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(72) Inventeurs/Inventors:
EBINUMA, HIROYASU, JP;
SUGITA, KOICHI, JP;
ETSUKO, MATSUNAGA, JP;
YAMAKADO, MIKIKO, JP

(73) Propriétaire/Owner:
NIPPON PAPER INDUSTRIES CO. LTD., JP

(74) Agent: G. RONALD BELL & ASSOCIATES

(54) Titre : VECTEUR POUR INTRODUIRE UN GENE DANS UNE PLANTE; METHODES POUR L'OBTENTION DE PLANTES TRANSGENIQUES ET INTRODUCTION MULTIPLE DE GENES DANS UNE PLANTE A L'AIDE DE CE VECTEUR

(54) Title: VECTOR FOR INTRODUCING A GENE INTO A PLANT, AND METHODS FOR PRODUCING TRANSGENIC PLANTS AND MULTITUDINOUSLY INTRODUCING GENES INTO A PLANT USING THE VECTOR

(57) Abrégé/Abstract:
A vector is provided for introducing a desired gene into a plant. The vector comprises the desired gene, at least one morphological abnormality induction (MAI) gene as a marker gene, and a removable element, wherein the MAI gene is positioned within the removable DNA element, and wherein the desired gene is positioned outside of the removable DNA element. A method is also provided for producing a transgenic plant free from the influence of a marker gene, as well as a method for multitudinously introducing desired genes into one plant.

ABSTRACT OF THE DISCLOSURE

A vector is provided for introducing a desired gene into a plant. The vector comprises the desired gene, at least one morphological abnormality induction (MAI) gene as a marker gene, and a removable element, wherein the MAI gene is positioned within the removable DNA element, and wherein the desired gene is positioned outside of the removable DNA element. A method is also provided for producing a transgenic plant free from the influence of a marker gene, as well as a method for multitudinously introducing desired genes into one plant.

The present invention relates to a novel vector for introducing a desired gene into a plant using genetic engineering methods to obtain a transgenic plant; a method for producing a transgenic plant free from the influence of a marker gene using the novel vector; and a method for introducing at least two desired genes into a plant using the novel vector.

Transformation of microorganisms and cultured cells using genetic engineering is currently applied to the production of physiologically active substances useful as medicines and thus greatly contributes to the industry. In the field of the plant breeding, industrial application of genetic engineering lags behind because the life cycles of plants are much longer than those of microorganisms. However, since this technology enables a desired gene to be directly introduced into plants to be bred, it has the following advantages compared to classical breeding which requires multiple crossing.

(a) It is possible to introduce only a characteristic to be improved.

(b) It is possible to introduce characteristics of species other than plants (such microorganisms and the like).

(c) It is possible to greatly shorten the breeding period.

Thus, generic engineering methods for plant breeding have been investigated vigorously.

The production of transgenic plants requires the following three steps.

(1) Introducing the desired gene into the plant cell (including introduction of the same into the chromosomes, nucleus and the like).

(2) Selecting plant tissue made only of cells into which the desired gene has been introduced.

(3) Regenerating a plant from the selected plant tissue.

In order to select transgenic tissues into which a desired gene has been introduced, visual identification of the tissue in which the desired gene is expressed without regenerating the new plant has been desired. To achieve this, the desired gene is typically introduced into the plant cell together with a marker gene of which the expression can be easily detected at the stage of cultivating the cell. That is, the expression of the marker gene is used as an index of the introduction of the desired gene. Examples of conventional marker genes include antibiotic-resistance genes such as a kanamycin-resistant gene (i.e., NPTII; neomycin phosphotransferase gene), a hygromycin-resistant gene (i.e., HPT; hygromycin phosphotransferase gene), an amino acid synthetase gene such as a nopaline synthetase gene (NOS), an octopine synthetase gene (OCS), and a sulfonylurea-resistant gene (i.e., ALS; acetoactate synthetase gene) that imparts agricultural chemical resistance.

However, the expression of a marker gene can be hazardous when such a transgenic plant is used for food. That is, it is difficult to ensure that a gene product produced by expressing a marker gene is safe for the human body. Consequently, if a transgenic plant containing a marker gene is to be sold as a food, a detailed investigation must be performed to determine the influence of the marker gene on the human body. For example, the NPTII gene has been used as a marker gene at the laboratory level since the early 1980s. In 1994, the product of that gene was finally accepted as a food additive by the U.S. Food

and Drug Administration (FDA). Since then, transgenic plants containing the NPTII gene as a marker gene have been used for food. However, some consumers of products containing the NPTII gene are still anxious about this gene's effect.

Moreover, marker genes which are practically used are only genes, such as the NPTII gene, which contribute to detoxification of a growth inhibitory substance in plant cells. Therefore, to select transgenic plant tissue into which a desired gene has been introduced, the tissue is cultivated in a culture medium containing the growth inhibitory substance, and the expression of the marker gene, namely the resistance of the tissue to the growth inhibitory substance is evaluated and used as an index. However, even when a tissue has such a resistance, cultivation in the presence of an inhibitory substance can result in undesirable side effects on the plant cells, such as a decrease in proliferation and redifferentiation of the transgenic tissue.

Further, the expression of a marker gene in a plant cell after the selection of transgenic tissue seriously obstructs plant breeding by subsequent gene introduction. That is, when another gene is introduced into a transgenic plant containing a marker gene, the gene introduction must be monitored using a different marker gene. However, the effectiveness of a marker gene varies with the plant species. Therefore, a preliminary test is required to set the conditions for each marker gene (for example, it is reported that the HPT gene is more effective in rice plants than the NPTII gene (K. Shimamoto *et al., Nature* (London), vol. 338, p.274, 1989)). Still further, since the varieties of marker genes are limited, the multiple introduction of a gene cannot be repeated

indefinitely simply by changing the marker gene. That is, the number of gene introductions into a certain plant is limited itself by the variety of marker genes which can be used in that plant. Besides, the kind of the marker gene which can be actually used is limited as mentioned above. Accordingly, it is desirable to find a method for removing the marker gene from the chromosome after selection of the transgenic plant tissue to exclude the influence of the marker gene from the cell, tissue and plant.

To eliminate the influence of a marker gene, two methods have been reported. In one method, a marker gene and a transposon of the plant are introduced into a plant chromosome and subsequently removed therefrom following introduction of the gene (International Laid-Open Patent Application No. WO 92/01370). In a second method, the site-specific recombination system of P1 phage is used instead of the transposon (International Laid-Open Patent Application No. WO 93/01283). Using these methods, it is possible to obtain a cell in which the marker gene has been removed from the plant chromosome at a given ratio after the introduction of the gene. Unfortunately, the probability that the marker gene is removed is very low.

Further, plant cells in which the marker genes have been removed from the chromosomes using theses methods are scattered among the cells in which the marker genes are still present and expressed. These two kinds of the cells cannot be distinguished visually.

Plant cells containing marker genes and a desired gene can be selected based on their chemical resistance, nutritional requirements and the like.

However, at the time of selection, the cells lacking marker genes exhibit serious growth inhibition and are destroyed in many cases. Accordingly, these selections cannot be applied to obtain cells lacking marker genes.

In order to obtain plants which lack a marker gene and which contain the desired gene using the above-mentioned methods, the tissue of the plant in which cells lacking the marker gene and cells containing the marker gene are mixed is proliferated, regenerated, and then analyzed for the selection, using methods such as Southern hybridization or polymerase chain reaction. This method is based on the premise that a regenerated individual is derived from a single cell and therefore all of the plant's cells should have the same characteristics. Thus, an individual derived from a cell lacking the marker gene is made only of such cells. Unfortunately, cells constituting such a regenerated individual are not necessarily uniform. Cells lacking the marker gene and cells containing the marker gene are coexistent and distributed quite irregularly even in the same individual regenerated plant and in the same tissue thereof. Thus, it is extremely difficult to obtain an individual made only of cells lacking the marker gene at the stage in which the cultured tissue is redifferentiated to regenerate the individual.

In addition, known analytical methods of selection use a tissue, such as a leaf, as a test sample (not a whole individual or a single cell). Consequently, only the overall tendency is analyzed with respect to the state of the marker gene present in one leaf. Furthermore, in this case, it is common that the marker gene-free cell and the marker gene-containing cell are both present in the same individual or tissue. So, even if an individual made only of cells lacking

the marker gene happens to be formed, it is difficult to select this. Even if the presence of the marker gene is not detected in this tissue, tissues in other sites of the same individual may contain the marker gene, or it simply shows that the amount of the marker gene is below the detectable limit. Therefore, it is impossible to determine if the test sample is completely free from the marker gene-containing cells.

Using the above-mentioned methods, an individual lacking the marker gene is obtained only from a germ cell, such as a pollen grain, an egg cell and the like. When self-pollination is conducted using the egg cell lacking the marker gene, a fertilized egg lacking the marker gene is obtained at a fixed ratio according to a classical hereditary law, and from this fertilized egg, an individual made only of cells having the same characteristics as the fertilized egg is produced. Conventional analytical methods such as Southern hybridization may be conducted using this individual. Namely, even if the cell lacking the marker gene is produced by the method described in the report referred to here, the individual made only of such a cell is obtained for the first time by redifferentiating the plant from the cultured tissue containing such a cell, conducting crossing of the regenerated plant and obtaining progeny of F_1 or later generations. The thus-obtained individual can be selected as an individual lacking the marker gene.

In order to remove the marker gene from the transgenic plant, JP-A-6-276872 (the term "JP-A" as used herein means a Japanese published patent application) reports a technique for gene introduction in which a marker gene is inserted into a separate plasmid vector different from the vector containing the

desired gene. The plasmid containing the marker gene is removed from the cell after the completion of the gene introduction. However, this technique requires a crossing step for the removal of the marker gene. In this respect, the technique is the same as those of the above-mentioned two reports.

The above methods are difficult to apply to woody plants that have a long growth period, sterile individuals or hybrid individuals in which F_1 is itself valuable. Further, when removable DNA elements, such as a transposon and the like, are used, the ratio at which these elements are removed from the chromosomal DNA, virus vector DNA and the like where these elements are present and function is typically extremely low. Accordingly, it is necessary that the removal of these elements (namely, the removal of the marker gene) can be easily detected at least at the stage of the cultured tissue. When this cannot be detected before redifferentiation of the cultured tissue and the formation of a later generation via the crossing of the regenerated individual, the method is impractical.

Accordingly, one object of the present invention is to provide a vector containing a gene desired to be introduced into a plant and a marker gene, wherein a plant containing the same has no adverse effect on the human body when ingested, even if the marker gene is expressed.

Another object of the present invention is to provide a vector for introducing a desired gene into a plant, wherein the vector contains a marker gene that enables selection of a transgenic tissue without the use of a plant cell growth inhibitory substance that decreases the activity of the plant cell.

Still another object of the present invention is to provide a vector for introducing a desired gene into a plant, wherein the vector contains a marker gene, and functions to exclude the influence of the marker gene by removing the marker gene from the DNA, where the marker gene is present and functions. Using this vector, a desired gene can be repeatedly introduced efficiently.

A further object of the present invention is to provide a method for producing a transgenic plant using such a vector, which can exclude the influence of the marker gene, without undergoing the step of the production of F_1 or later generations by crossing, and a method for multitudinously introducing genes into a plant by applying the above-described method.

These and other objects of the present invention have been achieved using a vector, which comprises a desired gene and at least one morphological abnormality induction (hereinafter referred to as "MAI") gene as a marker gene.

Furthermore, the invention contemplates using such a vector, wherein the marker gene is removed from the DNA after its expression. Expression of the marker gene and the disappearance of the function thereof are detectable by morphological changes in the tissue into which the marker gene has been introduced.

Accordingly, as an aspect of the present invention there is provided a vector for introducing a desired gene into a plant, which comprises the desired gene, at least one morphological abnormality induction gene as a marker gene, and a removable DNA element, wherein the morphological abnormality induction gene is positioned within the removable DNA element, and wherein the desired gene is positioned outside of the removable DNA element.

Another aspect of the present invention involves the use of a vector as defined herein.

A further aspect of the invention provides a method for producing a transgenic plant free from the influence of a marker gene, which comprises the following steps:

(A) introducing a vector into a plant cell, wherein the vector comprises a desired gene, at least one MAI gene as a marker gene, and a removable DNA element, wherein the MAI gene is positioned within the removable DNA element, and wherein the desired gene is positioned outside of the removable DNA element,

(B) cultivating the plant cell obtained in (A), detecting a morphologically abnormal plant tissue which appears during the cultivation, and selecting the morphologically abnormal tissue, and

(C) cultivating the morphologically abnormal tissue selected in (B), detecting a morphologically normal tissue which appears during the cultivation, and selecting the morphologically normal tissue.

Furthermore, another aspect of the invention provides a method for introducing at least two desired genes into a plant, which comprises conducting the following steps at least two times:

(A) introducing a vector into a plant cell, wherein the vector comprises a desired gene, at least one MAI gene as a marker gene, and a removable DNA element, wherein the MAI gene is positioned within the removable DNA element, and wherein the desired gene is positioned outside of the removable DNA element,

(B) cultivating the plant cell obtained in (A), detecting a morphologically abnormal plant tissue which appears during the cultivation, and selecting the morphologically abnormal tissue, and

(C) cultivating the morphologically abnormal tissue selected in (B), detecting a morphologically normal tissue which appears during the cultivation, and selecting the morphologically normal tissue.

As a further aspect, the present invention provides a cell from a transgenic plant free from the influence of a marker gene, produced by an asexual method comprising the following steps:

(A) introducing a vector into a plant cell, wherein the vector comprises a desired gene, at least one morphological abnormality induction gene as a marker gene, and a removable DNA element, wherein the morphological abnormality induction gene causes the formation of a morphologically abnormal plant tissue, and is positioned within the removable DNA element, and wherein the desired gene is positioned outside of the removable DNA element,

(B) cultivating the plant cell obtained in (A), detecting the morphologically abnormal plant tissue which appears during the cultivation, and selecting the morphologically abnormal plant tissue,

(C) cultivating the morphologically abnormal plant tissue selected in (B), detecting a morphologically normal plant tissue which appears during the cultivation, and selecting the morphologically normal plant tissue, and

(D) cultivating the morphologically normal plant tissue to regenerate the transgenic plant.

Finally, the present invention provides a cell from a plant containing two or

more desired genes, produced by an asexual method comprising the following steps:

(A) introducing a vector into a plant cell, wherein the vector comprises a desired gene, at least one morphological abnormality induction gene as a marker gene, and a removable DNA element, wherein the morphological abnormality induction gene causes the formation of a morphologically abnormal plant tissue, and is positioned within the removable DNA element, and wherein the desired gene is positioned outside of the removable DNA element,

(B) cultivating the plant cell obtained in (A), detecting the morphologically abnormal plant tissue which appears during the cultivation, and selecting the morphologically abnormal plant tissue,

(C) cultivating the morphologically abnormal plant tissue selected in (B), detecting morphologically normal plant tissue which appears during the cultivation, and selecting the morphologically normal plant tissue,

(D) repeating steps (A) to (C) at least one time, and

(E) cultivating the morphologically normal plant tissue to regenerate the plant.

Embodiments of the invention will now be described by way of example, with reference to the accompanying drawings, wherein:

Figure 1 is a diagram of the Ti plasmid and a restriciton endonuclease map of *Pst* I fragment on a T-DNA region of *A. tumefaciens* strain PO22;

Figure 2 is a diagram of the construction of pIPT2;

Figure 3 is a diagram of the construction of pIPT3 from pIPT2;

Figure 4 is a diagram of the construction of pIPT4 from pIPT3;

Figure 5 is the restriction endonuclease map of a T-DNA region in the structure of pIPT4;

Figure 6 is the result of a PCR analysis of an extreme shooty phenotype of tobacco into which a gene has been introduced using pIPT4;

Figure 7 is a diagram of the construction of pNPI102;

Figure 8 is a diagram of the construction of pNPI103 from pIPT4 and pNPI102;

Figure 9 is a diagram of the construction of pNPI106 from pNPI103;

Figure 10 is the restriction endonuclease map of a T-DNA region in the structure of pNPI106;

Figure 11 is a photograph of shoot No. 2 after one month of cultivation in Example 2;

Figure 12 is a photograph of shoot No. 8 after one month of cultivation in Example 2;

Figure 13 is the result of a PCR analysis of shoot No. 8 in Example 2;

Figure 14 is the result of a PCR analysis of normal individuals obtained from shoots Nos. 13-1 and 14-1 in Example 3;

Figure 15 is a photograph of normal shoots differentiated from an extreme shooty phenotype of tobacco in Example 3;

Figure 16 is the result of a PCR analysis of a normal individual which is obtained from a leaf formed from shoot No. 7 in Example 2;

Figure 17 is a diagram of the construction of pNPI128;

Figure 18 is a diagram of the construction of pNPI129 from pNPI128;

Figure 19 is a diagram of the construction of pNPI132 from pNPI101 and pNPI129;

Figure 20 is the restriction endonuclease map of the T-DNA region in the structure of pNPI132;

Figure 21 is the result of a PCR analysis of normal individuals obtained from shoots Nos. 15 to 21 in Example 5 using primers in which the existence of an *ipt* gene was detected;

Figure 22 is the result of a PCR analysis of normal individuals obtained from shoots Nos. 15 to 21 in Example 5 using primers in which the elimination of a region held by a couple of Rs's including an *ipt* gene was detected;

Figure 23 is the result of a PCR analysis of normal individuals obtained from shoots Nos. 15 to 21 in Example 5 using primers in which the existence of a GUS gene was detected;

Figure 24 is the result of a PCR analysis of normal individuals obtained from the line which could not form an extreme shooty phenotype in Example 5;

Figure 25 is a diagram of the construction of pNPI702;

Figure 26 is the restriction endonuclease map of the T-DNA region in the structure of pNPI702;

Figure 27 is a photograph of normal shoots differentiated from an extreme shooty phenotype of the hybrid aspen in Example 7;

Figure 28 is the restriction endonuclease map of the T-DNA region in the structure of pNPI140; and

Figure 29 is the result of a PCR analysis of a normal shoot differentiated from an extreme shooty phenotype after the multitudinous introduction of genes in Example 8.

As used therein, the MAI gene is a gene that induces into a tissue of a plant morphologically abnormal differentiation resulting in dwarfishness, destruction of apical dominance, change in pigments, formation of a crown gall, formation of hairy roots, waving of the leaves or the like. With respect to preferred MAI gene, those genes isolated from bacteria of the genus *Agrobacterium* or the like that induce tumor or teratoma (e.g., formation of

adventitious shoots and adventitious roots) in various plants can be used. Examples of these various MAI genes include cytokinin synthesis genes (e.g., *ipt* (isopentenyltranferase) gene (A.C. Smigocki, L.D. Owens, *Proc. Natl. Acad. Sci. U.S.A.,* vol.85, p. 5131, 1988)), *iaa*M (tryptophan monooxygenase) gene (H.J. Klee *et al.*, *GENES & DEVELOPMENT,* vol.1, p.86, 1987), gene 5 (H. Korber *et al., EMBO Journal,* vol.10, p.3983, 1991), gene *6b (*P.J.J. Hooykaas *et al., Plant Mol. Biol.,* vol.11, p.791, 1988) and *rol* genes such as *rolA, rolB, rolC* and *rolD* (F.F. White *et al., J. Bacteriol.,* vol.164, p.33, 1985). Furthermore, examples thereof include an *iaa*L (indoleacetic acid-lysine synthetase) gene as *Pseudomonas syringae* subsp. *savastanoi* (A. Spena *et al., Mol. Gen. Genet.,* vol.227, p.205, 1991), homeo box genes and phytochrome genes in various plants. Preferably, the cytokinin synthesis genes such as the *ipt* gene or at least one gene selected from the *rol* genes (more preferably, *rol* genes containing genes *rolA, rolB,* and *rolC)* are used. The *ipt* gene is present in the T-DNA of *Agrobacterium tumefaciens* and induces destruction of apical dominance. The *rol* genes *rolA, rolB* and *rolC* are present in the T-DNA of *Agrobacterium rhizogenes* and at least one of these induces the formation of hairy roots, dwarfishness, waving of the leaves and the like of a plant regenerated from a hairy root.

Using the techniques of the present invention, one can design a combination of these marker genes, so that a specific structure, such as adventitious shoot, an adventitious root or the like is redifferentiated in a specific plant into which these marker genes are introduced. In the present invention, such a combination of MAI genes can be used, according to the conditions of

producing the transgenic plant, such as the kind of plant into which the genes are to be introduced.

The morphologically abnormal tissue produced by introducing the MAI gene into the cell is made up only of the cells containing this gene. Therefore, using this gene as the marker gene, a vector is constructed together with the desired gene. When this vector is introduced into the plant cell and the transgenic cell is cultivated, the tissue made up only of this cell into which the marker gene and the desired gene are introduced can be selected by visually selecting the morphologically abnormal tissue formed from this cell.

Suitable vectors useful in accordance with the present invention have a DNA sequence which introduces a foreign gene into a host cell and which expresses the foreign gene within a cell of a host.

When the gene is introduced using the vector of the present invention, the plant tissue made up only of the transformed cell can be visually selected by merely cultivating the cell after the operation for the gene introduction in a common culture medium such as MS (Murashige-Skoog) culture medium under ordinary cultivation conditions. Since there is no need to use a special substance for selecting the transformed tissue, such as a plant cell growth inhibitory substance or the like, not only is the procedure simplified, but also the activity of the plant cell is not decreased through such a substance. In addition, the plant has inherently the MAI gene, or the MAI gene is spontaneously introduced into the plant through infection with bacteria or the like. Accordingly, a plant obtained using the vector of the present invention is no different from naturally occurring plants which have this morphologic abnormality.

Suitable vectors in accordance with the present invention include a vector where the MAI gene is positioned such that it behaves integrally with a removable DNA element and the desired gene is positioned such that it does not behave integrally with the removable DNA element.

As used herein, a removable DNA element is an element of a DNA sequence which itself is removable from the DNA wherein the DNA element exists and functions. In plants, a transposon present in a chromosome is known as this element. The structure, activity and behavior of transposons have been almost completely identified. For the transposon to function, two components are required in principle: (1) an enzyme which is expressed from the gene present therein and which catalyzes the excision and transposition of the transposon itself (transposase) and (2) DNA binding sequences which are present in the terminal region of the transposon and upon which the transposase acts. By these elements, the transposon is excised from the chromosome in which it exists, and is then usually transposed to a new position in the DNA. However, at a certain ratio, the transposon also disappears without being transposed. The present invention makes use of such a transposition error of the transposon.

The transposon can be of one of two types, either an autonomous transposon or a non-autonomous transposon. The autonomous transposon maintains the two elements, the transposase and the DNA binding sequence. In the autonomous transposon, the transposase is expressed and binds to the DNA binding sequence for action, whereby the transposon is autonomously excised from the chromosome. The non-autonomous transposon retains the terminal DNA binding sequence to which the transposase is bound for action.

In the non-autonomous transposon, the transposase gene undergoes mutation such that the transposase is not expressed; thus the transposon cannot be excised from the chromosome autonomously. However, when transposase is supplied to the non-autonomous transposon from the autonomous transposon or from an independent transposase gene, the non-autonomous transposon behaves similarly to the autonomous transposon.

Examples of autonomous transposons include *Ac* and *Spm* isolated from maize (A. Gierl and H. Saedler, *Plant Mol. Biol.*, vol. 19, p.39, 1992). *Ac* can be obtained by digesting *wx-m7* gene locus in the chromosome of the maize with restriction endonuclease *Sau*3A (U. Behrens *et al., Mol. Gen. Genet.*, vol.194, p. 346, 1984). This autonomous transposon is the most analyzed among plant transposons. In fact, the DNA sequence has already been determined (M. Müller-Neumann *et al., Mol. Gen. Genet.*, vol. 198, p.19, 1984).

Examples of non-autonomous transposons include *Ds* and *dSpm* obtained by deleting the inner regions of *Ac* and *Spm,* respectively (H.-P. Döring and P. Starlinger, *Ann. Rev. Genet.,* vol. 20, p. 175, 1986) and those isolated from many plants, other than maize, such as snapdragon, morning glory and the like (for example, Y. Inagaki *et al., Plant Cell,* vol.6, p.375, 1994). When these transposons are introduced into chromosomes of exogenous plants, these transposons are also excised from a chromosome and transposed upon exhibiting the activity (for example, B. Baker *et al., Proc. Natl. Acad. Sci. U.S.A.,* vol.83, p.4844, 1986).

In the present invention, both the autonomous and non-autonomous transposons can be used. The non-autonomous transposon may be used upon inserting thereinto a functioning transposase gene.

Another removable DNA element, which is not present in plants, but which can be used in accordance with the present invention is an element derived from a site-specific recombination system. A site-specific recombination system consists of two elements, (1) a recombination site (corresponding to the removable DNA element of the present invention) having a characteristic DNA sequence, and (2) an enzyme (recombinase) that binds to the DNA sequence specifically and catalyzes the recombination between DNA sequences if two or more of the sequences exist. When the two DNA sequences are oriented in the same direction at a given interval on the same DNA molecule, the region held by these DNA sequences is excised from the DNA molecule, such as a plasmid, chromosome or the like. When the two DNA sequences are oriented in opposite directions on the same DNA molecule, the region held by these DNA sequences is inverted.

