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(54) **EMPLOI DE PROMOTEURS E8 DERIVES DE LA TOMATE POUR L'EXPRESSION DE LA S-ADENOSYLMETHIONINE HYDROLASE DANS LE MURISSEMENT DU FRUIT**

(54) **USE OF TOMATO E8-DERIVED PROMOTERS TO EXPRESS S-ADENOSYLMETHIONINE HYDROLASE IN RIPENING FRUIT**

(57) L'utilisation d'AdoMétase pour réduire la biosynthèse de l'éthylène chez les plantes est rendue possible par l'exploitation des propriétés tissulaires et spécifiques aux étapes du promoteur de E8 de la tomate. L'expression de l'AdoMétase est limitée a la tomate mûrissante. Les propriétés fonctionnelles de plusieurs régions du promoteur de E8 sont décrites. Le promoteur de E8 et les variants décrits dans cette invention sont d'utiles promoteurs régulables employés pour exprimer d'autres gènes ainsi que le gène de l'AdoMétase.

(57) The use of AdoMetase to reduce ethylene biosynthesis in plants is facilitated by the exploitation of the tissue and stage specific properties of the E8 promoter from tomato. Expression of AdoMetase is shown to be limited to the ripening tomato fruit. The functional properties of several regions of the E8 promoter are described. The E8 promoter and variants described herein provide useful regulatable promoters for the expression of other genes as well as the AdoMetase gene.

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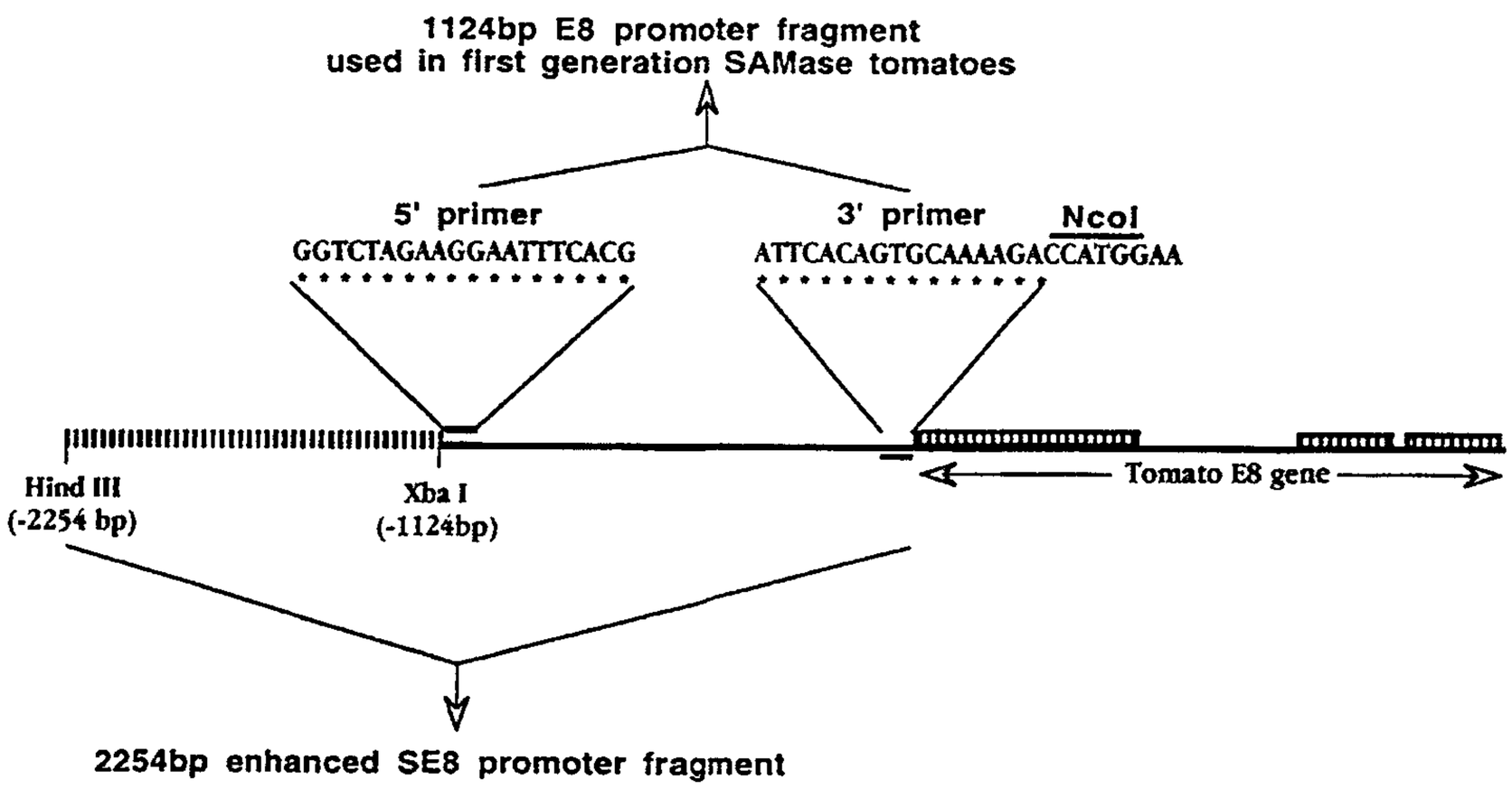
**(54) Title:** USE OF TOMATO E8-DERIVED PROMOTERS TO EXPRESS HETEROLOGOUS GENES, E.G. 5-ADENOSYLMETHIONINE HYDROLASE, IN RIPENING FRUIT




