced by biotic or abiotic stress, such as, for example, the pathogen-inducible promoter of the PRP1 gene (Ward et al., Plant Mol Biol 1993, 22: 361-366), the tomato heat-inducible hsp80 promoter (US
40 5,187,267), the potato chill-inducible alpha-amylase promoter (WO 96/12814) or the wound-induced pinII promoter (EP375091).

Other preferred promoters are promoters with specificity for the anthers, ovaries, pollen, the meristem, flowers, leaves, stems,
45 roots and seeds.

A development-regulated promoter is, inter alia, described by Baerson et al. (Baerson SR, Lamppa GK (1993) Plant Mol Biol 22(2):255-67).

5 Especially preferred promoters are those which ensure expression in tissues or plant parts in which the biosynthesis of starch and/or oils or their precursors takes place or in which the products are advantageously accumulated. The biosynthesis site of starch are the chloroplasts of the leaves or the amyloplasts of
10 the storage organs such as seeds, fruits or tubers. Within these organs, it is predominantly the cells of the endosperm or the cotyledons of the embryo in which synthesis takes place. Preferred promoters are thus in addition to the abovementioned constitutive promoters in particular seed-specific promoters such
15 as, for example, the phaseolin promoter (US 5,504,200, Bustos MM et al., Plant Cell. 1989;1(9):839-53), the promoter of the 2S albumin gene (Joseffson LG et al.(1987) J Biol Chem 262: 12196-12201), the legumin promoter (Shirsat A et al. (1989) Mol Gen Genet. 215(2):326-331), the USP (unknown seed protein)
20 promoter (Bäumlein H et al. (1991) Molecular & General Genetics 225(3):459-67); the napin gene promoter (US 5,608,152; Stalberg K, et al. (1996) L. Planta 199: 515-519), the sucrose binding protein promoter (WO 00/26388) or the legumin B4 promoter (LeB4; Bäumlein H et al. (1991) Mol Gen Genet 225:121-128; Baeumlein et
25 al. (1992) Plant Journal 2(2):233-239; Fiedler U et al. (1995) Biotechnology (NY) 13(10):1090-1093), the Ins Arabidopsis oleosin promoter (WO9845461), the Brassica Bce4 promoter (WO 91/13980). Further suitable seed-specific promoters are those of the genes encoding the "high-molecular-weight glutenin" (HMWG), gliadin,
30 branching enzyme, ADP-glucose pyrophosphatase (AGPase) or starch synthase. Furthermore preferred promoters are those which enable seed-specific expression in monocots such as maize, barley, wheat, rye, rice and the like. Promoters which may advantageously be employed are the promoter of the lpt2 or lpt1 gene
35 (WO 95/15389, WO 95/23230) or the promoters described in WO 99/16890 (promoters of the hordein gene, the glutelin gene, the oryzin gene, the prolamine gene, the gliadin gene, the glutelin gene, the zein gene, the kasirin gene or the secalin gene).

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Promoters which are preferred as genetic control elements are, furthermore, pollen-specific promoters such as, for example, the promoter of the B. campestris bgp1 gene (GenBank Acc.-No: X68210; Xu H et al. (1993) Mol Gen Genet 239(1-2):58-65; WO 94/13809), of
45 the Oryza sativa ory s 1 gene (GenBank Acc.-No.: AJ012760; Xu H et al. (1995) Gene 164 (2):255-259), of the pollen-specific maize gene ZM13 (Hamilton DA et al. (1998) Plant Mol Biol

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38(4):663-669; US 5,086,169), and of the B.napus gene Bp10 (GenBank Acc.-No.: X64257; Albani D (1992) Plant J 2(3):331-342; US 6,013,859).

- 5 Other preferred promoters are the Lcgl promoter for cell-specific expression in the male gametes (WO 99/05281; XU H et al. (1999) Proc. Natl. Acad. Sci. USA Vol. 96:2554-2558) and the promoter of the AtDMC1 gene (Klimyuk VI et al.(1997) Plant J. 11(1):1-14).
- 10 Further suitable promoters are, for example, specific promoters for tubers, storage roots or roots such as, for example, the class I patatin promoter (B33), the potato cathepsin D inhibitor promoter, the starch synthase (GBSS1) promoter or the sporamin promoter, and fruit-specific promoters such as, for example, the
- 15 tomato fruit-specific promoter (EP-A 409625).

Promoters which are furthermore suitable are those which ensure leaf-specific expression. Promoters which may be mentioned are the potato cytosolic FBPase promoter (WO 98/18940), the Rubisco

20 (ribulose-1,5-bisphosphate carboxylase) SSU (small subunit) promoter or the potato ST-LSI promoter (Stockhaus et al. (1989) EMBO J 8(9):2445-2451). Other preferred promoters are those which govern expression in seeds and plant embryos.

- 25 Further suitable promoters are, for example, fruit-maturation-specific promoters such as, for example, the tomato fruit-maturation-specific promoter (WO 94/21794), flower-specific promoters such as, for example, the phytoene synthase promoter (WO 92/16635) or the promoter of the P-rr gene
- 30 (WO 98/22593) or another node-specific promoter as described in EP-A 249676 may be used advantageously [sic].

In principle, all natural promoters together with their regulatory sequences, such as those mentioned above, may be used

35 for the method according to the invention. In addition, synthetic promoters may also be used advantageously.

Genetic control sequences also encompass further promoters, promoter elements or minimal promoters capable of modifying the

40 expression-specific characteristics. Thus, for example, the tissue-specific expression may take place in addition as a function of certain stress factors, owing to genetic control sequences. Such elements are, for example, described for water stress, abscisic acid (Lam E and Chua NH (1991) J Biol Chem

45 266(26):17131 -17135) and heat stress (Schoffl F et al. (1989) Molecular & General Genetics 217(2-3):246-53).

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Furthermore, other promoters which enable expression in further plant tissues or other organisms, such as, for example, *E.coli* bacteria, may be linked operably with the nucleic acid sequence to be expressed. Plant promoters which are suitable are, in principle, all of the above-described promoters.

Genetic control sequences furthermore also encompass the the 5'-untranslated region, introns or the noncoding 3'-region of genes. It has been demonstrated that they may play a significant role in the regulation of gene expression. Thus, it has been demonstrated that 5'-untranslated sequences are capable of enhancing the transient expression of heterologous genes. Furthermore, they may promote tissue specificity (Rouster J et al., *Plant J.* 1998, 15: 435-440.). Conversely, the 5'-untranslated region of the opaque-2 gene suppresses expression. Deletion of the region in question leads to an increased gene activity (Lohmer S et al., *Plant Cell* 1993, 5:65-73).

Genetic control sequences may also encompass ribosome binding sequences for initiating translation. This is preferred in particular when the nucleic acid sequence to be expressed does not provide suitable sequences or when they are not compatible with the expression system.

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The expression cassette can advantageously comprise one or more of what are known as enhancer sequences in operable linkage with the promoter, which enable the increased transgenic expression of the nucleic acid sequence. Additional advantageous sequences, such as further regulatory elements or terminators, may also be inserted at the 3' end of the nucleic acid sequences to be expressed recombinantly. One or more copies of the nucleic acid sequences to be expressed recombinantly may be present in the gene construct.

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Genetic control sequences are furthermore understood as meaning sequences which encode fusion proteins consisting of a signal peptide sequence.

Polyadenylation signals which are suitable as genetic control sequences are plant polyadenylation signals, preferably those which correspond essentially to T-DNA polyadenylation signals from *Agrobacterium tumefaciens*, in particular of gene 3 of the T-DNA (octopine synthase) of the Ti plasmids pTiACHS (Gielen et al., *EMBO J.* 3 (1984), 835 et seq.) or functional equivalents thereof. Examples of particularly suitable terminator sequences

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are the OCS (octopine synthase) terminator and the NOS (nopaline synthase) terminator.

As mentioned above, the recombination constructs according to the
5 invention may encompass further nucleic acid sequences. Such
nucleic acid sequences may preferably constitute expression
cassettes. The following may be mentioned by way of example of
the DNA sequences to be expressed in the expression constructs,
but not by way of limitation:

10

i) Positive selection markers:

As a rule, selection markers are required for selecting cells
which have successfully undergone homologous recombination or
15 transformation. The selectable marker which has been
introduced together with the expression construct confers
resistance to a biocide (for example a herbicide such as
phosphinothricin, glyphosate or bromoxynil), a metabolism
inhibitor such as 2-deoxyglucose-6-phosphate (WO 98/45456) or
20 an antibiotic such as, for example, tetracyclins [sic],
ampicillin, kanamycin, G 418, neomycin, bleomycin or
hygromycin to the cells which have successfully undergone
recombination or transformation. The selection marker permits
the selection of the transformed cells from untransformed
25 cells (McCormick et al., Plant Cell Reports 5 (1986), 81-84).
Especially preferred selection markers are those which confer
resistance to herbicides. Examples of selection markers which
may be mentioned are:

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- DNA sequences which encode phosphinothricin
acetyltransferases [sic] (PAT), which acetylates the free
amino group of the glutamine synthase inhibitor
phosphinothricin (PPT) and thus brings about
detoxification of the PPT (de Block et al. 1987, EMBO J.
35 6, 2513-2518) (also referred to as Bialophos[®]
resistance gene (bar)),

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- 5-enolpyruvylshikimate-3-phosphate synthase genes (EPSP
synthase genes), which confer resistance to Glyphosate[®]
(N-(phosphonomethyl)glycine),

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- the gox gene, which encodes the Glyphosate[®]-degrading
enzymes [sic] (Glyphosate oxidoreductase),

- the deh gene (encoding a dehalogenase which inactivates
Dalapon[®]),

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- acetolactate synthases which inactivate sulfonylurea and imidazolinone,
 - bxn genes which encode Bromoxynil[®]-degrading nitrilase enzymes,
 - the kanamycin, or G418, resistance gene (NPTII). The NPTII gene encodes a neomycin phosphotransferase which reduces the inhibitory effect of kanamycin, neomycin, G418 and paromomycin owing to a phosphorylation reaction,
 - the DOG^{R1} gene. The DOG^{R1} gene has been isolated from the yeast *Saccharomyces cerevisiae* (EP 0 807 836). It encodes a 2-deoxyglucose-6-phosphate phosphatase which confers resistance to 2-DOG (Randez-Gil et al. 1995, *Yeast* 11, 1233-1240).
- ii) Negative selection markers enable for example the selection of organisms with successfully deleted sequences which encompass the marker gene (Koprek T et al. (1999) *The Plant Journal* 19(6):719-726). TK thymidine kinase (TK) and diphtheria toxin A fragment (DT-A), *codA* gene encoding a cytosine deaminase (Gleve AP et al. (1999) *Plant Mol Biol.* 40(2):223-35; Perreat RI et al. (1993) *Plant Mol. Biol* 23(4): 793-799; Stougaard J; (1993) *Plant J* 3:755-761), the cytochrome P450 gene (Koprek et al. (1999) *Plant J.* 16:719-726), genes encoding a haloalkane dehalogenase (Naested H (1999) *Plant J.* 18:571-576), the *iaaH* gene (Sundaresan V et al. (1995) *Genes & Development* 9:1797-1810) or the *tms2* gene (Fedoroff NV & Smith DL 1993, *Plant J* 3: 273- 289).
- iii) Report genes which encode readily quantifiable proteins and which, via intrinsic color or enzyme activity, ensure the assessment of the transformation efficacy or of the location or timing of expression. Very especially preferred here are genes encoding reporter proteins (see also Schenborn E, Groskreutz D. *Mol Biotechnol.* 1999; 13(1):29-44) such as
- "green fluorescence [sic] protein" (GFP) (Chui WL et al., *Curr Biol* 1996, 6:325-330; Leffel SM et al., *Biotechniques.* 23(5):912-8, 1997; Sheen et al.(1995) *Plant Journal* 8(5):777-784; Haseloff et al.(1997) *Proc Natl Acad Sci USA* 94(6):2122-2127; Reichel et al.(1996) *Proc Natl Acad Sci USA* 93(12):5888-5893; Tian et al. (1997) *Plant Cell Rep* 16:267-271; WO 97/41228).

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- Chloramphenicol transferase,
- luciferase (Millar et al., Plant Mol Biol Rep 1992 10:324-414; Ow et al. (1986) Science, 234:856-859);
5 permits the detection of bioluminescence,
- β -galactosidase, encodes an enzyme for which a variety of chromogenic substrates are available,
- 10 - β -glucuronidase (GUS) (Jefferson et al., EMBO J. 1987, 6, 3901-3907) or the uidA gene, which encodes an enzyme for a variety of chromogenic substrates,
- 15 - R locus gene product: protein which regulates the production of anthocyanin pigments (red coloration) in plant tissue and thus makes possible the direct analysis of the promoter activity without the addition of additional adjuvants or chromogenic substrates (Dellaporta et al., In: Chromosome Structure and
20 Function: Impact of New Concepts, 18th Stadler Genetics Symposium, 11:263-282, 1988),
- β -lactamase (Sutcliffe (1978) Proc Natl Acad Sci USA 75:3737-3741), enzyme for a variety of chromogenic
25 substrates (for example PADAC, a chromogenic cephalosporin),
- xylE gene product (Zukowsky et al. (1983) Proc Natl Acad Sci USA 80:1101-1105), catechol dioxygenase capable of
30 converting chromogenic catechols,
- alpha-amylase (Ikuta et al. (1990) Bio/technol. 8:241-242),
- 35 - tyrosinase (Katz et al.(1983) J Gene Microbiol 129:2703-2714), enzyme which oxidizes tyrosine to give DOPA and dopaquinone which subsequently form melanine, which is readily detectable,
- 40 - aequorin (Prasher et al.(1985) Biochem Biophys Res Commun 126(3):1259-1268), can be used in the calcium-sensitive bioluminescence detection.

The recombination construct according to the invention and any
45 vectors derived from them [sic] may comprise further functional elements. The term "further functional elements" is to be understood in the broad sense. It preferably refers to all those

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elements which affect the generation, multiplication, function, use or value of the recombination system according to the invention, recombination construct according to the invention or cells or organisms comprising them. The following may be mentioned by way of example, but not by limitation, of the further functional elements:

- iv) Replication origins which ensure replication of the expression cassettes or vectors according to the invention in, for example, *E. coli*. Examples which may be mentioned are ORI (origin of DNA replication), the pBR322 ori or the P15A ori (Sambrook et al.: *Molecular Cloning. A Laboratory Manual*, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1989).
- v) Multiple cloning regions (MCS) enable and facilitate the insertion of one or more nucleic acid sequences.
- vi) Sequences which make possible homologous recombination or insertion into the genome of a host organism.
- vii) Elements, for example border sequences, which make possible the agrobacterium-mediated transfer in plant cells for the transfer and integration into the plant genome, such as, for example, the right or left border of the T-DNA or the vir region.

All of the abovementioned expression cassettes or further functional elements may be located, as mentioned, between the homology sequences A and B. However, they may also be located outside them. This is advantageous in particular in the case of border sequences.

A recombination cassette or expression construct according to the invention for a DSBI enzyme may advantageously be introduced into cells using vectors into which these constructs or cassettes are inserted. Examples of vectors may be plasmids, cosmids, phages, viruses, retroviruses or else agrobacteria.

Vectors for expression in *E. coli* are preferably pQE70, pQE60 and pQE-9 (QIAGEN, Inc.); pBluescript vectors, Phagescript vectors, pNH8A, pNH16a, pNH18A, pNH46A (Stratagene Cloning Systems, Inc.); ptrc99a, pKK223-3, pKK233-3, pDR540, pRIT5 (Pharmacia Biotech, Inc.).

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Preferred vectors for eukaryotic expression encompass pWLNE0, pSV2CAT, pOG44, pXT1 and pSG (Stratagene Inc.); pSVK3, pBPV, pMSG and pSVL (Pharmacia Biotech, Inc.). Inducible vectors which may be mentioned are pTet-Thia, Potter-Splice, pCDNA4/TO, pCDNA4/TO
5 /LacZ, pCDNA6/TR, pCDNA4/TO/Myc-His /LacZ, pCDNA4/TO/Myc-His A, pCDNA4/TO/Myc-His B, pCDNA4/TO/Myc-His C, pVgRXR (Invitrogen, Inc.) or the pMAM series (Clontech, Inc.; GenBank Accession No.: U02443). These already provide the inducible regulatory control element for example for a chemical inducible expression of a DSBI
10 enzyme. The nucleic acid sequence encoding a DSBI enzyme may be inserted directly into these vectors.

Vectors for the expression in yeast encompass by way of example pYES2, pYD1, pTEF1/Zeo, pYES2/GS, pPICZ, pGAPZ, pGAPZ α , pPIC9,
15 pPIC3.5, PHIL-D2, PHIL-S1, pPIC3SK, pPIC9K and PA0815 (Invitrogen, Inc.).

In an advantageous embodiment, the expression cassette is introduced by means of plasmid vectors. Preferred vectors are
20 those which enable the stable integration of the expression cassette into the host genome.

Another subject matter of the invention relates to eukaryotic transgenic organisms comprising the recombination system
25 according to the invention, and to cells, cell cultures, tissues, parts or propagation material – such as, for example, in the case of plant organisms leaves, roots, seeds, fruit, pollen and the like – derived from such organisms.

30 Eukaryotic organism, starting organism or host organism refers to higher and lower, single- and multi-celled eukaryotic organisms. Also encompassed are eukaryotic microorganisms such as, for example, yeasts, algae or fungi.

35 Preferred yeasts are *Candida*, *Saccharomyces*, *Hansenula* or *Pichia*, with *Saccharomyces cerevisiae* or *Pichia pastoris* (ATCC Accession No. 201178) being particularly preferred.

Preferred fungi are *Aspergillus*, *Trichoderma*, *Ashbya*, *Neurospora*,
40 *Fusarium*, *Beauveria* or other fungi described in Indian Chem Engr. Section B. Vol 37, No 1,2 (1995) on page 15, Table 6. The filamentous Hemiascomycete *Ashbya gossypii* is particularly preferred.

45 Host or starting organisms which are preferred in accordance with the invention are, furthermore, animal organisms and cells or tissue derived from them. Animal organisms encompass preferably

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vertebrates and invertebrates. Especially preferred vertebrates are mammals such as in [sic] dogs, cats, sheep, goats, chickens, mice, rats, bovines or horses. Preferred animal cells encompass CHO, COS and HEK293 cells. Preferred invertebrates encompass 5 insect cells such as Drosophila S2 and Spodoptera Sf9 or Sf21 cells.

Host organisms or starting organisms which are preferred as transgenic organisms are especially plants. Included within the 10 scope of the invention are all genera and species of higher and lower plants of the plant kingdom. Included are furthermore the mature plants, seed, shoots and seedlings, and parts, propagation material (for example seeds and fruit) and cultures, for example cell cultures, derived therefrom. Mature plants is to be 15 understood as meaning plants at any developmental stage beyond the seedling. Seedling is to be understood as meaning a young, immature plant in an early developmental stage.

The recombination system according to the invention may 20 preferably be used for the following plant families:
Amaranthaceae, Brassicaceae, Caryophyllaceae, Chenopodiaceae, Compositae, Cucurbitaceae, Labiatae, Leguminosae-Papilionoideae, Liliaceae, Linaceae, Malvaceae, Rosaceae, Saxifragaceae, Scrophulariaceae, Solanaceae [sic], Tetragoniaceae [sic].

25 Annual, perennial, monocotyledonous and dicotyledonous plants are preferred host organisms for the generation of transgenic plants. The use of the recombination system, or method according to the invention is furthermore advantageous in all ornamental plants, 30 useful or ornamental trees, flowers, cut flowers, shrubs or turf. Plants which may be mentioned by way of example but not by limitation are angiosperms, bryophytes such as, for example, Hepaticae (hepaticas) and Musci (mosses); pteridophytes such as ferns, horsetail and clubmosses; gymnosperms such as conifers, 35 cycads, ginkgo and Gnetales; algae such as Chlorophyceae, Phaeophyceae [sic], Rhodophyceae, Myxophyceae, Xanthophyceae, Bacillariophyceae (diatoms) and Euglenophyceae.