The present invention preferably utilizes the former excision. Both excision and inversion within the recombination site occur as a result of homologous recombination through the site-specific recombination system, which is different from the mechanism using the transposon. It is known that the recombinase gene is not necessarily present in the same DNA molecule in which the recombination site exists. The recombinase gene only needs to be present in the same cell and expressed to excise or invert the region held by two DNA sequences (N.L. Craig, *Annu. Rev. Genet.*, vol.22, p.77, 1988).

At present, site-specific recombination systems have been identified in microorganisms such as phage, bacterium (e.g., *E. coli),* yeast and the like. Examples thereof include a Cre/lox system, a pSR1 system, a FLP system, a *cer* system, and a *fim* system (for example, N.L. Craig, *Annu. Rev. Genet.,* vol.22, p.77, 1988). When a site-specific recombination system separated from these microorganisms, such as a Cre/lox system derived from P1 phage (WO 93/01283), is introduced into an organism (including plants) different from the organism from which this system was derived, it behaves in the same way as in the original organism. The site-specific recombination system of yeast (*Zygosaccharomyces rouxii)* (pSR1 system (H. Matsuzaki *et al., J. Bacteriology,* vol. 172, p. 610, 1990)) can also be used in accordance with the present invention. This pSR1 system also maintains its inherent function in higher plants (H. Onouchi *et al., Nucleic Acid Res.,* vol.19, p.6373, 1991).

In the present invention, the morphological abnormality induction (MAI) gene may be inserted into a position where this gene is excised along with the removable DNA element. For instance, when the transposon is used as the removable DNA element, the MAI gene can be inserted into a position which does not influence the excision of the transposon and which is upstream of the promoter region of the transposase gene but downstream of the terminal region to which the transposase binds. When the pSR1 system is used, the MAI gene can be inserted into any position within the region held by the couple of characteristic DNA sequences which does not inhibit the expression of the recombinase.

In the present invention, the MAI gene is preferably present within the removable DNA element. On the other hand, the position of the desired gene is not particularly limited; however, preferably, the desired gene is present outside of the removable DNA element.

Using the vector of such a structure after the desired gene introduction, the MAI gene can be removed at a certain frequency, along with the removable DNA element, from the DNA in which it is introduced and functions. The desired gene which does not behave integrally with the marker gene remains in the same DNA. The vector can be used to multiply introduce a desired gene into a certain plant. In addition, since the loss of the function of this MAI gene can be visually detected as a morphological change of the transgenic tissue during cultivation, the tissue made up only of the cells with the desired gene but without the marker gene can be selected with ease and without the need for a special procedure. Consequently, even when such cells are actually formed at a low ratio, the cells can be sufficiently selected to make the procedure practically useful. Further, not only can the multiple introduction of the gene using this vector be repeated many times, but this can be repeated before a mature plant is regenerated. Thus, multiple introduction can be conducted efficiently. In order to obtain the individual transgenic plant made up only of such cells, the plant may be regenerated from the thus-selected tissue without having to undergo the crossing step. The thus-obtained individual transgenic plant is completely free from any adverse effects on the human body caused by the marker gene as mentioned above. Moreover, the use of this vector does not

require a cell growth inhibitory substance in the step of selecting the transgenic tissue that might decrease the activity of the cell.

The vector of the present invention can be used in any plants into which the gene can be introduced by genetic engineering methods. The desired gene in accordance with the present invention can be any gene by which agriculturally desirable characteristics can be imparted and any gene which allows for studies of gene expression mechanism and the like, though agriculturally desirable characteristics are not necessarily imparted.

For producing a protein such as enzyme from a gene, a structural gene sequence encoding information for the polypeptide and regulatory sequences of the structural gene, such as a promoter sequence (expression initiation sequence), a terminator sequence (expression termination sequence) and the like, are generally required. Examples of suitable promoter sequences that function in plants include the 35S promoter of a cauliflower mosaic virus (J.T. Odell *et al., Nature* (London), vol. 313, p.810, 1985), the promoter of a nopaline synthetase (W.H.R. Langridge *et al., Plant Cell Rep.,* vol. 4, p.355, 1985), and the promoter of ribulose diphosphate carboxylase/oxygenase small subunit (R. Fluhr *et al., Proc. Natl. Acad. Sci. U.S.A.,* vol.83, p.2358, 1986). Examples of suitable terminator sequence include the polyadenylation signal of a nopaline synthetase (A. Depicker *et al., J. Mol. Appl. Gen.,* vol. 1, p. 561, 1982) and the polyadenylation signal of an octopine synthetase (J. Gielen *et al., EMBO J.,* vol.3, p. 835, 1984). Accordingly, when necessary, a gene on the vector of the present invention comprises a structural gene and the gene expression

regulatory sequences thereof. The gene, or gene and regulatory sequences, can be obtained by chemical synthesis or by cloning cDNA or genomic DNA.

The vector of the present invention can be indirectly introduced into the plant cell through viruses or bacteria with which plants are infected (I. Potrykus, *Annu. Rev. Plant Physiol. Plant Mol. Biol.*, vol.42, p.205, 1991). Examples of suitable viruses include cauliflower mosaic virus, geminivirus, tobacco mosaic virus and brome mosaic virus. Examples of suitable bacteria include *Agrobacterium tumefaciens* (hereinafter referred to as *A. tumefaciens),* and *Agrobacterium rhizogenes* (hereinafter referred to as *A. rhizogenes*). Dicotyledonous plants are generally known to be infected with bacteria of the genus *Agrobacterium.* Recently, the introduction of genes into monocotyledonous plants by infection with *Agrobacterium* has also been reported (for example, International Laid-Open Patent Application No. WO 94/00977).

The vector of the present invention can be directly introduced into the plant cell by physical and chemical methods such as microinjection, electroporation, a polyethylene glycol method, a fusion method and high-speed ballistic penetration (I. Potrykus, *Annu. Rev. Plant Physiol. Plant Mol. Biol.*, vol.42, p.205, 1991). Since the general indirect introduction method using the genus *Agrobacterium* cannot be applied to many of the monocotyledonous plants and the dicotyledonous plants which are resistant to infection with *Agrobacterium,* the above-mentioned plants direct introduction methods are effective for these plants.

The vector for use in the present invention is not particularly limited as long as the requirements of the present invention are satisfied. For example, if the vector is indirectly introduced into the plant cell, the vector may be a Ti vector or a virus vector. Examples of the Ti vector for use in the present invention include Bin19 (M. Bevan *et al., Nucleic Acids Res.,* vol.12, p.8711, 1984), pRAL3940 (A. Hoekema *et al., Plant Mol. Biol.,* vol.5, p.85, 1985), pGA492 and pGA482 (G. An, *Plant Physiol.,* vol.81, p.86, 1986), pC22 (C. Simoens *et al., Nucleic Acids Res.,* vol.14, p.8073, 1986), pAGS111 (P. van den Elzen *et al., Plant Mol. Biol.,* vol.5, p.149, 1985), pEND4K (H. J. Klee *et al., Bio/Technology,* vol.3, p.637, 1985), pGV831 (R. Delaere *et al., Nucleic Acids Res.,* vol.13, p.4777, 1985), and pMON200 (R.T. Fraley *et al., Bio/Technology,* vol.3, p.629, 1985). Examples of the virus vector for use in the present invention include cauliflower mosaic virus vector (N. Brisson *et al., Nature* (London), vol.310, p.511, 1984), geminivirus vector (R.J. Hayes *et al., Nature* (London), vol.334, p.179, 1988), brome mosaic virus vector (R. French *et al., Science,* vol.231, p.1294, 1986), tobacco mosaic virus vector (N. Takamatsu *et al. EMBO J.,* vol.6, p.307, 1987), and agroinfection vector (N. Grimsley *et al., Nature* (London), vol.325, p.177, 1987). However, the vectors for use in the present invention are not limited thereto.

Furthermore, the desired gene for use in the present invention is not particularly limited. The nature of the desired gene itself is not critical to the present invention. Examples of the desired gene for use in the present invention include genes for disease resistance (e.g., gene for endotoxin of *Bacillus thuringiensis,* WO 92/20802), herbicide resistance (e.g. mutant acetolactate

synthase gene, WO 92/08794), seed storage protein (e.g., glutelin gene, WO 93/18643), fatty acid synthesis (e.g., acyl-ACP thioesterase gene, WO 92/20236), cell wall hydrolysis (e.g., polygalacturonase gene (D. Grierson *et al., Nucleic Acids Res.,* vol.14, p.8595, 1986)), anthocyanin biosynthesis (e.g., chalcone synthase gene (H.J. Reif *et al., Mol. Gen. Genet.,* vol.199, p.208, 1985)), ethylene biosynthesis (e.g., ACC oxidase gene (A. Slater *et al., Plant Mol. Biol.,* vol.5, p.137, 1985)), active oxygen-scavenging system (e.g., glutathione reductase gene (S. Greer & R.N. Perham, *Biochemistry,* vol.25, p.2736, 1986)), and lignin biosynthesis (e.g., phenylalanine ammonia-lyase gene, cinnamyl alcohol dehydrogenase gene, *o*-methyltransferase gene, cinnamate 4-hydroxylase gene, 4-coumarate-CoA ligase gene, cinnamoyl CoA reductase gene (A.M. Boudet *et al., New Phytol,* vol. 129, p.203, 1995)). However, the desired genes for use in the present invention are not limited thereto.

Moreover, the host plant for use in the present invention is not particularly limited. Examples of herbaceous plants used as the host plant include tobacco (*Tabacum)*, tomato (*Lycopersicom)*, sweet potato (*Impoea),* potato (*Solanum),* carrot (*Dacus)*, lettuce (*Lactuca),* cauliflower (*Brassica)*, cabbage (*Brassica),* oilseed rape (*Brassica)* sunflower (*Helianthus),* sugar beat (*Bela),* asparagus (*Asparagus),* banana (*Musa*), cotton (*Gossypium*), arabidopsis (*Arabidopsis),* alfalfa (*Medicago),* peas (*Pisum)*, soybean (*Glycine),* rice (*Oryza),* corn (*Zea),* and rye (*Secale).* Examples of arboreous plants used as the host plant include poplar (*Populus),* eucalyptus (*Eucalyptus),* acacia (*Acacia),* pear (*Pyrus),* apple (*Malus),* grape (*Vitis),* walnut (*Juglans),* plum (*Prunus),* rose (*Rosa),* and spruce

(*Picea*). However, the host plants for use in the present invention are not limited thereto.

In the present invention, the MAI gene is expressed to make the inside of the cell physiologically abnormal. Physiological abnormalities include the production of plant growth hormone in a plant cell, with the result that proliferation and differentiation of the cell containing the MAI gene are confused, inducing various morphological abnormalities. For example, an aggregate of disordered shoots when apical dominance is destroyed (extreme shooty phenotype; ESP) or hairy roots or the like may be exhibited in a tissue into cells of which such an MAI gene is introduced. The phenotype is formed by abnormal proliferation and differentiation of the above-mentioned cell. Thus, this morphologically abnormal tissue is made up only of cells containing this gene. Accordingly, if the vector is constructed using this gene as the marker gene together with the desired gene and is introduced into the plant cell and the cell is cultivated, the tissue made only of the cell into which the marker gene and the desired gene have been introduced can be selected by merely visually selecting the morphologically abnormal tissue formed from the plant cell. Thus, it is possible to visually select the transgenic tissue without conducting any special procedures such as the addition of plant cell growth inhibitory substance and the like to a culture medium.

Whereas conventional marker genes, such as the NPTII gene, are not introduced into plants without genetic engineering; the MAI gene of the present invention is a gene which plants inherently retain or which is naturally introduced

into plants by infection with bacteria or the like. For this reason, the safety of that gene product to the human body is considered to be quite high.

Further, in the present invention, the MAI gene is inserted into a position such that it behaves integrally with the removable DNA element. After the vector having such a structure is introduced into the plant, the MAI gene used as the marker gene is removed from DNA along with the removable DNA element at a fixed frequency resulting in the loss of its function. Meanwhile, the desired gene which does not behave integrally with the removable DNA element remains in the same DNA and maintains its function. So, the expression of the same marker gene can be used as an index for the introduction of a desired gene repeatedly. Accordingly, this vector permits multiple introduction of a desired gene into a certain plant by merely changing the structure related to the desired gene to be introduced without imparting any change on the structures of the marker gene and other genes. For this reason, the vector can be repetitively used for an unlimited number of times.

Since the loss of the function of the marker gene, that is, the loss of the function of the MAI gene, can be visually detected, the tissue made up of cells lacking the marker gene and containing the desired gene can be obtained readily and confidently. Thus, cultivation, visual selection and separation of the tissue may be performed without the need for any special procedure. Further, the plant made up only of the cells containing the desired gene can be obtained by simply regenerating the plant from the obtained tissue, without having to undergo the crossing step. Still further, although a transposon may not be completely

removed from DNA because of its high transposability, the invention is sufficiently practical because selection of the desired tissue is highly efficient.

The present invention will now be illustrated by referring to the following Examples, but the present invention should not to be construed as being limited thereto.

In the following Examples, experiments were conducted according to the instruction of *Molecular Cloning,* 2nd edition (Sambrook *et al.* eds., *Cold Spring Harbor Laboratory Press,* New York, 1989) or as otherwise specified.

EXAMPLE 1

I. Construction of a vector

An *ipt* gene present in T-DNA of pathogenic *A. tumefaciens* strain P022 (H. Wabiko, *Chemical Regulation of Plants,* vol.24, p.35, 1989 (see Figure1)) was cut out with restriction endonuclease *PstI*, and plasmid *pIPT1* was obtained by ligating the *ipt* gene into the *PstI* restriction endonuclease site of plasmid puC7 (*Molecular Cloning,* 2nd edition, vol.1, 4.10). From this plasmid, an *ipt* gene containing a native promoter and a native polyadenylation signal was cut out with restriction endonucleases *Bam*HI and *Pst*I, and plasmid pIPT2 was obtained by ligating the *ipt* gene into the *Bam*HI-*Pst*I restriction endonuclease sites of plasmid pUC119 (obtained from Takara Shuzo Co., Ltd.). From this plasmid, the structural gene and the native polyadenylation signal of the *ipt* gene were cut out with restriction endonuclease *Rsa*I, and plasmid pIPT3 was obtained by ligating the *ipt* gene into the *Sma*I restriction endonuclease site of plasmid pUC119. Further, the *ipt* gene inserted into pIPT3 was cut out with restriction endonucleases *Bam*HI and *Sac*I, and plasmid pIPT4 was obtained by ligating the

fragment into the *Bam*HI-*Sac*I restriction endonuclease sites of vector plasmid pBI121 (obtained from Clontech Co.) which is useful for gene introduction into a plant. When a plant is infected with A. *tumefaciens* having the plasmid pIPT4, a T-DNA region which exists between an LB site and an RB site, here a region of approximately 5 kb having the NPTII gene and the *ipt* gene, is integrated into the chromosome of the plant.

This plasmid pIPT4 was introduced into *E. coli (Escherichia coli)* JM109 strain, and it was deposited in accordance with the Budapest Treaty as E. coli JM109 (pIPT4) under Deposit No. FERM BP-5063.

The strategy for constructing the plasmid pIPT4 is schematically shown in Figures 2 to 4. The restriction endonuclease map of the T-DNA region thereof is shown in Figure 5. In Figures 2 to 4 and 5, encircled "P" and "T" indicate a native promoter and a native polyadenylation signal of the *ipt* gene, respectively. 35S-P indicates a 35S promoter of a cauliflower mosaic virus, and Nos-P indicates a promoter of a nopaline synthetase gene. T (Figure 4) or Nos-T (Figure 5) indicates a polyadenylation signal of the nopaline synthetase gene.

In this Example, as shown in Figure 5, for the MAI gene as the marker gene, the *ipt* gene which contributes to formation of an ESP by inducing the destruction of apical dominance was used, and the NPTII gene was used as a model of the desired gene. The *ipt* gene is a member of oncogenes that pathogenic *A. tumefaciens* retains. A plant cell into which this *ipt* gene is introduced differentiates, leading to the formation of an ESP through the overproduction of cytokinin, which is a plant hormone.

In this Example, 35S promoter of a cauliflower mosaic virus was used for a promoter sequence of the *ipt* gene, and the native polyadenylation signal of the *ipt* gene itself was used for a terminator sequence.

II. Introduction of pIPT4 into *Agrobacterium*

A. tumefaciens strain LBA4404 (obtained from Clontech Co.) was inoculated into 10ml of YEB liquid culture medium (containing 5 g/liter of beef extract, 1 g/liter of yeast extract, 1 g/liter of peptone, 5 g/liter of sucrose, and 2 mM $MgSO_4$, pH of 7.2 at 22°C (the pH at 22°C is applied to the following unless otherwise specified)), and was cultivated at 28°C until OD_{630} was within the range of from 0.4 to 0.6. Then, the culture was centrifuged at 6,900 x g for 10 minutes at 4°C to collect the cells. The cells were suspended in 20 ml of 10-mM Tris-HCl (pH 8.0), and the suspension was recentrifuged at 6,900 x g for 10 minutes at 4°C. Subsequently, the collected cells were resuspended in 200μl of YEB liquid culture medium, and this suspension was used as a cell solution for introducing a plasmid.

In a 15ml tube (made by Falcon), 200μl of the cell solution for introducing the plasmid was mixed with 6μg of plasmid pIPT4 obtained in the above-described step I, and the mixture was cooled by dipping it for 5 minutes in ethanol which had been cooled in liquid nitrogen for 30 to 40 minutes. The cooled solution, together with the tube, was allowed to stand in a water bath of 29°C for 25 minutes. Then, 750μl of YEB liquid culture medium was added thereto, and the mixed solution was cultivated at 29°C for 1 hour while being shaken. This cell solution was spread on YEB agar culture medium (containing 1.2 w/v% agar and the same ingredients as those of the above-mentioned

culture medium) to which 50 mg/ liter of kanamycin were added, and cultivated at 28°C for 2 days. The obtained cell colonies were inoculated into YEB liquid culture medium and further cultivated. Thereafter, plasmids were extracted from cells by an alkali method, and cleaved with restriction endonucleases *PstI*, *Bam*HI and *Eco*RI. The obtained fragments of the plasmid were analyzed by agarose gel electrophoresis, and it was confirmed that the plasmid pIPT4 was introduced into *A. tumefaciens* strain LBA4404.

III. Introduction of pIPT4 from Agrobacterium into tobacco

Matured leaves of tobacco (*Nicotiana tabacum* cv. xanthi, hereinafter tobacco means this variety unless otherwise indicated) grown in a greenhouse were dipped in a 1 v/v% sodium hypochlorite aqueous solution for sterilization, and washed three times with sterile water. Then, the midrib of the leaf was removed to form leaf discs of approximately 8 mm square. The leaf discs were then dipped for approximately 1 minute in a cell suspension of *A. tumefaciens* strain LBA4404 containing pIPT4 in the above-described step II, and was infected therewith (the suspension was diluted with sterilized water at OD_{630}=0.25 after overnight cultivation in YEB liquid culture medium). The infected leaf disc was put on sterilized filter paper to remove any extra cell suspension. It was then laid on hormone-free MS agar culture medium (T. Murashige and F. Skoog, *Physiol. Plant.*, vol.15, p.473, 1962 (provided that a 0.8 w/v% agar was added thereto)) containing 50 mg/liter of acetosyringone with the back of the leaf facing upward, and was cultivated for 3 days, at 25°C in full light (cultivation of explants, plant tissues and plants were conducted under these temperature and lighting conditions unless otherwise specified). The cultivated

leaf disc was then transplanted into hormone-free MS agar culture medium containing only 500 mg/liter of carbenicillin, and the cultivation was continued. As a result, 22 adventitious shoots were redifferentiated. These adventitious shoots were separated and further cultivated in a culture medium having the above-mentioned composition to obtain 6 ESP lines. These ESP lines were subcultured in the same culture medium every month, and were subcultured in hormone-free MS agar culture medium not containing carbenicillin several times 3 months after an infection. After the proliferation of *Agrobacterium* was not observed, a test for kanamycin resistance and PCR analysis were carried out.

IV. Analysis of tobacco into which a gene has been introduced

A. Test for kanamycin resistance

The 6 ESP lines obtained in the above-described step III were cultivated as such without subculture. Leaves developed from these ESP lines were cut out to form leaf discs of approximately 3 mm square. The thus-obtained leaf discs were laid on MS agar culture medium (1 mg/liter of benzyl adenine and 0.2 mg/liter of α-naphthalene acetic acid were added thereto) containing 200 mg/liter of kanamycin. After cultivation in this kanamycin containing culture medium for 1 month, the formation of ESP lines was also observed on the leaf discs obtained from these ESP lines.

B. PCR analysis

Chromosomal DNA was extracted from all of the 6 ESP lines obtained in the above-described step III, and the genes introduced thereinto were analyzed by the PCR method.

The chromosomal DNA was extracted by the following modified CTAB method.

First, approximately 1 g of the leaves developed from the ESP was ground in liquid nitrogen using a chilled mortar and pestle, and suspended in 5 ml of a buffer (containing 2 w/v% CTAB (hexadecyltrimethylammonium bromide), 1.4 M NaCl, 0.2 v/v% β-mercaptoethanol, 20 mM EDTA, and 100 mM Tris-HCl (pH 8.0)) which had been heated at 60°C . This suspension was heated at 60°C for 30 to 60 minutes while being gently shaken, and was then cooled to room temperature. To this suspension a mixture of chloroform and isoamyl alcohol (24:1) at an equal volume was added, and these were gently mixed. Then, the mixture was centrifuged at 1,600 x g for 5 minutes to recover a supernatant. Subsequently, 2/3 volume of isopropyl alcohol was added to the supernatant, and these were gently mixed again. The mixture was allowed to stand on ice for 10 minutes to precipitate the chromosomal DNA. This chromosomal DNA was collected by centrifugation at 1,600 x g for 10 minutes. The thus-collected chromosomal DNA was washed with 70 v/v% ethanol, then vacuum-dried, and dissolved in 300μl of TE (comprising 10 mM Tris-HCl and 1 mM EDTA).

Meanwhile, in order to detect the *ipt* gene by the PCR method, a pair of primers (oligonucleotide) were synthesized by a DNA synthesizer (manufactured by Applied Biosystems Co.) When they were binding to the *ipt* gene the distance between the two primers became approximately 800 bp. To amplify the *ipt* gene, 1μg of the extracted chromosomal DNA was dissolved in 50 μl of a mixed solution containing 0.2 μM of these primers, 10 mM Tris-HCl (pH of 8.8 at 25°C), 50 mM KCl, 1.5 mM $MgCl_2$, 1 w/v% Triton X-100, 0.1 mM dNTP and

1.25 units of Tag polymerase (obtained from CETUS CO.) . After the mixture was heated at 94°C for 1.5 minutes, a three-part heating cycle, namely 94°C for 1 minute, 55°C for 2 minutes and 72°C for 3 minutes was repeated for a total of 30 times to complete the reaction. The obtained reaction mixture was analyzed by agarose gel electrophoresis to detect the presence of the *ipt* gene in the chromosomal DNA.

The results are shown in Figure 6. As is clear from Figure 6, the amplification of the gene of approximately 800 bp was observed in all of the 6 ESPs. In Figure 6, the values shown on the left side indicate the length of bases of band ingredients detected (hereafter referred to as the "band") in the electrophoresis of the DNA size marker.

COMPARATIVE EXAMPLE 1

The analysis was conducted with respect to 16 shoots which lacked the ability to form an ESP and were obtained from the adventitious shoots redifferentiated from the *A. tumefaciens*-infected leaf in step III of Example 1. That is, at the time the 22 adventitious shoots were cultivated and the 6 ESP lines were selected in step III of Example 1, those showing morphologically normal shoots (hereinafter referred to as "non-ESP") were also freed from *A. tumefaciens* and subjected to the test for kanamycin resistance in the same manner as in steps III and IV of Example 1. Further, 9 non-ESP lines were subjected to PCR analysis. However, with respect to these non-ESP lines, the leaf discs laid on the kanamycin-containing culture medium all turned brown and withered after approximately 3 months. Further, in the PCR analysis,

amplification of a DNA fragment of approximately 800 bp which proves the presence of the *ipt* gene was not detected in any of the analyzed nine lines.

The results of the PCR analysis are shown in Figure 6.

EXAMPLE 2

I. Construction of a vector

Plasmid pHSG398 (obtained from Takara Shuzo Co., Ltd.) was digested with restriction endonuclease *Bam*HI. The cohesive termini produced by the digestion were changed into blunt-ended termini with T4DNA polymerase I (large subunit), and plasmid pNPI100 was obtained by ligating these termini. That is, the pNPI100 was the pHSG398 losing the *Bam*HI restriction endonuclease site. Meanwhile, plasmid pCKR97 (T. Izawa *et al., Mol. Gen. Genet.*, vol.227, p.391, 1991) was digested with restriction endonuclease *Pst*I. Transposon *Ac* of maize was cut out, and inserted into the *Pst*I restriction endonuclease site of the pNPI100 to obtain plasmid pNPI102.