**(57) Abstract**

The use of AdoMetase to reduce ethylene biosynthesis in plants is facilitated by the exploitation of the tissue and stage specific properties of the E8 promoter from tomato. Expression of AdoMetase is shown to be limited to the ripening tomato fruit. The functional properties of several regions of the E8 promoter are described. The E8 promoter and variants described herein provide useful regulatable promoters for the expression of other genes as well as the AdoMetase gene.

# Use of Tomato E8-derived Promoters to Express S-adenosylmethionine Hydrolase in Ripening Fruit

## Field of the Invention

The present invention describes the use of the E8 promoter, and variants described herein, as useful regulatable promoters for the expression of heterologous genes, including the AdoMetase gene.

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**Background of the Invention**

Ethylene is a plant hormone which is a powerful regulator of plant metabolism, acting, and interacting with other plant hormones in trace amounts. Ethylene is a gas under normal physiological conditions. Even at low concentrations, ethylene has profound hormonal effects on plants.

The effects of ethylene, whether produced by the plant itself or applied exogenously, are numerous, dramatic, and of considerable commercial importance. Among the diverse physiological effects are the following: leaf abscission; fading in flowers; flower wilting; leaf yellowing; leaf epinasty; and stimulation of ripening in fruits and vegetables. Ethylene promotes senescence in plants, both in selected groups of cells and in whole organs, such as, fruits, leaves, or flowers. Senescence is the natural, genetically controlled degenerative process which usually leads to death in plants.

Normally, ethylene production from plant tissue is low. Large quantities of ethylene, however, are produced during ripening and senescence processes. A large amount of ethylene is also produced following trauma caused by chemicals, temperature extremes, water stress, ultraviolet light, insect damage, disease, or mechanical wounding. Ethylene produced by plants under such trauma conditions is referred to as "wound ethylene" or "stress ethylene". In fruits and vegetables, the stimulation of ethylene production by cuts or bruises may be very large and bear considerably on storage effectiveness. Ethylene-induced leaf browning is a common basis for loss in many plants, including lettuce and tobacco. In some tissues, exposure to only a small

amount of ethylene may cause an avalanche of ethylene production in adjacent plants or plant tissues such as fresh produce. This autocatalytic effect can be very pronounced and lead to loss of fruit quality during transportation and storage.