Plants for the purposes of the invention comprise by way of 40 example and not by way of limitation the families of the Rosaceae such as rose, Ericaceae such as rhododendrons and azaleas, Euphorbiaceae such as poinsettias and croton, Caryophyllaceae such as pinks, Solanaceae such as petunias, Gesneriaceae such as African violet, Balsaminaceae such as touch-me-not, Orchidaceae 45 such as orchids, Iridaceae such as gladioli, iris, freesia and crocus, Compositae such as marigold, Geraniaceae such as

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geraniums, Liliaceae such as drachaena, Moraceae such as ficus, Araceae such as philodendron and many others.

Flowering plants which may be mentioned by way of example but not
5 by limitation are the families of the Leguminosae such as pea, alfalfa and soya; Gramineae such as rice, maize, wheat; Solanaceae such as tobacco and and many others; the family of the Umbelliferae, particularly the genus *Daucus* (very particularly the species *carota* (carrot)) and *Apium* (very particularly the
10 species *graveolens dulce* (celery)) and many others; the family of the Solanaceae [sic], particularly the genus *Lycopersicon*, very particularly the species *esculentum* (tomato) and the genus *Solanum*, very particularly the species *tuberosum* (potato) and *melongena* (aubergine) and many others; and the genus *Capsicum*,
15 very particularly the species *annum* (peppers) and many others; the family of the Leguminosae, particularly the genus *Glycine*, very particularly the species *max* (soybean) and many others; and the family of the Cruciferae, particularly the genus *Brassica*, very particularly the species *napus* (oilseed rape), *campestris*
20 (beet), *oleracea cv Tastié* (cabbage), *oleracea cv Snowball Y* (cauliflower) and *oleracea cv Emperor* (broccoli); and the genus *Arabidopsis*, very particularly the species *thaliana* and many others; the family of the Compositae, particularly the genus *Lactuca*, very particularly the species *sativa* (lettuce) and many
25 others.

The transgenic plants according to the invention are selected in particular among monocotyledonous crop plants, such as, for example, cereals such as wheat, barley, sorghum and millet, rye,
30 triticale, maize, rice or oats, and sugar cane. The transgenic plants according to the invention are furthermore selected in particular from among dicotyledonous crop plants such as, for example,

35 Brassicaceae such oilseed rape, cress, *Arabidopsis*, cabbages or canola, Leguminosae such as soya, alfalfa, peas, beans or peanut

Solanaceae such as potato, tobacco, tomato, aubergine or peppers, Asteraceae such as sunflower, *Tagetes*, lettuce or *Calendula*,

40 Cucurbitaceae such as melon, pumpkin/squash or courgette,

and linseed, cotton, hemp, flax, red pepper, carrot, carrot, [sic] sugar beet and the various tree, nut and wine species.

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Especially preferred are *Arabidopsis thaliana*, *Nicotiana tabacum* and oilseed rape and all genera and species which are used as food or feedstuffs, such as the above-described cereal species, or which are suitable for the production of oils, such as oil
5 crops (such as, for example, oilseed rape), nut species, soya, sunflower, pumpkin/squash and peanut.

Plant organisms are furthermore, for the purposes of the invention, other organisms which are capable of photosynthetic
10 activity, such as, for example, algae or cyanobacteria, and also mosses.

Preferred algae are green algae, such as, for example, algae of the genus *Haematococcus*, *Phaedactylum tricornatum*, *Volvox* or
15 *Dunaliella*.

The generation of a transformed organism or a transformed cell requires introducing the DNA in question into the host cell in question. A multiplicity of methods is available for this
20 procedure, which is termed transformation (see also Keown et al. 1990 Methods in Enzymology 185:527-537). For example, the DNA can be introduced directly by microinjection or by bombardment with DNA-coated microparticles. Also, the cell can be permeabilized chemically, for example using polyethylene glycol, so that the
25 DNA can enter the cell by diffusion. The DNA can also be introduced by protoplast fusion with other DNA-containing units such as minicells, cells, lysosomes or liposomes. Another suitable method of introducing DNA is electroporation, where the cells are permeabilized reversibly by an electrical pulse.
30 Preferred general methods which may be mentioned are the calcium-phosphate-mediated transfection, the DEAE-dextran-mediated transfection, the cationic lipid-mediated transfection, electroporation, transduction and infection. Such methods are known to the skilled worker and described, for
35 example, in Davis et al., Basic Methods In Molecular Biology (1986).

In plants, methods for transforming and regenerating plants from plant tissues or plant cells with which the skilled worker is
40 familiar are exploited for transient or stable transformation. Suitable methods are especially protoplast transformation by means of polyethylene-glycol-induced DNA uptake, biolistic methods such as the gene gun ("particle bombardment" method), electroporation, the incubation of dry embryos in DNA-containing
45 solution, sonication and microinjection, and the transformation of intact cells or tissues by micro- or macroinjection into tissues or embryos, tissue electroporation, incubation of dry

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embryos in DNA-containing solution or vacuum infiltration of seeds. In the case of injection or electroporation of DNA into plant cells, the plasmid used need not meet any particular requirement. Simple plasmids such as those of the pUC series may
5 be used. If intact plants are to be regenerated from the transformed cells, the presence of an additional selectable marker gene on the plasmid is useful.

Any plant tissue may act as target material. Likewise, expression
10 may take place in callus, embryogenic tissue or somatic embryos.

In addition to these "direct" transformation techniques, transformation can also be carried out by bacterial infection by means of *Agrobacterium tumefaciens* or *Agrobacterium rhizogenes*.
15 These strains contain a plasmid (Ti or Ri plasmid). Part of this plasmid, termed T-DNA (transferred DNA), is transferred to the plant following agrobacterial infection and integrated into the genome of the plant cell.

20 The recombination construct or the expression cassette for the DSBI enzyme is preferably integrated into specific plasmids, either into a shuttle, or intermediate, vector or into a binary vector). If, for example, a Ti or Ri plasmid is to be used for the transformation, at least the right border, but in most cases
25 the right and the left border, of the Ti or Ri plasmid T-DNA is linked with the expression cassette to be introduced as a flanking region. Binary vectors are preferably used. Binary vectors are capable of replication both in *E. coli* and in *Agrobacterium*. As a rule, they contain a selection marker gene
30 and a linker or polylinker flanked by the right or left T-DNA flanking sequence. They can be transformed directly into *Agrobacterium* (Holsters et al., *Mol. Gen. Genet.* 163 (1978), 181-187). The selection marker gene permits the selection of transformed agrobacteria and is, for example, the nptII gene,
35 which imparts resistance to kanamycin. The agrobacterium, which acts as host organism in this case, should already contain a plasmid with the vir region. The latter is required for transferring the T-DNA to the plant cell. An agrobacterium thus transformed can be used for transforming plant cells.

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The use of *Agrobacterium tumefaciens* for the transformation of plants using tissue culture explants has been described by Horsch et al. (Horsch RB (1986) *Proc Natl Acad Sci USA* 83(8):2571-2575), Fraley et al. (Fraley et al. 1983, *Proc. Natl. Acad. Sci. USA* 80,
45 4803-4807) and Bevens et al. (Bevens et al. 1983, *Nature* 304, 184-187). Many strains of *Agrobacterium tumefaciens* are capable of transferring genetic material - for example the recombination

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constructs according to the invention -, such as, for example, the strains EHA101[pEHA101], EHA105[pEHA105], LBA4404[pAL4404], C58C1[pMP90] and C58C1[pGV2260]. The strain EHA101[pEHA101] has been described by Hood et al. (Hood EE et al. (1996) J Bacteriol
5 168(3):1291-1301), the strain EHA105[pEHA105] by Hood et al. (Hood et al. 1993, Transgenic Research 2, 208-218), the strain LBA4404[pAL4404] by Hoekema et al. (Hoekema et al. 1983, Nature 303, 179-181), the strain C58C1[pMP90] by Koncz and Schell (Koncz and Schell 1986, Mol. Gen. Genet. 204, 383-396), and the strain
10 C58C1[pGV2260] by Deblaere et al. (Deblaere et al. 1985, Nucl. Acids Res. 13, 4777-4788).

The agrobacterial strain employed for the transformation comprises, in addition to its disarmed Ti plasmid, a binary
15 plasmid with the T-DNA to be transferred, which, as a rule, comprises a gene for the selection of the transformed cells and the gene to be transferred. Both genes must be equipped with transcriptional and translational initiation and termination signals. The binary plasmid can be transferred into the
20 agrobacterial strain for example by electroporation or other transformation methods (Mozo & Hooykaas 1991, Plant Mol. Biol. 16, 917-918). Coculture of the plant explants with the agrobacterial strain is usually performed for two to three days.

25 A variety of vectors could, or can, be used. In principle, one differentiates between those vectors which can be employed for the agrobacterium-mediated transformation or agroinfection, i.e. which comprise the recombination constructs, or the expression cassette, for the expression of the DSB1 enzyme within a T-DNA,
30 which indeed permits stable integration of the T-DNA into the plant genome. Moreover, border-sequence-free vectors may be employed, which can be transformed into the plant cells for example by particle bombardment, where they can lead both to transient and to stable expression.

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The use of T-DNA for the transformation of plant cells has been studied and described intensively (EP 120516; Hoekema, In: The Binary Plant Vector System, Offsetdrukkerij Kanters B. V., Alblasterdam, Chapter V; Fraley et al., Crit. Rev. Plant. Sci.,
40 4:1-46 and An et al., EMBO J. 4 (1985), 277-287). Various binary vectors are known, some of which are commercially available such as, for example, pBIN19 (Clontech Laboratories, Inc. USA).

To transfer the DNA to the plant cell, plant explants are
45 cocultured with *Agrobacterium tumefaciens* or *Agrobacterium rhizogenes*. Starting from infected plant material (for example leaf, root or stalk sections, but also protoplasts or suspensions

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of plant cells), intact plants can be regenerated using a suitable medium which may contain, for example, antibiotics or biocides for selecting transformed cells. The plants obtained can then be screened in the presence of the DNA introduced, in this case the recombination construct or the expression cassette for the DSBI enzyme according to the invention. As soon as the DNA has integrated into the host genome, the genotype in question is, as a rule, stable and the insertion in question is also found in the subsequent generations. As a rule, the expression cassette integrated contains a selection marker which confers a resistance to a biocide (for example a herbicide) or an antibiotic such as kanamycin, G 418, bleomycin, hygromycin or phosphinotricin and the like to the transformed plant. The selection marker permits the selection of transformed cells (McCormick et al., Plant Cell Reports 5 (1986), 81-84). The plants obtained can be cultured and hybridized in the customary fashion. Two or more generations should be grown in order to ensure that the genomic integration is stable and hereditary.

- 20 The abovementioned methods are described, for example, in B. Jenes et al., Techniques for Gene Transfer, in: Transgenic Plants, Vol. 1, Engineering and Utilization, edited by S.D. Kung and R. Wu, Academic Press (1993), 128 - 143 and in Potrykus, Annu. Rev. Plant Physiol. Plant Molec. Biol. 42 (1991), 205-225).
- 25 The construct to be expressed is preferably cloned into a vector which is suitable for the transformation of *Agrobacterium tumefaciens*, for example pBin19 (Bevan et al., Nucl. Acids Res. 12 (1984), 8711).
- 30 *Agrobacterium*-mediated transformation is suited best to dicotyledonous plant cells, whereas the direct transformation techniques are suitable for any cell type.

Transformed cells, i.e. those which comprise the DNA integrated into the DNA of the host cell, can be selected from untransformed cells if a selectable marker is part of the DNA introduced. A marker can be, for example, any gene which is capable of conferring a resistance to antibiotics or herbicides. Transformed cells which express such a marker gene are capable of surviving in the presence of concentrations of a suitable antibiotic or herbicide which kill an untransformed wild type. Various positive and negative selection markers are described hereinabove. Examples are the bar gene, which confers resistance to the herbicide phosphinotricin (Rathore KS et al., Plant Mol Biol. 1993 Mar;21(5):871-884), the nptII gene, which confers resistance to kanamycin, the hpt gene, which confers resistance to

hygromycin, or the EPSP gene, which confers resistance to the herbicide Glyphosate.

As soon as a transformed plant cell has been generated, an intact
5 plant can be obtained using methods known to the skilled worker. For example, callus cultures are used as starting material. The formation of shoot and root can be induced in this as yet undifferentiated cell biomass in the known fashion. The shoots obtained can be planted and cultured.

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Also in accordance with the invention are cells, cell cultures, parts – such as, for example, in the case of transgenic plant organisms, roots, leaves and the like – derived from the above-described transgenic organisms, and transgenic propagation
15 material (such as seeds or fruits).

Genetically modified plants according to the invention which can be consumed by humans or animals can also be used as food or feedstuffs, for example directly or following processing known
20 per se. Here, the deletion of, for example, resistances to antibiotics and/or herbicides, as are frequently introduced when generating the transgenic plants, makes sense for reasons of customer acceptance, but also product safety.

25 A further subject matter of the invention relates to the use of the above-described transgenic organisms according to the invention and the cells, cell cultures, parts – such as, for example, in the case of transgenic plant organisms, roots, leaves and the like – derived from them, and transgenic propagation
30 material such as seeds or fruits, for the production of food or feedstuffs, pharmaceuticals or fine chemicals. Here again, the deletion of, for example, resistances to antibiotics and/or herbicides is advantageous for reasons of customer acceptance, but also product safety.

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Fine chemicals is understood as meaning enzymes, vitamins, amino acids, sugars, fatty acids, natural and synthetic flavors, aromas and colorants widely usable [sic]. Especially preferred is the production of tocopherols and tocotrienols, and of carotenoids.
40 Culturing the transformed host organisms, and isolation from the host organisms or from the culture medium, is performed by methods known to the skilled worker. The production of pharmaceuticals such as, for example, antibodies or vaccines, is described by Hood EE, Jilka JM. (1999) Curr Opin Biotechnol.
45 10(4):382-386; Ma JK and Vine ND (1999) Curr Top Microbiol Immunol.236:275-92).

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The recombination system or method according to the invention furthermore offers various advantageous uses which can not be achieved with the deletion methods described in the prior art. Various use examples are described hereinbelow by way of example, 5 but not by limitation:

1. Simple deletion of a nucleic acid sequence from the chromosomal DNA of an organism:

10 Using any homology sequences A and B, nucleic acid sequences located between them can be deleted. The sequence which is the result of the recombination of the homology sequences A and B remains in the genome. The method is suitable for example for removing, from the chromosomal DNA, selection 15 markers after a transgenic organism, for example a transgenic plant, has been generated. The method is shown schematically in Figs. 2 and 3, Fig. 2 showing the variant with one recognition sequence for the site-directed induction of DNA double-strand breaks and Fig. 3 showing the variant with two 20 recognition sequences for the site-directed induction of DNA double-strand breaks.

2. Complete deletion of recombinantly introduced heterologous nucleic acid sequences from the chromosomal DNA of an 25 organism:

Using homology sequences A and B, which are homologous to certain sequences of the organism, the expression construct can be introduced into the organism by homologous 30 recombination. Using the recombination system or method according to the invention, the nucleic acid sequences located between the homology sequences would be deleted. The induced homologous recombination between homology sequences A and B restores the original sequence. All of the construct is 35 removed from the chromosomal DNA. The method is suitable for example for removing selection markers from the chromosomal DNA after a transgenic plant has been generated. Furthermore, the system or method according to the invention is suitable for expressing certain proteins transiently in order to 40 achieve an advantageous effect and then to switch them off using an induced DSBI enzyme expression or activity by irreversibly removing the gene in question from the genome. The method is shown schematically in Fig. 4, the variant with two recognition sequences for the site-directed induction of 45 DNA double-strand breaks being shown. The system can also be realized using one recognition sequence; however, two cleavage sites are advantageous in the case of larger

43

insertions between the homology sequences A and B since this allows the deletion efficacy and homologous recombination efficacy to be increased further (further recognition sequences may be located within the sequence region to be deleted).

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3. Induced gene activation by the site-directed deletion of nucleic acid sequences:

10 Using homology sequences A and B, whose homologous recombination restores for example a complete open reading frame of a protein, or a functional promotor, the inducible expression of target proteins can be realized as a function of the presence of the DSBI enzyme. Using the recombination
15 system or method according to the invention, the nucleic acid sequences located between the homology sequences would be deleted. The method is shown schematically in Figs. 5 and 6, Fig. 6 showing a specific embodiment of the general method shown in Fig. 5 in which the recombination construct is
20 inserted into an endogenous gene at an earlier point in time by means of homologous recombination, thus enabling this gene to be inducibly activated as a function of the presence of the DSBI enzyme. Fig. 7a illustrates the system of gene activation with reference to a specific use example where the
25 β -glucuronidase (GUS) gene is reconstituted using the system or method according to the invention, enabling a color reaction to take place (see description to Fig. 7a and Examples).

- 30 4. Readily selectable system for the deletion of a nucleic acid sequence from the chromosomal DNA of an organism:

In a preferred embodiment, the recombination construct comprises a positive and a negative selection marker (and, if
35 appropriate, further nucleic acid sequences to be deleted) in such a way that both markers are deleted when the double-strand breaks are induced. Such a system is shown in Figs. 8 and 9 (A). Moreover, the expression cassette for the DSBI enzyme may also be present between the homology
40 sequences (Fig. 10 (B)), expression preferably being effected under the control of an inducible promotor (Pi) (for example: Aoyama T and Chua NH (1997) Plant J 11:605-612; Caddick MX et al. (1998) Nat. Biotechnol 16:177-180). As already described, further nucleic acid sequences may be present (Fig. 9 (C)).

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Expression of the DSBI enzyme leads in both cases to the elimination of the DNA sequences located between the two recognition sequences and the recombination of the homologous sequences. Since the cells simultaneously lose a negative selection marker, the cells with a successful deletion can be identified by means of selection (Gleave AP et al.(1999) Plant Mol Biol. 40:223-235).

In the case of plant cells, for example, the resulting cells can be used for regenerating and propagating the corresponding intact plants, which now no longer contain any marker genes.

5. Genetic manipulation of the host genome:

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The recombination system or method according to the invention can be used for in-situ modifications of the host genome. Thus, for example, a homology sequences [sic] may already exist endogenously in the genome. After insertion of the second homology sequence, which is linked with a DSBI enzyme recognition sequence, any regulatory or coding sequences located between the homology sequences A and B are eliminated from the genome.

At the same time, it is conceivable that the recombination construct encompasses regulatory or coding sequences which are eliminated from the organism once deletion has taken place. Thus, it is possible for example to regulate transiently an endogenous gene in a site-directed fashion.

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In a further preferred embodiment, the efficacy of the recombination system is increased by combination with systems which promote homologous recombination. Such systems are described and encompass, for example, the expression of proteins such as RecA or the treatment with PARP inhibitors. It has been demonstrated that the intrachromosomal homologous recombination in tobacco plants can be increased by using PARP inhibitors (Puchta H et al. (1995) Plant J. 7:203-210). Using these inhibitors, the homologous recombination rate in the recombination constructs after induction of the sequence-specific DNA double-strand break, and thus the efficacy of the deletion of the transgenesequences, can be increased further. Various PARP inhibitors may be employed for this purpose. Preferably encompassed are inhibitors such as 3-aminobenzamide, 8-hydroxy-2-methylquinazolin-4-one (NU1025), 1,11b-dihydro-[2H]benzopyrano[4,3,2-de]isoquinolin-3-one (GPI 6150), 5-aminoisoquinolinone,

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3,4-dihydro-5-[4-(1-piperidinyl)butoxy]-
1(2H)-isoquinolinone, or the compounds described in WO 00/26192,
WO 00/29384, WO 00/32579, WO 00/64878, WO 00/68206, WO 00/67734,
WO 01/23386 and WO 01/23390.