Subsequently, from the plasmid pIPT4 constructed in Example 1, a cauliflower mosaic virus 35S promoter and an *ipt* gene linked thereto were cut out with restriction endonucleases *Hind*III and *Sac*I. The cohesive termini of the thus-obtained fragment were changed into blunt-ended termini with T4DNA polymerase I, and the fragment was inserted into the *Hinc*II restriction endonuclease site of the plasmid pUc119 to obtain plasmid pNPI101. From this plasmid pNPI101, the cauliflower mosaic virus 35S promoter and the *ipt* gene were cut out again with restriction endonucleases *Pst*I and *Eco*RI, and the cohesive termini of the fragment were changed into blunt-ended termini with T4DNA polymerase I. Further, plasmid pNPI103 was obtained by ligating the

fragment into the blunt-ended *Bam*HI endonuclease site of pNPI102. That is, in the plasmid pNPI103, the 35S promoter and the *ipt* gene linked thereto existed in the old *Bam*HI restriction endonuclease site within transposon *Ac*.

The desired vector was obtained by cutting out transposon *Ac* containing the cauliflower mosaic virus 35S promoter and the *ipt* gene from the plasmid pNPI103 with restriction endonuclease *Pst*I, and inserting this transposon *Ac* into the *Sse*I restriction endonuclease site of vector plasmid pBI121. This was designated as plasmid pNPI106.

This plasmid pNPI106 was also introduced into *E. coli* JM109 strain, and it was deposited in accordance with the Budapest Treaty as E. coli JM109 (pNPI106) under Deposit No. FERM BP-5064.

The strategy for constructing the plasmid pNPI106 is schematically shown in Figures 7 to 9. A restriction endonuclease map of the T-DNA region thereof is shown in Figure 10. In Figures 7 to 9 and 10, the terminal region of transposon *Ac* is shown by opposite black triangles, respectively. In Figure 10, Ac-P is a native promoter present within *Ac.* Other symbols are the same as those shown in Figures 2 to 5.

As is clear from Figure 10, this plasmid has the *ipt* gene as the marker gene, and the NPTII gene and GUS (β-galactosidase) gene as a model of the desired gene in the T-DNA region, namely in the region to be integrated into the chromosome of the plant. Further, the *ipt* gene is present as inserted within the transposon *Ac.* Since the cell having the GUS gene metabolizes a special substrate to produce a blue pigment, the expression of the gene can be

identified by detecting this pigment. Thus, the GUS gene is often used for analysis of the expression of a gene in a plant.

II. Introduction of pNPI106 into tobacco and analysis of tobacco into which the gene has been introduced

A. Introduction of pNPI106 into tobacco and test for expression of the introduced gene

In the same manner as in steps II and III of Example 1, pNPI106 was introduced into *A. tumefaciens* strain LBA4404, and leaf discs of tobacco were infected with this *A. tumefaciens*. The infected tobacco leaf was cultivated into hormone-free MS agar culture medium containing 50 mg/liter of acetosyringone, and then in the hormone-free MS agar culture medium to which 500 mg/liter of carbenicillin were added. After two months of such cultivation, 63 ESP lines were separated.

These ESP lines were transplanted in the culture medium of the same composition (hormone-free MS agar culture medium containing 500 mg/liter of carbenicillin). One month later, from among the shoots of the ESPs which had grown slightly, 9 shoots (those which are generated from ESPs are called simply "shoots" hereinafter) were selected visually which had grown approximately two or more times in comparison to the other shoots, in which the growth of the side shoots was not observed and in which the influence of the *ipt* gene appeared to be decreased. The leaves of those shoots were subjected to the same test for kanamycin resistance as conducted in Step IV-A of Example 1 and the test for the expression of the GUS gene (test for GUS activity) based on the method of Jefferson et al. (Plant Mol. Biol. Rep., (1987) 5:387-405). The shoots obtained after the leaves were cut off were transplanted in hormone-free MS agar culture medium and cultivated.

One month later, the shoots were observed for their ability to form ESPs, thus exhibiting expression of the *ipt* gene.

The results are shown in Table 1.

TABLE 1

Results of a test for expression of a gene introduced into tobacco by vector pNPI106

	Shoot No.	Kanamycin resistance	GUS activity	Morphology after 1 month of cultivation
Example 2	1	+	+	ESP
	2	+	+	ESP
	3	+	-	ESP
	4	+	+	ESP
	5	+	-	ESP
	6	+	-	ESP
	7	+	+	ESP
	8	+	+	normal
	9	+	+	ESP
Comparative Example 2	10	-	-	normal
	11	-	-	normal
	12	-	-	normal

Notes: 1. In the kanamycin resistance, + is “resistant”, and - is “not resistant”.

2. In the GUS activity, + is “active”, and - is “inactive”.

3. “normal” refers to an individual that exhibits dominant growth of an apical shoot and the formation of roots.

As is apparent from Table 1, although the leaf of shoot No. 8 has kanamycin resistance and GUS activity, an ESP is not formed even if the shoot is cultivated for 1 month. This is presumably because the *ipt* gene that causes the formation of the ESP is present in the inserted form within the transposon *Ac* in the plasmid pNPI106. That is, the *ipt* gene, which is introduced into a chromosome of the tobacco cell by infection with *A. tumefaciens* containing this plasmid and expressed at the initial stage of the tissue cultivation just after the infection, is removed together with *Ac* through the action of *Ac* during the subsequent cultivation. Meanwhile, in the same vector, the NPTII gene and the GUS gene are inserted into a position where they do not behave integrally with *Ac*, so that these genes still remain in the plant chromosome and are expressed.

In Table 1, although kanamycin resistance and ESP-forming ability are observed in shoots Nos. 3, 5 and 6, only the GUS activity is negative. This means that in these shoots only the GUS gene of the genes introduced by using pNPI106 is not expressed. This is considered to be owing to the erroneous integration that occurred when these genes were integrated into the plant chromosome. That is, when the gene is introduced via *A. tumefaciens* containing the plasmid which has the structure of pNPI106 or the like, the T-DNA region, namely, the overall inner region between the RB site and the LB site, must be normally integrated into the plant chromosome. However, this region is sometimes not completely integrated, but is torn and the deficient piece lacking some portion of the LB terminal is inserted. In the T-DNA region of the pNPI106, the GUS gene is present in the closest position to the LB site. Accordingly, it is possible that due to erroneous integration during the gene

introduction, the GUS gene is integrated into the chromosome in a condition in which it is broken apart and its function is lost, or the GUS gene is not inserted thereinto, so that the GUS gene is not expressed in these shoots and the activity thereof is not observed.

Photographs of shoot No. 2 and No. 8 after one month of cultivation are shown in Figures 11 and 12.

With respect to the leaf grown from shoot No. 8 and subjected to the test for kanamycin resistance, cultivation was further continued after the test. Five adventitious shoots were obtained from this leaf, and these were all non-ESPs.

B. PCR analysis

On shoots Nos. 1 to 9 in Table 1, after ESP-forming ability was observed, PCR analysis was conducted in the same manner as in step IV-B of Example 1 to further examine the presence of *ipt* gene in the chromosome, provided that the couple of primers which were designed to be bound to the NPTII gene and the GUS gene respectively were used in addition to the primers used in step IV-B of Example 1. In the case where these primers were used, when the *Ac* and the *ipt* gene inserted thereinto (*Ac-ipt* gene complex) are excised from the T-DNA region of the pNPI106, a DNA fragment of approximately 3 kb is amplified in the PCR. Accordingly, the excision of the *Ac-ipt* gene complex from the DNA can be detected using this amplification as an index.

The results of the PCR analysis on shoot No. 8 are shown in Figure 13, in which the values indicated on the left side are the same as those shown in Figure 6.

As is apparent from the results, in the chromosomal DNA extracted from shoot No. 8, the amplification of the DNA fragment of approximately 3 kb which proves the excision of the *Ac-ipt* gene complex is observed, while the amplification of a DNA fragment of approximately 800 bp which proves the presence of the *ipt* gene is not observed. This means that the *ipt* gene is excised from the chromosomal DNA of this shoot together with the *Ac* and disappears.

On the other hand, with respect to shoots Nos. 1 to 7 and 9, the amplification of the DNA fragment of approximately 3 kb was not detected in any of the chromosomal DNA samples thereof, while the amplification of the DNA fragment of approximately 800 bp was detected in all of the chromosomal DNA samples thereof. Accordingly, it is concluded that in these shoots, the *ipt* gene is still present in the chromosomal DNA along with the *Ac*.

COMPARATIVE EXAMPLE 2

In the cultivation of the *A. tumefaciens*-infected leaf in step II-A of Example 2, the 3 non-ESPs redifferentiated along with the ESPs were separated, and subjected to the test for kanamycin resistance, the test for GUS activity, visual observation after 1 month of cultivation and PCR analysis in the same manner as in II of Example 2.

The results are shown in Table 1. The shoots obtained from these non-ESPs did not possess any of the kanamycin resistance, the GUS activity and ESP-forming ability. Further, the amplification of the DNA fragments of approximately 800 bp and approximately 3 kb was not detected in the PCR analysis.

EXAMPLE 3

The cultivation of 63 ESP lines separated in Example 2 were continued in hormone-free MS agar culture medium. Approximately two months after the separation, a total of seven shoots Nos. 13-1 to 13-3 and 14-1 to 14-4 which were normal shoots identified visually, that is, which exhibited the apical dominance, were obtained from the 2 ESP lines. These shoots were separated to transplant in the culture medium having the above-mentioned composition, and they were normally extended and rooted. Of these , the individuals obtained from shoots Nos. 13-1 and 14-1 were subjected to PCR analysis in the same manner as in step II-B of Example 2. As a result, amplification of a DNA fragment of approximately 800 bp was not observed in either of shoot Nos. 13-1 and 14-1. Meanwhile, amplification of a DNA fragment of approximately 3 kb was observed in both of these shoots. It was thus determined that the *ipt* gene had been excised from the chromosomal DNA of these individuals along with the *Ac* and had disappeared. The results are shown in Figure 14, in which the values indicated on the left side are the same as those shown in Figure 6. Further, the expression of the GUS gene was detected in all of the individuals obtained from the seven shoots.

Figure 15 shows the state of a normal shoot differentiated from an ESP.

EXAMPLE 4

The leaf obtained from shoot No. 7 in Table 1 was cultivated in hormone-free MS agar culture medium for approximately 1 month. One normal shoot was visually selected and separated from 6 adventitious shoots which were redifferentiated from the cultivated leaf. This normal shoot was transplanted in

a culture medium of the above-mentioned composition, then a normal extended and rooted individual was obtained. Further, this individual was subjected to PCR analysis in the same manner as in step II-B of Example 2, and on the grounds of the disappearance of the DNA fragment of approximately 800 bp and the amplification of the DNA fragment of approximately 3 kb, it was determined that the *ipt* gene had been excised from the chromosomal DNA along with the *Ac* and had disappeared. The results are shown in Figure 16 , in which the values indicated on the left side are the same as those shown in Figure 6. Furthermore, in the same individuals, the expression of the GUS gene was also detected.

EXAMPLE 5

I. Separation of a site-specific recombination system (pSR1 system) from yeast

Yeast (*Zygosaccharomyces rouxii* (obtained from the Institute for Fermentation)) was inoculated in 5 ml of YPAD liquid culture medium (containing 10 g/liter of yeast extract, 20 g/liter of polypeptone, 0.4 g/liter of adenine and 20 g/liter of glucose), and was cultivated at 30°C for 24 hours. The culture solution was centrifuged at 6,900 x g for 3 minutes at 20°C to collect the cells (hereinafter, the cells were collected under the same conditions). The obtained cells were suspended in 2 ml of a solution comprising 0.2 M Tris-HCl (pH 8.0) and 5 v/v% mercaptoethanol. The cell suspension was allowed to stand at 25°C for 30 minutes while being gently stirred sometimes, and cells were then collected. Further, the collected cells were suspended in 1 ml of a solution (pH 6.8) containing 2.5 mg/ml of Zaimolyeis-20T (obtained from SEIKAGAKU CORPORATION), 10 w/v% sorbitol and 5 w/v% KPO_4. The suspension was

allowed to stand at 30°C for 90 minutes, and recentrifuged to collect the cells again. The collected cells were resuspended in 1 ml of a solution containing 0.2 M NaCl, 0.1 M EDTA, 5 w/v% SDS, 50 mM Tris-HCl (pH 8.5), and Proteinase K was added to be 20 mg/ml thereto. The mixed solution was allowed to stand at 60°C for 1 hour, then returned to room temperature, and extracted with a mixture of phenol and chloroform and then with chloroform to purify. To the supernatant was added in an equal volume of isopropanol to precipitate chromosomal DNA and plasmid pSR1. The mixture was centrifuged at 6,900 x g for 10 minutes at 4°C to collect the DNA, and the collected DNA was washed with 70 v/v% ethanol, then vacuum-dried and dissolved in 100 μl of TE.

Using the extracted DNA (the mixture of the chromosomal DNA and the plasmid pSR1) as a template, only a site-specific recombination system which was present in the plasmid pSR1 (hereinafter referred to as "pSR1 system") was amplified by the PCR method. The pSR1 system consists of an R gene which is a recombinase gene and a recombination sequence Rs, and their DNA sequences have been already determined (H. Araki *et al. J. Mol. Biol.,* vol.182, p.191, 1985). In the present invention, in order to amplify the R gene, a primer in which an *Xba*I restriction endonuclease site was added to a 5' - position of 22 bases, namely, 5,596th - 5,617th bases in the sequence of the plasmid pSR1 (5' - CCTCTAGAATGCAATTGACCAAGGATACTG -3') and a primer in which the *Sac*I restriction endonuclease site was added to the 5' - position of 22 bases, namely, 4,126th - 4,147th bases in the sequences of plasmid pSR1 (5' - CCGAGCTCTTAATCTTGTCAGGAGGTGTCA - 3'), were synthesized. To amplify Rs, two couples of primers each comprising 30 bases (a total of four

types) were synthesized. That is, one couple was composed of a primer in which three of the 287th-316th bases of the sequence of the plasmid pSR1 were replaced and an *Sse*I restriction endonuclease site was introduced (5'-AGGATTGAGCTACTGGACGGGAATCCTGCA- 3') and a primer in which four of the 690th - 719th bases of the sequence of the plasmid pSR1 were replaced and the *Hin*dIII restriction endonuclease site and the *Xho*I restriction endonucleases site were introduced (5'-CAACTCCGAGCAATCAAAGCTTCTCGTAGTC-3'). Rs to be amplified with this primer set was called "Rs1". Another couple was composed of a primer in which three of the 287th - 316th bases of the sequence of the plasmid pSR1 were replaced and an *Xho*I restriction endonuclease site and an *Eco*RI restriction endonuclease site were introduced (5' -AGGATTGAGCTACTCGAGGGGAATTCTGGA-3') and a primer in which five of the 688th - 717th bases of the sequence of the plasmid pSR1 were replaced and the *Sse*I restriction endonuclease site was introduced (5'-ACTGGACCAATCCCTGCAGGTCGTAGTCAA-3'). Rs to be amplified with this primer set was called "Rs2".

In order to amplify the R gene and the Rs's, 1 μl of the extracted DNA solution was added to every 50 μl of the mixed solution used in step IV-B of Example 1 containing 0.2 μM each primer set respectively. A three-part heating cycle, namely, 95°C for 30 seconds, 55°C for 30 seconds and 72°C for 1.5 minutes was repeated on the mixture a total of 30 times. The thus-obtained reaction mixture was analyzed through agarose gel electrolysis to confirm the amplification of the R gene and the Rs's.

II. Construction of a vector

The Rs1 amplified by the PCR method was digested with restriction endonucleases *Pst*I and *Xho*I, and plasmid pNPI126 was obtained by inserting this Rs1 into *Pst*I-*Xho*I restriction endonuclease sites of pSL1180 (obtained from Pharmacia Biotech Co.).

Subsequently, in order to eliminate the *Eco*RI restriction endonuclease site and the *Hind*III restriction endonuclease site of pHSG398, digestion of these restriction endonucleases, changing the digested termini into blunt-ended termini with T4 polymerase I (large subunit) and ligation of the blunt-ended termini were repeated in sequence. In this way, plasmid pNPI121 with these restriction endonuclease sites eliminated was obtained. Plasmid pNPI127 was produced by digesting the Rs2 amplified by the PCR method with restriction endonucleases *Xho*I and *Pst*I, and inserting this Rs2 into the *Sa1*I-*Pst*I restriction endonuclease sites of the plasmid pNPI121.

Plasmid pNPI128 was obtained by cutting out Rs1 from the pNPI126 with restriction endonucleases *Sma*I and *Spe*I and inserting this fragment into the *Sma*I-*Xba*I restriction endonuclease site of the pNPI127.

The R gene amplified by the PCR method was digested with restriction endonucleases *Xba*I and *Sac*I, and inserted into the *Xba*I-*Sac*I restriction endonuclease sites of pHSG398. The thus-obtained plasmid was designated pNPI124.

Then, pBI221 (obtained from Clontech Co.) was digested with restriction endonuclease *Pst*I. The digested termini were changed into blunt-ended termini and then ligated in the above-described manner. Thus, the plasmid pNPI111

with the *Sse*I and *Pst*I restriction endonuclease sites eliminated was obtained. Thereafter, the R gene cut out from the pNPI124 with restriction endonucleases *Xba*I and *Sac*I was inserted into the *Xba*I-*Sac*I restriction endonuclease sites of the pNPI111 replacing the GUS gene to produce plasmid pNPI125. Further, a cauliflower mosaic virus 35S promoter, the R gene linked to the promoter and a polyadenylation signal of nopaline synthetase were cut out with restriction endonucleases *Hind*III and *Eco*RI and inserted into the *Hind*III-*Eco*RI restriction endonuclease sites of the pNPI128 to obtain plasmid pNPI129.

pNPI101 was digested with restriction endonuclease *Sma*I, and a 5'-phospholylated *Hind*III linker (obtained from Takara Shuzo Co., Ltd.) was inserted into the digestion site to obtain plasmid pNPI122. That is, this pNPI122 is one in which the *Sma*I restriction endonuclease site of the pNPI101 was replaced with the *Hind*III restriction endonuclease site. Further, the pNPI122 was digested with restriction endonuclease *Pst*I, and the digested termini were changed into blunt-ended termini and ligated to produce plasmid pNPI123 with the *Sse*I and *Pst*I restriction endonuclease sites eliminated. From this plasmid pNPI123, a cauliflower mosaic virus 35S promoter and *ipt* gene linked to the promoter were cut out with restriction endonuclease *Hind*III, and inserted into the *Hind*III restriction endonuclease site of the pNPI129 to obtain the plasmid pNPI130.

The desired vector was obtained by cutting out the fragment containing the *ipt* gene, and the R gene, the cauliflower mosaic virus 35S promoters linked to them and the Rs's present on both terminals of these genes with restriction endonuclease *Pst*I, and inserting this fragment into the *Sse*I restriction

endonuclease site of the pBI121. The thus-obtained plasmid was designated pNPI132.

This plasmid pNPI132 was also introduced into *E. coli* JM109 strain, and was deposited in accordance with the Budapest Treaty as E. coli JM109 (pNPI132) under Deposit No. FERM BP-5065.

The strategy for constructing the plasmid pNPI132 is schematically shown in Figures 17-19. The restriction endonuclease map of the T-DNA region thereof is shown in Figure 20. In Figures 17 to 19 and 20, a hatched triangle indicates the recombination sequence Rs derived from the plasmid pSR1 of yeast and the direction of its sequence. Other symbols are the same as those shown in Figures 2 to 5.

As is apparent from Figure 20, the plasmid pNPI132, as the same as the plasmid pNPI106, has the *ipt* gene as the marker gene and the NPTII gene and the GUS gene as models of the desired gene in the T-DNA region. However, in this case, the region between the two recombination sequence Rs's of the pSRI system behaves as the removable DNA element. Therefore, the *ipt* gene is inserted such that it is held by the two same directed recombination sequences.

III. Introduction of pNPI132 into tobacco and analysis of the tobacco into which the gene has been introduced

In the same manner as in steps II and III of Example 1, the plasmid pNPI132 was introduced into *A. tumefaciens* strain LBA4404, and leaf discs of tobacco (*Nicotiana tabacum* cv. SR1) were infected with this *A. tumefaciens.* Then, the infected leaves were cultured in hormone-free MS agar culture medium containing 50 mg/liter of acetosyringone and then in hormone-free MS agar culture medium containing 500 mg/ liter of carbenicillin.

One month later, the cultured leaves were transplanted in the culture medium of the same composition, and the cultivation was further continued for 1 month. Then, 48 ESP lines were separated.

These ESP lines were transplanted again in the culture medium of the same composition, and the cultivation was further continued. Approximately one month later (namely, approximately 3 months after the infection with *A. tumefaciens*), shoots which were visually detectable to have a normal morphology were generated from seven of 48 ESP lines. These shoots were separated and transplanted into a culture medium of the same composition, and ten normal individuals were obtained.

These individuals were subjected to the PCR analysis in the same manner as in step II-B of Example 2, provided that a couple of primers for detection of the GUS gene were used in addition to the primers used in step II-B of Example 2. By conducting PCR using these primers, a DNA fragment of approximately 800 bp was simplified when the *ipt* gene was present; a DNA fragment of approximately 3 kb was amplified when the *ipt* gene was excised from the T-DNA region of the plasmid pNPI132 through the excision of the portion held by Rs's (these results are the same as in the analysis of step II-B in Example 2); and a DNA fragment of approximately 1.7 kb was amplified when the GUS gene was present. The results are shown in Figures 21-23 and Table 2. In Figures 21-23, the values indicated on the left side are the same as those shown in

Figure 6.

TABLE 2

Results of analysis of a transgenic gene in tobacco into which the gene is introduced with vector pNPI132

ESP line No.	Individual plant No.	re *ipt* 800bp	re *ipt* 3kb	re GUS 1.7kb
15	1	-	+	+
	2	-	+	+
16	1	-	+	+
17	1	-	+	+
18	1	-	+	+
19	1	-	+	+
	2	-	+	+
20	1	-	+	+
	2	-	+	+
21	1	-	+	+

Notes: + indicates that the corresponding DNA fragment is amplified, and - indicates that it is not amplified.

As is apparent from Table 2, the presence of the *ipt* gene which was the marker gene was not detected in any of chromosomes of the individuals that had been selected simply by visual observation of their morphology, and instead, the amplification of the DNA fragment which indicates the excision of the *ipt* gene was detected. Meanwhile, the presence of the GUS gene used as the desired gene was detected in all of the individuals.

Kanamycin resistance was examined using the terminal buds of individuals, which were obtained from non-ESPs differentiating almost simultaneously with the 48 ESP lines and showed normal elongation and rooting, with the use of a hormone-free MS agar medium containing 200 mg/l of kanamycin. As a result, it was found that two among 16 individuals had resistance to kanamycin.

Subsequently, these kanamycin-resistant individuals were further examined by subjecting them to PCR analysis together with three individuals selected among 14 kanamycin-sensitive individuals in the same manner as that employed for individuals obtained from the ESPs. Figure 24 shows the results wherein the values indicated on the left side are the same as those shown in Figure 6.

As Figure 24 clearly shows, each of the two kanamycin-resistant individuals exhibited the amplification of a DNA fragment which indicated the excision of a region containing the *ipt* gene and held by Rs's, and the presence of the GUS gene. Thus it was proved that genes originated in pNPI132 had been integrated into these chromosomes. In contrast, no such amplification was observed in the three kanamycin-sensitive individuals. Further, none of these

individuals (namely, neither kanamycin-resistant individuals nor kanamycin-sensitive individuals) showed amplification of a DNA fragment indicating the presence of the *ipt* gene.

It is assumed that these kanamycin-resistant individuals, obtained from a strain lacking an ability to from ESPs, as the same as the kanamycin-sensitive individuals, originated from cells into the chromosomes of which pNPI132 had not been introduced when infected with *A. tumefaciens*. Based on this assumption, the genes originating in this vector must not have been contained in the chromosomes. Moreover, it is unreasonable that individuals, which lacked the *ipt* gene but contained the NPTII gene (as indicated by the fact that these individuals were resistant to kanamycin) and the GUS gene each in the complete form in the chromosomes, should appear at such a frequency considering that all these genes originated in the same vector.

Therefore, it is reasonable to conclude that, in these kanamycin-resistant individuals, pNPI132 had been introduced into the chromosomes. That is to say, it is probable that the T-DNA region of pNPI132 was introduced into the chromosome during infection with *A. tumefaciens*, but the excessively efficient function of the pSR1 system used for this vector induced the excision of the *ipt gene* prior to the formation of ESPs following the infection with *A. tumefaciens*. As a result, the NPTII gene and the GUS gene exclusively remained in the chromosome. The fact that the kanamycin-resistant individuals showed excision of the region containing the *ipt* gene and held by Rs's in PCR analysis also supports this conclusion.

EXAMPLE 6

I. Construction of a vector

Rol genes (S. Kiyokawa , *Plant Physiol.*, vol. 104, p.801, 1994) containing *rolA, rolB* and *rolC* and having a total size of 7.6 kb, which genes had been inserted into the *Eco*RI restriction endonuclease site of pBluescriptII SK÷ (made by Toyobo Co., Ltd.), was cut out with a restriction endonucelase *Eco*RI. This fragment was inserted into the *Eco*RI restriction endonuclease site of the pNPI129 to produce plasmid pNPI700.