Current technologies that specifically address post-harvest storage life have been in existence for decades and are hampered by such problems as high cost, side effects, and an inability to completely shut off ethylene production. Included in this group are controlled atmosphere (CA) storage, chemical treatment, packaging, and irradiation.

CA facilities slow ethylene biosynthesis through: (i) low temperature, (ii) reducing the oxygen level below 3%, and (iii) elevating the carbon dioxide level in the storage area to the 3%-5% range. Expensive scrubbers are sometimes added which reduce ethylene already respired to the atmosphere. Drawbacks are that CA facilities are expensive to construct, have a high utility cost, and are unable to completely eliminate ethylene production and side effects. Also, CA storage techniques can only control external ethylene and not that which resides inside the plant tissue. CA storage can also lead to undesirable side effects: injury can result from high $CO_2$ levels, low $O_2$ levels, or low temperature.

Another treatment is to limit the ethylene biosynthesis in the plant tissue through chemical treatment. Aminoethoxyinylglycine (AVG), an analog of the antibiotic rhizobitoxine, is one such inhibitor. However, AVG cannot be used as a chemical additive in foods due to its high toxicity. Silver thiosulfate (STS) is also effective in slowing fruit ripening and flower fading, but is also toxic and cannot be used on foods. Further, STS only works

with certain flowers and often causes black spotting.

Recently, molecular genetic approaches leading to transgenic plants with impaired biosynthesis of ethylene have been reported. Hamilton, *et al.*, identified a cDNA clone for tomato EFE (pTOM13) by inhibiting ethylene synthesis with an antisense gene expressed in transgenic plants. Oeller, *et al.*, showed that expression of antisense RNA to the rate-limiting enzyme in the biosynthetic pathway of ethylene, 1-aminocyclopropane-1-carboxylate synthase, inhibits fruit ripening in tomato plants. Klee, *et al.*, cloned the gene encoding ACC deaminase, from soil bacteria, and introduced it into tomato plants. Reduction in ethylene synthesis in transgenic plants did not cause any apparent vegetative phenotypic abnormalities. However, fruits from these plants exhibited significant delays in ripening, and the mature fruits remained firm for at least 6 weeks longer than the non-transgenic control fruit.

**Summary of the Invention**

The present invention describes the development of transgenic plants, particularly, fruit-bearing transgenic plants. These plants contain a DNA sequence which encodes a desired gene product, such as a S-adenosylmethionine hydrolase enzyme (Ado-Metase). Adometase is capable of hydrolyzing S-adenosylmethionine to homoserine and 5'-methylthioadenosine. In the transgenic plants of the present invention the expression of the desired gene product is under the transcriptional control of an E8-derived promoter. The DNA sequence encoding the desired gene product is not the DNA sequence normally adjacent (homologous sequences) the E8 promoter,

rather, the sequences are heterologous to the E8 promoter.

The E8 gene promoter comprises the regulatory region located 5′ to the coding sequence of the tomato E8 gene. Promoters homologous to the E8 promoter can be identified by standard hybridization or DNA amplification methods. Figure 13 presents a representative nucleotide sequence of a portion of the E8 promoter. Two E8 promoter regions have been defined by the present invention, the SE8 promoter, which includes the entire region represented in Figure 13, and the lower E8 promoter, which includes the region represented as bases 1090 to 2214. Either of these promoters can be used in generating the transgenic plants of the present invention.

AdoMetase enzyme coding sequences can be obtained from a number of bacteriophage including the following: *Escherichia coli* bacteriophage T3, coliphage BA14, *Klebsiella* phage K11, and *Seratti* phage IV. An exemplary AdoMetase enzyme coding sequence was derived from *Escherichia coli* bacteriophage T3 and a representative coding sequence is presented in Figure 11.

One embodiment of the present invention is a transgenic tomato plant containing a heterologous DNA sequence whose expression is under the control of an E8 promoter. The invention also includes transgenic tomato-fruit cells. Included in the present invention, transgenic tomato plant cells and tomato fruit cells containing a DNA sequence which encodes and expresses AdoMetase, where expression of AdoMetase is under the transcriptional control of an E8 promoter.