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In addition, it was possible to increase the frequency of various
homologous recombination reactions in plants by expressing the E.
coli RecA gene (Reiss B et al. (1996) Proc Natl Acad Sci USA
93(7):3094-3098). Also, the presence of the protein shifts the
10 ratio between homologous and illegitimate DSB repair in favor of
homologous repair (Reiss B et al. (2000) Proc Natl Acad Sci USA
97(7):3358-3363). Reference may also be made to the methods
described in WO 97/08331 for increasing the homologous
recombination in plants. A further increase in the efficacy of
15 the recombination system might be achieved by the simultaneous
expression of the RecA gene or other genes which increase the
homologous recombination efficacy (Shalev G et al. (1999) Proc
Natl Acad Sci USA 96(13):7398-402). The above-stated systems for
promoting homologous recombination can also be advantageously
20 employed in cases where the recombination construct is to be
introduced in a site-directed fashion into the genome of a
eukaryotic organism by means of homologous recombination.

Sequences

25

1. SEQ ID NO:1
Nucleic acid sequence for the I-SceI homing endonuclease.

30

2. SEQ ID NO:2
Protein sequence for the I-SceI homing endonuclease.

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3. SEQ ID NO:3
Nucleic acid sequence for fusion protein of I-ChuI homing
endonuclease and N-terminal nuclear localization sequence.

40

4. SEQ ID NO:4
Protein sequence for fusion protein of I-ChuI homing
endonuclease and N-terminal nuclear localization sequence.

45

5. SEQ ID NO:5
Nucleic acid sequence for fusion protein of I-CreI homing
endonuclease and N-terminal nuclear localization sequence.

6. SEQ ID NO:6
Protein sequence for fusion protein of I-CreI homing
endonuclease and N-terminal nuclear localization sequence.

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7. SEQ ID NO:7
Nucleic acid sequence for fusion protein of I-CpaI homing
endonuclease and N-terminal nuclear localization sequence.
- 5 8. SEQ ID NO:8
Protein sequence for fusion protein of I-CpaI homing
endonuclease and N-terminal nuclear localization sequence.
9. SEQ ID NO:9
- 10 10. SEQ ID NO:10
Nucleic acid sequence for fusion protein of I-CpaII homing
endonuclease and N-terminal nuclear localization sequence.
- 15 10. SEQ ID NO:10
Protein sequence for fusion protein of I-CpaII homing
endonuclease and N-terminal nuclear localization sequence.
11. SEQ ID NO: 11: oligonucleotide primer OPN1
5'-CGG CTC GAG CTA CGG GGA CGA TTT CTT TTT TTC AC-3'
- 20 12. SEQ ID NO: 12: oligonucleotide primer OPN2
5'-CGG CTC GAG TAC CTA GAA TAC AAA GAA GAG GAA GAA GAA ACC
TCT ACA GAA GAA GCC ATG GGT CCA AAG AAA AAG AGA AAG GTT ATC
AT GAA TAC AAA ATA TAA TAA AGA GTT CTT ACT C-3'
- 25 13. SEQ ID NO: 13: oligonucleotide primer OPN3
5'-CGG CTC GAG TAC CTA GAA TAC AAA GAA GAG GAA GAA GAA ACC
TCT ACA GAA GAA GCC ATG GGT CCA AAG AAA AAG AGA AAG GTT ATC
ATG GAC ATT AAT CCT CAA TGG ATT ACA GG- 3'
- 30 14. SEQ ID NO: 14: oligonucleotide primer OPN4
5'-CGG CTC GAG TTA CTC GCC AGT TTC TTC AAA ACG-3'
15. SEQ ID NO: 15: oligonucleotide primer OPN5
5'-CGG CTC GAG TAC CTA GAA TAC AAA GAA GAG GAA GAA GAA ACC
35 TCT ACA GAA GAA GCC ATG GGT CCA AAG AAA AAG AGA AAG GTT ATC
ATG ACC GAT TCT AAA TCT AGA AAC AAC-3'
16. SEQ ID NO: 16: oligonucleotide primer OPN6
5'-CGG CTC GAG CTA AAG GTG GCC TTT ATT GCC ATC AG-3'
- 40 17. SEQ ID NO: 17: oligonucleotide primer OPN7
5'-CGG CTC GAG TAC CTA GAA TAC AAA GAA GAG GAA GAA GAA ACC
TCT ACA GAA GAA GCC ATG GGT CCA AAG AAA AAG AGA AAG GTT ATC
ATG TCA TTA ACA CAA CAA CAA AAA GAC-3'

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18. SEQ ID NO: 18: oligonucleotide primer OPN8
5'-CGG CTC GAG CTA AAG GTG GCC TTT ATT GCC ATC AG-3'
19. SEQ ID NO: 19: oligonucleotide primer OPN9
5
5'-CGG CTC TAG AGC GGC CGC CTA GGG ATA ACA GGG TAA TAG AAT
CCC ACA AAA ATC TGA GCT TAA CAG 3'
20. SEQ ID NO: 20: oligonucleotide primer OPN10
5'-CGG CTC TAG ACT ATT ACC CTG TTA TCC CTA GGC CCG ATC TAG
10 TAA CAT AGA TGA CAC CGC GCG CG 3'
21. SEQ ID NO: 21: oligonucleotide primer OPN11
5'- CGG AAG CTT CGT CAC CAA TCC CAA TTC GAT CTA C - 3'
- 15 22. SEQ ID NO: 22: oligonucleotide primer OPN12
5'- CGG AAG CTT CCA CTT GCA AAG TCC CGC TAG TGC C - 3'
23. SEQ ID NO: 23: oligonucleotide primer OPN13
5'- CGG AAG CTT CGT CAC CAA TCC CAA TTC GAT CTA C - 3'
20
24. SEQ ID NO: 24: oligonucleotide primer OPN14
5'- CGG AAG CTT CCA CTT GCA AAG TCC CGC TAG TGC C - 3'
25. SEQ ID NO: 25: oligonucleotide primer OPN15
25 5'- CTA GTA CAA AAC GTC GTG AGA CAT TTT AAT CTG AAG GTT TGG
CAC CTC GAT GTC GGC TCA TC-3'
26. SEQ ID NO: 26: oligonucleotide primer OPN16
5'-CTA GGA TGA GCC GTC ATC GAG GTG CCA AAC CTT CAG ATT AAA
30 ATG TCT CAC GAC GTT TTG TA-3'
27. SEQ ID NO: 27: oligonucleotide primer OPN17
5'-CTA GTC CGA AAA CGC CGT GAG ACA TAT TGG TTA CGA TCC TAA
GGT AGC GAA ATT CAC CCG GTA ACT CTG TGC CAG-3'
35
28. SEQ ID NO: 28: oligonucleotide primer OPN18
5'-CTA GCT GGC ACA GAG TTA CCG GGT GAA TTT CGC TAC CTT AGG
ATC GTA ACC AAT ATG TCT CAC GGC GTT TTC GGA-3'
- 40 29. SEQ ID NO: 29: nuclear localization sequence NLS1
N-Pro-lys-Thr-Lys-Arg-Lys-Val-C
30. SEQ ID NO: 30: nuclear localization sequence NLS2
N-Pro-Lys-Lys-Lys-Arg-Lys-Val-C (SEQ ID NO: 30)
45

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Figures

The following abbreviations apply to the figures in general:

- 5 H1: Homology sequence A
 H2: Homology sequence B
 H1/2: Sequence as the result of homologous recombination of H1
 and H2
 S1: First recognition sequence for the site-directed
 10 induction of DNA double-strand breaks
 S2: Second recognition sequence for the site-directed
 induction of DNA double-strand breaks
 E: DSBI enzyme
 P: Promotor or other genetic control element
 15 N: Further nucleic acid sequence
 NS: Negative selection marker
 PS: Positive selection marker
 T1: Front part, for example of a gene or open reading frame
 T2: Back part, for example of a gene or open reading frame
 20 STOP: Interruption of a gene or open reading frame by, for
 example, stop codons or reading-frame shifts.

Fig. 1: Diagram of the principle of the invention
 Sequences in the genome can be eliminated efficiently
 25 when they are flanked by the homology sequences H1 and H2
 and when a cleavage site (S1) for a DSBI enzyme is
 located between the homology sequences. Owing to the
 action of the DSBI enzyme (E) on this recombination
 cassette (H1-S1-H2), double-strand breaks are formed at
 30 the cleavage site S1 and the sequences located between H1
 and H2 are eliminated.

Fig. 2: Preferred embodiment
 Sequences – in the present case for example an expression
 35 cassette consisting of a promotor (P) and a further
 nucleic acid sequence (N) to be expressed (for example a
 selection marker) – can be eliminated efficiently from
 the chromosomal DNA when they are flanked by the homology
 sequences H1 and H2 and when a cleavage site (S1) for a
 40 DSBI enzyme is located between the homology sequences.
 Owing to the action of the DSBI enzyme (E) on this
 recombination cassette (H1-S1-P-N-H2), double-strand
 breaks are formed at the cleavage site S1 and the
 sequences located between H1 and H2 are eliminated. The
 45 cleavage site S1 may also be located behind or within the
 expression cassette.

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Fig. 3: Preferred embodiment

Sequences – in the present case for example an expression cassette consisting of a promotor (P) and a further nucleic acid sequence (N) to be expressed (for example a selection marker) – can be eliminated particularly efficiently from the chromosomal DNA when they are flanked by the homology sequences H1 and H2 and when in each case one cleavage site (S1 and S2) for a DSBI enzyme is located before and after the nucleic acid sequence to be deleted. Owing to the action of the DSBI enzyme (E) on this recombination cassette (H1-S1-P-N-S2-H2), double-strand breaks are formed at the cleavage sites S1 and S2 and the sequences located between H1 and H2 are eliminated.

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Fig. 4: Preferred embodiment

Sequences – in the present case for example an expression cassette consisting of a promotor (P) and a further nucleic acid sequence (N) to be expressed (for example a selection marker) can be eliminated virtually without trace from the chromosomal DNA when the recombination construct comprising them has previously been inserted into the host genome, for example by homologous recombination. In doing so, the gene consisting of the sequence segments T1, H1/2 and T2 is interrupted. The recombination construct is flanked by two parts of the interrupted gene (T1-H1 or H2-T2), the middle part (H1 or H2) having been duplicated in order to enable homologous recombination to take place. The action of the DSBI enzyme (E) on the cleavage sites (S1 and S2) induces double-strand breaks and induces the homologous recombination between the homology sequences H1 and H2, whereby firstly the sequences located between H1 and H2 are deleted and secondly the original gene is restored.

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Fig. 5: Preferred embodiment

Nucleic acid sequences (in the present case a gene with the sequence T1-H1/2-T2 under the control of a promotor P) can be expressed inducibly by reconstituting the intact gene only by applying the recombination system. The gene, consisting of the sequence segments T1, H1/2 and T2 is inactivated, for example by the insertion of stop codons or other interruptions of the reading frame within the recombination construct. The recombination construct is flanked by two parts of the interrupted gene (T1-H1 or H2-T2), the middle part (H1 or H2) having been duplicated in order to enable homologous

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recombination to take place. The action of the DSBI enzyme (E) on the cleavage sites (S1 and S2) induces double-strand breaks and induces the homologous recombination between the homology sequences H1 and H2, whereby firstly the sequences located between H1 and H2 are deleted and secondly the intact gene is restored.

Fig. 6: Preferred embodiment

The figure shows a method which is identical to that described in Fig. 5, only that in the present case an endogenous gene is to be activated in a site-directed manner by introducing the recombination construct for example by means of homologous recombination.

15 Fig. 7a: Exemplary embodiment

The figure illustrates a specific embodiment of the method described in Fig. 6. A recombination construct is introduced via agrobacterium-mediated transfection. Flanked by the right and left border sequence (RB and LB, respectively), the construct comprises the interrupted reading frame of the GUS gene (β -glucuronidase) under the control of the 35S promotor (P) and the nopaline sythase [sic] (nos) terminator. The middle region of the GUS gene (U) was duplicated and constitutes the homology sequences A and B. Located between these sequences is the codA gene as negative selection marker under the control of the Cauliflower Mosaic Virus (CaMV) 35S promotor and the nopaline synthase (nos) terminator, flanked by two recognition sequences of the DSBI enzyme (S1 and S2). The recombination construct furthermore additionally comprises the BAR gene under the control of the 35S promotor (P) and the 35S terminator, as positive selection marker.

Fig.7a illustrates the appearance of double-strand breaks and the homologous recombination between the homologous U sequences, brought about by the action of the DSBI enzyme, whereby firstly the sequences located between the homologous U sequences are deleted and secondly the GUS gene is restored. The length of the Acc65I fragment is thus reduced from 7.3 kb to 3.7 kb.

Fig.7b: Shows the same system as described under Fig.7a. Fig.7a illustrates the appearance of double-strand breaks as the result of the action of the DSBI enzyme. In contrast to Fig.7a, no homologous recombination takes place in the present case, but illegitimate recombination by

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non-homologous end-joining. While the region between S1 and S2 is deleted owing to the two cleavage sites, the GUS gene is not restored. The length of the Acc65I fragment is thus reduced from 7.3 kb to 4.4 kb.

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Fig. 7c: The figure is another representation of the two end products of the processes described under Fig.7a and Fig.7b.

- 10 A: Result of the homologous recombination; Acc65I fragment has a length of 3.7 kb; the size of the fragment amplified with the aid of the primers OPN13 and OPN14 (shown by the arrows) is 0.7 kb.
- 15 B: Result of the illegitimate recombination (non-homologous end-joining); Acc65I fragment has a length of 4.4 kb; the size of the fragment amplified with the aid of the primers OPN13 and OPN14 (shown by the arrows) is 1.4 kb.

Fig. 8: Preferred embodiment

20 The recombination cassettes advantageously encompass both a positive and a negative selection marker (PS and NS, respectively), in each case under the control of a promoter. The positive selection marker is useful for facilitating and detecting the introduction of the construct into the genome. The negative selection marker is useful for detecting the deletion of the construct from the genome. Both markers are eliminated efficiently from the chromosomal DNA when they are flanked by the

25 homology sequences H1 and H2 and when in each case one cleavage site (S1 and S2, respectively) for a DSBI enzyme is located before and/or after the nucleic acid sequence to be deleted. Owing to the effect of the DSBI enzyme (E) on this recombination cassette, double-strand breaks appear at the cleavage sites S1 and/or S2 and the

30 sequences located between H1 and H2 are then eliminated.

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The effect of one of the abovementioned DSBI enzymes brings about site-directed double-strand breaks and induces the homologous recombination between the

40 homologous U sequences, whereby firstly the sequences located between the homologous U sequences are deleted and secondly the GUS gene is restored.

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Fig.9: Readily selectable systems for deleting a nucleic acid sequence from the chromosomal DNA of an organism. The constructs comprise a positive selection marker (PS) and negative selection marker (NS), in each case under the control of a promotor (P).

5 (B) additionally comprises an expression cassette for the DSBI enzyme, expression preferably taking place under the control of an inducible promotor (Pi).

(C) Further nucleic acid sequences may be present.

10 Expression of the DSBI enzyme leads in all cases to the elimination of the DNA sequences located between the two recognition sequences and to the recombination of the homologous sequences. Since the cells simultaneously lose a negative selection marker, the cells where successful deletion has taken place can be identified by means of selection (Gleave AP et al.(1999) Plant Mol Biol. 40:223-235).

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Fig. 10: The figure illustrates the two constructs (SI construct (A) and SD construct (B)) which was [sic] used for proving that homologous recombination by double-strand breaks can be induced with different restriction enzymes. The constructs are introduced via agrobacterium-mediated transfection. The constructs, which are flanked by the right and left border sequence (RB and LB, respectively) contain the interrupted reading frame of the GUS gene (β -glucuronidase) under the control of the 35S promotor (P) and the nopaline synthase (nos) terminator. The middle region of the GUS gene (U) was duplicated and constitutes the homology sequences A and B. Located between these sequences are, in the case of the SI construct (A), the recognition sequences of the DSBI enzymes I-SceI, I-CpaI, I-CpaII and I-CreI, and, in the case of the SD construct (B), the recognition sequence of the I-ChuI enzyme. The recombination constructs furthermore additionally contain the BAR gene under the control of a promotor (P) as positive selection marker.

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Fig. 11: Representative histochemical analysis of tobacco calli obtained after the induction of double-strand breaks. A blue coloration (here shown as dark coloration) indicates the expression of the β -glucuronidase gene, and thus the elimination of the selection marker by homologous recombination. Blue (dark colorations) can be seen in the case of the calli in the wells A2, A5, A6, B2, C1, C6 and D2.

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Fig. 12: PCR analysis for detecting homologous recombination. PCR on DNA from tobacco calli using the primers OPN13 and OPN14.

5 The PCR product (size 0.7 kb) which indicates homologous recombination can be seen in lanes 1, 2 and 3. The corresponding calli were blue following histochemical staining, and the corresponding PCR bands were sequenced in order to demonstrate that the open reading frame (ORF) of β -glucuronidase was indeed restored by homologous recombination.

10 Lanes 4 and 5: PCR products (1.4 kb) of calli which did not turn blue upon staining, where the transgene was eliminated by non-homologous end-joning.

15 Fig. 13: Southern blots which indicate the complete elimination of the transgene sequence in question.

The lanes of blots A to D comprise in each case:

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Lane	Line	Description	
1	GU.C.USB 1	Original line	
2	GU.C.USB 1-61	Non-homologous end-joining	
25	3	GU.C.USB 1-83	Homologous recombination
	4	GU.C.USB 3	Original line
	5	GU.C.USB 3-1	Non-homologous end-joining
	6	GU.C.USB 3-3	Homologous recombination
	7	GU.C.USB 7	Original line
30	8	GU.C.USB 7-14	Non-homologous end-joining
	9	GU.C.USB 7-34	Homologous recombination

A: HindIII-digested DNA hybridized with a β -glucuronidase-specific sample [sic].

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B: HindIII-digested DNA hybridized with a codA-specific sample [sic].

C: Acc65I-digested DNA hybridized with a β -glucuronidase-specific sample [sic].

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D: Acc65I-digested DNA hybridized with a codA-specific sample [sic].

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The analysis demonstrates that, following the induction of DNA double-strand breaks by means of expression of the restriction enzyme, not only with

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[sic] homologous recombination (lanes 3, 6 and 9), but also with [sic] illegitimate recombination (lanes 2, 5 and 8) may occur, the transgene sequence (codA) located between the restriction cleavage sites always having been eliminated from the plant genome.

Examples

General methods:

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The chemical synthesis of oligonucleotides can be effected for example in the known manner using the phosphoramidite method (Voet, Voet, 2nd edition, Wiley Press New York, pages 896-897). The cloning steps carried out for the purposes of the present invention, such as, for example, restriction cleavages, agarose [sic] gel electrophoresis, purification of DNA fragments, the transfer of nucleic acids to nitrocellulose and nylon membranes, the linkage of DNA fragments, the transformation of E. coli cells, bacterial cultures, the propagation of phages and the sequence analysis of recombinant DNA are carried out as described by Sambrook et al. (1989) Cold Spring Harbor Laboratory Press; ISBN 0-87969-309-6. Recombinant DNA molecules were sequenced using an ALF Express laser fluorescence DNA sequencer (Pharmacia, Upsala [sic], Sweden) following the method of Sanger (Sanger et al., Proc. Natl. Acad. Sci. USA 74 (1977), 5463-5467).

Example 1: Cloning of the homing endonucleases

The open reading frames (ORFs) of the homing endonucleases I-CreI (Wang J et al. (1997) Nucleic Acids Res 25: 3767-3776), I-ChuI (Cote V et al. (1993) Gene 129:69-76), I-CpaI (Turmel M et al. (1995a) Nucleic Acids Res 23:2519-2525) and I-CpaII (Turmel M et al. (1995b) Mol. Biol. Evol. 12, 533-545) were cloned from the corresponding Chlamydomonas strains.

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To ensure optimal translation of the gene, the ORFs of the endonucleases were linked to the leader sequence of a plant virus (CaMV gene V, as has proven useful in the case of I-SceI; Puchta H (1993) Nucl Acids Res 21:5034-5040). Also, a nuclear localization sequence (NLS2; SEQ ID NO: 30) was placed in front of the ORFs in order to efficiently transport the protein to the intended site of action. The two elements (leader sequence and nuclear localization sequence) were introduced via PCR by means of the oligonucleotide primers used.