From plasmid pNPI700, the *rol* genes, the cauliflower mosaic virus 35S promoter, the R gene linked to the promoter and the Rs's present on both terminals of these genes were cut out with restriction endonuclease *Sse*I, and inserted into the *Sse*I restriction endonuclease site of the pBI121 to obtain the desired plasmid pNPI702.

Plasmid pNPI702 was introduced into *E. coli* JM109 strain, and was deposited in accordance with the Budapest Treaty as E. coli JM109 (pNPI702) under Deposition No. FERM BP-5066.

The strategy for constructing the plasmid pNPI702 is schematically shown in Figure 25. The restriction endonuclease map of the T-DNA region thereof is shown in Figure 26. The symbols in Figures 25 and 26 are the same as those in Figures 2 to 5.

As is apparent from Figure 26, the plasmid pNPI702 is similar to pNPI132, but only the marker gene was changed from the *ipt* gene to the *rol* genes. The *rol* genes used for this vector are present in the T-DNA of *A. rhizogenes* in nature. It is known that when the *rol* genes are introduced into plant cells, hairy

roots are generated in the plant tissue and that the plant regenerated from this hairy root shows morphological abnormality such as dwarfishness or the like.

II. Introduction of pNPI702 into tobacco and analysis of the tobacco into which the gene has been introduced

In the same manner as in steps II and III of Example 1, the plasmid pNPI702 was introduced into *A. tumefaciens* strain LBA4404, and leaf discs of a tobacco were infected with this *A. tumefaciens.*

The thus-infected tobacco leaf disc was cultivated in hormone-free MS agar culture medium containing 50mg/liter of acetosyringone in a dark place for 3 days, and then in hormone-free MS agar culture medium to which 400 mg/liter of ticarcillin were added. Approximately 15 days from the beginning of cultivation, differentiation of hairy roots was observed. The hairy roots were separated, and laid on a shoot induction culture medium (MS agar culture medium containing 0.1 mg/liter of α-naphthaleneacetic acid, 2.0 mg/liter of benzyladenine and 400 mg/liter of ticarcillin). From among the redifferentiated shoots, 18 shoots considered to have normal morphology were visually selected, and subjected to PCR analysis in the same manner as in step II-B of Example 2, using the primers to detect for eliminating the region containing the *rol* genes and held by Rs's through the amplification of a DNA fragment of approximately 3 kb (using the same primers as in Examples 2 to 5 and Comparative Example 2) and the primers for detection of the presence of the *rol* genes through the amplification of a DNA fragment of approximately 1.1 kb. As a result, it was confirmed that the region containing the *rol* genes and held by Rs's was excised from the chromosomes of the 9 shoots.

EXAMPLE 7

By using the vector pNPI106 constructed in the above Example 2, a hybrid aspen (*Populus Sieboldii* x *Populus grandidentata;* a woody plant) was subjected to gene introduction.

The stem of the hybrid aspen strain Y63 (harvested in the experimental forest of Akita Jujo Chemicals Co., Ltd.) grown in a sterilized flask was cut into a nod-free section of 5 mm in length. Then it was further vertically cut into two pieces to use as a sample, and the sample was infected with the pNPI106-introduced *A. tumefaciens* strain LBA4404 in the same manner as in step of Example 1. After the infection, the stem section was placed on a hormone-free modified MS agar culture medium (containing 2 w/v% sucrose and 0.8 w/v% agar) to which 40 mg/liter of acetosyringone was added and cultivated therein for 3 days. Subsequently it was transplanted into the same medium but containing 500 mg of carbenicillin instead of acetosyringone and further cultivated therein. The modified MS culture medium employed herein is one prepared by changing the concentrations of ammonia-nitrogen and nitrate-nitrogen of the usual MS culture medium to 10 mM and 30 mM, respectively.

After approximately two months, the adventitious buds growing from this section were separated and further cultivated for 2 months. Thus 6 ESP lines were obtained. These lines were further subcultured, and approximately 4 months thereafter (i.e., approximately 8 months after the infection with *A. tumefaciens)*, morphologically normal shoots growing from the ESPs were observed for the first time. Then these shoots were transplanted into a 2/3 diluted MS gellan gum medium (containing 2 w/v% sucrose and 0.3 w/v% gellan

gellan gum) to which 0.05 mg/liter of IBA (indolebutyric acid) were added and cultivated therein. Thus, several normal individuals growing roots were obtained in total from the 2 EPS lines within ten months following the infection with *A. tumefaciens*.

These individuals were then subjected to the PCR analysis in the same manner as in step III of Example 5. As a result, the *ipt* gene was detected from none of them. On the other hand, the presence of the GUS gene was detected in two individuals among them. Thus it was confirmed that the vector of the present invention would work efficiently in a woody plant too.

In the remaining 5 normal individuals, only a part of the GUS gene was detected. It seems that when the transposon *Ac* introduced by the vector pNPI106 was excised from the chromosome together with the *ipt* gene, part of the GUS gene located in the vicinity thereof was involved in the excision.

Figure 27 shows the state of a normal shoot differentiated from an ESP.

EXAMPLE 8

The normal individual (obtained via ESP) having the genes introduced thereinto by pNPI132 in Example 5 was further subjected to gene introduction with the use of the vector of the present invention.

The GUS gene of pNPI132 was replaced by an HPT gene (hygromycin resistance gene). Using the thus-obtained vector (pNPI140, Figure 28), the above-mentioned normal individuals were subjected to gene introduction in the same manner as in steps II and III of Example I. Thus 10 ESP lines were obtained 40 days after the infection with *A. tumefaciens* having this vector introduced thereinto. These ESP lines were separated, transplanted into a

culture medium of the same composition (hormone-free MS agar culture medium containing 500 mg/liter of carbenicillin) and further cultivated therein. In the resulting differentiation of shoots, visually normal morphology was observed in one of these lines 20 days thereafter (namely, approximately 2 months after the infection with *A. tumefaciens)*.

One of these normal shoots thus differentiated was subjected to PCR analysis in the same manner as in steps of IV-B of Example 1. Figure 29 shows the result. It is to be noted that a couple of primers designed to bind onto the NPTII gene and the HPT gene respectively, for detecting the excision of the region containing the *ipt* gene and held by Rs's, from the chromosomal DNA, and another couple of primers for detecting the presence of the HPT gene (detected through the amplification of DNA fragments of approximately 4 kb and approximately 1 kb, respectively) were employed herein in addition to the primers used in step IV-B of Example 1. In Figure 29, the values indicated on the left side are the same as those shown in Figure 6.

As Figure 29 clearly shows, in the PCR analysis, the chromosomal DNA extracted from the shoot showed the amplification of the DNA fragment of approximately 4 kb, which indicated that the *ipt* gene had been excised through the excision of the region held by Rs's, and the amplification of the DNA fragment of approximately 1 kb, which indicated the presence of the HPT gene. On the other hand, the amplification of the DNA fragment of approximately 800 bp, which indicated the presence of the *ipt* gene, was not detected in the same DNA. These results suggest that the *ipt* gene, which had been previously introduced into the chromosomal DNA of this shoot, was excised therefrom

through the excision of the region held by Rs's and thus disappeared, while the HPT gene still remained in the DNA. That is to say, an individual introduced with the desired genes (i.e., the NPTII gene and the GUS gene) by pNPI132 was further introduced with a novel desired gene (i.e., the HPT gene), by using a vector, wherein the construction relating to the desired gene was exclusively altered (i.e., the same *ipt* gene used as the marker gene), with repeating the usual cultivation, the visual selection and the separation. Moreover, the results suggest that third, fourth or more desired genes could be introduced with the use of the same marker gene.

As is apparent from the above-described Examples, the obtained ESP lines always had the *ipt* gene within their chromosomes as shown in Figure 6. Further, the ESPs, which showed remarkable morphological abnormality that could be visually identified, exhibited kanamycin resistance, without exception, by the expression of the NPTII gene which was the desired gene as a model and introduced together with the *ipt* gene. This proves that such an MAI gene is fully available as a marker gene for introducing a gene into a plant, and that the vector of the present invention containing this MAI gene as the marker gene is also available as a vector for introducing a gene into a plant.

When a gene was introduced into a plant using the vector pNPI106 in which the *ipt* gene was integrated within the transposon *Ac*, a shoot or the like that lost its ESP-forming ability as a result of the *ipt* gene disappearance from the chromosome, while retaining characteristics provided by the desired gene (NPTII gene and/or GUS gene), was obtained from the tissue that once formed the ESP at the initial stage of the cultivation just after the operation of introducing

the gene, as shown in Table 1 and Figures 13, 14 and 16. The morphology of this obtained tissue (i.e., shoot or the like that lost its ESP-forming ability) could be visually identified as shown in Figures 15 and 27. Further, when this tissue was selected, separated and cultivated, an extended and rooted individual having a normal morphology was obtained. Furthermore, tissues which were redifferentiated from the tissue obtained from the shoot that lost the ESP-forming ability also showed normal morphologies without having ESP-forming ability. This proves that such a shoot or the like consisted of uniform cells.

The same results were also observed when a DNA element derived from the site-specific recombination system was used as the removable DNA element and when the *rol* genes were used as the MAI gene. That is, when a gene was introduced into a plant using a vector, in which the construction relating to the transposon or the transposon and the *ipt* gene of the vectors used in Examples 1 to 4 and 7 was replaced with that relating to the above-mentioned recombination system and/or *rol* genes as described in Examples 5 and 6, the morphologically normal tissue and plant in which the MAI gene disappeared from its chromosome, while maintaining the desired gene, was obtained from the tissue that showed the abnormal morphology immediately after the gene introduction (Figures 21-23, Table 2). Further, it is also possible to multitudinously introduce desired genes into the same individual, repeating the steps of gene introduction, cultivation and visual selection, using the vector wherein the construction relating to the desired gene is exclusively altered while the same morphological abnormality-inducing gene is employed as the marker gene (Example 8, Figure 29).

Accordingly, when such a vector is used, in which the MAI gene is used as the marker gene and is inserted into the position such that it behaves integrally with the removable DNA element, a tissue made only of cells in which the desired gene alone remains in the chromosome or the like and maintains its function is obtained only by conducting the following steps: (1) cultivating the cells just after the operation of introducing the gene, and visually selecting a morphologically abnormal tissue which appears during the cultivation, (2) further cultivating that morphologically abnormal tissue, and visually selecting a morphologically normal tissue which appears during the cultivation. Further, a plant made only of such cells can also be obtained from that morphologically normal tissue.

Further, when a DNA element derived from the site-specific recombination system was used as the removable DNA element, the excision thereof occurred rapidly and with a high frequency, such that the morphologically normal tissue appearing from the morphologically abnormal tissue could be detected quickly, and many normal individuals were obtained therefrom with high efficiency.

Table 3 shows the efficiency at which the normal individual was obtained from the morphologically abnormal tissue when the transposon or one derived from the site-specific recombination system was used as the removable DNA element in the vector of the present invention.

TABLE 3

Difference in efficiency of obtaining normal individuals depending on the type of removable DNA element:

	Vector	Removable DNA element	Number of ESP lines	Number of lines in which normal individuals regenerate
Example 3	pNPI106	*Ac* (transposon)	63	2 (7 individuals)
Example 5	pNPI132	the region held by Rs's of pSR1 system (site-specific recombination system)	48	7 (10 individuals)

Notes:

1. The ESP was separated after two months of cultivation.
2. The normal shoot could be detected after four months of cultivation in Example 3 and after three months of cultivation in Example 5.
3. Each of the above-mentioned normal individuals contained a GUS gene as a model of the desired gene.

In Examples 1 to 5, under the hormone-free conditions, the tissue containing the transgenic cells proliferated, differentiated the adventitious shoot and regenerated the plant. This is presumably ascribable to the action of the *ipt* gene which was introduced into the chromosome in the transgenic cell as the marker gene. That is, by the expression of this gene, the plant hormone was overproduced within the cell. Consequently, the plant hormone produced in the cell containing the *ipt* gene influenced not only that cell to differentiate the tissue such as the ESP or the like, but also the tissue adjacent to the cell to some extent, whereby the same state as that given by the artificial addition of the plant hormone to the culture medium was created.

In the vector of the present invention, the MAI gene is used as the marker gene. Therefore, when the gene introduction is conducted on the plant using this vector, the tissue into which the desired gene is introduced may be selected by cultivating the cell subjected to the treatment for the gene introduction, in a common culture medium under common cultivation conditions without adding any chemical substance for selection, and visually identifying the resulting morphologically abnormal tissue. Accordingly, the procedure is simplified, and the activity of the plant cell does not decrease during the selection.

Further, such an MAI gene is inherent in the plant or is introduced into the plant by infection with bacteria or the like in nature. For this reason, even if the MAI gene is expressed within the plant cell into which the gene has been introduced, its safety is considerably high when ingested into the human body.

Still further, when the *ipt* gene is used as the MAI gene, the tissue containing the transgenic cell proliferated and differentiated the adventitious

shoot by the action of this gene, making it possible to eliminate the need for the addition of plant hormones to the culture medium which is generally deemed indispensable for the proliferation and differentiation in the cultivation of the plant cell.

In addition, in this vector, the MAI gene used as the marker gene is inserted into the position such that it behaves integrally with the removable DNA element, whereby the marker gene is removed form the DNA where this gene exists and functions through the excision of the DNA element at a given ratio after the gene has been introduced into the plant cell, and thus loses its function. Thus, only the desired gene present in a position of the vector such that it does not behave integrally with the removable DNA element remains in the DNA, and maintains the ability to express its function. Accordingly, in this structure, the vector causes the multiple introduction relating to the gene into a certain plant by merely changing the portion of the desired gene to be introduced without changing the structures of the marker gene and the others. Thus, introduction can be conducted an unlimited number of times.

Furthermore, the loss of the function of the MAI gene as the marker gene can be visually detected through the morphological change of the transgenic tissue. Therefore, tissue made up only of the cells in which the desired gene remains in the chromosome and maintains its function can be selected accurately and easily. As a result, the multiple introduction of the gene can be conducted at high efficiency, and the transgenic plant made only of such cells, namely, the individual free from the influence of the marker gene and completely

free from any health risks posed by the marker gene product can be obtained without having to undergo a crossing step.

THE EMBODIMENTS OF THE PRESENT INVENTION IN WHICH AN EXCLUSIVE PROPERTY OR PRIVILEGE IS CLAIMED ARE DEFINED AS FOLLOWS:

1. A vector for introducing a desired gene into a plant, which comprises said desired gene, at least one marker gene that induces morphologically abnormal cellular differentiation in a tissue of the plant, and a removable DNA element having an element of a DNA sequence which itself is excisable from a genome in the plant, wherein said marker gene is positioned within the removable DNA element, and wherein said desired gene is positioned outside of the removable DNA element.

2. A vector according to claim 1, wherein said removable DNA element is a transposon.

3. A vector according to claim 1, wherein said removable DNA element is derived from a site-specific recombination system.

4. A vector according to any one of claims 1 to 3, wherein expression of said marker gene in said plant induces morphologically abnormal cellular differentiation resulting in one or more of dwarfishness, destruction of apical dominance, change in pigmentation, formation of crown gall, and formation of hairy roots or wavy leaves.

5. A vector according to any one of claims 1 to 4, wherein said marker gene is obtained from a microorganism of the genus *Agrobacterium*.

6. A vector according to any one of claims 1 to 5, wherein said marker gene is a cytokinin synthesis gene.

7. A vector according to claim 6, wherein said cytokinin synthesis gene is an *ipt*, isopentenyltransferase, gene which is present in the T-DNA of *Agrobacterium tumefaciens*.

8. A vector according to any one of claims 1 to 5, wherein said marker gene is at least one gene selected from *rol* genes.

9. A vector according to claim 8, wherein said marker gene comprises the genes *rolA*, *rolB* and *rolC*, which are present in the T-DNA of *Agrobacterium rhizogenes*.

10. Use of a vector as defined in any one of claims 1 to 9, for introducing a desired gene into a plant.

11. A method for producing a transgenic plant free from the influence of a marker gene, which comprises the following steps:

(A) introducing a vector into a plant cell, wherein said vector comprises a desired gene, at least one marker gene that induces

morphologically abnormal cellular differentiation in a tissue of the plant, and a removable DNA element having an element of a DNA sequence which itself is excisable from a genome in the plant, wherein said marker gene is positioned within the removable DNA element, and wherein said desired gene is positioned outside of the removable DNA element,

(B) cultivating the plant cell obtained in (A), detecting the morphologically abnormal plant tissue which appears during the cultivation, and selecting said morphologically abnormal plant tissue, and

(C) cultivating said morphologically abnormal plant tissue selected in (B), detecting a morphologically normal plant tissue which appears during the cultivation, and selecting said morphologically normal plant tissue.

12. A method according to claim 11, wherein said removable DNA element is a transposon.

13. A method according to claim 11, wherein said removable DNA element is derived from a site-specific recombination system.

14. A vector according to any one of claims 11 to 13, wherein expression of said marker gene in said plant induces morphologically abnormal cellular differentiation resulting in one or more of dwarfishness, destruction of apical dominance, change in pigmentation, formation of crown gall, and formation of hairy roots or wavy leaves.

15. A method according to any one of claims 11 to 14, wherein said marker gene is obtained from a microorganism of the genus *Agrobacterium*.

16. A method according to any one of claims 11 to 15, wherein said marker gene is a cytokinin synthesis gene.

17. A method according to claim 16, wherein said cytokinin synthesis gene is an *ipt,* isopentenyltransferase, gene which is present in the T-DNA of *Agrobacterium tumefaciens*.

18. A method according to any one of claims 11 to 15, wherein said marker gene is at least one gene selected from *rol* genes.

19. A method according to claim 18, wherein said marker gene comprises the genes *rolA, rolB* and *rolC*, which are present in the T-DNA of *Agrobacterium rhizogenes*.

20. A method for introducing at least two desired genes into a plant, which comprises conducting the following steps at least two times:

(A) introducing a vector into a plant cell, wherein said vector comprises a desired gene, at least one marker gene that induces morphologically abnormal cellular differentiation in a tissue of the plant, and a removable DNA element having an element of a DNA sequence which itself is excisable from a genome in the plant, wherein said marker gene is positioned

within the removable DNA element, and wherein said desired gene is positioned outside of the removable DNA element,

(B) cultivating the plant cell obtained in (A), detecting the morphologically abnormal plant tissue which appears during the cultivation, and selecting said morphologically abnormal plant tissue, and

(C) cultivating said morphologically abnormal plant tissue selected in (B), detecting a morphologically normal plant tissue which appears during the cultivation, and selecting said morphologically normal plant tissue.

21. A method according to claim 20, wherein said removable DNA element is a transposon.

22. A method according to claim 20, wherein said removable DNA element is derived from a site-specific recombination system.

23. A vector according to any one of claims 20 to 22, wherein expression of said marker gene in said plant induces morphologically abnormal cellular differentiation resulting in one or more of dwarfishness, destruction of apical dominance, change in pigmentation, formation of crown gall, and formation of hairy roots or wavy leaves.

24. A method according to any one of claims 20 to 23, wherein said marker gene is obtained from a microorganism of the genus *Agrobacterium.*

25. A method according to any one of claims 20 to 24, wherein said marker gene is a cytokinin synthesis gene.

26. A method according to claim 25, wherein said cytokinin synthesis gene is an *ipt*, isopentenyltransferase, gene which is present in the T-DNA of *Agrobacterium tumefaciens*.

27. A method according to any one of claims 20 to 24, wherein said marker gene is at least one gene selected from *rol* genes.

28. A method according to claim 27, wherein said marker gene comprises the genes *rolA*, *rolB* and *rolC*, which are present in the T-DNA of *Agrobacterium rhizogenes*.

29. A cell from a transgenic plant free from the influence of a marker gene, produced by an asexual method comprising the following steps:

(A) introducing a vector into a plant cell, wherein said vector comprises a desired gene, at least one marker gene that induces morphologically abnormal cellular differentiation in a tissue of the plant, and a removable DNA element having an element of a DNA sequence which itself is excisable from a genome in the plant, wherein said marker gene is positioned within the removable DNA element, and wherein said desired gene is positioned outside of the removable DNA element,

(B) cultivating the plant cell obtained in (A), detecting the morphologically abnormal plant tissue which appears during the cultivation, and selecting said morphologically abnormal plant tissue,

(C) cultivating said morphologically abnormal plant tissue selected in (B), detecting a morphologically normal plant tissue which appears during the cultivation, and selecting said morphologically normal plant tissue, and

(D) cultivating said morphologically normal plant tissue to regenerate said transgenic plant.

30. A cell from a plant containing two or more desired genes, produced by an asexual method comprising the following steps:

(A) introducing a vector into a plant cell, wherein said vector comprises a desired gene, at least one marker gene that induces morphologically abnormal cellular differentiation in a tissue of the plant, and a removable DNA element having an element of a DNA sequence which itself is excisable from a genome in the plant, wherein said marker gene is positioned within the removable DNA element, and wherein said desired gene is positioned outside of the removable DNA element,

(B) cultivating the plant cell obtained in (A), detecting the morphologically abnormal plant tissue which appears during the cultivation, and selecting said morphologically abnormal plant tissue,

(C) cultivating said morphologically abnormal plant tissue selected in (B), detecting morphologically normal plant tissue which appears during the cultivation, and selecting said morphologically normal plant tissue,

(D) repeating steps (A) to (C) at least one time, and

(E) cultivating said morphologically normal plant tissue to regenerate said plant.

FIG. 1

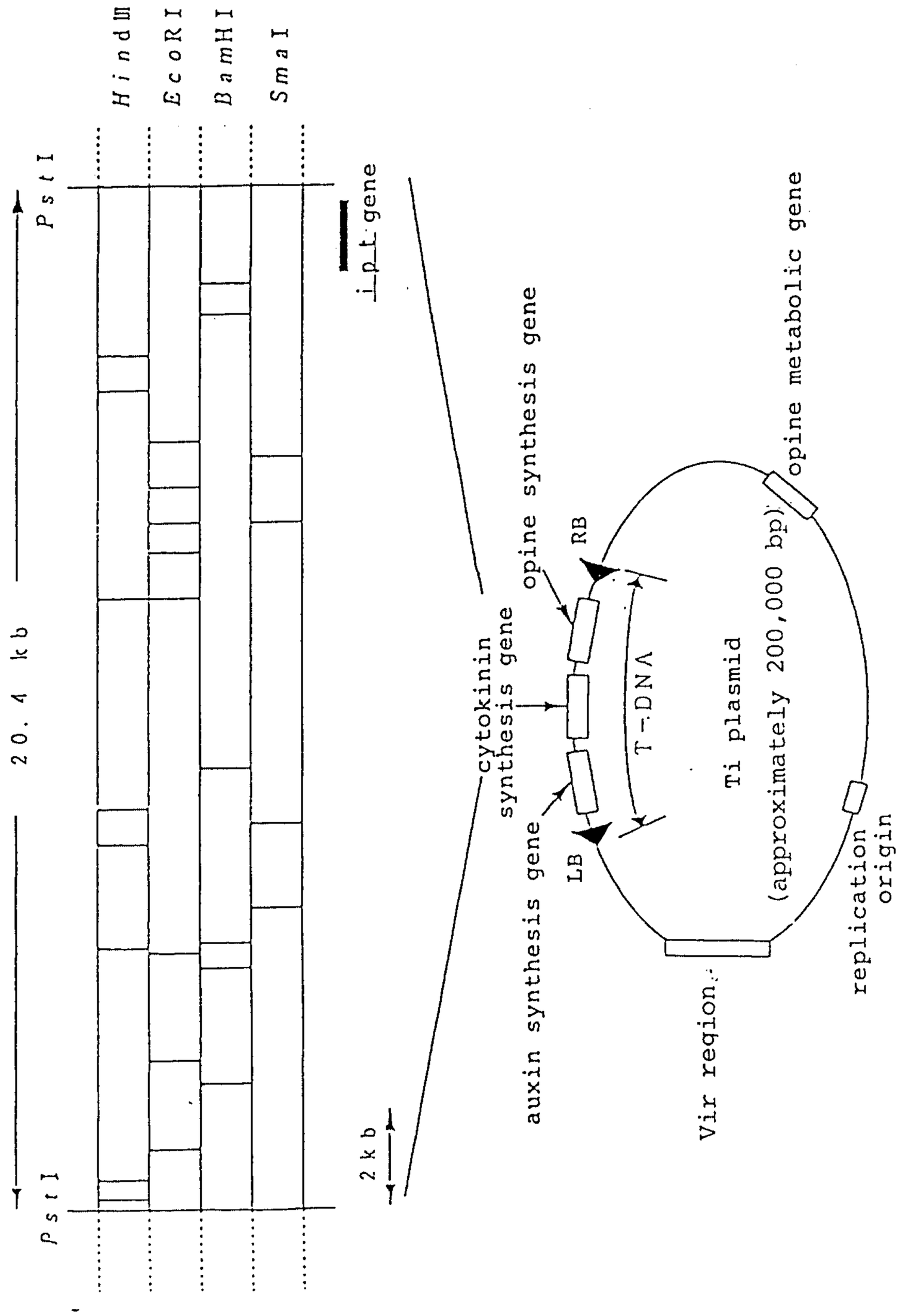
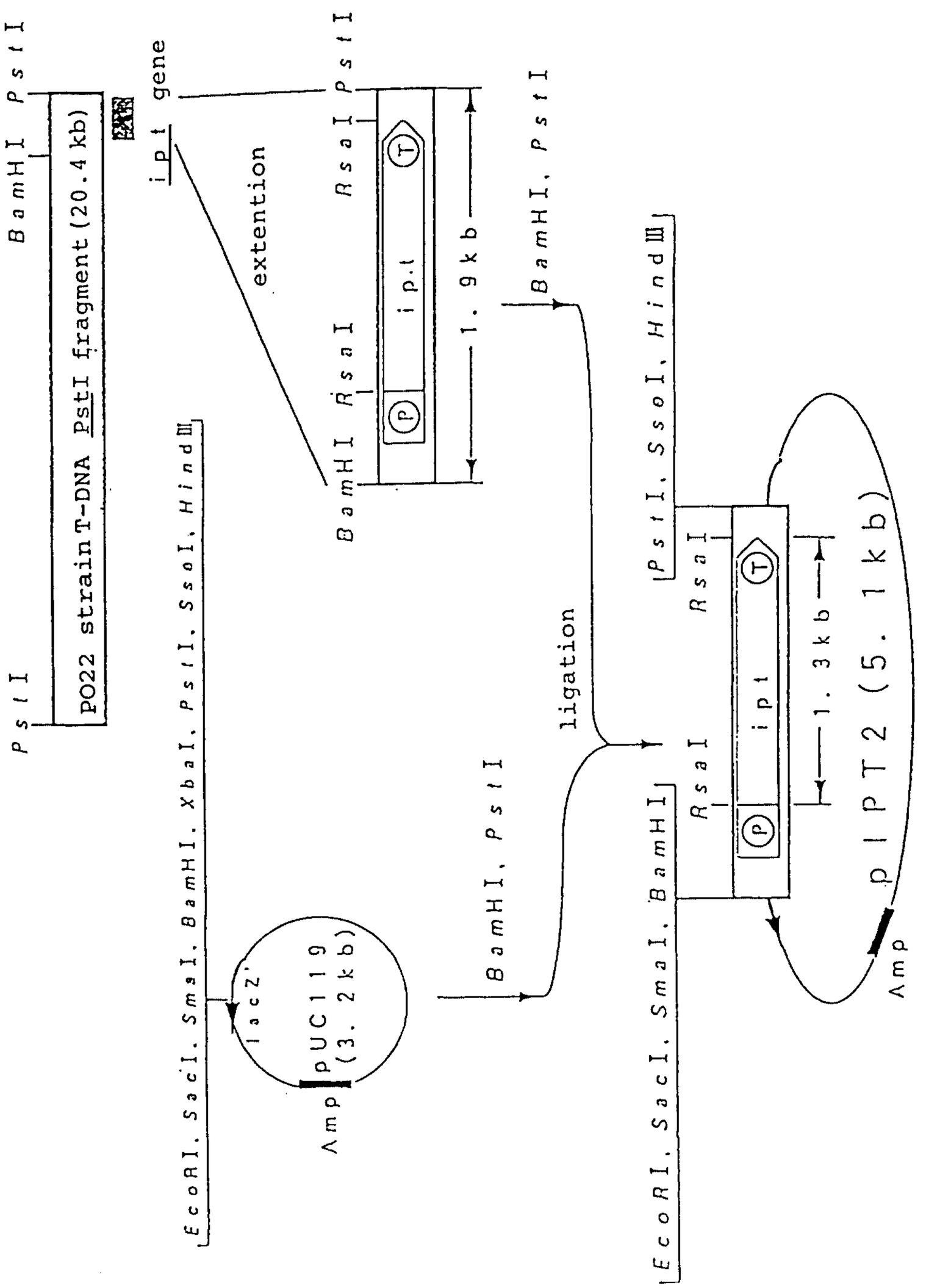