The present invention also includes a method for regulating expression of a gene product in cells of a plant. In this method a vector is provided containing a first DNA sequence containing a gene

useful for genetic selection in plant cells, where this sequence is flanked by regulatory elements effective to allow expression of the sequence in plant host cells. The vector also includes a second DNA sequence that encodes a desired gene product, where expression of said second DNA sequence is under the transcriptional control of an E8 promoter, and where said DNA sequence is not normally contiguous with the E8 promoter. In one embodiment, the second DNA sequence encodes a S-adenosylmethionine hydrolase enzyme which hydrolyses S-adenosylmethionine to homoserine and 5'-methylthioadenosine.

The vector described above is used to transform plant host cells. These cells are cultivated to generate transgenic plants. When the vector is used to generate transgenic plants that bear fruit, the fruit cells can express the desired gene product, *e.g.*, the AdoMetase enzyme. AdoMetase enzyme coding sequences can be obtained from the same sources as described above.

The vector can be introduced into host plant cells by a number of transformation methods including, Agrobacterium-mediated transformation, electroporation, microinjection, and microprojectile bombardment. A typical gene useful for genetic selection in plant cells is a gene which confers kanamycin resistance.

The invention includes the above described vectors useful in plant transformation methods.

Expression of a selected gene, for example, the AdoMetase gene, can be regulated by a tissue or stage specific promoter, including an E8 promoter, or derivatives thereof.

The invention also includes the use of variants of the E8 promoter, described herein, to confer tissue and/or stage specific expression to any gene placed under their control.

**Brief Description of the Figures**

Figure 1 schematically illustrates the metabolic reactions for the synthesis of ethylene from methionine under both normal and stress conditions.

Figure 2 schematically illustrates the steps described for the genetic engineering of the AdoMetase-encoding gene.

Figure 3 illustrates the elements of the tomato E8 promoter and the primers used to amplify and isolate the promoter sequences.

Figure 4 outlines the steps involved in the construction of pGA-SESKN from pGA-ESKN and shows the elements of the E8 gene adjacent the AdoMetase (SAMase) coding sequences which are followed by *nosT* transcription termination sequences.

Figure 5A schematically represents the structure of the pGA-ESKN vector. Figure 5B schematically represents the structure of the pGA-SESKN vector.

Figure 6 shows the photograph of an autoradiogram which demonstrates the AdoMetase mRNA levels in fruit derived from two different transgenic plants.

Figure 7 shows a quantitation of the results presented in Figure 6. These results illustrate the effect of variations of the E8 promoter on AdoMetase mRNA levels in ripening tomatoes.

Figure 8 is a graph representing the relative levels of AdoMetase activity in ripening tomatoes at different stages.

Figure 9 presents the data for ethylene production in the fruit of 4 different transgenic plants (Figure 9A, ES 19-2; Figure 9B, LS 4-2; Figure 9C, ES 35-1; and Figure 9D, ES22 A-1) over a ten day period after entry of the fruit into breaker stage.

Figure 10 illustrates the post-harvest shelf life of tomatoes obtained from SESKN transgenic plants.

Figure 11 presents the sequence of the SAM-K modification of the AdoMetase gene derived from bacteriophage T3.

Figure 12A outlines the steps involved in the construction of the vector pESKN. Figure 12B outlines the steps involved in the modification of pESKN to the vector pGA-ESKN.

Figure 13 presents the sequence of the upstream minus 2216 base pair region of the tomato E8 gene.

**Detailed Description of the Invention**

The present application describes use of variants of the E8 promoter, described herein, to confer tissue and/or stage specific expression to any gene placed under their control.