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To isolate the open reading frames (ORFs) of the endonucleases from *Chlamydomonas*, the algal cultures *Chlamydomonas reinhardtii*/Smith (strain no. 11-32b), *Chlamydomonas applanata*/Lucksch (strain no.: 11-9) and *Chlamydomonas* 5 *segris*/King (strain no.: 9.83) were obtained from the algal culture collection at Göttingen (University of Göttingen, experimental phycology and collection of algal cultures, Albrecht-von-Haller Institute for Plant Sciences, Untere Karspüle 2, D-37073 Göttingen). The cultures were grown with the aid of a 10 shake culture in MS medium, and DNA was obtained using the DNeasy Plant Maxi Kit (Qiagen, Hilden).

The I-CreI ORF (GenBank Acc. No.: X01977) was amplified from a sample of the algal culture 11-32b *Chlamydomonas* 15 *reinhardtii*/Smith with the aid of the oligonucleotides OPN1 and OPN2 (SEQ ID NO: 11 and 12).

OPN1 (SEQ ID NO: 11):

5'-CGG CTC GAG CTA CGG GGA CGA TTT CTT TTT TTC AC- 3'

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OPN2 (SEQ ID NO: 12):

5'- CGG CTC GAG TAC CTA GAA TAC AAA GAA GAG GAA GAA GAA ACC TCT
ACA GAA GAA GCC ATG GGT CCA AAG AAA AAG AGA AAG GTT ATC AT GAA
TAC AAA ATA TAA TAA AGA GTT CTT ACT C 3'

25

2 µl (corresponding to approximately 100 ng DNA) of the DNA preparation were employed in the PCR reaction. The following were combined in a total volume of 50 µl in accordance with the manufacturer's instructions (Life Technologies):

30

5 µl 10X PCR buffer [200 mM Tris-HCl (pH 8.4), 500 mM KCl]
1.5 µl 50 mM MgCl₂
1 µl 10 mM dNTP mix (10 mM of each dATP, dCTP, dGTP and dTTP)
1 µl primer OPN1 (10 µM)
35 1 µl primer OPN2 (10 µM)
0.4 µl Taq DNA polymerase (5 U/µl)
2 µl DNA preparation
38.1 µl autoclaved distilled water

40 The reaction mixture is covered with approx. 50 µl of silicone oil and subjected to the following temperature program (Thermocycler: MWG Biotech Primus HT; MWG Biotech, Germany):

1 cycle of 180 sec at 95°C
45 30 cycles of 60 sec at 92°C, 60 sec at 54°C and 3 min at 72°C

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1 cycle of 5 min at 72°C.

The PCR fragment was purified via agarose gel electrophoresis using the QIAquick® Gel Extraction Kit (Qiagen, Hilden, Germany) and cloned into the pGEM-T Easy vector (Promega, Madison, USA). Next, a sequence analysis is carried out using the ALF-Express DNA sequencer (Pharmacia, Upsala, Sweden). The sequence is shown in SEQ ID NO: 5.

10 Cloning of the ORF of I-CpaI from the algal culture 9.83 *Chlamydomonas segrisi*/King (Genbank Acc. No.: L36830) was carried out analogously to the description given for I-CreI. The oligonucleotides OPN3 and OPN4 were used for the PCR. The sequence is shown in SEQ ID NO: 7.

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OPN3 (SEQ ID NO: 13):

5'-CGG CTC GAG TAC CTA GAA TAC AAA GAA GAG GAA GAA GAA ACC TCT
ACA GAA GAA GCC ATG GGT CCA AAG AAA AAG AGA AAG GTT ATC ATG GAC
ATT AAT CCT CAA TGG ATT ACA GG- 3'

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OPN4 (SEQ ID NO: 14):

5'-CGG CTC GAG TTA CTC GCC AGT TTC TTC AAA ACG-3'

Cloning the ORF of I-CpaII was also carried out analogously as described for I-CreI (Genbank Acc. No: L39865). A sample of the algal culture 9.83 *Chlamydomonas segrisi*/King was used for this purpose. The oligonucleotides OPN5 and OPN6 were used for the PCR. The sequence is shown in SEQ ID NO: 9.

30 OPN5 (SEQ ID NO: 15):

5'-CGG CTC GAG TAC CTA GAA TAC AAA GAA GAG GAA GAA GAA ACC TCT
ACA GAA GAA GCC ATG GGT CCA AAG AAA AAG AGA AAG GTT ATC ATG ACC
GAT TCT AAA TCT AGA AAC AAC-3'

35 OPN6 (SEQ ID NO: 16):

5'-CGG CTC GAG CTA AAG GTG GCC TTT ATT GCC ATC AG-3'

Cloning of the ORF of I-ChuI from the algal culture 11-9 *Chlamydomonas applanata*/Lucksch (Genbank Acc. No.: L06107) was carried out analogously to the description given for I-CreI. The oligonucleotides OPN7 and OPN8 were used for the PCR. The sequence is shown in SEQ ID NO: 3.

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OPN7 (SEQ ID NO: 17):

5'-CGG CTC GAG TAC CTA GAA TAC AAA GAA GAG GAA GAA GAA ACC TCT ACA
GAA GAA GCC ATG GGT CCA AAG AAA AAG AGA AAG GTT ATC ATG TCA TTA
ACA CAA CAA CAA AAA GAC-3'

5

OPN8 (SEQ ID NO: 18):

5'-CGG CTC GAG CTA AAG GTG GCC TTT ATT GCC ATC AG-3')

The ORF of the individual homing endonucleases (with the nuclear
10 localization signal) was in each case excised from the pGEM-T
Easy vector by means of restriction digestion with SallI, purified
by gel electrophoresis and in each case cloned into the SallI
restriction cleavage site of the binary vector pBinAR (Höfgen and
Willmitzer (1990) Plant Science 66:221-230). Expression of the
15 individual enzymes takes place under the control of the 35S
promotor and the octopine synthase terminator.

The binary I-SceI expression vector pCISceI (Puchta H et al.
(1996) Proc. Natl. Acad. Sci. USA 93:5055-5060) contains a
20 synthetic I-SceI ORF under the control of the CaMV 35S promotor
(Puchta H et al. (1993) Nucl Acids Res 21: 5034-5040) between the
T-DNA borders.

All of the five plasmids were multiplied in *E. coli*, purified by
25 means of the QIAfilter Plasmid Midi kit (Qiagen, Hilden) and
transferred into the agrobacterial strain C58 by means of
electroporation.

Example 2: Generation of the construct pGU.I.USB

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The plasmid pGU.US (Tinland B et al. (1994) Proc. Natl. Acad.
Sci. USA 91:8000-8004) was used for constructing the
recombination substrates. Within the T-DNA region, the plasmid
contain two overlapping halves of the β -glucuronidase (GUS) gene
35 with a 557 bp overlap. A hygromycin gene is integrated in a
unique XbaI cleavage site between the GUS sequences.

In a first step, the BAR gene together with promotor and
terminator sequences was excised from the vector pRC (Puchta H et
40 al. (1996) Proc Natl Acad Sci USA 93:5055-5060) in the form of an
isolated HindIII fragment, separated from the vector sequence via
agarose gel electrophoresis, excised from the gel, isolated with
the aid of the QIAquick® Gel Extraction Kit (Qiagen, Hilden,
Germany) and thereafter inserted into the unique HindIII cleavage
45 site of pGU.US. To this end, the vector pGU.US was previously cut
with HindIII and dephosphorylated with alkaline phosphatase (calf
intestinal alkaline phosphatase (CIP), New England Biolabs,

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Frankfurt, Germany) in order to prevent recircularization. The resulting vector is termed pGU.US-BAR.

In the vector pNE3 (Stougaard J (1993) Plant J 3:755-761), the XbaI cleavage site was first removed by a Klenow filling-in reaction. The open reading frame (ORF) of the negative selection marker gene cytosine deaminase (*codA*) under the control of the Cauliflower Mosaic Virus (CaMV) 35S promoter and of the nopaline synthase [sic] (*nos*) terminator was amplified from the resulting vector pNE3-XBA by means of PCR using the oligonucleotide primers ONP9 (SEQ ID NO: 16) and ONP10 (SEQ ID NO: 17). Owing to the oligonucleotide primers OPN9 and OPN10 used, in each case one I-SceI cleavage site (emphasized in bold in the sequences stated hereinbelow) and an NotI or XbaI cleavage site were added to the two ends of the amplificate.

OPN9 (SEQ ID NO: 19):

5'-CGG CTC TAG AGC GGC CGC CTA **GGG ATA ACA GGG TAA TAG AAT CCC**
ACA AAA ATC TGA GCT TAA CAG 3'

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OPN10 (SEQ ID NO: 20):

5'-CGG CTC TAG ACT **ATT ACC CTG TTA TCC CTA GGC CCG ATC TAG TAA**
CAT AGA TGA CAC CGC GCG CG 3'

25 2 μ l (corresponding to approximately 100 ng) of a plasmid preparation of pNE3-XBA were employed for the PCR reaction. The following were combined in a total volume of 50 μ l in accordance with the manufacturer's instructions (Life Technologies):

30 5 μ l 10X PCR buffer [200 mM Tris-HCl (pH 8.4), 500 mM KCl]
1.5 μ l 50 mM MgCl₂
1 μ l 10 mM dNTP mix (10 mM of each dATP, dCTP, dGTP and dTTP)
1 μ l primer OPN1 (10 μ M)
1 μ l primer OPN2 (10 μ M)
35 0.4 μ l Taq DNA polymerase (5 U/ μ l)
2 μ l plasmid preparation of pNE3-XBA
38.1 μ l autoclaved distilled water

The reaction mixture is covered with approx. 50 μ l of silicone oil and subjected to the following temperature program (Thermocycler: MWG Biotech Primus HT; MWG Biotech, Germany):

1 cycle of 180 sec at 95°C
30 cycles of 60 sec at 92°C, 60 sec at 54°C and 3 min at
45 72°C
1 cycle of 5 min at 72°C.

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The PCR product was digested with XbaI and NotI. The vector pGU-US-BAR was likewise digested with XbaI and NotI (which resulted in the deletion of the hygomycin marker gene), and the vector fragment was purified by agarose gel electrophoresis using the QIAquick® Gel Extraction Kit (Qiagen, Hilden, Germany).
5 Ligation of the digested PCR fragment and vector gave rise to the binary vector pGU.C.USB (see Fig. 7a). The vector contains a marker gene (cytosine deminase (codA)) on a T-DNA between two I-SceI cleavage sites. The I-SceI cleavage sites are outwardly
10 flanked by homologous sequence regions 557 bp in size of the β -glucuronidase gene (GUS). The GUS gene acts as homologous restoration marker (Swoboda P et al. (1994) EMBO J 13:481- 489). If the gene is restored by homologous recombination, the expression can be detected histochemically. Elimination of the
15 marker gene gives rise to 5-FC (fluorocytosine)-resistant tobacco cells, which can then regenerate it to give calli (Salomon S and Puchta H (1998) EMBO J 17:6086-6095).

Example 3: Plant transformation with pGU.I.USB

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Nicotiana tabacum L. cv. Petite Havana Line SR1 seedlings were transformed with the agrobacterial strain C58, which contained the binary vector pGU.C.USB.

25 To this end, seeds were placed on moistened filter paper under sterile conditions and the seedlings were harvested after 2 weeks, all as described by Puchta H. (1999) Methods Mol Biol 113: 447-451 (25°C, 16 hours light/8 hours dark rhythm).

30 For the inoculation, the agrobacterial strain containing the binary transformation plasmid was first grown overnight in a shake culture at 28°C in YEB medium. Then, the agrobacterial suspension was centrifuged for 10 minutes at 15.000 g and the cells were taken up in in 10 mM MgSO₄ so that the final optical
35 density of the suspension had a value of approximately 0.5. In a reaction vessel, the seedlings were then placed into the bacterial solution under sterile conditions and a vacuum of 0.15 at was applied in a sterile desiccator. After 10 minutes, the seedlings were then placed on MS plates supplemented with BAP
40 (6-benzylaminopurine 5 µg/ml) and NAA (1-naphthaleneacetic acid 0.5 µg/ml) and left for 3 days in a growth cabinet (25°C, 16 hours light/8 hours dark rhythm). The seedlings were then placed on MS medium supplemented additionally with phosphinothricin (100 µg/ml), vancomycin (1 µg/ml) and cefotaxim (0.5 µg/ml) in
45 addition to NAA and BAP. Every 10 days, the seedlings were transferred to freshly made plates. Eventually, the resulting calli formed shoots. As soon as the shoots had attained a certain

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size (1 to 2 cm), they were excised from the callus material and planted in magenta boxes comprising MS medium supplemented with phosphinothricin, vancomycin and cefotaxin (concentrations as above). After a short time, the shoots developed roots; they were transferred into soil after 2 to 4 weeks. The plants were made to flower in the greenhouse and were then selfed, and the seeds formed were left to ripen in the capsules. The seeds were then placed on MS medium supplemented with 300 µg of phosphinothricin (for the positive selection) or 500 µg of 5-FC (fluorocytosin; for the negative selection) per ml in order to carry out the segregation analyses. By determining the ratio of resistant to sensitive seedlings (3:1 in the case of positive selection and 1:3 in the case of negative selection), it was possible to demonstrate that the recombination constructs were inserted at a locus in the three selected lines.

Example 5: Induction of gene deletion by introducing the DSBI enzyme I-SceI

In the experiments, F1 seedlings of the transgenic lines GU.C.USB 1, 3 and 7, each of which comprises a copy of the T-DNA GU.C.USB shown in Fig. 2, were inoculated with an agrobacterial strain which transiently expressed I-SceI and which comprised the plasmid pCISceI (Puchta H et al. (1996) Proc Natl Acad Sci USA 93, 5055-5060) in the abovedescribed manner (see also Puchta, 1999b). After 3 days, the seedlings were plated on MS medium supplemented with BAP and NAA (concentrations as above) medium to the same medium additionally in the presence of 100 µg of 5-FC and 100 µg of phosphinothricin per ml incubated in order to detect plant cells in which the marker gene to be eliminated (in this case the *codA* gene) was deleted. After 6 weeks, the calli growing on the medium were divided into two, and one part was used for the regeneration of shoot axes while the other was used for isolating DNA and for the β-glucuronidase assay. The resulting 5-FC-resistant transgenic calli were analyzed for homologous recombination events by means of histochemical staining. A blue staining indicated restoration of the callus (see Fig. 11).

The histochemical staining of the calli was carried out as described by Swoboda et al., 1994. To this end, the calli were introduced into staining solution (0.3 mg X-Gluc [Duchefa, Harlem, NL] per ml of 100 mM sodium phosphate buffer pH 7.0; 0.1% Triton; 0.05% NaN₃). A vacuum was applied for 15 minutes to the desiccator, and the calli were subsequently incubated in the solution for 48 hours at 37°C. After the staining solution was poured off, the remaining chlorophyll was removed from the plant

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material by repeated shaking in 80% ethanol. The blue staining obtained indicated the β -glucuronidase activity.

In approximately one quarter of the cases, the marker gene was eliminated successfully by homologous recombination (Fig. 11, Table 2).

Table 2. Number of 5-FC-resistant tobacco calli following transient DSB induction

10

Transgenic line	Seedlings	resistant calli	GUS-positive	GUS-positive (% of resistant calli)
15 GU.C.USB 1	290	56	22	39
GU.C.USB 3	490	90	24	27
GU.C.USB 7	370	59	11	19

20 Molecular analyses confirm the facts: since the line GU.C.USB 1 contained a single copy of the transgene, the calli were analyzed directly for recombination results by means of PCR.

A random fraction of calli was then analyzed at the molecular level by means of PCR. Molecular analysis with the primer pairs

OPN11 (SEQ ID NO: 21)

5'- CGG AAG CTT CGT CAC CAA TCC CAA TTC GAT CTA C - 3' and

30 OPN12 (SEQ ID NO: 22)

5'- CGG AAG CTT CCA CTT GCA AAG TCC CGC TAG TGC C - 3'

allowed the isolation of the newly-formed linkage sites from the tobacco genome (Fig. 12; Table 3).

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Table 3. Molecular analysis of recombination events by means of PCR

Transgenic line	Calli	PCR fragment(s)		
		0.7 kb	1.4 kb	none/other
40 GU.C.USB 1	30	10	12	7

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Three 0.7 kb PCR fragments were selected and sequenced. In all three cases, sequencing confirmed the functional sequence of the β -glucuronidase gene, i.e. the restoration of the gene did indeed take place accurately by homologous recombination.

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When five 1.4 kb PCR bands were sequenced, it was found that these bands were formed after excision of the *codA* gene by reparation of the two I-SceI cleavage sites (by non-homologous end-joining, NHEJ) without homologous recombination taking place.

10 In most cases, minor deletions at the I-SceI cleavage site resulted.

Southern blots demonstrated that, as expected, complete elimination of the sequence located between the I-SceI cleavage sites took place in the recombinants with the 0.7 and 1.4 kb bands, respectively. No *codA*-specific DNA whatsoever was detectable any longer in the genome of the regenerated plants (Fig. 13 B and D, lanes 2 and 3).

20 The DNA was isolated with the aid of the DNeasy Plant Mini Kit (Quiagen, Hilden). To detect the recombination products, genomic DNA was analyzed by means of PCR using the oligonucleotides OPN13 and OPN14.

25 OPN13 (SEQ ID NO: 23):

5'- CGG AAG CTT CGT CAC CAA TCC CAA TTC GAT CTA C - 3'

OPN14 (SEQ ID NO: 24):

5'- CGG AAG CTT CCA CTT GCA AAG TCC CGC TAG TGC C - 3'

30

5 μ l 10X PCR buffer [200 mM Tris-HCl (pH 8.4), 500 mM KCl]

1.5 μ l 50 mM MgCl₂

1 μ l 10 mM dNTP mix (10 mM of each dATP, dCTP, dGTP and dTTP)

1 μ l primer OPN1 (10 μ M)

35 1 μ l primer OPN2 (10 μ M)

0.4 μ l Taq DNA polymerase (5 U/ μ l)

2 μ l DNA preparation

38.1 μ l autoclaved distilled water

40 The reaction mixture is covered with approx. 50 μ l of silicone oil and subjected to the following temperature program (Thermocycler: MWG Biotech Primus HT; MWG Biotech, Germany):

1 cycle of 180 sec at 95°C

45 30 cycles of 60 sec at 92°C, 60 sec at 54°C and 3 min at 72°C

1 cycle of 5 min at 72°C.

The PCR products were sequenced using the "ABI Prism Dye Terminator Cycle Sequencing Reaction Kit" (PE Applied Biosystems, Weiterstadt).

For the Southern blotting, the DNA was cut with HindIII or Acc65I and subjected to electrophoresis in a 0.8% agarose gel. The DNA in the gel was then transferred to the hybridization membrane 'Hybond N' (Amersham, Little Chalfont, UK) by means of capillary blotting as described in the manufacturer's instructions. For the molecular hybridization, *codA*- or GUS-specific gene fragments were isolated from the starting plasmids (XbaI/XhoI fragment as PNE3; Stougaard, 1993, and KpnI/SacI fragment from pGUS23, Puchta and Hohn, 1991, isolated using the QIAquick Gel Extraction Kit [Qiagen, Hilden]) and labeled with the aid of a "Random Priming Labeling Kit" (Megaprime DNA labeling system RPN1607, Amersham, Little Chalfont, UK) and [α -³²P]dATP (Amersham, Little Chalfont, UK). The hybridization reactions were carried out at 65° C.

Since in each case 2 genetically linked transgene copies were integrated in the case of lines GU.C.USB 3 and GU.C.USB 7, a representative number of plants was regenerated from callus in the case of these lines, DNA was obtained and then analyzed per Southern blot (Table 4).

In the case of Acc65I, the presence of a GUS-specific 3.7 kb band suggests a homologous recombination, while a 4.4 kb band suggests an NHEJ event ("non-homologous end-joining"; NHEJ) (Fig. 7b and c; Fig. 13 C).

Table 4. Molecular analysis of recombination events by means of Southern blots

Transgenic line	Calli	Acc65I fragment (kb)		
		3.7	4.4	Deletion
GU.C.USB 3	39	6	18	15
GU.C.USB 7	14	2	5	7

Interestingly, the same type of linkage in the two transgene copies was found in all cases. In other words, either only homologous recombinations or only NHEJ events occurred. In no case did both possibilities exist in parallel, i.e. for example a homologous recombination in the one transgene and an NHEJ event in the other.