Pst I
Hin
Eco
Bam
Sma
20.4 kb
int gene
2 kb
opine synthesis gene
RB
cytokinin synthesis gene
auxin synthesis gene
LB
T-DNA
opine metabolic gene
Ti plasmid
(approximately 200,000 bp)
Vir region
replication origin

FIG. 2


Psti
PO22 strain T-DNA PstI fragment (20.4 kb)
BamHI Psti
ipt gen
extention
BamHI RsaI
RsaI Psti
P
ipt
T
1.9kb
BamHI, PstI, SsoI, HindIII
EcoRI, SacI, SmaI, BamHI, XbaI, PstI, SsoI, HindIII
lacZ'
pUC119 (3.2kb)
Amp
BamHI, PstI
ligation
PstI, SsoI, HindIII
RsaI
ipt
1.3kb
pIPT2 (5.1kb)
Amp
EcoRI, SacI, SmaI, BamHI

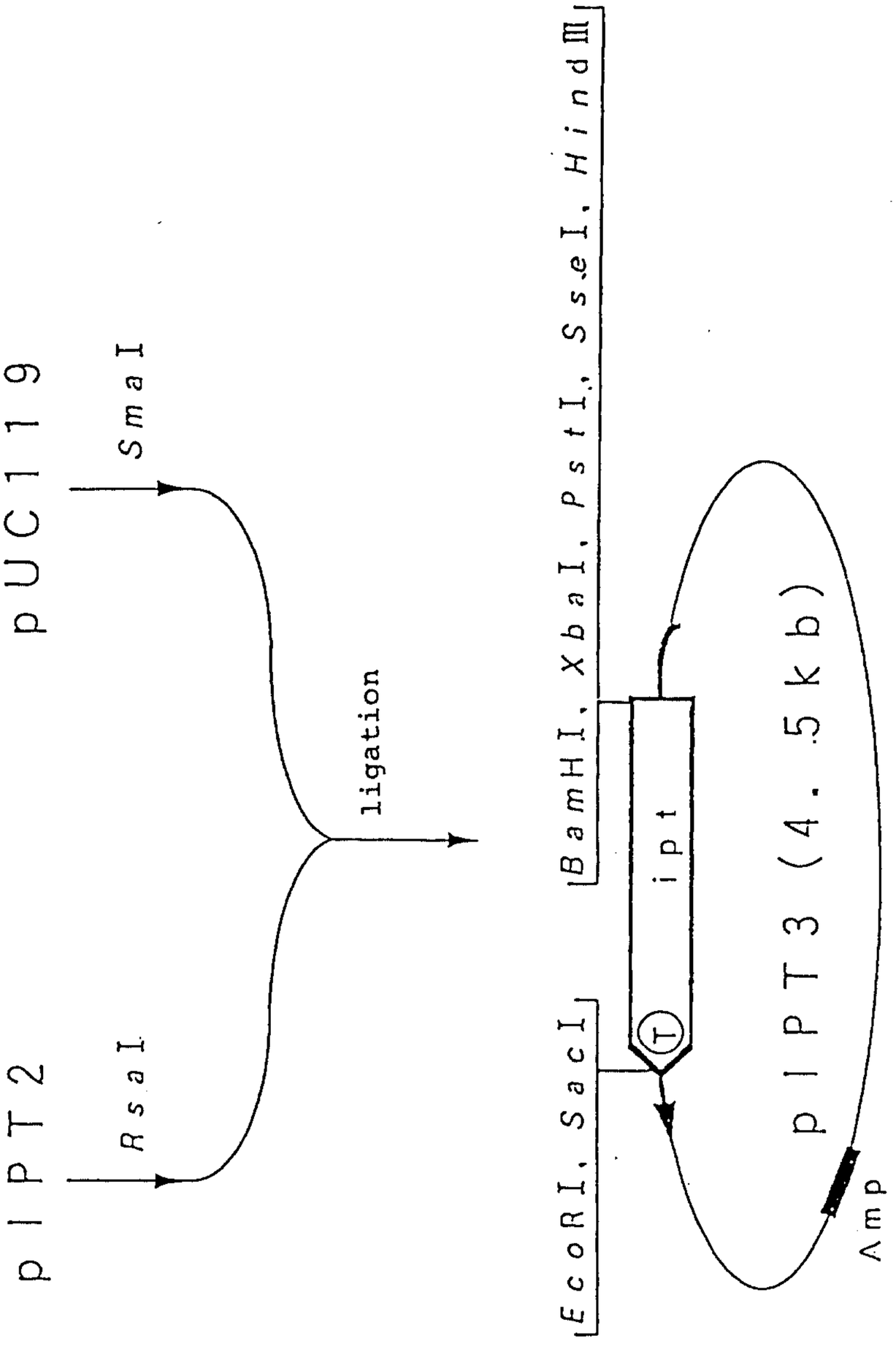

pUC119
SmaI
pIPT2
RsaI
ligation
EcoRI, SacI
BamHI, XbaI, PstI, SseI, HindIII
ipt
T
pIPT3 (4.5kb)
Amp

FIG. 3

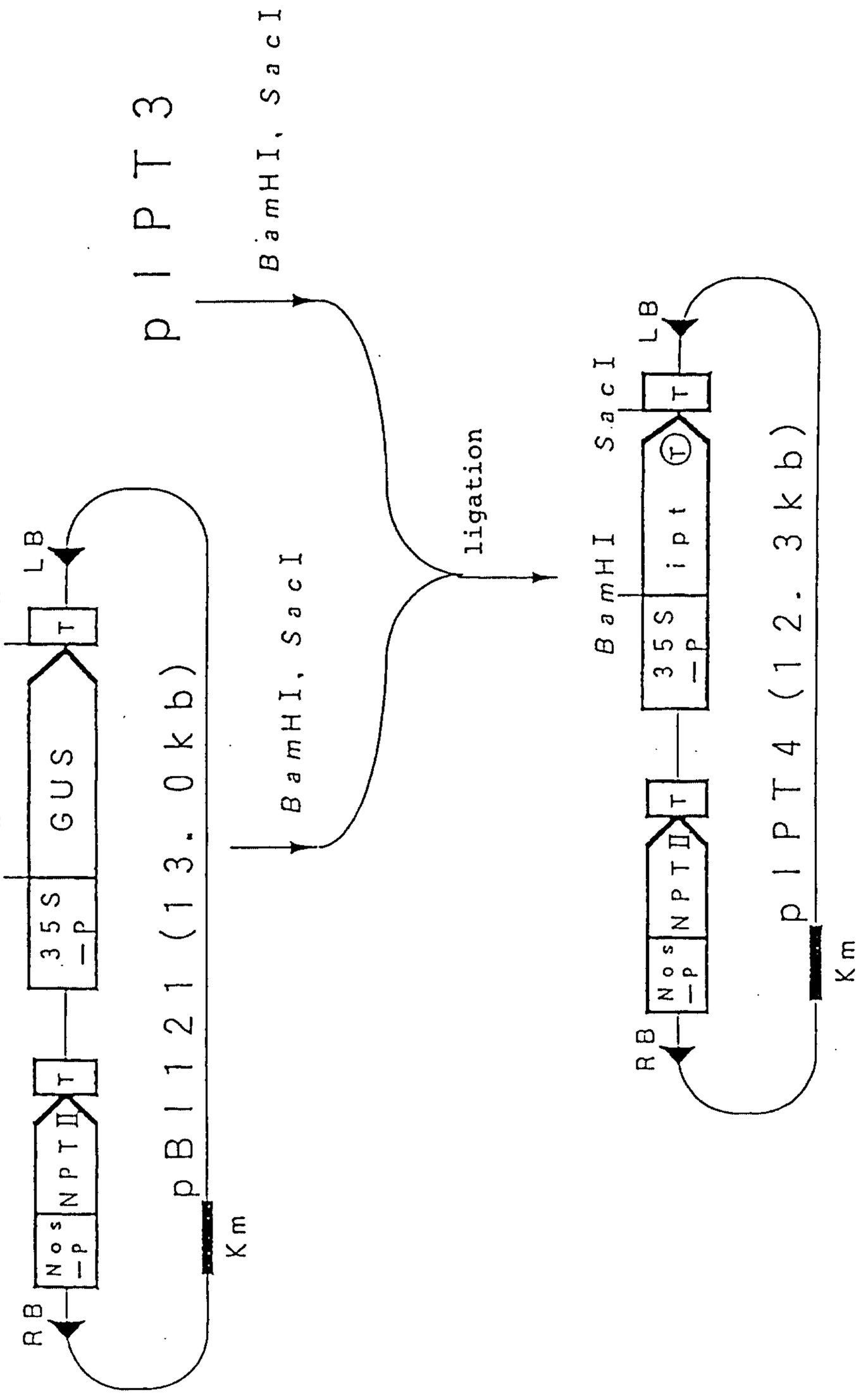

pIPT3
BamHI, SacI
ligation
pBI121 (13.0kb)
BamHI
SacI
LB
35S
—P
GUS
T
NPTII
Nos
—P
RB
Km
BamHI, SacI
pIPT4 (12.3kb)

FIG. 4

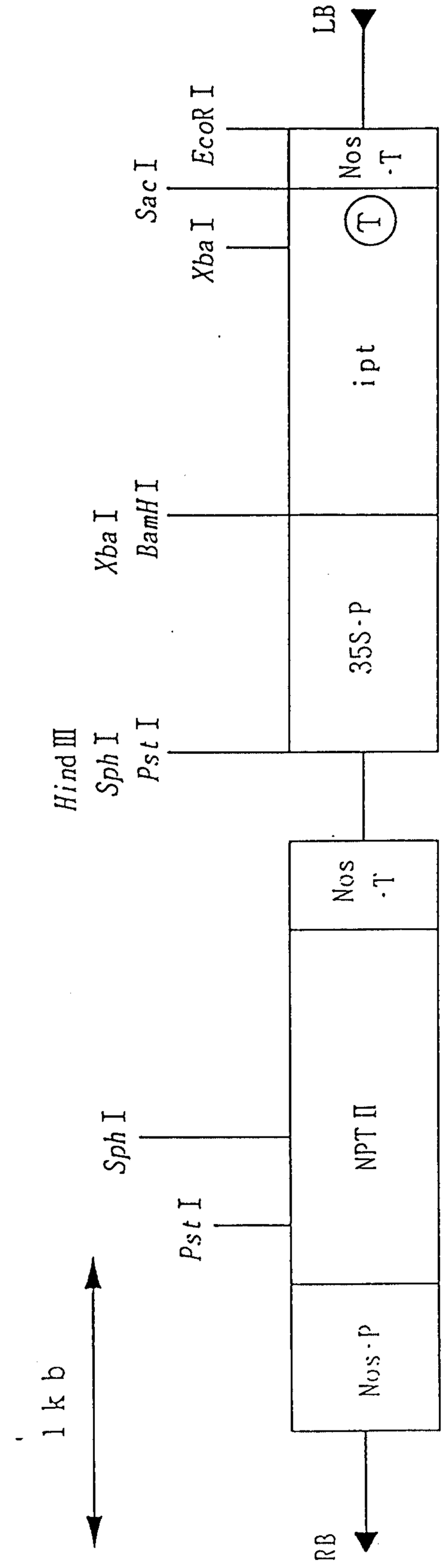
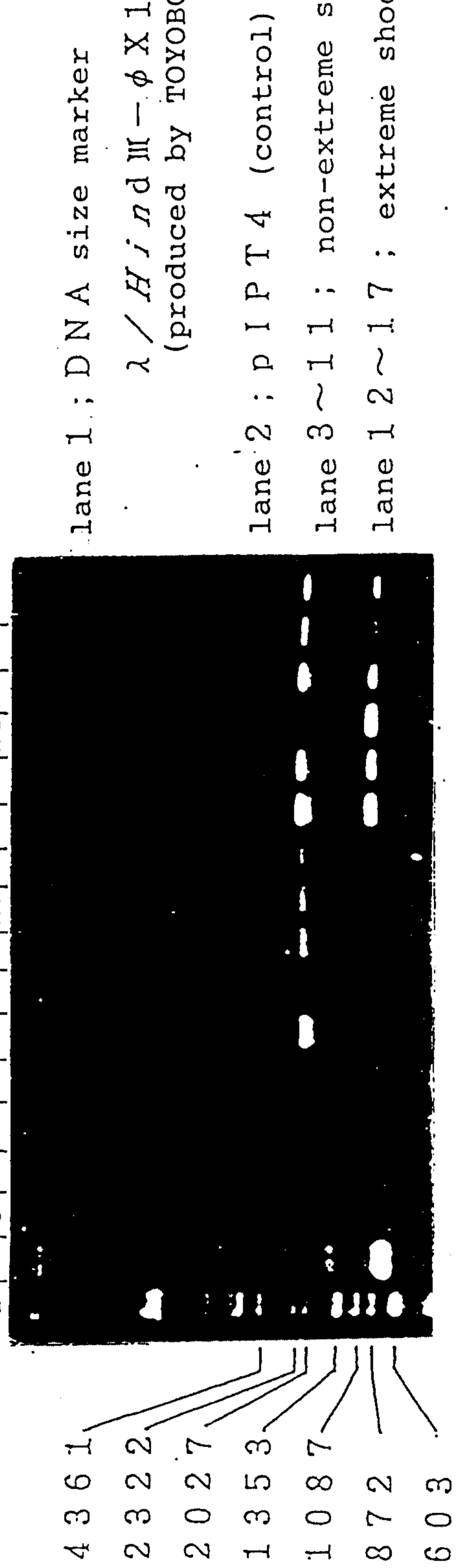


FIG. 5

pIPT4 (12.3kb)

FIG. 6


lane 1 ; D N A size marker
λ / Hind III — φ X 174 / Hae III
(produced by TOYOBO CO., LTD.)
lane 2 ; p I P T 4 (control)
lane 3 ~ 11 ; non-extreme shooty phenotype
lane 12 ~ 17 ; extreme shooty phenotype
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17
4361
2322
2027
1353
1087
872
603

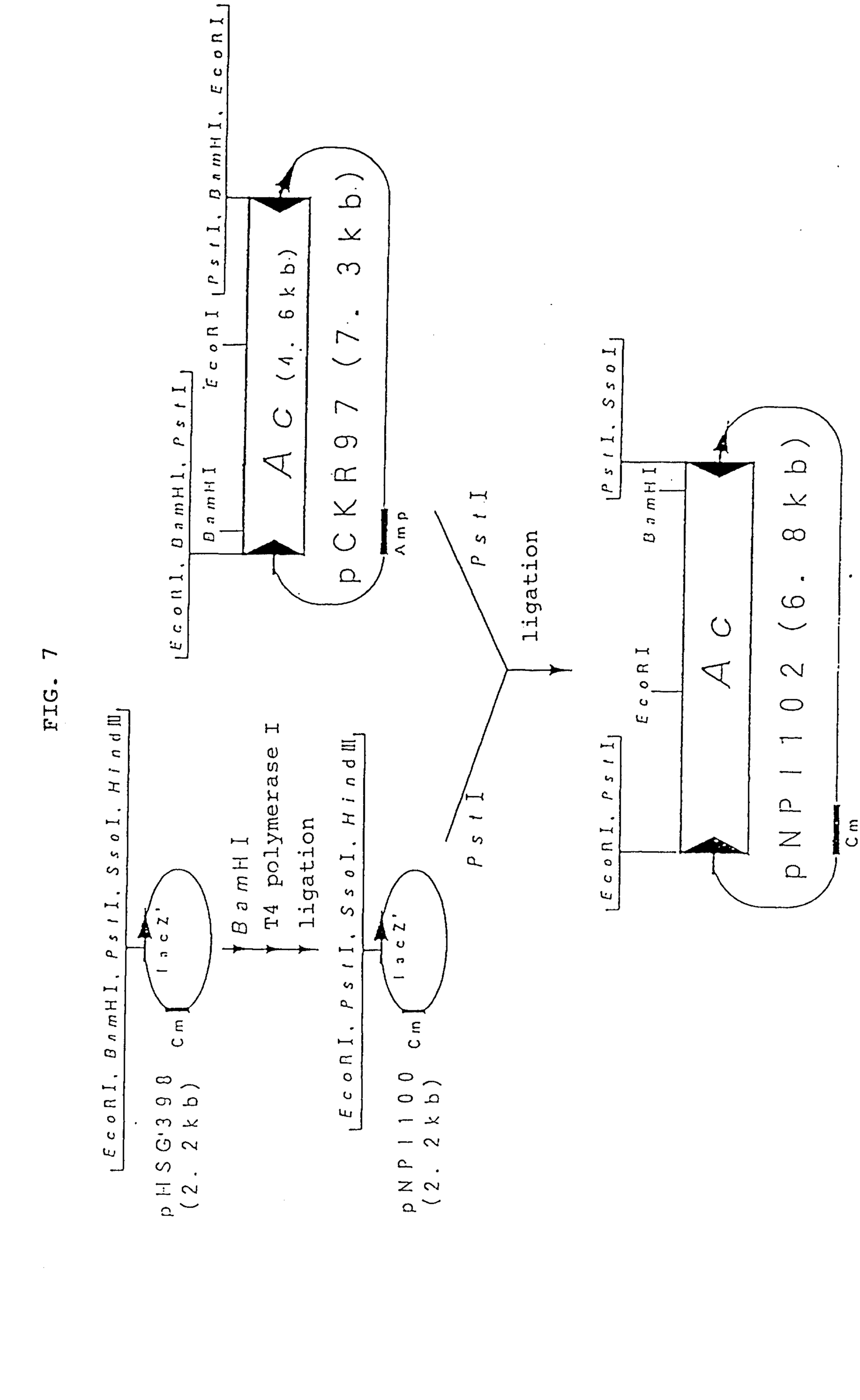
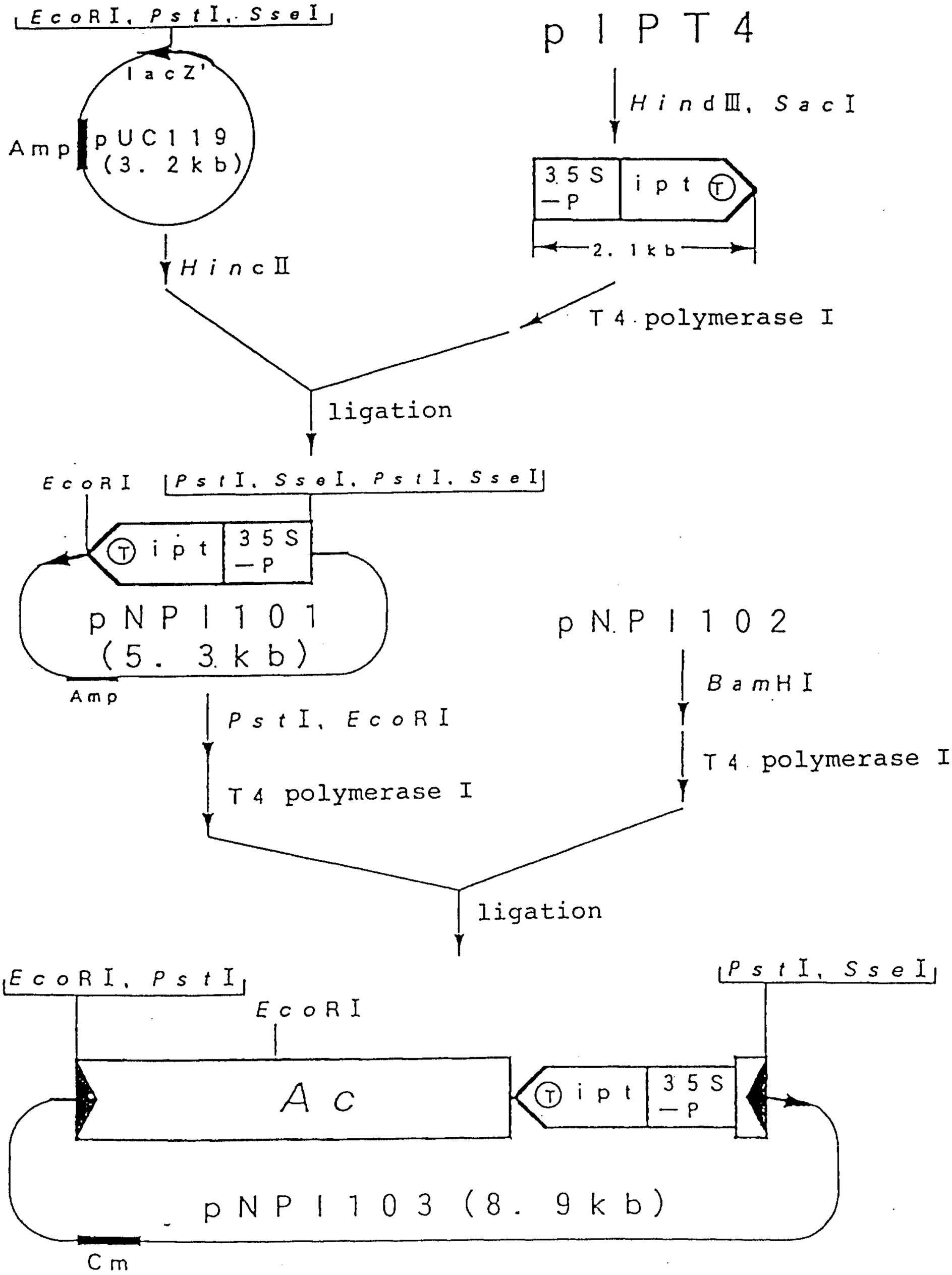