I. Use of S-Adenosylmethionine Hydrolase in Plants.

The amino acid methionine has been shown to be a precursor of ethylene in plant tissues (reviewed by Imaseki). Methionine, however, is not the immediate precursor but first must be converted to the sulfonium compound S-adenosylmethionine (SAM) and, subsequently, aminocyclopropane-1-carboxylic acid (ACC) prior to conversion to ethylene. The metabolic reactions for the synthesis of ethylene from methionine under both normal and stress conditions are presented in Figure 1 and summarized as follows:

Methionine → SAM → ACC → Ethylene

ACC synthase catalyzes the degradation of SAM to ACC and 5′-methylthioadenosine (MTA). This enzymatic reaction appears to be the rate limiting

step in ethylene formation. For example, the natural plant hormone indoleacetic acid (IAA or auxin) stimulates ethylene production by inducing the synthesis of ACC synthase. Conversely, the synthesis of SAM from methionine and the production of ethylene from ACC do not require auxin induction.

In addition, wounding and fruit ripening induces the formation of ACC synthase and, therefore, the conversion of SAM to ACC. The other product of the ACC synthase reaction, MTA, must be recycled back into methionine so as to provide an adequate supply of methionine for continual ethylene production. This recycling pathway from MTA to methionine has been shown to exist in plant tissue (Adams, *et al.*; Kushad, *et al.*). The degradation of MTA has added significance in light of the finding that MTA is a potent inhibitor of ACC synthase. The importance of the degradation and recycling of MTA in normal plant tissues is, therefore, twofold: 1) to prevent the direct inhibition of ethylene synthesis by MTA, and 2) to provide adequate methionine for continual ethylene synthesis. A summary of this metabolic pathway is shown in Figure 1a.

The first step in the degradation of MTA in plant tissue is the hydrolysis of this nucleoside to 5-methylthioribose (MTR) by a specific MTA nucleosidase. MTR not only provides its methylthio moiety for the formation of methionine, but also contributes four carbons from its ribose towards the synthesis of this amino acid. Therefore, the methylthio group is conserved by recycling. It should be noted that this pathway merely maintains a methionine supply for ethylene biosynthesis, but does not result in a net increase in methionine synthesis.

The approach to reduce ethylene biosynthesis in plants reported here and in co-owned PCT Interna-

tional Publication No. WO 91/09112 published 27 June 1991, utilizes a gene that encodes the enzyme S-adenosylmethionine hydrolase. This enzyme, encoded by the *E. coli* bacteriophage T3, hydrolyses AdoMet to homoserine and MTA. The enzyme is known as its recommended name, AdoMet hydrolase (AdoMetase), or by its other name, S-adenosylmethionine cleaving enzyme (SAMase) (Studier, *et al.*). Both products of the reaction (*i.e.*, homoserine and MTA) are recycled to methionine; MTA as previously shown (Figure 1) and homoserine via a metabolism pathway known to exist in plant tissues.

The AdoMetase gene has been identified, isolated, cloned, and sequenced (Hughes, *et al.*, 1987a; Hughes, *et al.*, 1987b). The gene contains two in-frame reading sequences that specify polypeptides of 17105 and 13978 daltons. Both polypeptides terminate at the same ochre codon. This results in the 14 kd polypeptide being identical to 82% of the 17kd polypeptide starting at the carboxyl end of the longer polypeptide. Both polypeptides are present in partially purified cells and from *E. coli* expressing the cloned gene (Hughes, *et al.*, 1987b; Studier, *et al.*, 1976). Other bacteriophages that encode the AdoMetase or SAMase genes are coliphage BA14, *Klebsiella* phage K11, and *Serratia* phage IV (Mertens, *et al.*; Horsten, *et al.*).

The effect AdoMetase expression in plant cells has on the plant methionine recycling pathway is shown schematically in Figure 1b. Experiments performed in support of the present invention, using transgenic plants expressing an AdoMetase gene and monitoring ethylene production, have demonstrated that the effect of AdoMetase on the pathway is to "short circuit" the branch that produces ethylene: ethylene production is reduced in such transgenic

plants, including production in leaf tissue and fruit.