* Trademark

64

In both lines, PCR analyses were also carried out, and in each case three 0.7 kb PCR fragments were selected and sequenced. In all three cases, sequencing revealed the functional sequence of the β -glucuronidase gene, i.e. the restoration of the gene did indeed occur by means of homologous recombination.

When a total of nine 1.4 kb PCR bands of the two lines were sequenced, it was furthermore found that these bands indeed originated after excision of the *codA* gene by repair of the two I-SceI cleavage sites (by "non-homologous end-joining" NHEJ). Again, minor deletions resulted at the I-SceI cleavage site in most cases.

Southern blots demonstrated that, as expected, the sequence between the I-SceI cleavage sites were eliminated completely in the recombinant. No *codA*-specific DNA whatsoever was detected any longer in the genome of the regenerated plants (Fig. 13 B and D, lanes 5, 6 and 8, 9).

20 Example 5:

Various transgenic tobacco plant lines were generated which, between the halves of the β -glucuronidase gene (arrangement as described above) also contained cleavage sites for the abovementioned restriction enzymes in addition to a I-SceI cleavage site by means of cloning synthetic oligonucleotides (Fig. 10). Seedlings of this tobacco line were inoculated in each case in direct comparison with agrobacteria capable of expressing either I-SceI or the corresponding enzyme in plant cells. The resulting calli were then stained histochemically after 2 weeks. The results are shown in Table 4.

The plasmid pGU.C.US.B was cut with I-SceI so that the *codA* gene was excised from the plasmid. The digested DNA was separated by means of agarose gel electrophoresis, the larger band was excised and purified by means of the QIAquick Gel Extraction Kit (Qiagen, Hilden) and subsequently ligated and transformed into *E. coli*. The resulting plasmid was then cut with XbaI.

The complementary single-stranded oligonucleotides OPN25 and OPN26 were made double-stranded by briefly heating to 92°C and subsequent cooling and then subsequently ligated with the XbaI-cut plasmid. The resulting SI construct (pSI) contains the cleavage sites for I-SceI, I-CpaI, I-CpaII and I-CreI ((see Fig. 10 (A)).

65

OPN15 (SEQ ID NO: 25):

5'-CTA GTA CAA AAC GTC GTG AGA CAT TTT AAT CTG AAG GTT TGG CAC
CTC GAT GTC GGC TCA TC-3'

5 OPN16 (SEQ ID NO: 26):

5'-CTA GGA TGA GCC GTC ATC GAG GTG CCA AAC CTT CAG ATT AAA ATG
TCT CAC GAC GTT TTG TA-3'

The complementary single-stranded oligonucleotides OPN27 and
10 OPN28 were made double-stranded by briefly heating to 92°C and
subsequent cooling and then subsequently ligated with the
XbaI-cut plasmid. The resulting SD construct (pSD) contains the
cleavage sites for I-SceI and I-ChuI (see Fig. 10 (B)).

15 OPN17 (SEQ ID NO: 27):

5'-CTA GTC CGA AAA CGC CGT GAG ACA TAT TGG TTA CGA TCC TAA GGT
AGC GAA ATT CAC CCG GTA ACT CTG TGC CAG-3'

OPN18 (SEQ ID NO: 28):

20 5'-CTA GCT GGC ACA GAG TTA CCG GGT GAA TTT CGC TAC CTT AGG ATC
GTA ACC AAT ATG TCT CAC GGC GTT TTC GGA-3'

Transgenic tobacco plants with the two constructs were generated
as described further above by means of agrobacterium
25 transformation. Lines which only contained transgenic sequences
at one locus were used for the further experiments. These lines
were determined by the 3:1 segregation into
phosphinothricin-resistant and nonresistant plants. The selfed
seedlings were then inoculated with agrobacterial strains which
30 comprised one of the four constructs for expressing the
restriction endonucleases or, as the vector control, the plasmid
BinAR or, as the positive control, a 1:1 mixture of BinAR and
CISce-I. The inoculations were carried out as described above
(Puchta H (1999) Methods Mol. Biol. 113:447-451), and for
35 selection purposes the seedlings were grown over several weeks on
MS medium supplemented with 100 µg kanamycin per ml, which also
contained BAP and NAA, vancomycin and cefotaxin (concentrations
as above). The resulting calli were then subjected to
histochemical β-glucuronidase staining as described above.

40

All four of the tested restriction enzymes were capable of
inducing homologous recombination in the same order of magnitude
as I-SceI (which was employed here in a coinoculation with the
selection vector pBinAR [AR]) (Table 5). This demonstrates that
45 homologous recombination can be induced efficiently when using
any restriction endonucleases.

66

Table 5. Induction of homologous recombination in plants by means of various endonucleases I-CreI, I-CpaI, I-CpaII and I-ChuI. [Sectors/calli] refers to the number of areas stained blue in the resistant calli.

5

Transgenic line	Enzyme	Sectors/calli	Ratio
SI5	I-SceI/AR	42/31	1.35
	I-CreI	77/50	0.54
	I-CpaII	51/50	1.02
SI2	I-SceI/AR	8/9	0.89
	I-CreI	40/18	2.22
	I-CpaII	9/20	0.45
SI2	I-CpaI	144/106	1.36
SD2	I-ChuI	166/100	1.66

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45

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<110> SunGene GmbH & Co. KGaA
 Institut F. Pflanzengenetik

<120> Recombination systems and a method for removing
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 of eukaryotic organisms

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<141> 2002-07-02

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aaa ctg ctg aaa gaa tac aaa tcc cag ctg atc gaa ctg aac atc gaa 157
Lys Leu Leu Lys Glu Tyr Lys Ser Gln Leu Ile Glu Leu Asn Ile Glu
          20          25          30
cag ttc gaa gca ggt atc ggt ctg atc ctg ggt gat gct tac atc cgt 205
Gln Phe Glu Ala Gly Ile Gly Leu Ile Leu Gly Asp Ala Tyr Ile Arg
          35          40          45
tct cgt gat gaa ggt aaa acc tac tgt atg cag ttc gag tgg aaa aac 253
Ser Arg Asp Glu Gly Lys Thr Tyr Cys Met Gln Phe Glu Trp Lys Asn
          50          55          60
aaa gca tac atg gac cac gta tgt ctg ctg tac gat cag tgg gta ctg 301
Lys Ala Tyr Met Asp His Val Cys Leu Leu Tyr Asp Gln Trp Val Leu
          65          70          75          80
tcc ccg ccg cac aaa aaa gaa cgt gtt aac cac ctg ggt aac ctg gta 349
Ser Pro Pro His Lys Lys Glu Arg Val Asn His Leu Gly Asn Leu Val
          85          90          95
atc acc tgg ggc gcc cag act ttc aaa cac caa gct ttc aac aaa ctg 397
Ile Thr Trp Gly Ala Gln Thr Phe Lys His Gln Ala Phe Asn Lys Leu
          100          105          110
gct agc ctg ttc atc gtt aac aac aaa aaa acc atc ccg aac aac ctg 445
Ala Ser Leu Phe Ile Val Asn Asn Lys Lys Thr Ile Pro Asn Asn Leu
          115          120          125
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gta ctg aac acc cag tct ttc act ttc gaa gaa gta gaa tac ctg gtt 589
Val Leu Asn Thr Gln Ser Phe Thr Phe Glu Glu Val Glu Tyr Leu Val
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aag ggt ctg cgt aac aaa ttc caa ctg aac tgt tac cta aaa atc aac 637
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aaa aac aaa ccg atc atc tac atc gat tct atg tct tac ctg atc ttc 685
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ccg aac act atc tcc tcc gaa act ttc ctg aaa taataagtcg agtactggat 786
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788

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      35              40              45
Ser Arg Asp Glu Gly Lys Thr Tyr Cys Met Gln Phe Glu Trp Lys Asn
      50              55              60
Lys Ala Tyr Met Asp His Val Cys Leu Leu Tyr Asp Gln Trp Val Leu
      65              70              75              80
Ser Pro Pro His Lys Lys Glu Arg Val Asn His Leu Gly Asn Leu Val
      85              90              95
Ile Thr Trp Gly Ala Gln Thr Phe Lys His Gln Ala Phe Asn Lys Leu
      100              105              110
Ala Ser Leu Phe Ile Val Asn Asn Lys Lys Thr Ile Pro Asn Asn Leu
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Val Glu Asn Tyr Leu Thr Pro Met Ser Leu Ala Tyr Trp Phe Met Asp
      130              135              140
Asp Gly Gly Lys Trp Asp Tyr Asn Lys Asn Ser Thr Asn Lys Ser Ile
145              150              155              160
Val Leu Asn Thr Gln Ser Phe Thr Phe Glu Glu Val Glu Tyr Leu Val
      165              170              175
Lys Gly Leu Arg Asn Lys Phe Gln Leu Asn Cys Tyr Leu Lys Ile Asn
      180              185              190
Lys Asn Lys Pro Ile Ile Tyr Ile Asp Ser Met Ser Tyr Leu Ile Phe
      195              200              205
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Gly Ser Val Gly Arg Thr Trp Arg Tyr Arg Ala Leu His Lys Ser Glu
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cat cag aca tac tta ttt cat aag tat gaa atc tta aag ccg ctt tgt      248
His Gln Thr Tyr Leu Phe His Lys Tyr Glu Ile Leu Lys Pro Leu Cys
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Gly Glu Asn Thr Leu Pro Thr Glu Ser Ile Val Phe Asp Glu Arg Thr
                    70                      75                      80
aac aag gag gtt aaa cgt tgg ttt ttc aac aca tta acc aat cct tcc      344
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                    85                      90                      95
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Lys Trp Val Lys Asp Val Pro Val Lys Val Gln Thr Phe Leu Thr Pro
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aat aag tct aac gct atg caa att tgt act gaa agt ttc agt caa ggg      536
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ata gct att cct gaa gcc agt agc ggt gct ttt cgt gaa gtc att aaa      680
Ile Ala Ile Pro Glu Ala Ser Ser Gly Ala Phe Arg Glu Val Ile Lys
                    195                      200                      205
cct ttt cta gtt gat tgt atg aga tac aaa gtt tct gat ggc aat aaa      728
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          35          40          45
Glu His Gln Thr Tyr Leu Phe His Lys Tyr Glu Ile Leu Lys Pro Leu
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Cys Gly Glu Asn Thr Leu Pro Thr Glu Ser Ile Val Phe Asp Glu Arg
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Thr Asn Lys Glu Val Lys Arg Trp Phe Phe Asn Thr Leu Thr Asn Pro
          85          90          95
Ser Leu Lys Phe Phe Ala Asp Met Phe Tyr Thr Tyr Asp Gln Asn Thr
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Pro Gln Ala Leu Ala Tyr Phe Tyr Ile Asp Asp Gly Ala Leu Lys Trp
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Leu Asn Lys Ser Asn Ala Met Gln Ile Cys Thr Glu Ser Phe Ser Gln
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Gly Gly Thr Ile Arg Ile Gln Lys Ala Leu Lys Thr Leu Tyr Asn Ile
          165          170          175
Asp Thr Thr Leu Thr Lys Lys Thr Leu Gln Asp Gly Arg Ile Gly Tyr
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Arg Ile Ala Ile Pro Glu Ala Ser Ser Gly Ala Phe Arg Glu Val Ile
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gac aaa tta gtg gat gaa att ggg gtt ggt tat gta aga gat agg ggt 297
Asp Lys Leu Val Asp Glu Ile Gly Val Gly Tyr Val Arg Asp Arg Gly
              70              75              80
agc gtt tcg gat tat att cta agc gaa atc aag cct ttg cat aat ttt 345
Ser Val Ser Asp Tyr Ile Leu Ser Glu Ile Lys Pro Leu His Asn Phe
              85              90              95
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aat gat tcg aag acg cgt aaa aca act tct gaa acc gtt cgt gct gtg 537
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Leu Ser Leu Ala Phe Gln Val Thr Gln Lys Thr Gln Arg Arg Trp Phe
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Gly Ser Val Ser Asp Tyr Ile Leu Ser Glu Ile Lys Pro Leu His Asn
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 135 140 145
 gag aaa aac tac cag gaa tct atc aaa cgt ttt gaa gaa act ggc gag 537
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 Asp Tyr Phe Lys Val Gly Ser Val Val Val Asn His Gly Glu Arg Leu
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 Cys Tyr Lys Val Lys Asn Ile Asp His Phe Ile Thr Val Ile Ile Pro
 85 90 95
 Phe Phe Glu Lys His Glu Leu Lys Thr Lys Arg Arg Ile Glu Phe Leu
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 Arg Phe Arg Lys Ile Cys Leu Leu Leu Lys Ala Gly Arg His Leu Glu

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Gly	Glu															

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Pro	Lys	Lys	Lys	Arg	Lys	Val	Ile	Met	Thr	Asp	Ser	Lys	Ser	Arg	Asn	
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Asn	Asn	Asn	Phe	Leu	Ser	Asn	Asn	Leu	Leu	Pro	Leu	Thr	Asp	Asp	Glu	
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Lys	Ala	Leu	Ile	Ala	Gly	Thr	Leu	Leu	Gly	Asp	Ala	His	Ile	Gln	Lys	
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Arg	Gly	Asp	Ser	Tyr	Arg	Leu	Lys	Ile	Ala	His	Gly	Leu	Asp	His	Glu	
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gag	ctt	gtc	gtc	tgg	aag	tat	aac	cgt	tta	atc	agg	ttg	tgt	caa	aca	298
Glu	Leu	Val	Val	Trp	Lys	Tyr	Asn	Arg	Leu	Ile	Arg	Leu	Cys	Gln	Thr	
			70				75						80			
aca	caa	ccc	cca	agg	gtg	gaa	acc	tac	tca	aca	aag	tta	aag	tct	ggc	346
Thr	Gln	Pro	Pro	Arg	Val	Glu	Thr	Tyr	Ser	Thr	Lys	Leu	Lys	Ser	Gly	
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Val	Leu	Pro	Gln	Gly	Val	Val	Phe	Tyr	Thr	Ser	Ser	Gly	Lys	Tyr	Leu	
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Leu	Val	Leu	Ala	Ala	Phe	Phe	Met	Asp	Asp	Gly	Ser	Val	Arg	Ser	Asp	
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Cys	Tyr	Ser	Gly	Lys	Ile	Ala	Thr	Pro	Gly	Phe	Ala	Gly	Lys	Glu	Glu	
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Ser	Gln	Leu	Leu	Cys	Asn	Tyr	Leu	His	Ser	Trp	Asp	Val	Gln	Ala	Asn	
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His Glu Glu Leu Val Val Trp Lys Tyr Asn Arg Leu Ile Arg Leu Cys
 65                70                75                80
Gln Thr Thr Gln Pro Pro Arg Val Glu Thr Tyr Ser Thr Lys Leu Lys
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Ser Gly Val Leu Pro Gln Gly Val Val Phe Tyr Thr Ser Ser Gly Lys
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His Pro Leu Val Leu Ala Ala Phe Phe Met Asp Asp Gly Ser Val Arg
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Ser Asp Cys Tyr Ser Gly Lys Ile Ala Thr Pro Gly Phe Ala Gly Lys
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Leu Pro Ala Lys Thr Phe Gly Arg Phe Ile Asn Ile Ile Glu Pro Tyr
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WHAT IS CLAIMED IS:

1. A recombination system which comprises, in a plant cell,
- I) a transgenic recombination construct inserted into the chromosomal DNA of the plant cell comprising a sequence consisting, in the 5'/3'-direction, of
- a1) a first homology sequence A;
- b1) at least one recognition sequence (b) for the site-directed induction of DNA double-strand breaks; and
- a2) a second homology sequence B, the homology sequences A and B having a sufficient length and sufficient homology in order to ensure homologous recombination,

10

together with

- II) an enzyme suitable for inducing DNA double-strand breaks at the recognition sequence (b) for the site-directed induction of DNA double-strand breaks or a nucleic acid sequence encoding an enzyme suitable for inducing DNA double-strand breaks at the recognition sequence (b),

wherein all recognition sequences for site-directed induction of DNA double-strand breaks are located between homology sequences A and B;

wherein the homology sequences A and B have at least 20 base pairs and at least 70% homology that allow for homologous recombination to each other; and

20

wherein the enzyme suitable for inducing DNA double-strand breaks at the recognition sequence for the site-directed induction of DNA double-strand breaks is an homing endonuclease, a chimeric nuclease or a combination thereof.

2. The recombination system as claimed in claim 1, wherein the recombination construct is constructed as follows:

- a1) the first homology sequence A;
- b1) the recognition sequence for the site-directed induction of DNA double-strand breaks;

- c) a further nucleic acid sequence; and
- a2) the second homology sequence B, the homology sequences A and B having a sufficient length and sufficient homology in order to ensure homologous recombination.

3. The recombination system as claimed in claim 2, wherein the recombination construct is constructed as follows:

- a1) the first homology sequence A;
- b1) the first recognition sequence for the site-directed induction of DNA double-strand breaks;
- 10 c) the further nucleic acid sequence;
- b2) a second recognition sequence for the site-directed induction of DNA double-strand breaks; and
- a2) the second homology sequence B, the homology sequences A and B having a sufficient length and sufficient homology in order to ensure homologous recombination.

4. The recombination system as claimed in any one of claims 1 to 3, wherein the recombination construct or the further nucleic acid sequence encompasses at least one of the following elements

- i) positive selection markers;
- 20 ii) negative selection markers;
- iii) reporter genes;
- iv) replication origins;
- v) multiple cloning regions;
- vi) border sequences for Agrobacterium transfection;
- vii) sequences which enable homologous recombination or insertion into the genome of a host organism; or

- viii) expression cassette for the enzyme suitable for inducing DNA double-strand breaks at the recognition sequence for the site-directed induction of DNA double-strand breaks.

5. The recombination system as claimed in any one of claims 1 to 4, wherein the enzyme suitable for inducing DNA double-strand breaks at the recognition sequence for the site-directed induction of DNA double-strand breaks is one of the following homing endonucleases: F-Scel, F-Scell, F-Suvl, F-Tevl, F-Tevll, I-Amal, I-Anil, I-Ceul, I-CeuAIIIP, I-Chul, I-Cmoel, I-Cpal, I-Cpall, I-Crel, I-CrepsbIP, I-CrepsbIIP, I-CrepsbIIIP, I-CrepsbIIVP, I-Csml, I-Cvul, I-CvuAIP, I-Ddil, I-Ddill, I-Dirl, I-Dmol, I-Hmul, I-Hmull, I-HspNIP, I-Llal, I-Msol, I-Naal, I-Nanl, I-NclIP, I-NgrIP, I-Nitl, I-Njal, I-Nsp236IP, I-Pakl, I-PbolP, I-PculP, I-PcuAl, I-PcuVI, I-PgrIP, I-PoblP, I-Porl, I-PorIIP, I-PpbIP, I-Ppol, I-SPBetaIP, I-Scal, I-Scel, I-Scell, I-Scelll, I-ScelV, I-SceV, I-SceVI, I-SceVII, I-SexIP, I-SnelP, I-SpomCP, I-SpomIP, I-SpomIIP, I-SqulP, I-Ssp6803I, I-SthPhiJP, I-SthPhiST3P, I-SthPhiS3bP, I-TdelP, I-Tevl, I-Tevll, I-Tevlll, I-UarAP, I-UarHGPA1P, I-UarHGPA13P, I-VinIP, I-ZbilP, PI-Mtul, PI-MtuHIP, PI-MtuHIIP, PI-Pful, PI-Pfull, PI-Pkol, PI-Pkoll, PI-Pspl, PI-Rma43812IP, PI-SPBetaIP, PI-Scel, PI-Tful, PI-Tfull, PI-Thyl, PI-Tlil or PI-TIill.

6. The recombination system as claimed in any one of claims 1 to 5, wherein the enzyme suitable for inducing DNA double-strand breaks at the recognition sequence for the site-directed induction of DNA double-strand breaks is one of the homing endonucleases as shown in SEQ ID NO: 2, 4, 6, 8 or 10.