EcoRI, BamHI, PstI
BamHI
EcoRI
PstI, BamHI
pCKR97 (7.3kb)
AC (4.6kb)
Amp
PstI
ligation
PstI
BamHI, PstI, SsoI, HindIII
lacZ'
Cm
BamHI
T4 polymerase I
ligation
EcoRI, PstI, SsoI, HindIII
lacZ'
Cm
pHSG398
(2.2kb)
EcoRI, BamHI, PstI
PstI, SsoI
BamHI
EcoRI
AC (6.8kb)
pNP1102
Cm
EcoRI, PstI
pNP1100
(2.2kb)

FIG. 7

FIG. 8


EcoRI, PstI, SseI
lacZ'
Amp
pUC119
(3.2kb)
HincII
pIPT4
HindIII, SacI
3.5S
—P
ipt
T
2.1kb
T4 polymerase I
ligation
EcoRI
PstI, SseI, PstI, SseI
T
ipt
3.5S
—P
pNPI101
(5.3kb)
Amp
pNPI102
BamHI
PstI, EcoRI
T4 polymerase I
T4 polymerase I
ligation
EcoRI, PstI
EcoRI
PstI, SseI
Ac
T
ipt
3.5S
—P
pNPI103 (8.9kb)
Cm

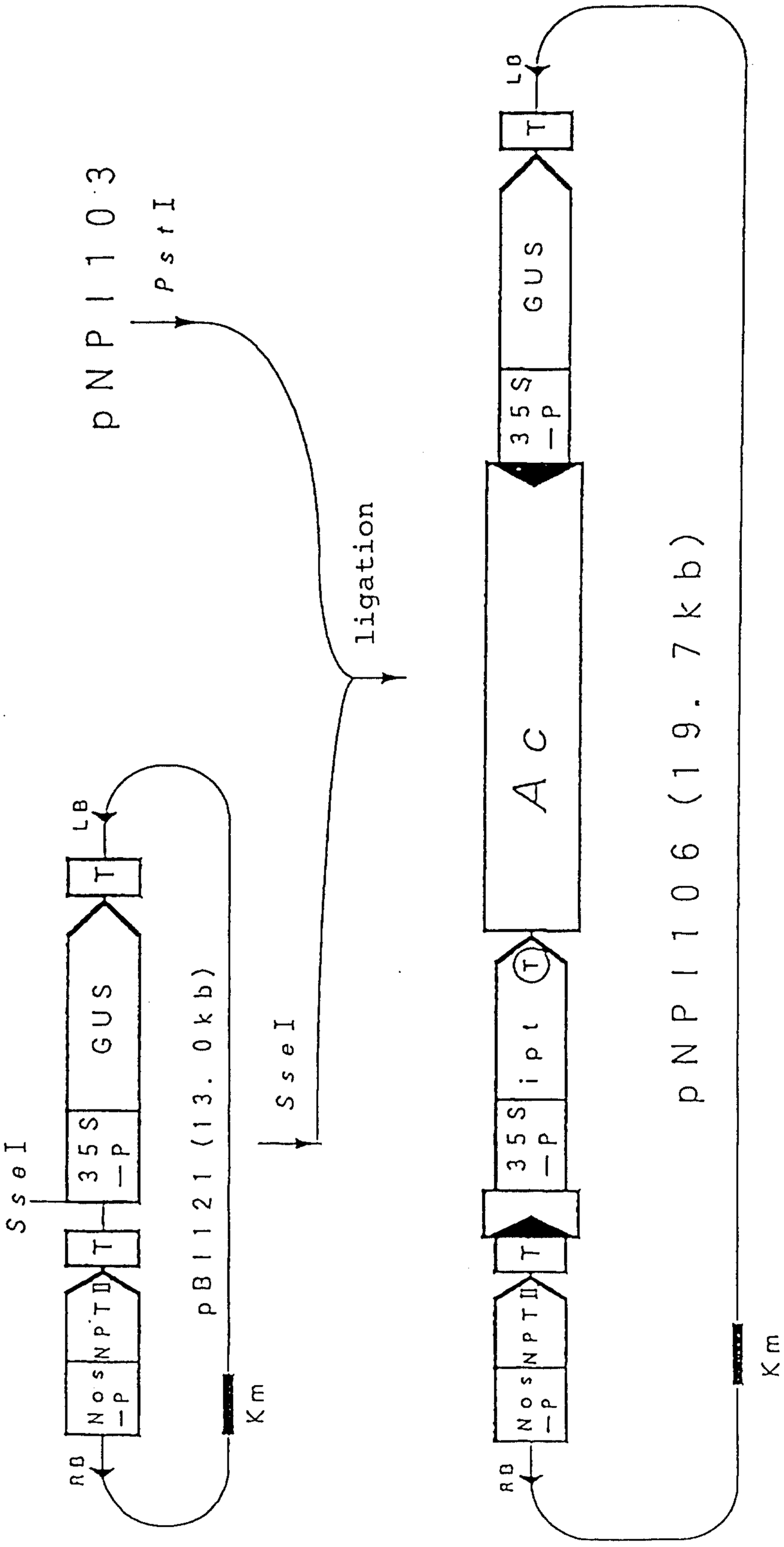
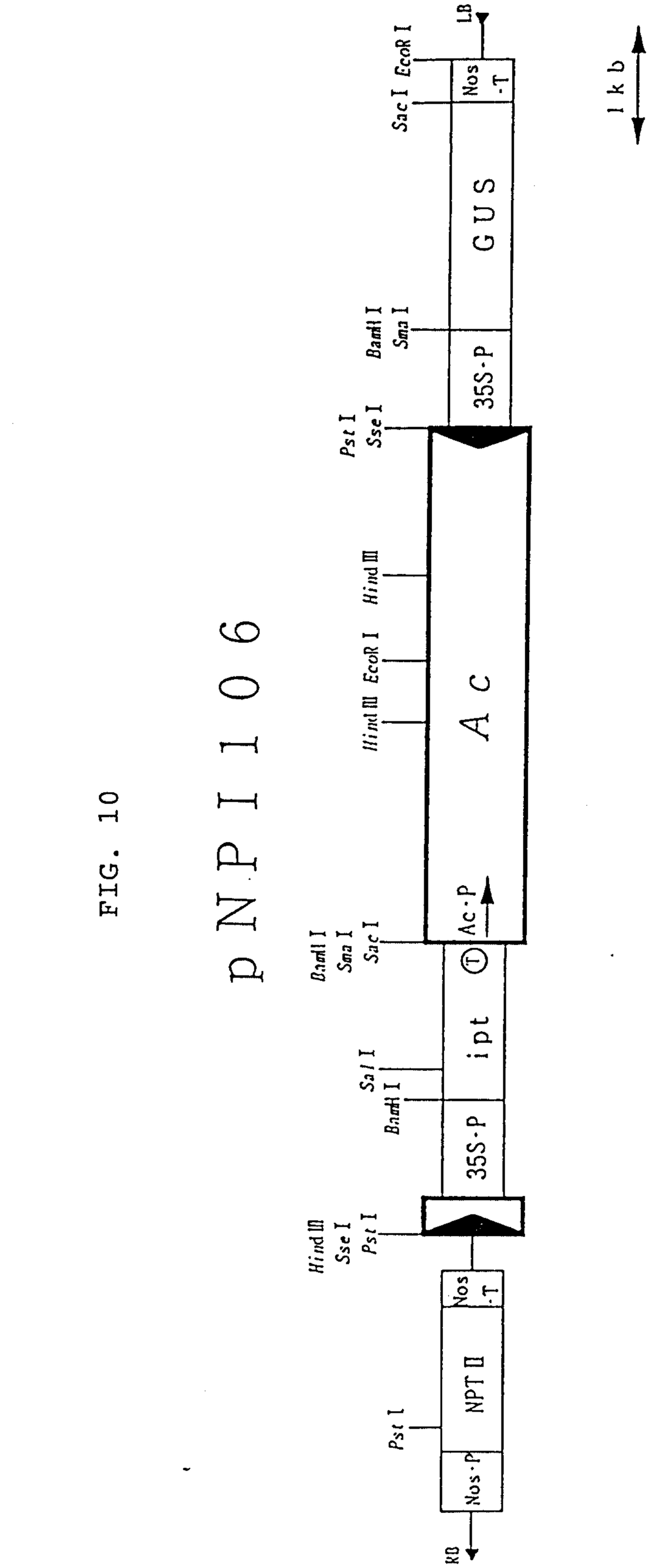

pNPI103
Pst I
ligation
LB
T
35S —P
GUS
NOS —P
NPT II
RB
pBI121 (13.0kb)
Sse I
Km
pNPI1106 (19.7kb)
ACC
ipt

FIG. 9

FIG. 10


pNPI106
LB
Sac I
EcoR I
Nos-T
GUS
1kb
Pst I
Sse I
BamH I
Sma I
35S-P
Hind III
EcoR I
Hind III
Ac
Bam H I
Sma I
Sac I
Ac-P
Sal I
BamH I
ipt
35S-P
Hind III
Sse I
Pst I
Nos-T
Pst I
NPT II
Nos-P
RB

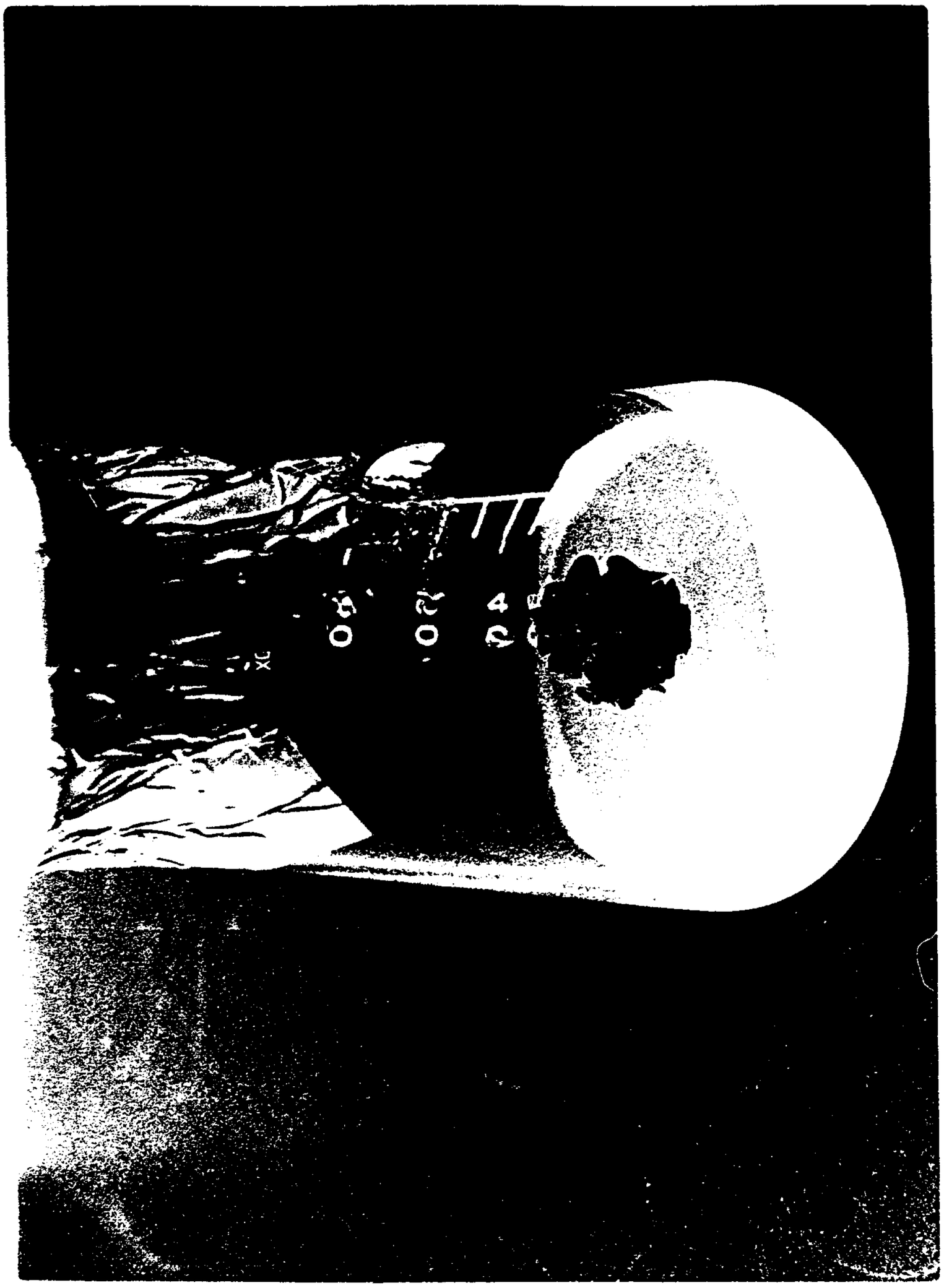


FIG. 11

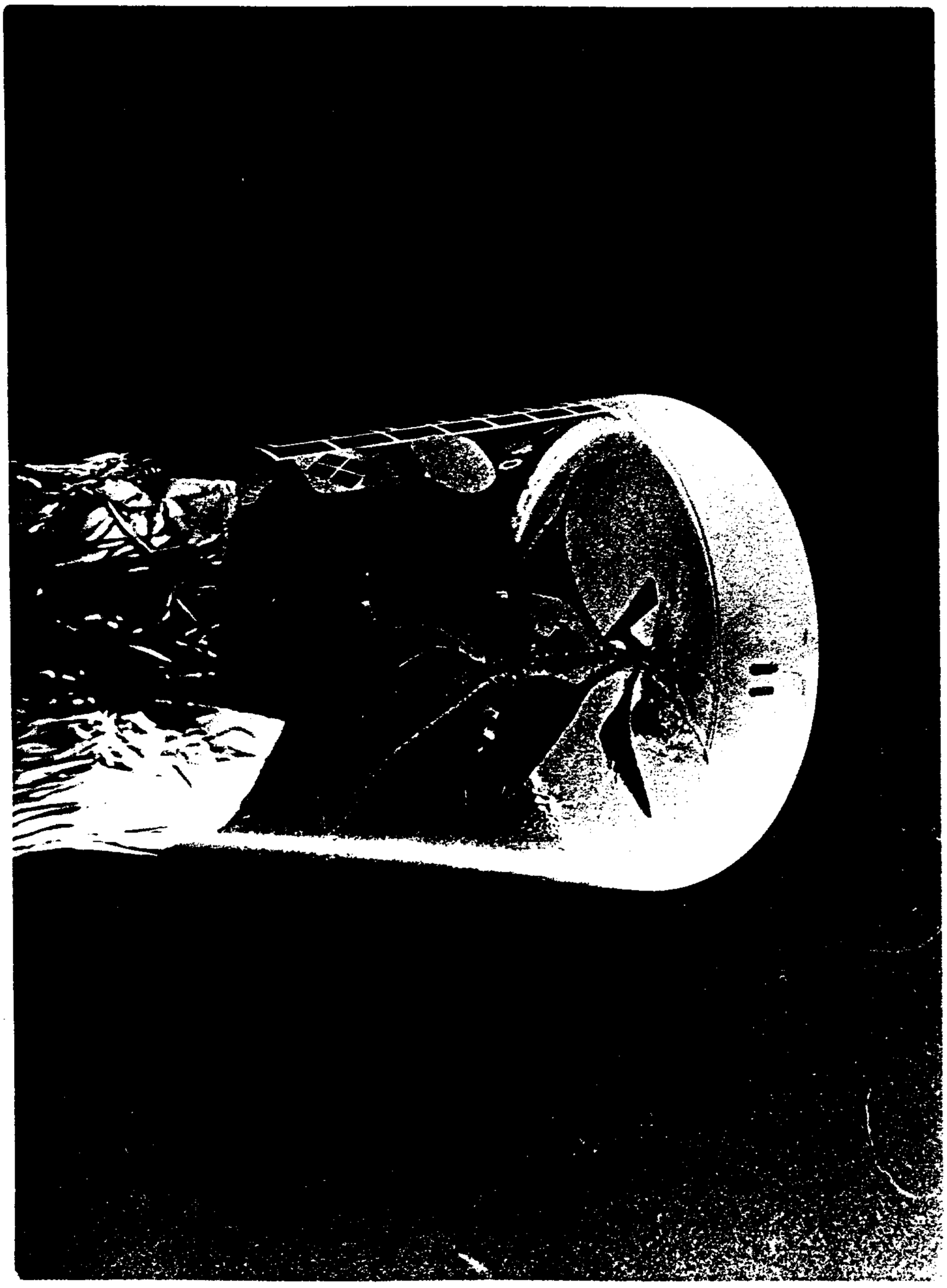
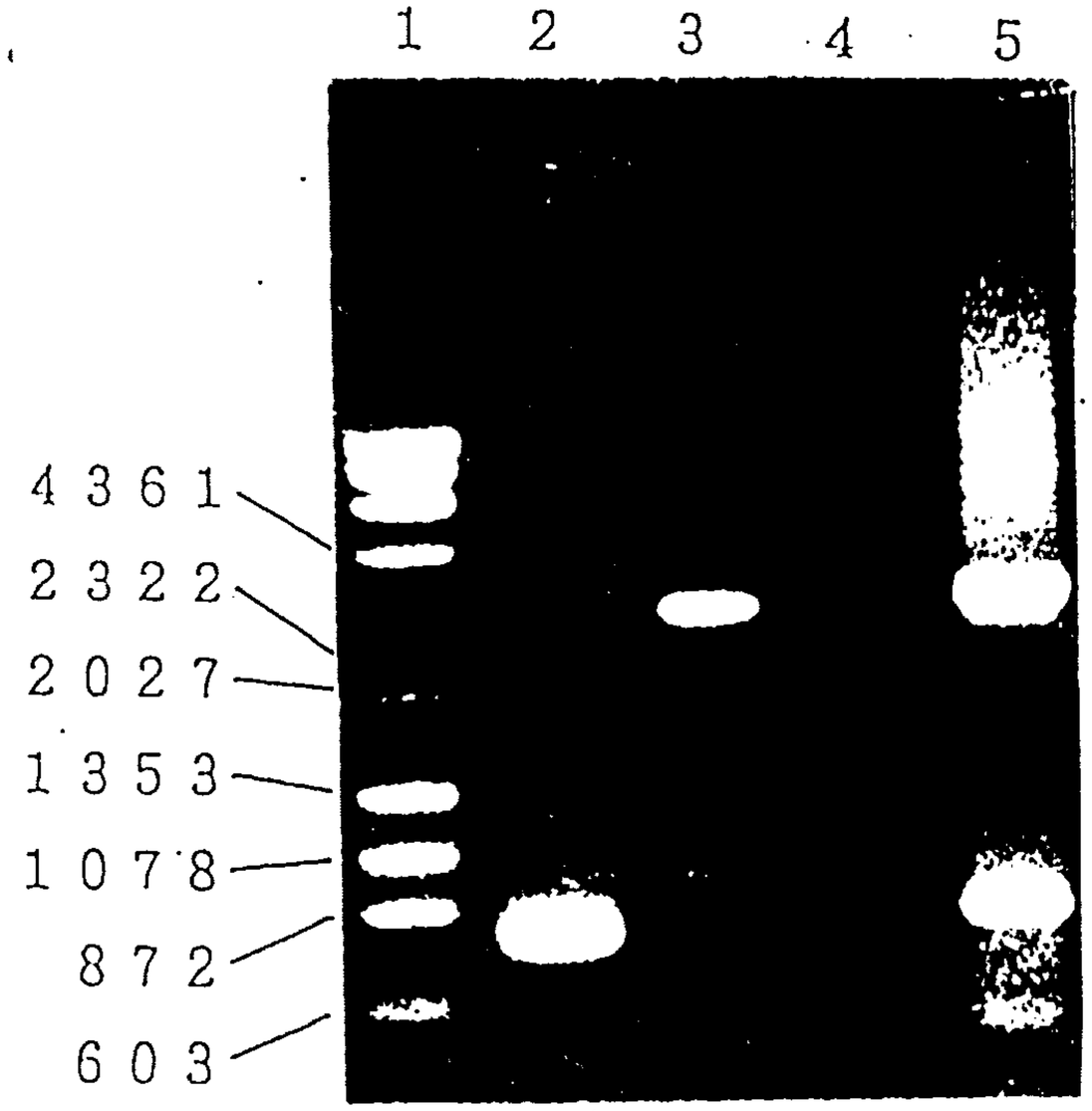


FIG. 12

FIG. 13



lane 1 ; DNA size marker

λ／*Hin*dⅢ－φX174／*Hae*Ⅲ
(produced by TOYOBO CO., LTD.)

lane 2 ; pNPI106 (control)

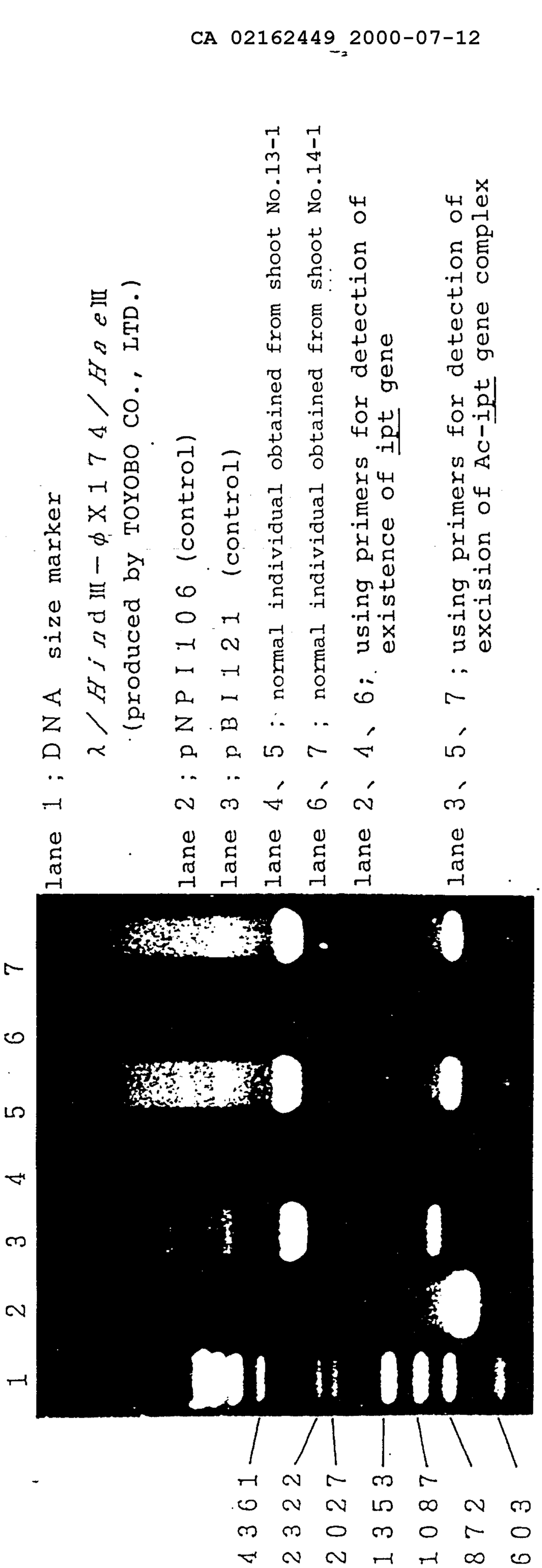
lane 3 ; pBI121 (control)

lane 4、5 ; shoot No. 8

lane 2、4 ; using primers for detection of existence of ipt gene

lane 3、5 ; using primers for detection of excision of Ac-ipt gene complex

FIG. 14


lane 1 ; DNA size marker
λ / Hind III − φX174 / Hae III
(produced by TOYOBO CO., LTD.)
lane 2 ; pNPI106 (control)
lane 3 ; pBI121 (control)
lane 4、5 ; normal individual obtained from shoot No.13-1
lane 6、7 ; normal individual obtained from shoot No.14-1
lane 2、4、6; using primers for detection of existence of ipt gene
lane 3、5、7 ; using primers for detection of excision of Ac-ipt gene complex
1
2
3
4
5
6
7
4361
2322
2027
1353
1087
872
603

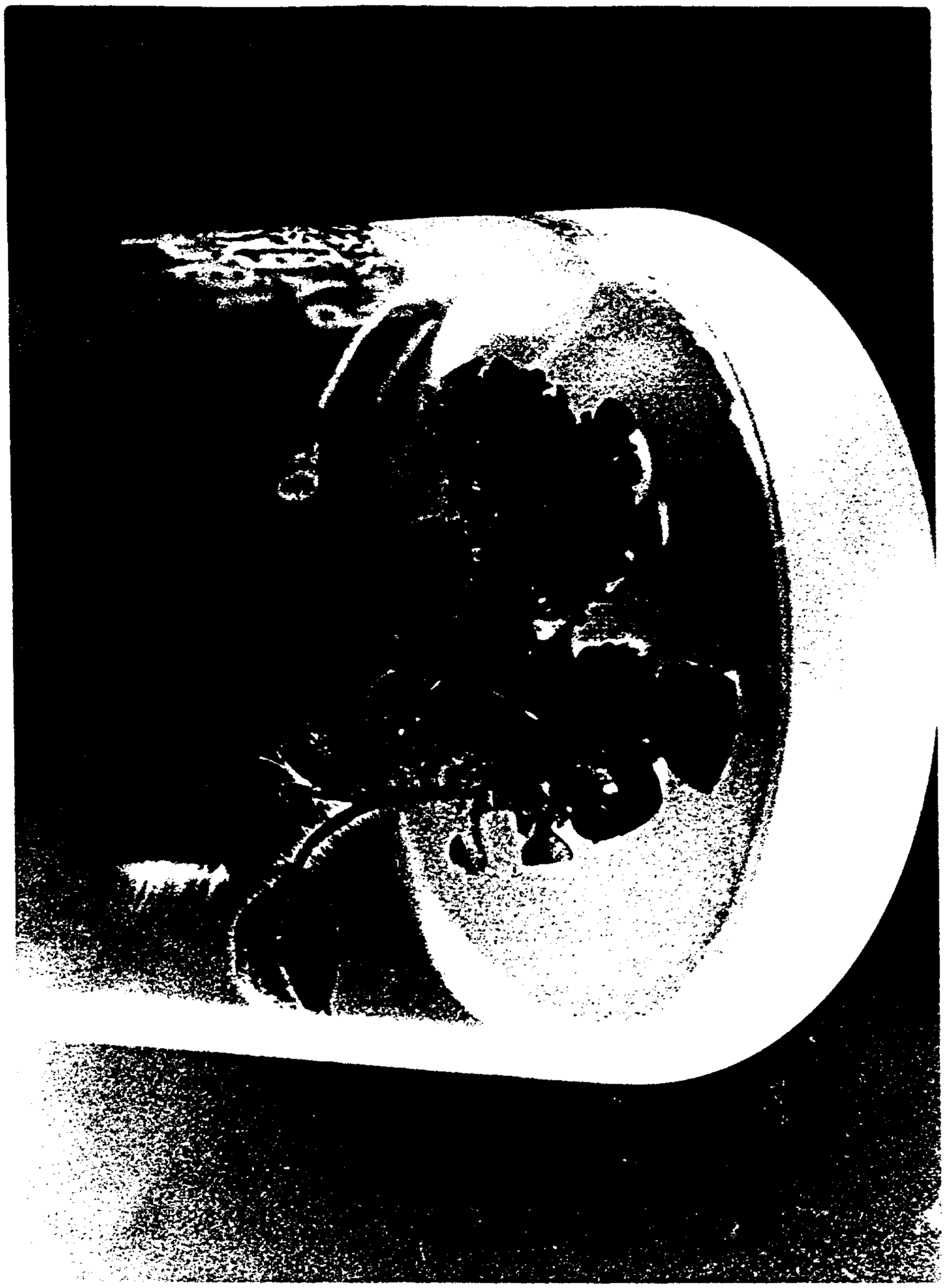


FIG. 15

FIG.