II. AdoMetase Encoding Genes.

Different bacteriophages may be expected to contain AdoMetase genes with variations in their DNA sequences. The isolation of AdoMetase coding sequences from bacteriophage coding sequences can be accomplished as previously described for AdoMetase from bacteriophage T3. Alternatively, degenerative hybridization probes for AdoMetase coding sequences can be generated and used to screen plasmids carrying fragments of a selected bacteriophage's genome for the presence of homologous sequences. AdoMetase enzymatic activity can be evaluated by standard biochemical tests (see for example, Example 5).

Furthermore, the amino acid sequence of AdoMetase may be modified by genetic techniques to produce enzymes with altered biological activities (see below). An increase in the biological activity could permit the use of lower amounts of the enzyme to control ethylene biosynthesis in plants.

A series of recombinant DNA manipulations were performed in the AdoMetase gene prior to placement in an Agrobacterium expression vector. Initially, a *MaeIII* to *BamHI* fragment from M13HB1 (Hughes, *et al.*, 1987a) was subcloned into the pUC19 plasmid vector to produce pUC19-SAM (Example 1). To increase the translational efficiency of the AdoMetase gene in plants, site directed mutagenesis of the nucleic acid sequences surrounding the ATG start codon was performed. A synthetic double stranded 39 base pair oligonucleotide was synthesized and substituted for the *BamHI* to *XmnI* fragment at the 5' end of the gene (Figure 2). The net effect of this substitution was to change the CACCAAATGA in the native T3 sequence to GCCACCATGG which an optimal

eukaryotic translational initiation sequence (Kozak, *et al.*; Lutcke, *et al.*).

The change also introduces an *NcoI* site (CCATGG) at the SAMase start codon which facilitates fusions to different promoters. The only alteration to the AdoMetase coding sequence is the amino acid at amino acid position two which is changed from isoleucine to valine: this is a highly conservative amino acid change. The altered form of AdoMetase was named SAM-K (Figure 11).

A recombinant vaccinia vector with SAM-K (vv:SAM-K) was constructed. Expression of this vector in African green monkey cells or T3-infected bacterial cells was compared with the gene to the native T3 gene when expressed in the same cells. The specific activity of AdoMetase was higher in the vv:SAM-K infected cells than in the T3 infected bacterial cells demonstrating that SAM-K encodes a fully functional version of AdoMetase.

Experiments performed in support of the present invention have demonstrated constitutive expression of AdoMetase in transgenic tomato and tobacco plants. In these plants there was a significant reduction in the ability of these plants to synthesize ethylene as measured in a leaf disk assay.

III. <u>Promoter Regulated SAMase Gene Expression</u>.

Regulatable promoters have been employed in the method of the present invention. One exemplary regulatable promoter is the tomato E8 gene promoter. Expression of the E8 gene has been shown to be induced (i) at the onset of ripening, and (ii) by treatment of tomatoes with ethylene (Deikman, *et al.*, 1988; Lincoln, *et al.*; Giovannoni, *et al.*). The sequence of the E8 promoter has been published (Deikman, *et al.*, 1988; Deikman, *et al.*, 1992) and

the DNA sequence of the minus 2216 base pair region is presented in Figure 13.

Using the sequence shown in Figure 13 primers were prepared for use in the polymerase chain reaction (PCR) to amplify the 1124 base pair promoter from tomato genomic DNA (Example 1). The primers were designed with unique restriction sites at each end and were used to place the promoter in the proper orientation 5′ of the SAM-K gene in pUC19 (Figure 3). The 3′ end of the promoter fragment had an *NcoI* site (CCATGG) placed such that the ATG start codon of the E8 gene product was used as the ATG in the *NcoI* site. This allowed precise placement of the entire E8 promoter directly in front of the SAM-K amino acid coding sequences with no intervening sequences (Example 1, Figure 12A).