7. The recombination system as claimed in any one of claims 1 to 6, wherein the enzyme suitable for inducing DNA double-strand breaks at the recognition sequence for the site-directed induction of DNA double-strand breaks is encoded by the expression cassette and expressed in the plant cell, using an expression cassette encompassing a nucleic acid sequence encoding said enzyme.

8. The recombination system as claimed in any one of claims 1 to 7, wherein the enzyme suitable for inducing DNA double-strand breaks at the recognition sequence for the site-directed induction of DNA double-strand breaks is encoded by the expression cassette and expressed in the plant cell, using an expression cassette encompassing a nucleic acid sequence as shown in SEQ ID NO: 1, 3, 5, 7 or 9.

9. A method for removing a DNA sequence from the chromosomal DNA of a plant cell, which comprises combining, in the plant cell,

10 I) a transgenic recombination construct inserted into the chromosomal DNA of the plant cell comprising a sequence consisting, in the 5'/3'-direction, of

a1) a first homology sequence A;

b1) at least one recognition sequence (b) for the site-directed induction of DNA double-strand breaks; and

a2) a second homology sequence B, the homology sequences A and B having a sufficient length and sufficient homology in order to ensure homologous recombination,

together with

20 II) an enzyme suitable for inducing DNA double-strand breaks at the recognition sequence (b) for the site-directed induction of DNA double-strand breaks,

and the induction of DNA double-strand breaks at the recognition sequence for the site-directed induction of DNA double-strand breaks and the homologous recombination taking place between the homology sequences A and B,

wherein all recognition sequences for site-directed introduction of DNA double-strand breaks are located between homology sequences A and B;

wherein the homology sequences A and B have at least 20 base pairs and at least 70% homology that allows for homologous recombination to each other; and

wherein the enzyme suitable for inducing DNA double-strand breaks at the recognition sequence for the site-directed induction of DNA double-strand breaks is an homing endonuclease, a chimeric nuclease or a combination thereof.

10. The method as claimed in claim 9, wherein the recombination construct is constructed as follows:

- a1) the first homology sequence A;
- b1) the first recognition sequence for the site-directed induction of DNA double-strand breaks;
- c) a further nucleic acid sequence; and
- 10 a2) the second homology sequence B, the homology sequences A and B having a sufficient length and sufficient homology in order to ensure homologous recombination.

11. The method as claimed in claim 10, wherein the recombination construct is constructed as follows:

- a1) the first homology sequence A;
- b1) the first recognition sequence for the site-directed induction of DNA double-strand breaks;
- c) the further nucleic acid sequence;
- 20 b2) the second recognition sequence for the site-directed induction of DNA double-strand breaks; and
- a2) the second homology sequence B, the homology sequences A and B having a sufficient length and sufficient homology in order to ensure homologous recombination.

12. The method as claimed in any one of claims 9 to 11, wherein the recombination construct or the further nucleic acid sequence encompasses at least one of the following elements:

- i) positive selection markers;
- ii) negative selection markers;

- iii) reporter genes;
- iv) replication origins;
- v) multiple cloning regions;
- vi) border sequences for *Agrobacterium* transfection;
- vii) sequences which enable homologous recombination or insertion into the genome of a host organism; and
- viii) expression cassette for the enzyme suitable for inducing DNA double-strand breaks at the recognition sequence for the site-directed induction of DNA double-strand breaks.

10 13. The method as claimed in any one of claims 9 to 12, wherein the enzyme suitable for inducing DNA double-strand breaks at the recognition sequence for the site-directed induction of DNA double-strand breaks is one of the following homing endonucleases: F-Scel, F-Scell, F-Suvl, F-Tevl, F-Tevll, I-Amal, I-Anil, I-Ceul, I-CeuAIIIP, I-Chul, I-Cmoel, I-Cpal, I-Cpall, I-Crel, I-CrepsbIP, I-CrepsbIIP, I-CrepsbIIIP, I-CrepsbIVP, I-Csml, I-Cvul, I-CvuAIP, I-Ddil, I-Ddill, I-Dirl, I-Dmol, I-Hmul, I-Hmull, I-HspNIP, I-Llal, I-Msol, I-Naal, I-Nanl, I-NclIP, I-NgrIP, I-Nitl, I-Njal, I-Nsp236IP, I-Pakl, I-PbolIP, I-PculP, I-PcuAl, I-PcuVI, I-PgrIP, I-PoblP, I-Porl, I-PorIIP, I-PpbIP, I-Ppol, I-SPBetaIP, I-Scal, I-Scel, I-Scell, I-Scelll , I-SceIV, I-SceV, I-SceVI, I-SceVII, I-SexIP, I-SnelP, I-SpomCP, I-SpomIP, I-SpomIIP, I-SqulP, I-Ssp6803I, I-SthPhiJP, 20 I-SthPhiST3P, I-SthPhiS3bP, I-TdelP, I-Tevl, I-Tevll, I-TevIII, I-UarAP, I-UarHGPA1P, I-UarHGPA13P, I-VinIP, I-ZbilP, PI-Mtul, PI-MtuHIP, PI-MtuHIIP, PI-Pful, PI-Pfull, PI-Pkol, PI-Pkoll, PI-Pspl, PI-Rma43812IP, PI-SPBetaIP, PI-Scel, PI-Tful, PI-Tfull, PI-Thyl, PI-TIil or PI-TIill.

14. The method as claimed in any one of claims 9 to 13, wherein the enzyme suitable for inducing DNA double-strand breaks at the recognition sequence for the site-directed induction of DNA double-strand breaks is one of the homing endonucleases as shown in SEQ ID NO: 2, 4, 6, 8 or 10.

15. The method as claimed in any one of claims 9 to 14, wherein the enzyme suitable for inducing DNA double-strand breaks at the recognition sequence for the site-directed induction of DNA double-strand breaks is encoded by the expression cassette and expressed in the plant cell, using an expression cassette encompassing a nucleic acid sequence encoding said enzyme.

16. The method as claimed in any one of claims 9 to 15, wherein the enzyme suitable for inducing DNA double-strand breaks at the recognition sequence for the site-directed induction of DNA double-strand breaks is encoded by the expression cassette and expressed in the plant cell, using an expression cassette encompassing
10 a nucleic acid sequence encoding said enzyme as shown in SEQ ID NO: 1, 3, 5, 7 or 9.

17. A plant cell comprising the recombination system as defined in any one of claims 1 to 8.

18. The plant cell as claimed in claim 17, wherein the plant cell is from *Arabidopsis thaliana*, tobacco, wheat, rye, barley, oats, oilseed rape, maize, potato, sugar beet, soybean, sunflower, pumpkin/squash or peanut.

19. Use of the plant cell as defined in claim 17 or 18, for the production of a medicament.

1/15

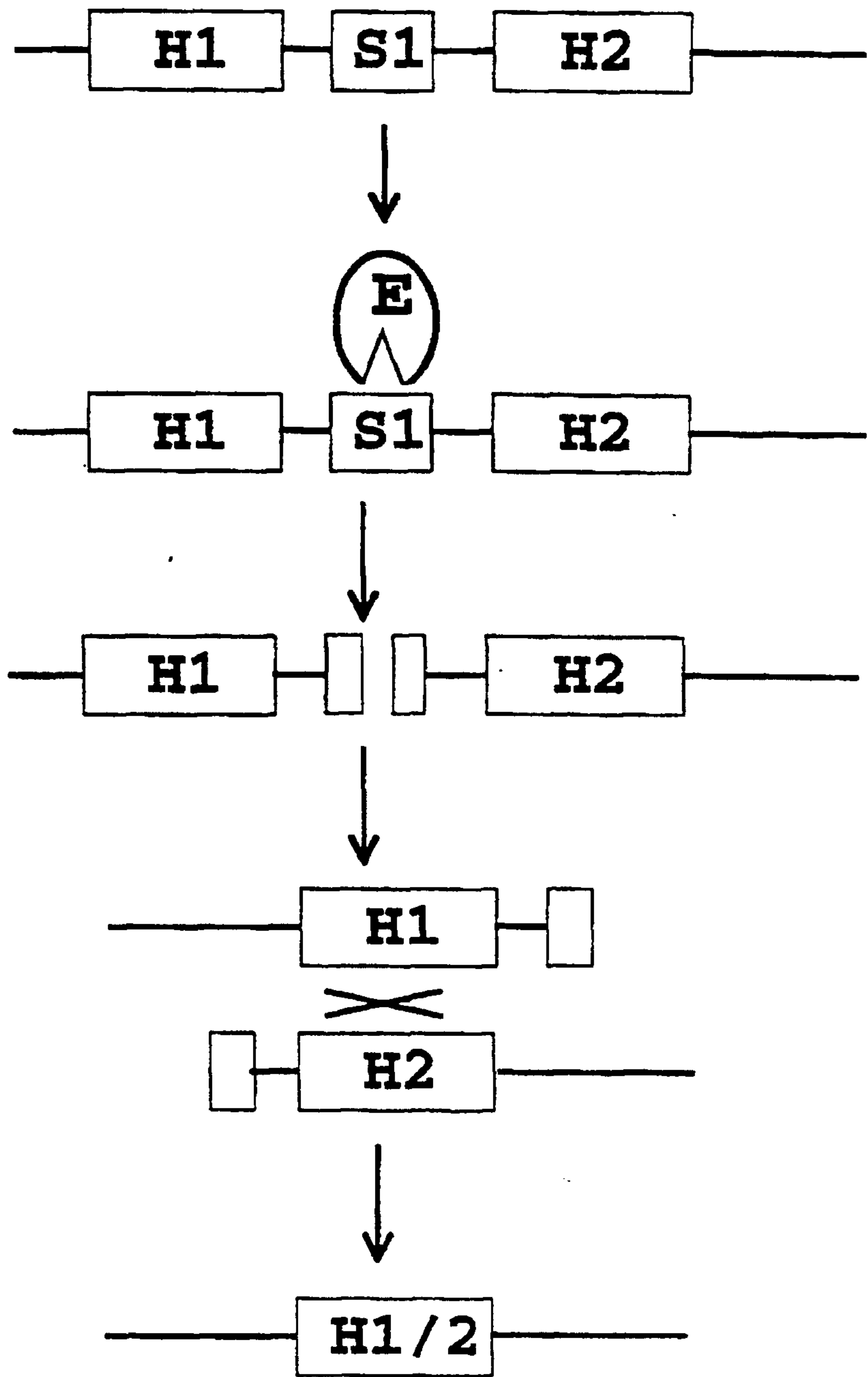


Fig. 1

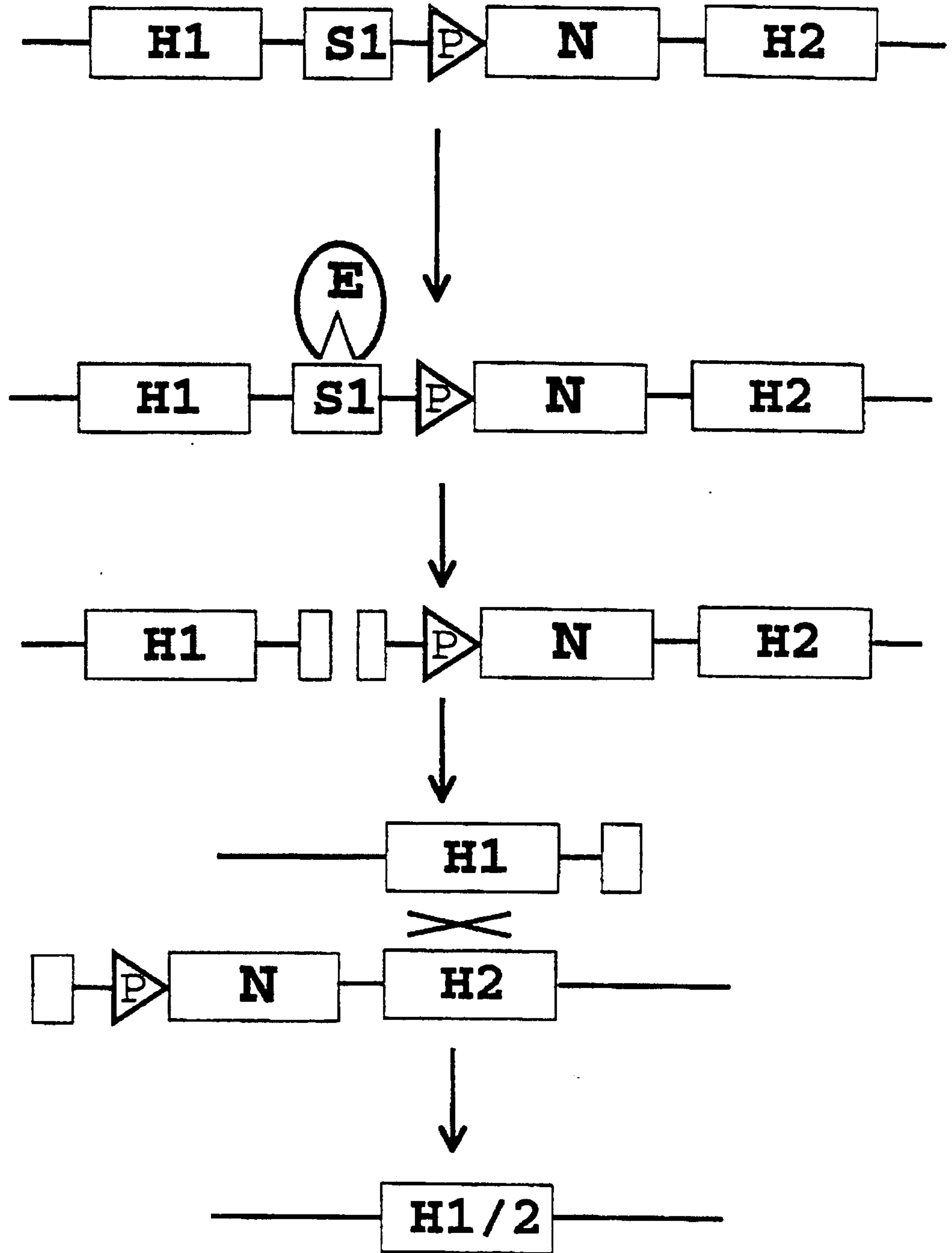


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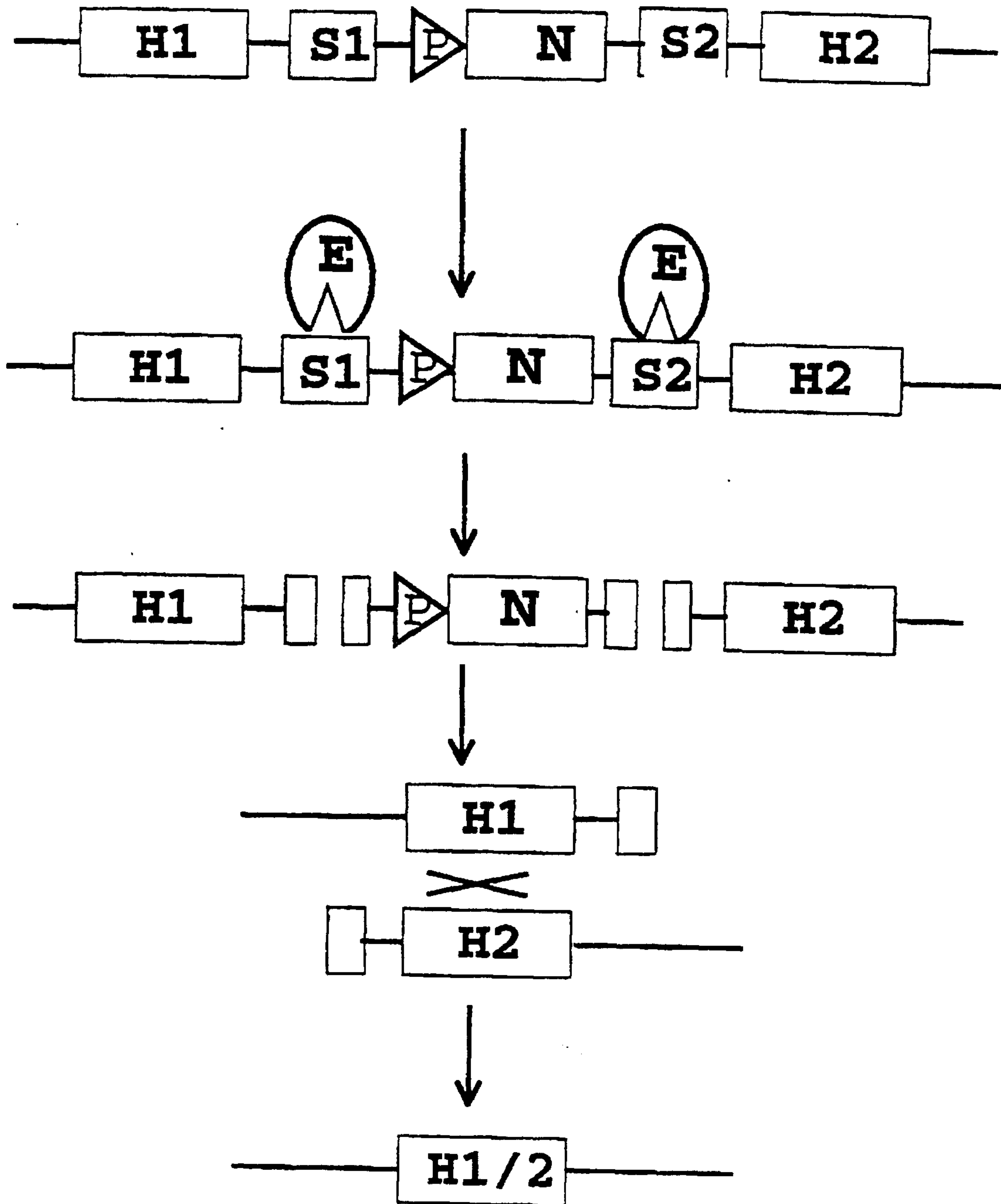


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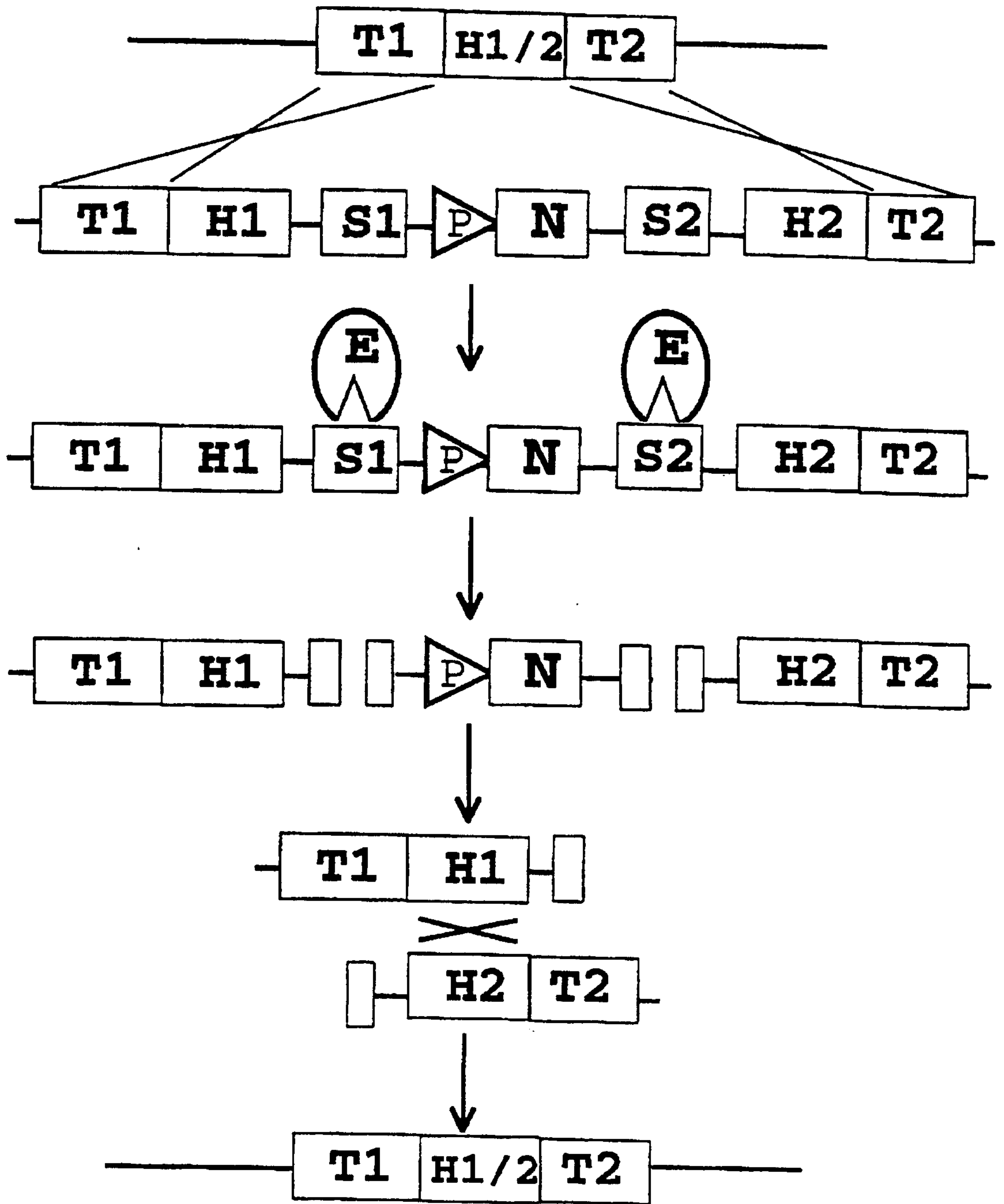