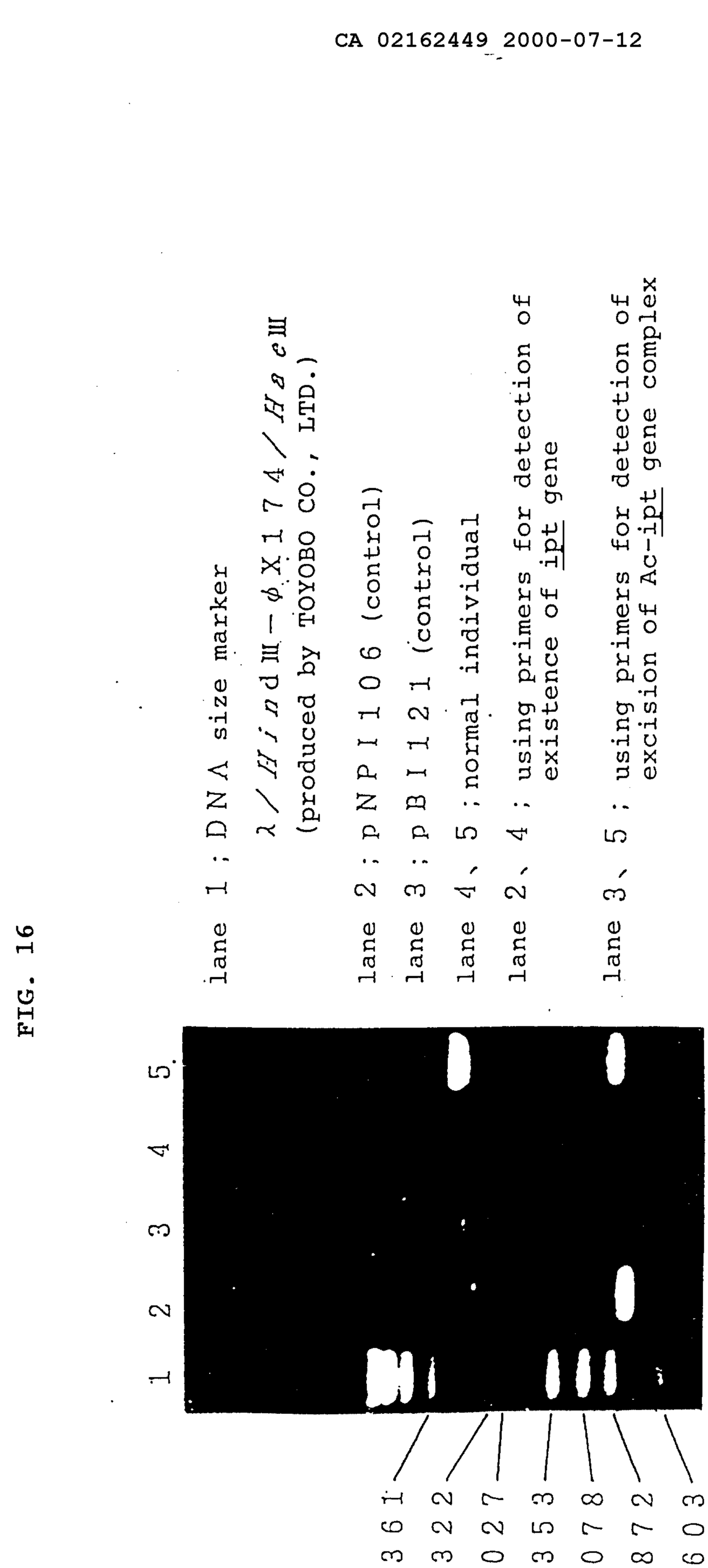

lane 1 ; DNA size marker
λ / Hind III − φX174 / Hae III
(produced by TOYOBO CO., LTD.)
lane 2 ; pNPI106 (control)
lane 3 ; pBI121 (control)
lane 4、5 ; normal individual
lane 2、4 ; using primers for detection of existence of ipt gene
lane 3、5 ; using primers for detection of excision of Ac-ipt gene complex
1
2
3
4
5
4361
2322
2027
1353
1078
872
603

FIG. 17

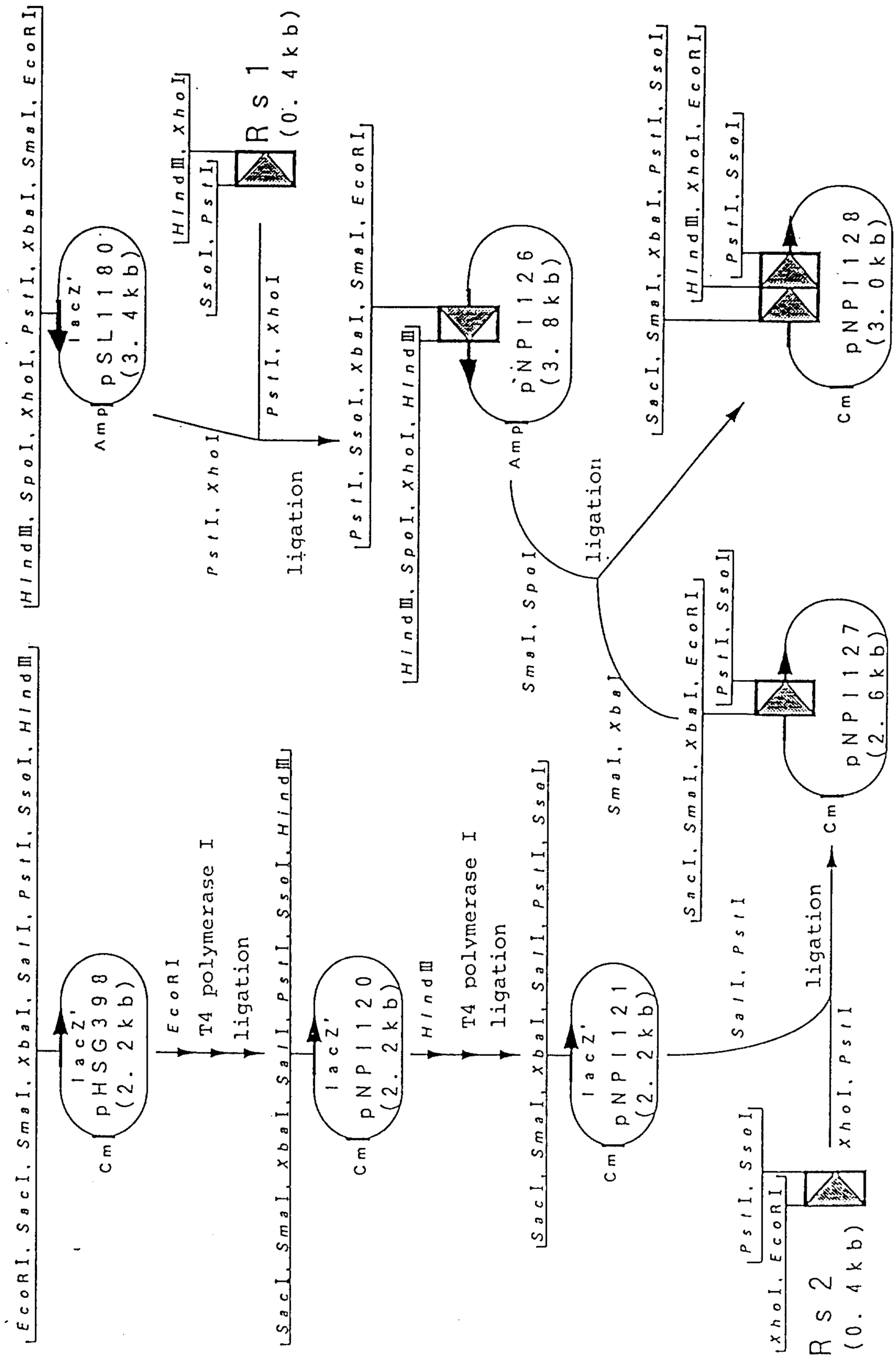
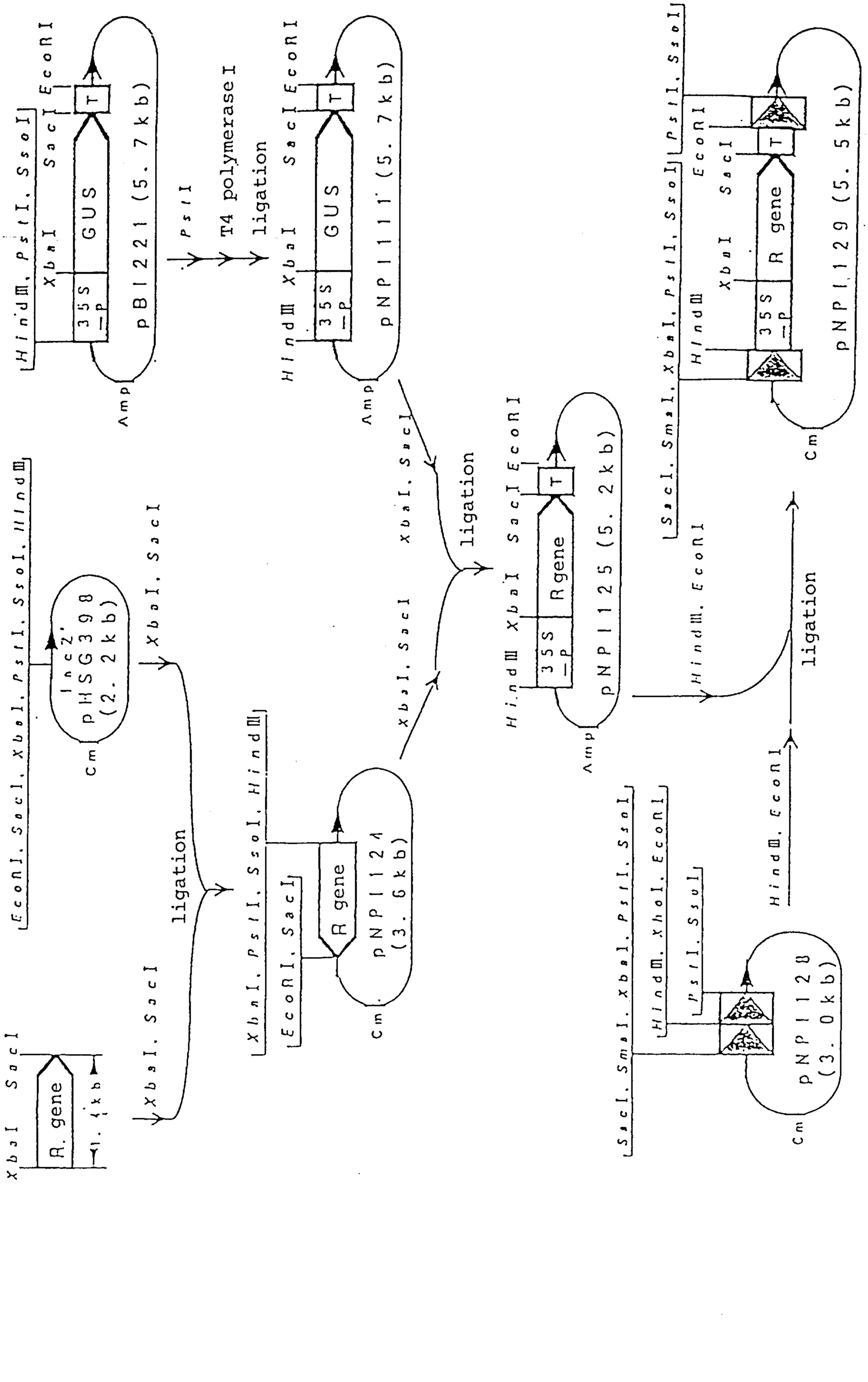

EcoRI, SacI, SmaI, XbaI, SalI, PstI, SsoI, HindIII
lacZ'
pHSG398
(2.2kb)
Cm
EcoRI
T4 polymerase I
ligation
SacI, SmaI, XbaI, SalI, PstI, SsoI, HindIII
lacZ'
pNP1120
(2.2kb)
Cm
HindIII
T4 polymerase I
ligation
SmaI, XbaI, SalI, PstI, SsoI
lacZ'
pNP1121
(2.2kb)
Cm
SacI, SmaI, XbaI, SalI, PstI, SsoI
SmaI, XbaI
SalI, PstI
ligation
SacI, SmaI, XbaI, EcoRI
PstI, SsoI
Cm
pNP1127
(2.6kb)
XhoI, PstI
ligation
PstI, SsoI
XhoI, EcoRI
RS2
(0.4kb)
HindIII, XhoI, PstI, SsoI
RS1
(0.4kb)
PstI, XhoI
ligation
PstI, XhoI
SacI, SmaI, XbaI, PstI, SsoI, HindIII
lacZ'
pSL1180
(3.4kb)
Amp
SacI, SmaI, XbaI, SalI, PstI, SsoI, HindIII
SmaI, SpoI, XhoI, PstI, XbaI, SmaI, EcoRI
HindIII, XhoI, PstI, SsoI
RS1
(0.4kb)
PstI, XhoI
ligation
PstI, SsoI, XhoI, HindIII
lacZ'
pNP1126
(3.8kb)
Amp
SpoI
ligation
SacI, SmaI, XbaI, PstI, SsoI, HindIII
lacZ'
pNP1128
(3.0kb)
Cm

FIG. 18


pBI221 (5.7kb)
HindIII, PstI, SsoI
XbaI
SacI
EcoRI
35S —P
GUS
T
Amp
PstI
T4 polymerase
ligation
pNPI1111 (5.7kb)
pNPI1129 (5.5kb)
SacI, SmaI, XbaI, PstI, SsoI
R gene
Cm
pHSG398 (2.2kb)
XbaI, SacI
pNPI1124 (3.6kb)
pNPI1125 (5.2kb)
pNPI1128 (3.0kb)
R. gene
1.4kb

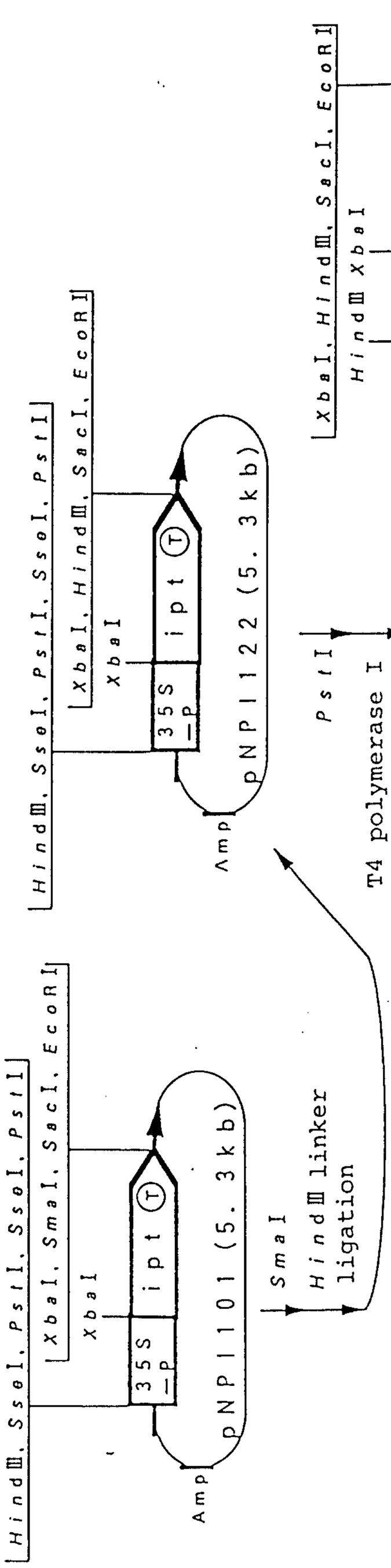
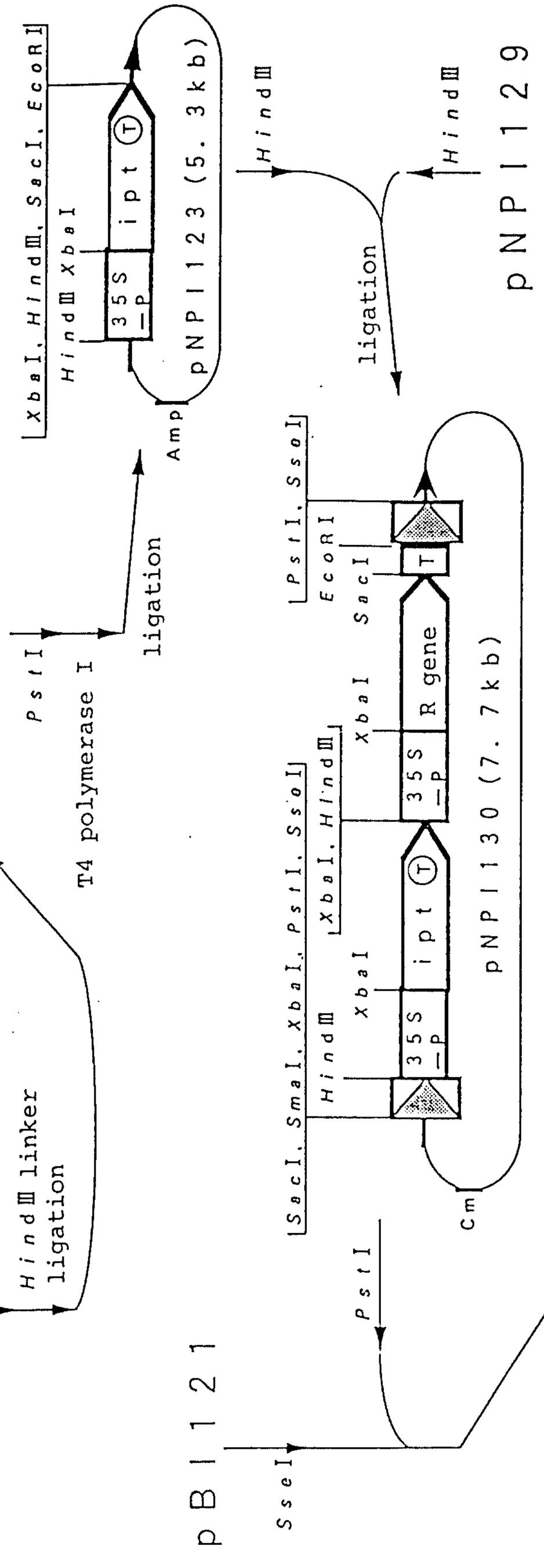

HindIII, SseI, PstI, SseI, PstI
XbaI, HindIII, SacI, EcoRI
XbaI
35S —P
ipt
T
pNPI1122 (5.3kb)
Amp
T4 polymerase I
PstI
ligation
HindIII, SseI
XbaI, SmaI, SacI, EcoRI
XbaI
35S —P
ipt
T
pNPI1101 (5.3kb)
SmaI
HindIII linker ligation

FIG. 1


XbaI, HindIII, SacI, EcoRI
HindIII XbaI
35S —P
ipt
T
pNPI1123 (5.3kb)
Amp
PstI
T4 polymerase I
ligation
HindIII
HindIII
ligation
pNPI129
PstI, SseI
EcoRI
SacI
XbaI
35S —P
R gene
T
pNPI1130 (7.7kb)
Cm
SacI, SmaI, XbaI, PstI, SseI
HindIII
XbaI
35S —P
ipt
T
XbaI, HindIII
pNPI1132 (18.5kb)
PstI
pBI121
SseI
ligation

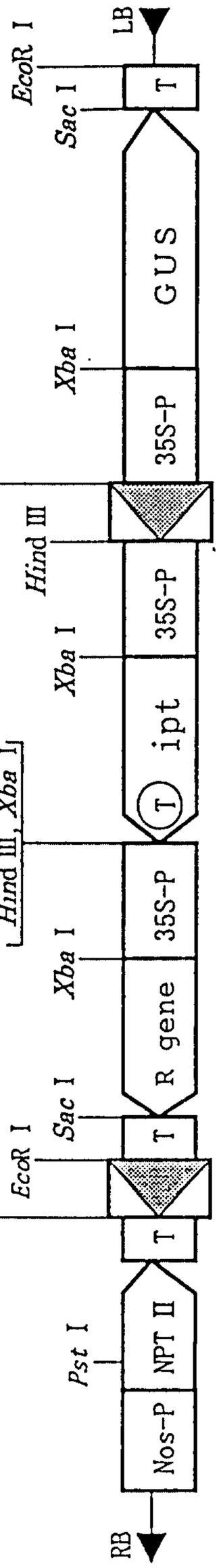
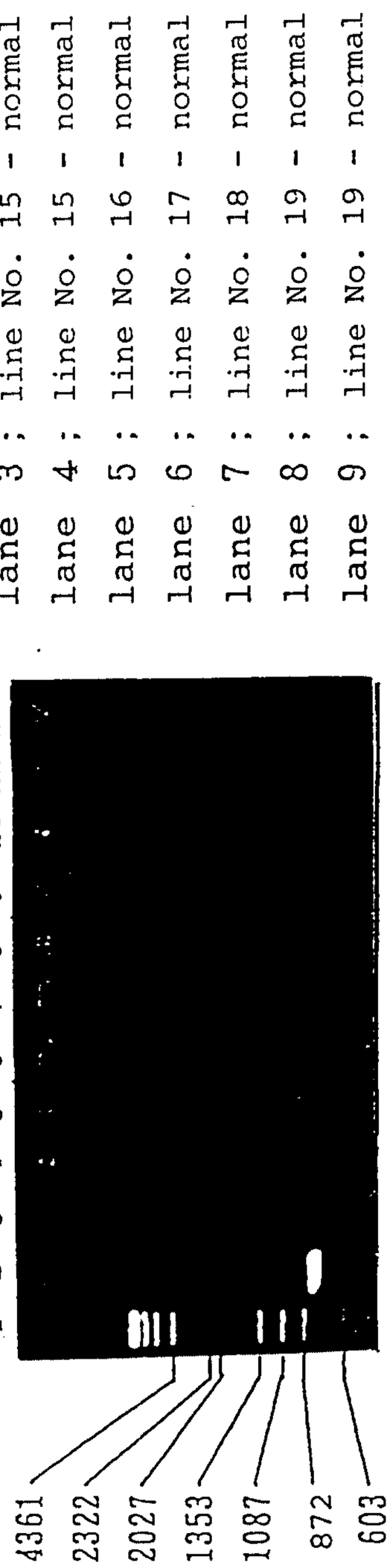


FIG. 20

pNPI132 (18.5 kb)

Using primers for detection of existence of ipt gene.