Fig. 4

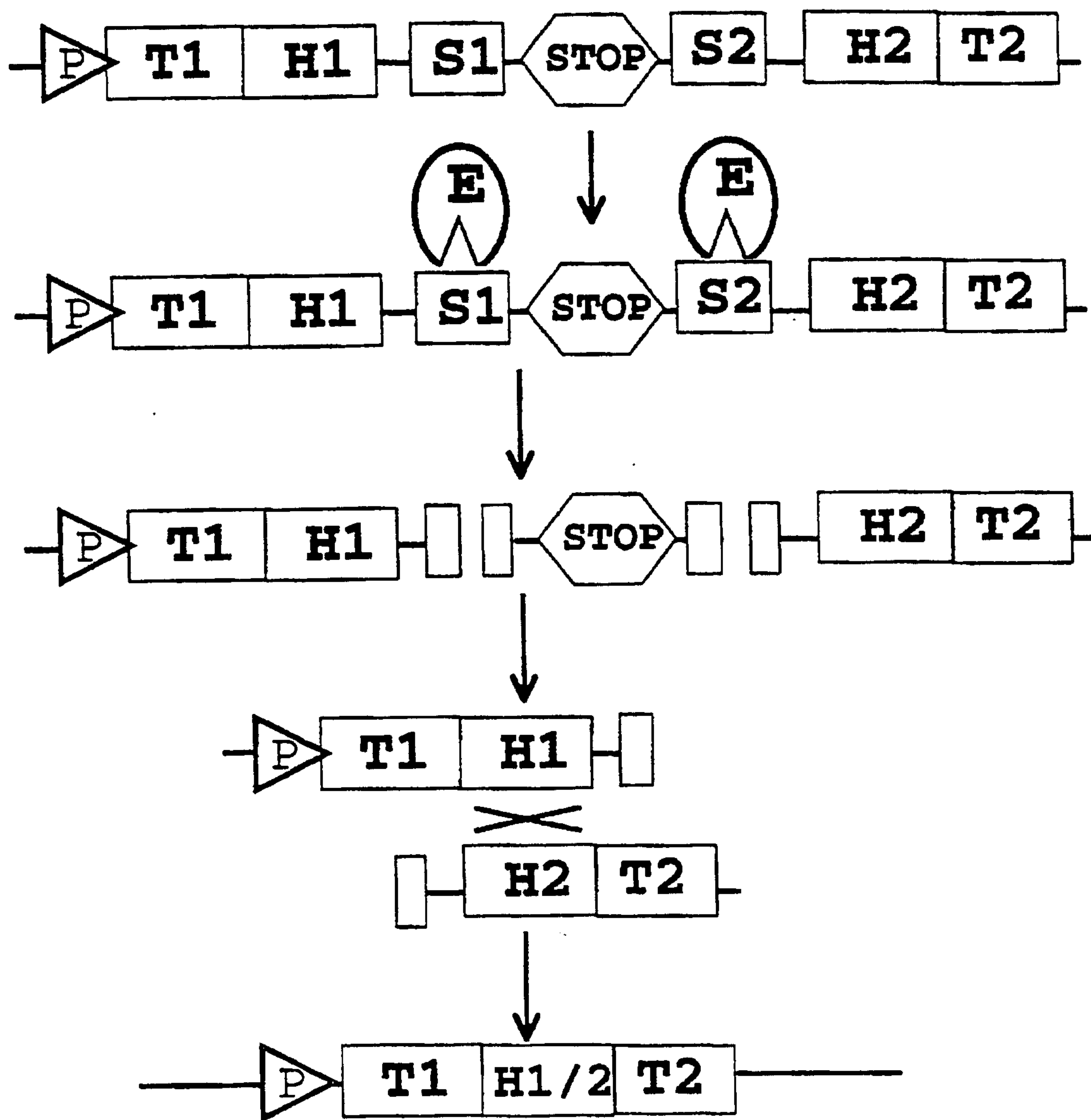


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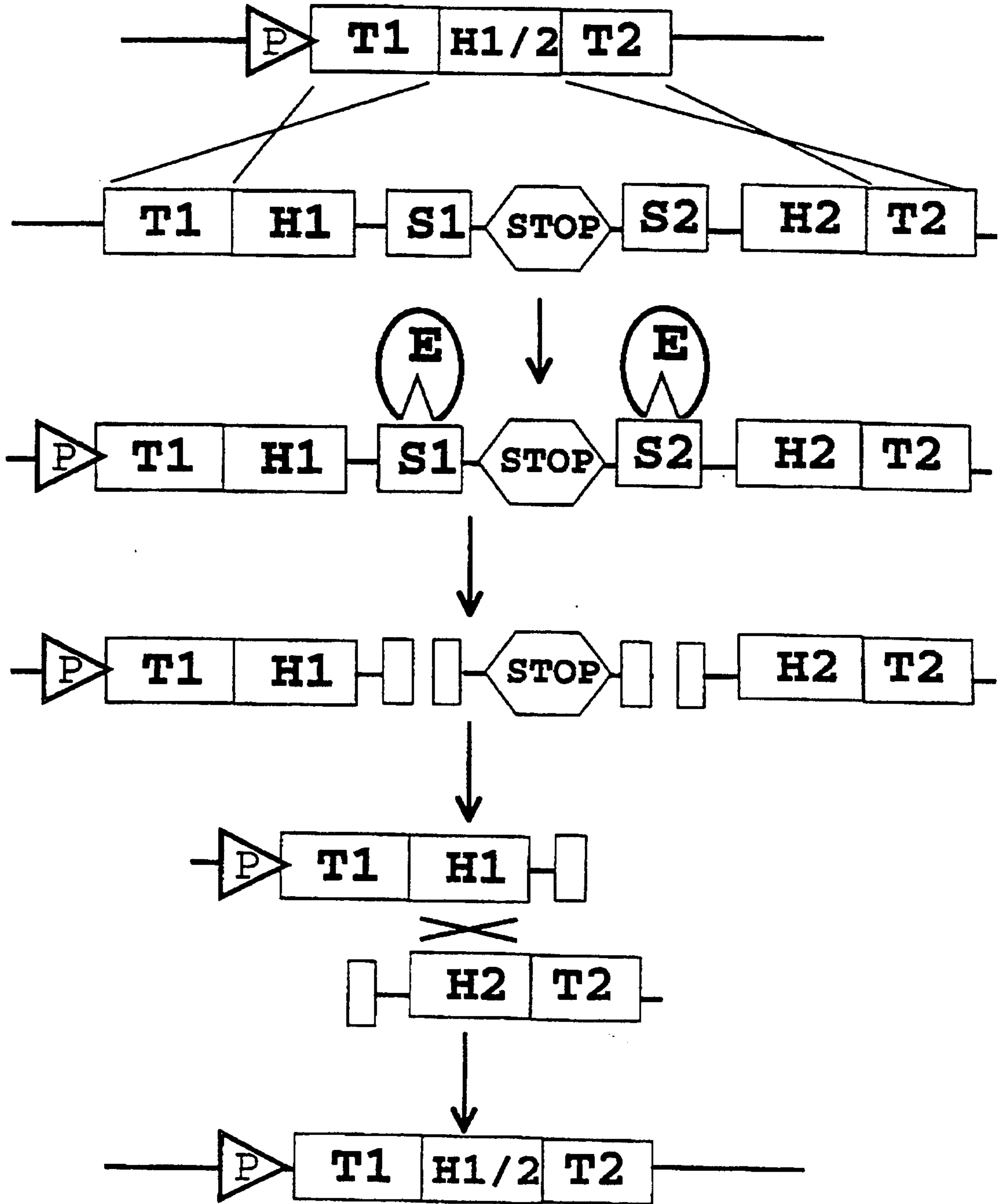


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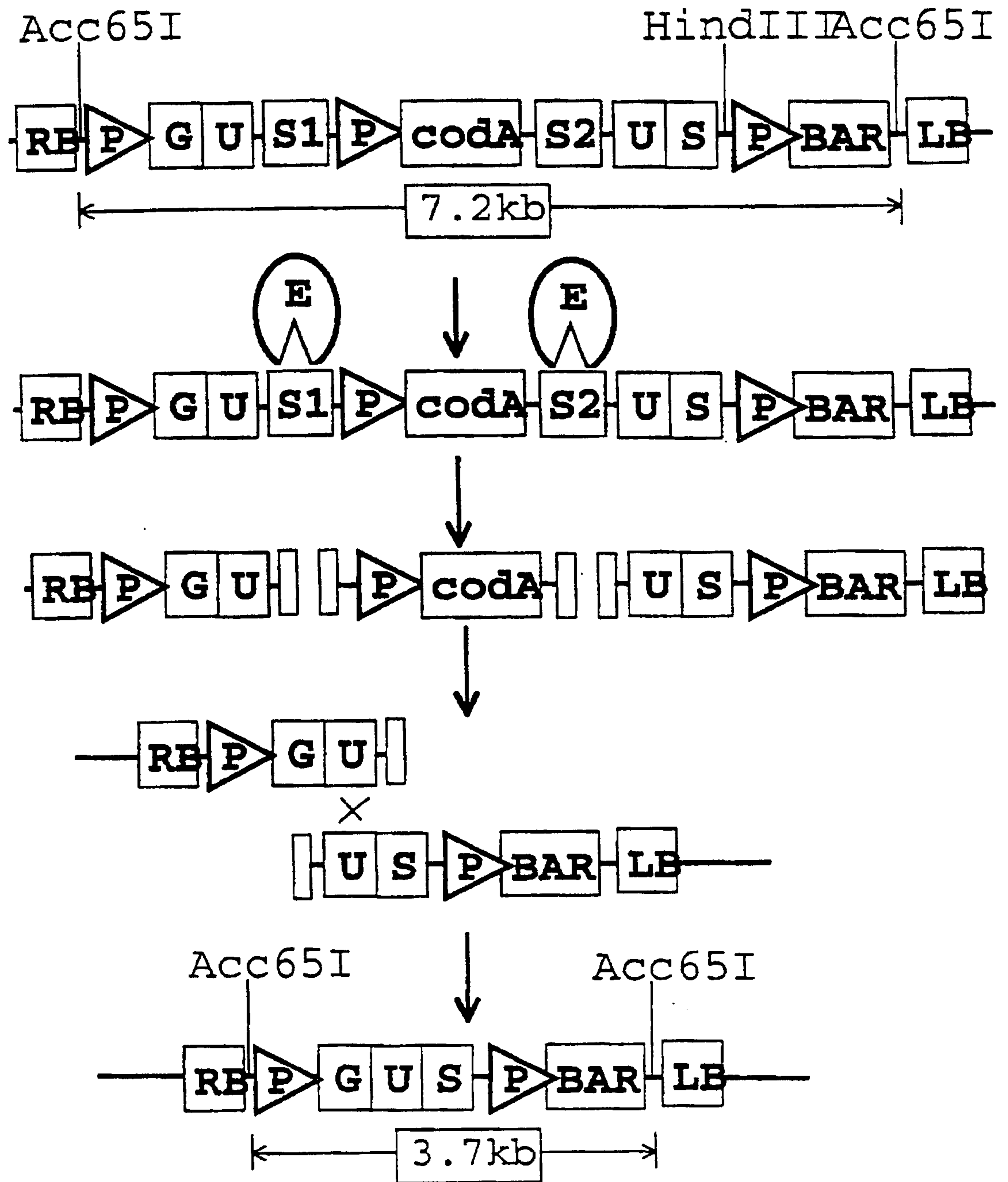


Fig. 7a

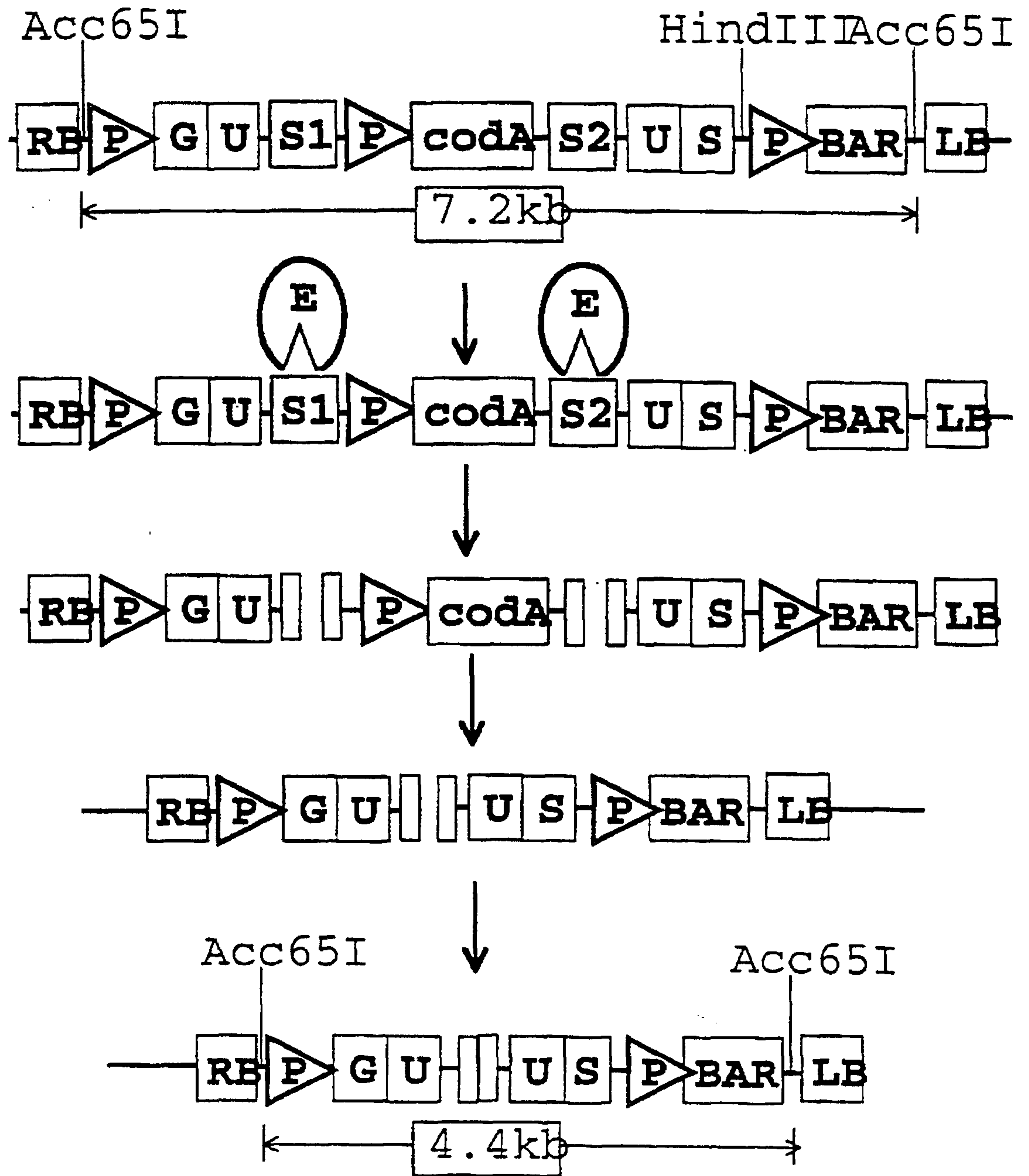
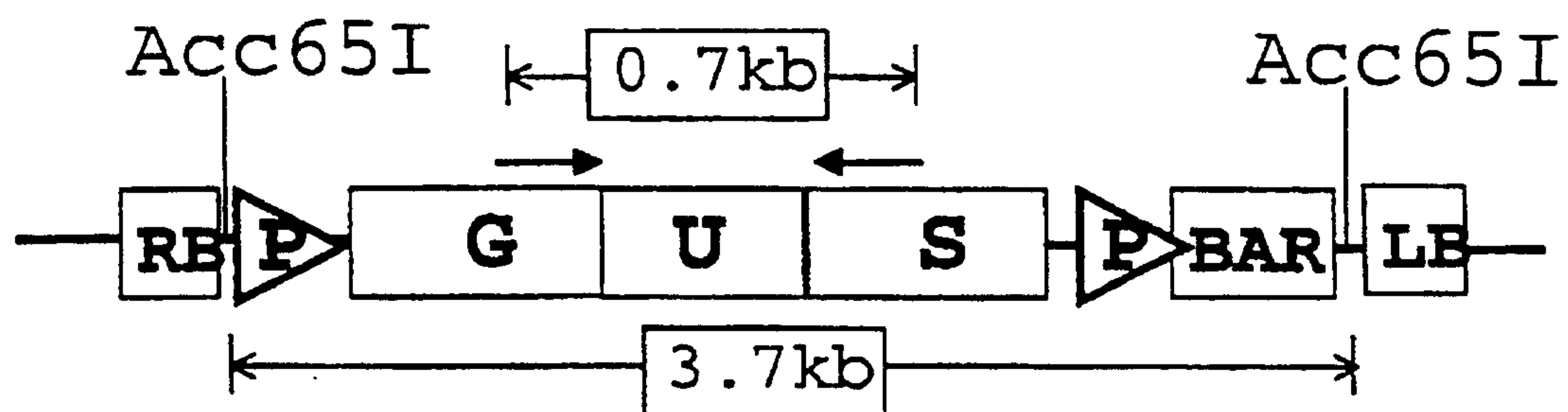
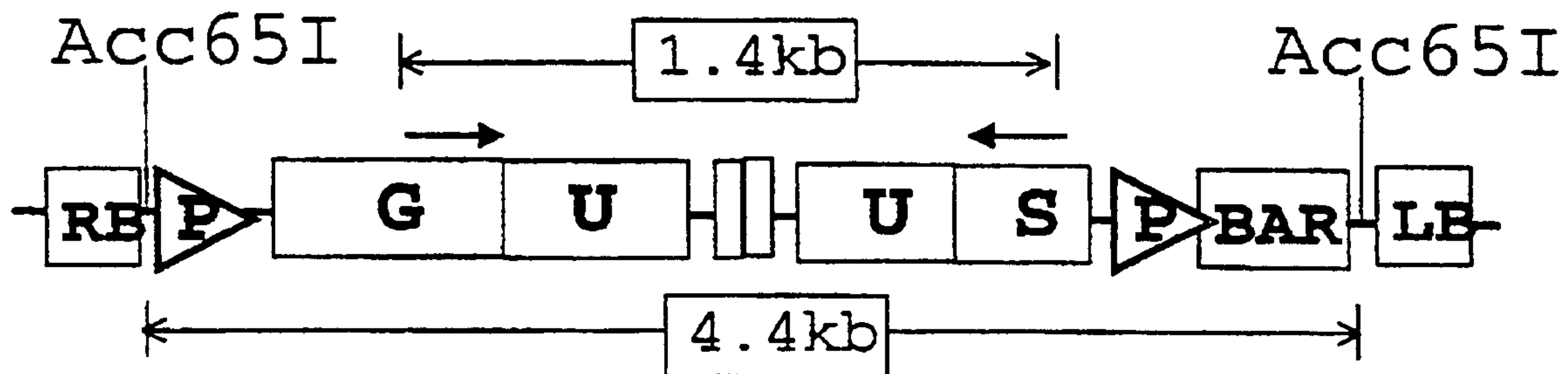


Fig. 7b

9/15

A**B****Fig. 7c**

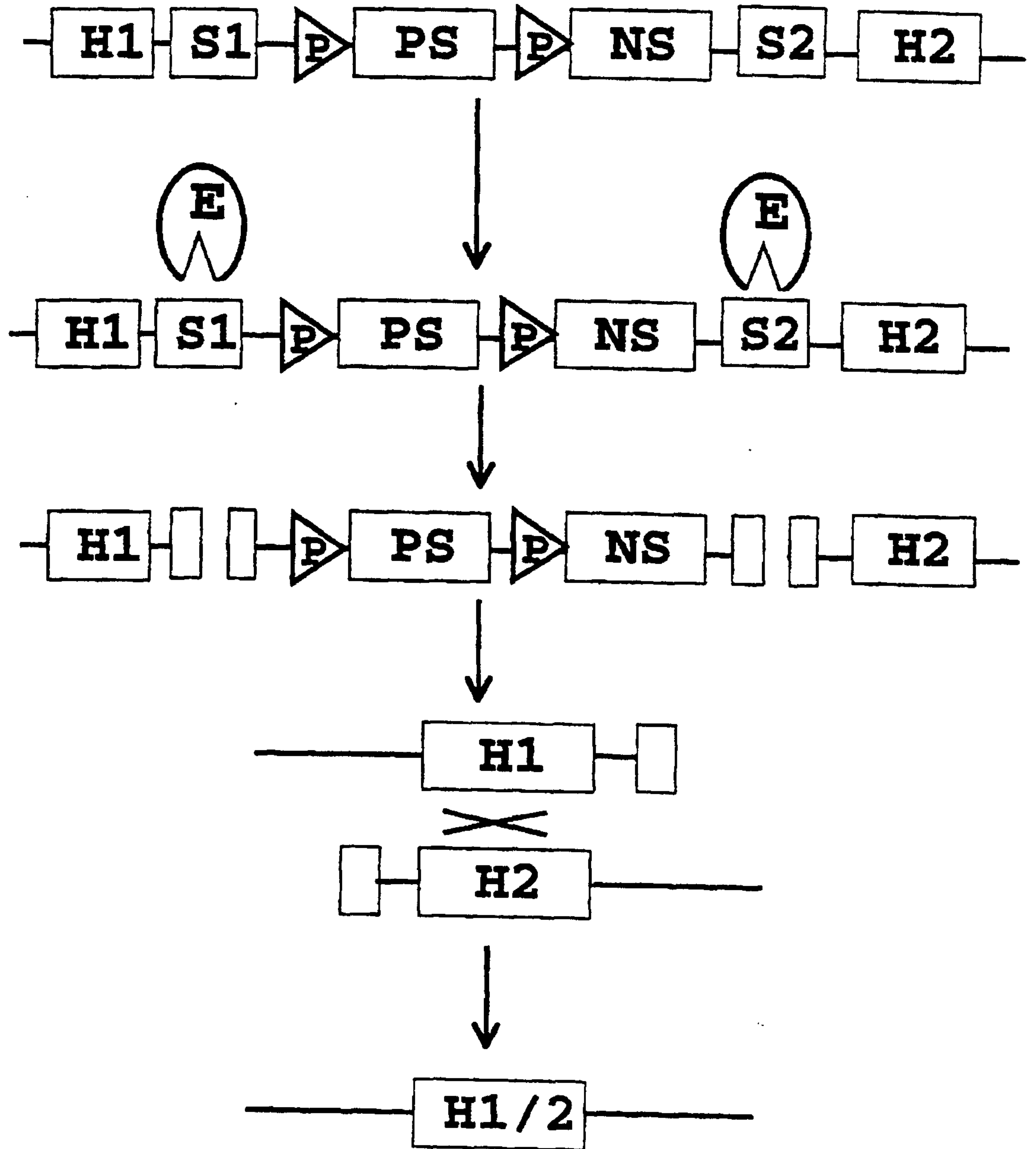


Fig. 8

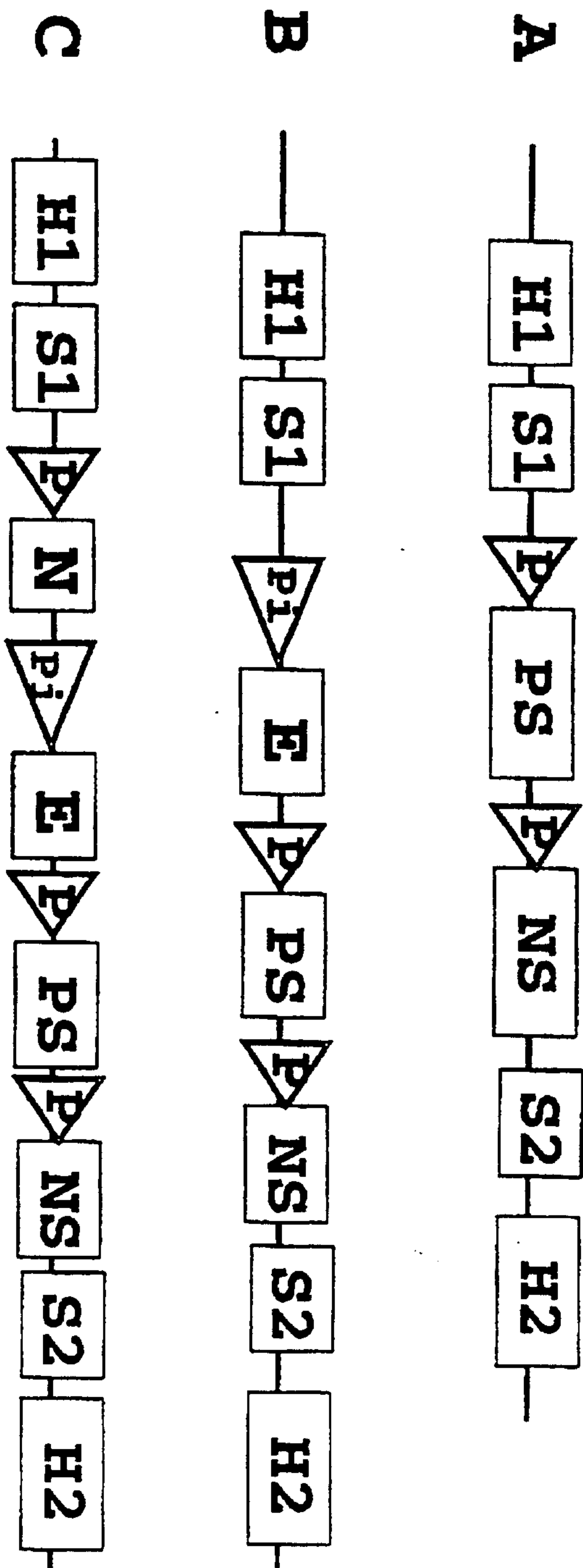
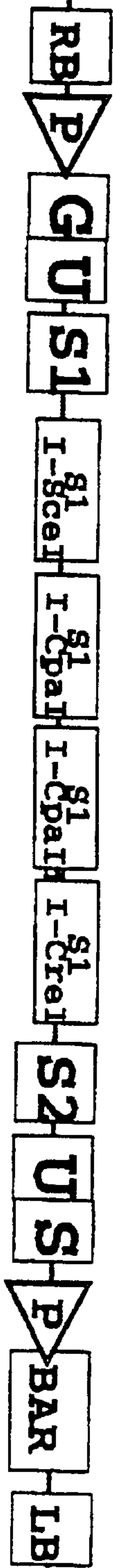


Fig. 9

A



B

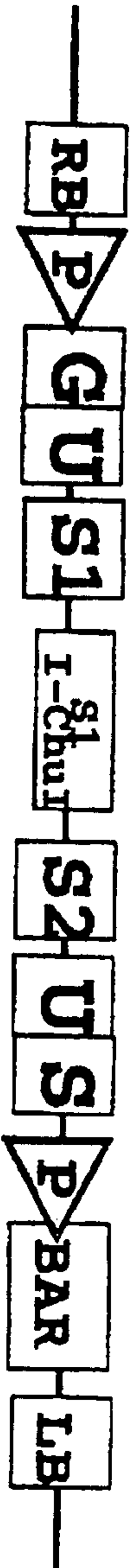


Fig. 10

0817/00023

13/15

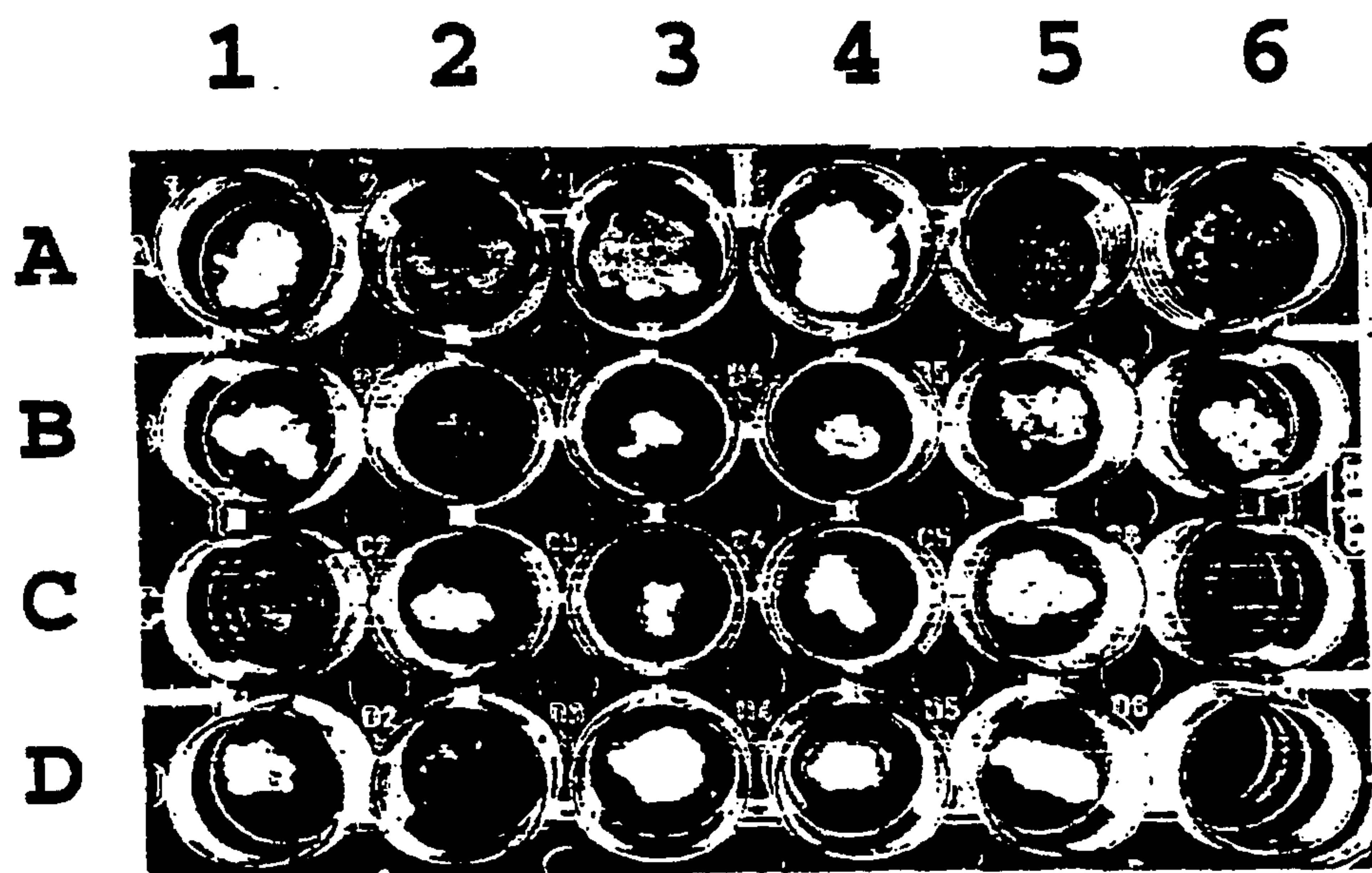


Fig. 11

0817/00023

14/15

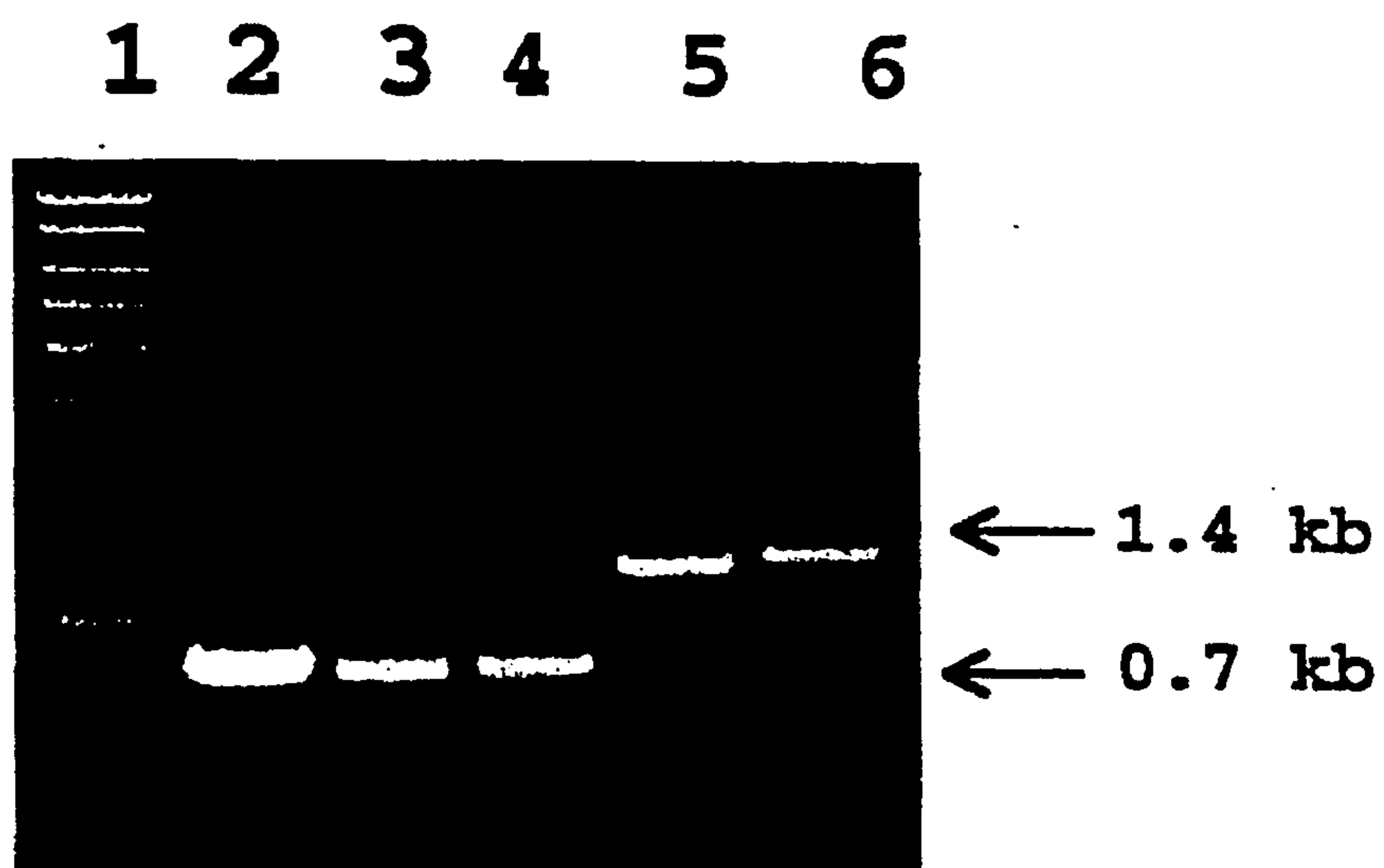


Fig. 12

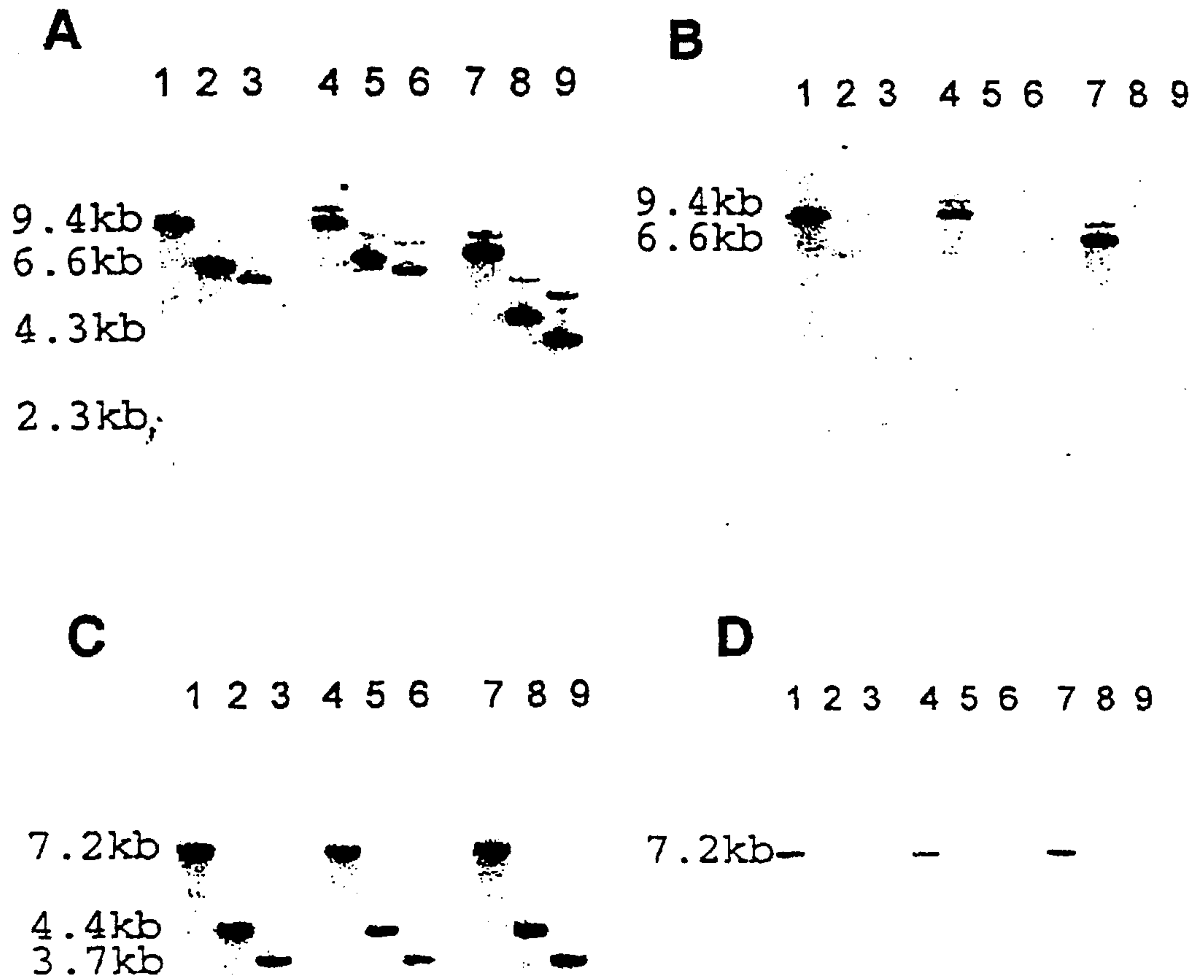


Fig. 13