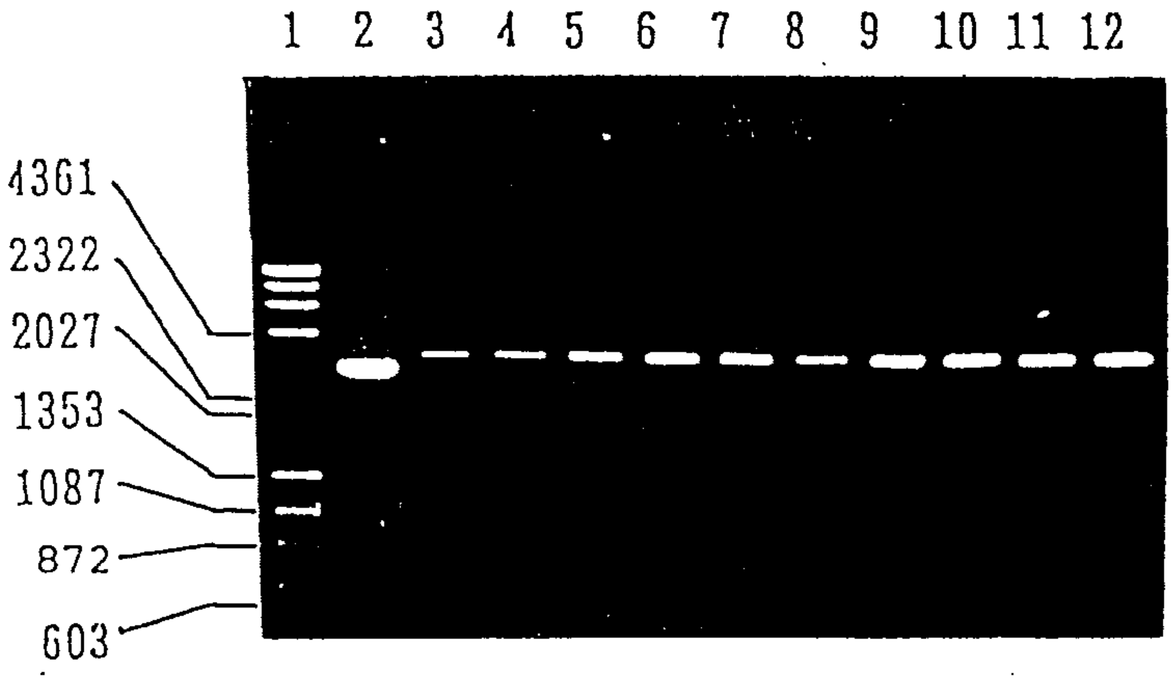


lane 1 ; DNA size marker
λ/HindIII−φX174/HaeIII
(produced by TOYOBO CO., LTD)
lane 2 ; pNPI132 (control) individual No. 1
lane 3 ; line No. 15 - normal individual No. 2
lane 4 ; line No. 15 - normal individual No. 1
lane 5 ; line No. 16 - normal individual No. 1
lane 6 ; line No. 17 - normal individual No. 1
lane 7 ; line No. 18 - normal individual No. 1
lane 8 ; line No. 19 - normal individual No. 2
lane 9 ; line No. 19 - normal individual No. 1
lane 10 ; line No. 20 - normal individual No. 2
lane 11 ; line No. 20 - normal individual No. 1
lane 12 ; line No. 21 - normal individual No. 1

FIG. 21

FIG. 22

Using primers for detection of excision of the region containing ipt gene and held by Rs's

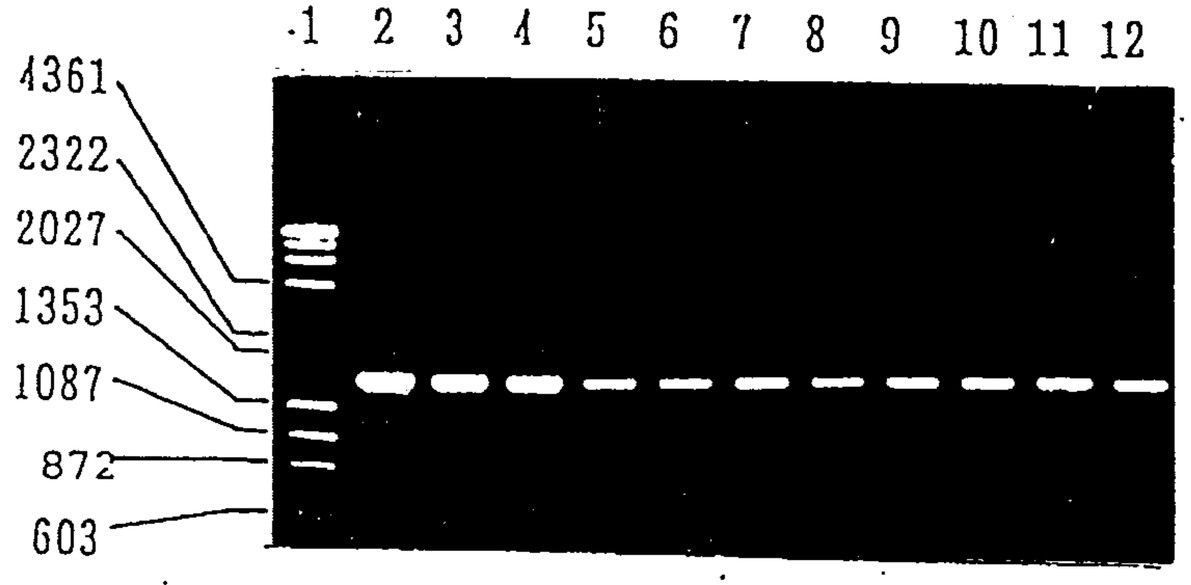


lane 1 ; DNA size marker
λ/HindIII−φX174/HaeIII
(produced by TOYOBO CO., LTD)

lane 2 ; pBI121 (control)
lane 3 ; line No. 15 - normal individual No. 1
lane 4 ; line No. 15 - normal individual No. 2
lane 5 ; line No. 16 - normal individual No. 1
lane 6 ; line No. 17 - normal individual No. 1
lane 7 ; line No. 18 - normal individual No. 1
lane 8 ; line No. 19 - normal individual No. 1
lane 9 ; line No. 19 - normal individual No. 2
lane 10 ; line No. 20 - normal individual No. 1
lane 11 ; line No. 20 - normal individual No. 2
lane 12 ; line No. 21 - normal individual No. 1

FIG. 23

Using primers for detection of existence of GUS gene



lane 1 ; DNA size marker
λ/HindIII−φX174/HaeIII
(produced by TOYOBO CO., LTD)

lane 2 ; pNPI132 (control)

lane 3 ; line No. 15 - normal individual No. 1

lane 4 ; line No. 15 - normal individual No. 2

lane 5 ; line No. 16 - normal individual No. 1

lane 6 ; line No. 17 - normal individual No. 1

lane 7 ; line No. 18 - normal individual No. 1

lane 8 ; line No. 19 - normal individual No. 1

lane 9 ; line No. 19 - normal individual No. 2

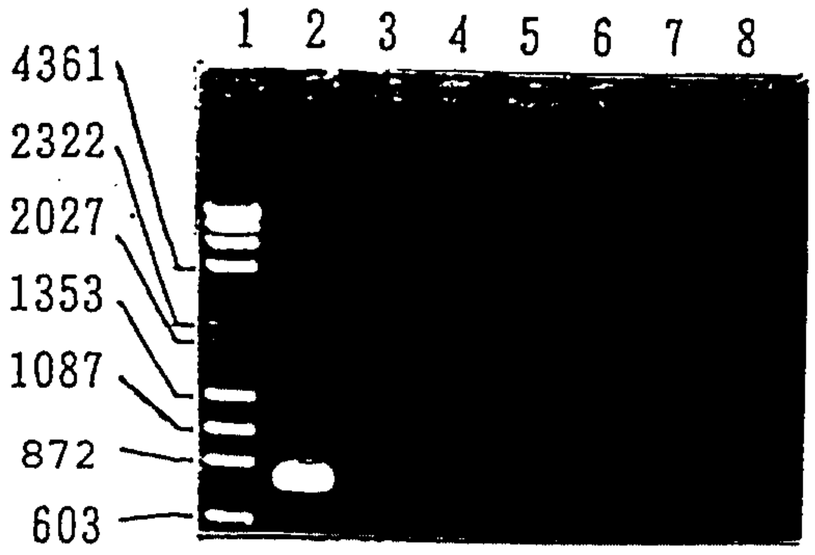
lane 10 ; line No. 20 - normal individual No. 1

lane 11 ; line No. 20 - normal individual No. 2

lane 12 ; line No. 21 - normal individual No. 1

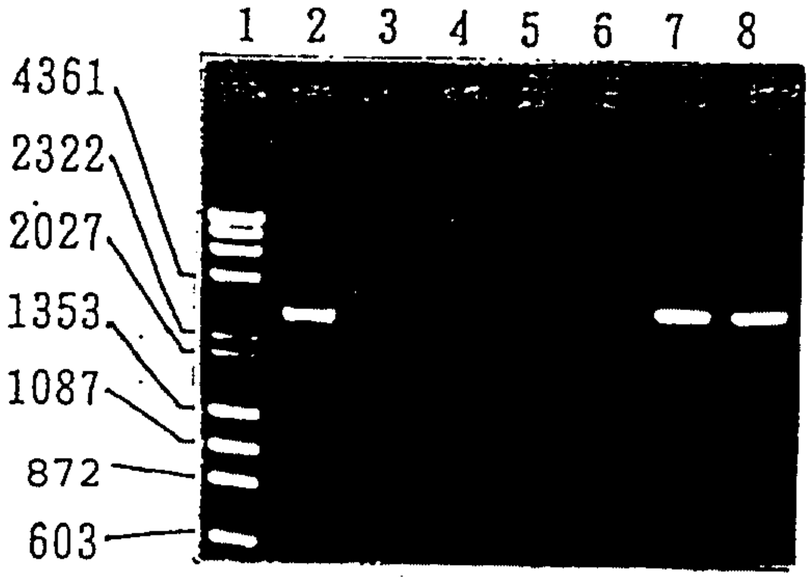
FIG. 24

(A) Using primers for detection of existence of ipt gene



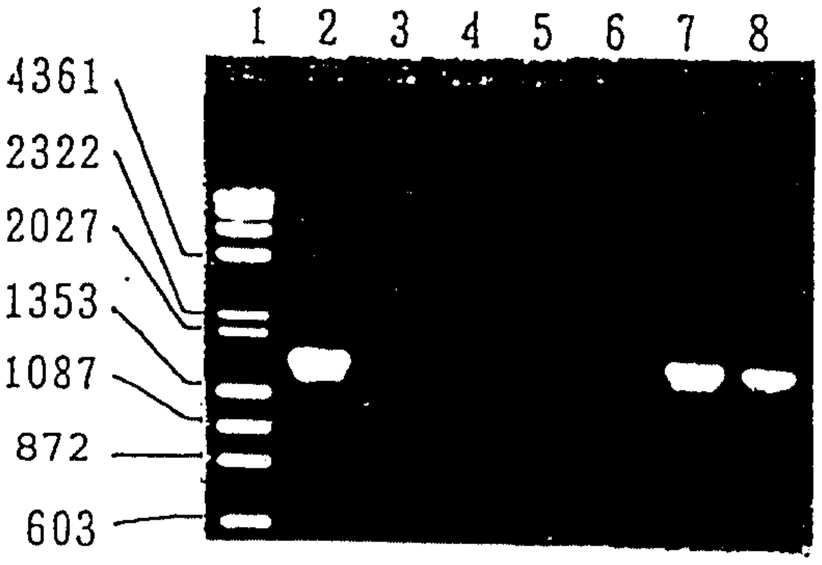
lane 1 ; DNA size marker
λ/Hind Ⅲ − φX174/HaeⅢ
(produced by TOYOBO CO., LTD.)
lane 2 ; pNPI132 (control)
lane 3 ; uninfected individual
lane 4～6 ; kanamycin sensitive-individual
lane 7、8 ; kanamycin resistant-individual

(B) Using primers for detection of excision of the region containing ipt gene and held by Rs's



lane 1 ; DNA size marker
λ/Hind Ⅲ − φX174/HaeⅢ
(produced by TOYOBO CO., LTD.)
lane 2 ; pBI121(control)
lane 3 ; uninfected individual
lane 4～6 ; kanamycin sensitive-individual
lane 7、8 ; kanamycin resistant-individual

(C) Using primers for detection of existence of GUS gene



lane 1 ; DNA size marker
λ/Hind Ⅲ − φX174/HaeⅢ
(produced by TOYOBO CO., LTD.)
lane 2 ; pNPI132 (control)
lane 3 ; uninfected individual
lane 4～6 ; kanamycin sensitive-individual
lane 7、8 ; kanamycin resistant-individual

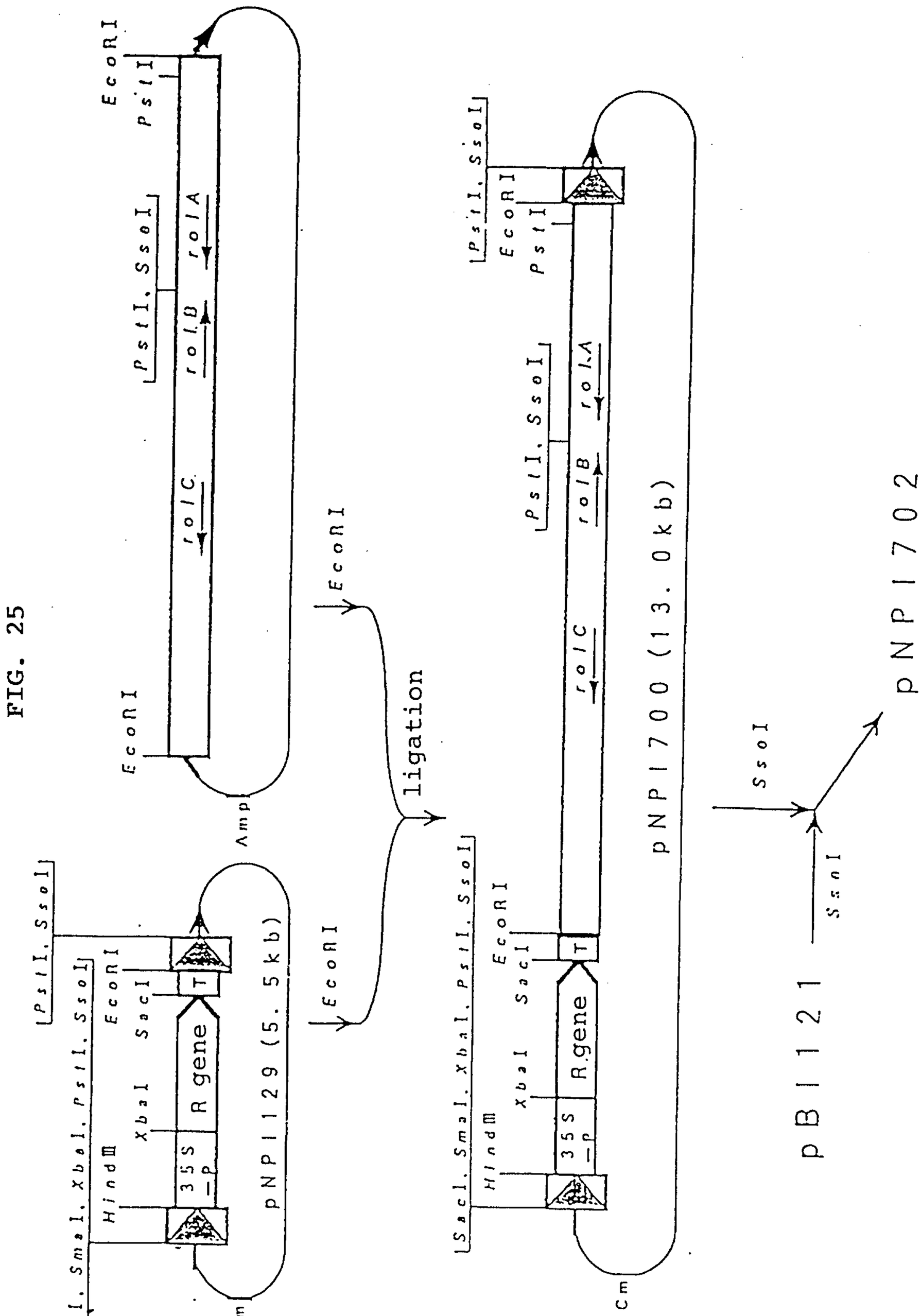

pNPI1700 (13.0kb)
EcoRI
PstI, SsoI
rolC
rolA
rolB
R.gene
35S —P
HindIII
SacI, SmaI, XbaI, PstI, SsoI
EcoRI
SacI
XbaI
T
pNPI1129 (5.5kb)
Amp
Cm
ligation
pNPI1702
SsoI
SacI
pBI121
Cm

FIG. 25

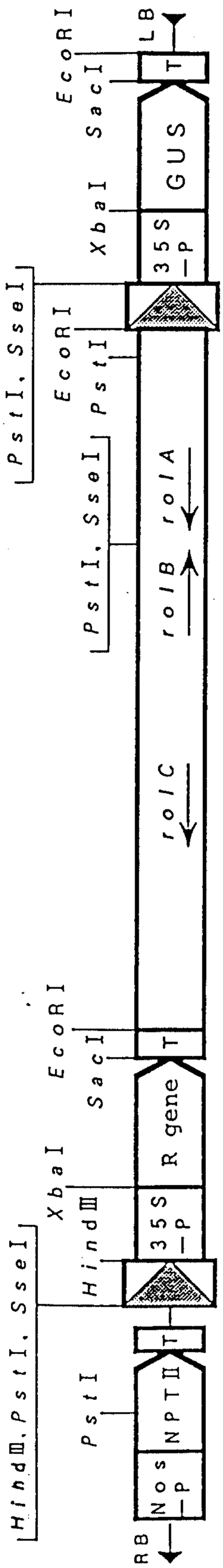


FIG. 26

pNPI1702 (23.9kb)

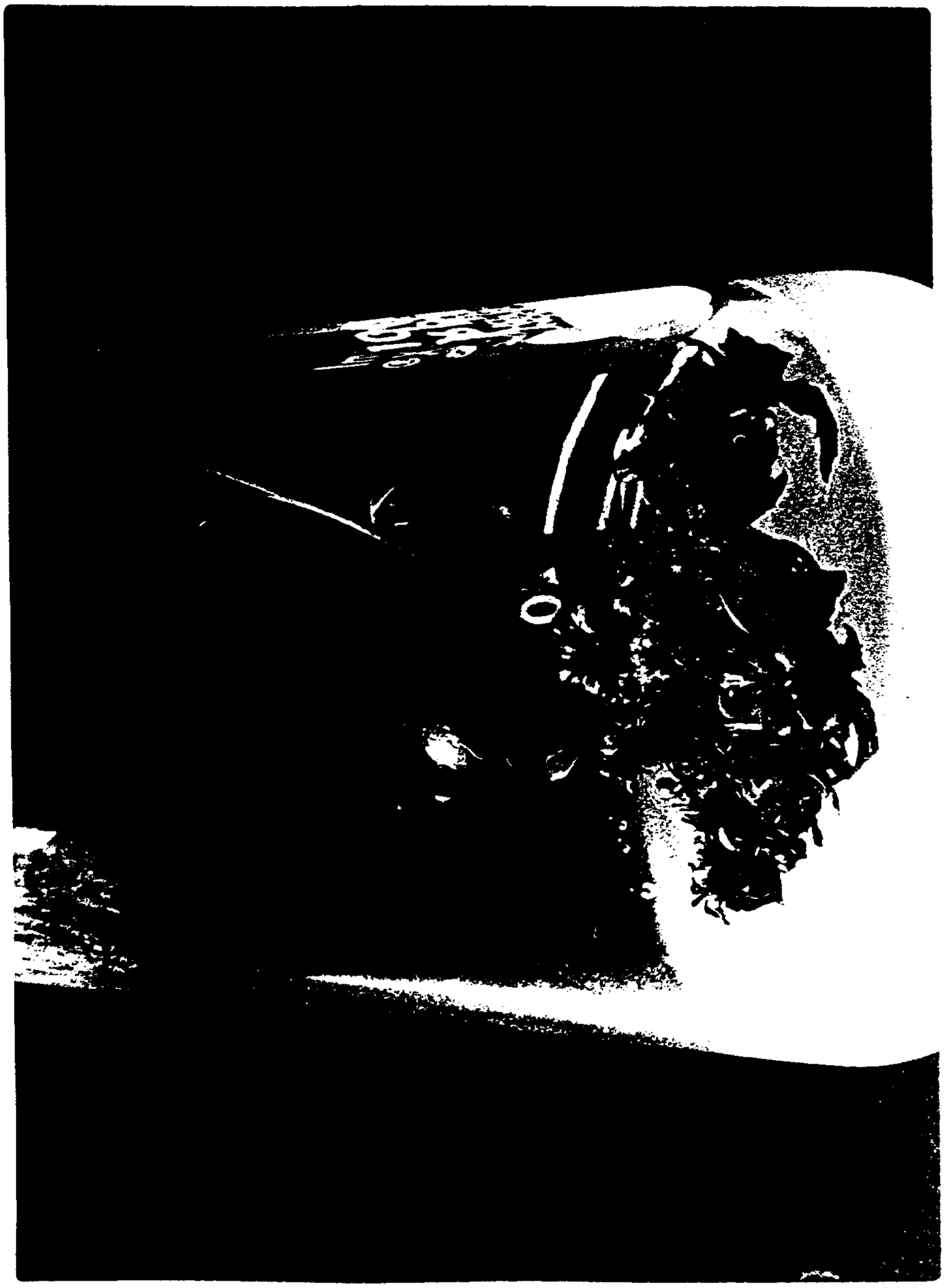
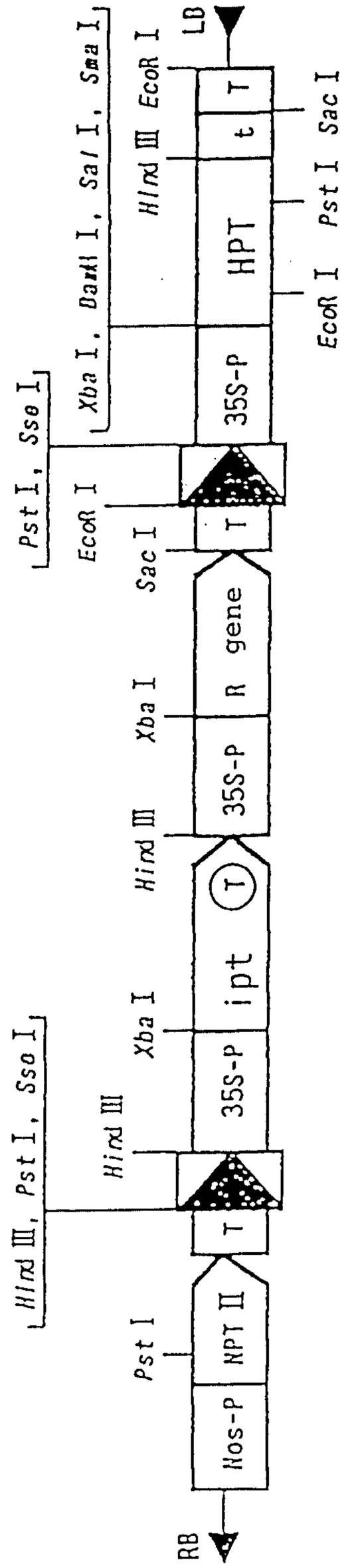


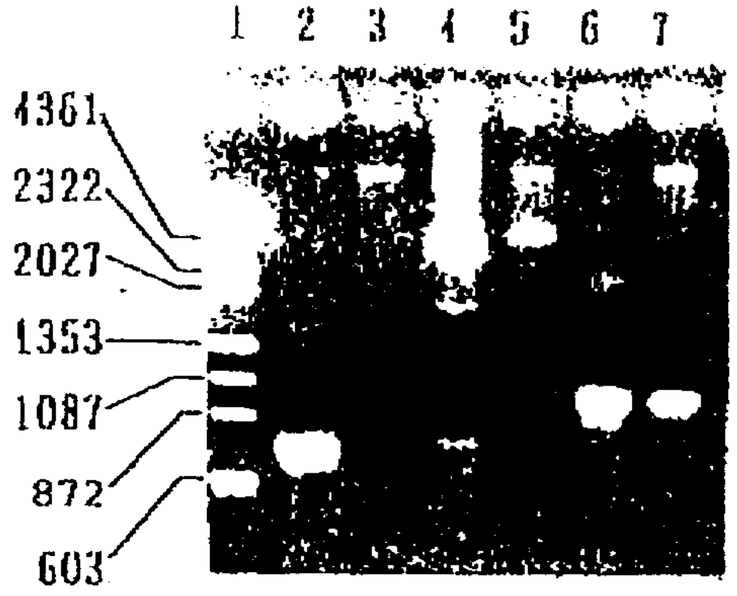
FIG. 27



pNPI140 (17.8kb)

FIG. 28

FIG. 29



lane 1 ; D N A size marker
λ/Hind III − φX174/Dae III
(produced by TOYOBO CO., LTD.)

lane 2 ; p N P I 1 4 0 (control)

lane 4 ; p N P I 1 3 9 (control)

lane 6 ; p N P I 1 4 0 (control)

lane 3、5、7 ; normal shoot after multiple introduction

lane 2、3 ; using primers for detection of existence of ipt gene

lane 4、5 ; using primers for detection of excision of the region containing ipt gene and held by Rs's

lane 6、7 ; using primers for detection of existence of HPT gene