Two AdoMetase expressing vectors were constructed (Example 1), the pGA-ESKN vector (Figures 12A, 12B and Figure 5A) and the pGA-SESKN vector (Figure 4 and Figure 5B). The pGA-ESKN vector contains a portion of the E8 promoter (Figure 4, lower E8 promoter) adjacent the AdoMetase coding sequences. A lambda EMBL-3 clone containing genomic tomato sequences that hybridize to the -1124 E8 region was isolated and used as the source for a region upstream of the -1124 E8 (lower E8) promoter. Restriction mapping analysis and subcloning allowed identification of an approximately 1200 bp *HindIII* to *XbaI* fragment as the region immediately upstream of the original -1124 bp E8 promoter (Figure 4). This region was added to the pGA-ESKN construct to yield pGA-SESKN, which contained the approximately -2254 bp E8 promoter fused to the AdoMetase gene (Figure 4, SE8).

Both of these vectors were transferred to tomato plants (Example 2) to generate transgenic plants expressing AdoMetase. A number of methods,

in addition to Agrobacterium-based methods, may be employed to elicit transformation of the plant host, such as electroporation, microinjection, and microprojectile bombardment. These methods are well known in the art (Klein, *et al.*; Miki, *et al.*; Bellini, *et al.*). Further, these methods provide the means to introduce selected DNA into plant genomes: such DNA may include a DNA cassette which consisting of the E8 gene promoter functionally adjacent AdoMetase coding sequences.

Several transgenic plants were assayed for their ability to synthesize AdoMetase mRNA using a sensitive RNAase protection assay (RPA) (Example 3). Figures 6 and 7 show the results of an RPA using the fruit from two transgenic plants (ESKN and SESKN) at different stages of fruit ripening. Other tissues from these plants including immature and mature leaves, flowers and stems were negative for the presence of AdoMetase RNA. Although the expression of AdoMetase in ESKN transgenic plants was regulated to the post mature green fruit, it was repeatedly observed (as shown in Figures 6 and 7) that the expression of AdoMetase turned off in the fully ripe fruit. On the other hand, the SESKN transgenic fruit maintained AdoMetase mRNA expression in ripe fruit.

To determine whether the presence of AdoMetase enzyme activity correlated with the level of AdoMetase mRNA, an AdoMetase assay was performed using extracts from four fruit obtained at different stages from an ESKN transgenic plant (Example 5). Figure 8 shows the level of AdoMetase activity in mature green, breaker, orange, and ripe fruit from a single pGA-ESKN transgenic plant. These data demonstrate that AdoMetase activity follows roughly the same expression pattern in ripening fruit as the AdoMetase mRNA levels.

The data presented above suggest that inclusion of the upstream region of the native E8 promoter in the AdoMetase expression construct enhances long-lived AdoMetase gene expression in ripening transgenic tomatoes. Figure 6 shows the RPA results from pGA-SESKN line 22A-1 and from pGA-ESKN line 18. ESKN line 18 had one of the highest levels of AdoMetase expression of all the ESKN transgenic lines. Quantitative measurement of AdoMetase mRNA is shown in Figure 7. The results show that the -2254 bp E8 promoter expression is maintained through the fully ripe stage of fruit development. This expression pattern is in sharp contrast to the -1124 bp E8 promoter (ESKN) mRNA levels also shown in Figure 6.

Ethylene evolution measurements from fruit picked at breaker and analyzed daily are shown in Figure 9. The rate at which fruit from SESKN lines 22A and 35-1 produced lycopene was reduced as evidenced by the time required for orange fruit development. Furthermore, the total amount of ethylene produced from these tomatoes was reduced by approximately 80%. The expression of AdoMetase and a reduction in ethylene biosynthesis was strictly correlated in the 25 SESKN transgenic plants analyzed.

The SESKN tomatoes that synthesized less ethylene were assessed for their shelf life properties when stored at room temperature (22°C) (Example 5). Three fruit each from SESKN lines 22A-1 and 35-1 were compared with untransformed normal tomatoes. Senescence was determined by visually observing contraction and wrinkles on the tomato skin. Firmness was not measured but was noted to be much greater in the transgenic lines. The results of these senescence assessments are shown in Figure 10. Even at 55 days post-breaker, the 22A-1 tomatoes

remained firm and appeared to be suffering more from dehydration than from the softening-induced senescence of the normal tomatoes.

These results demonstrate the ability to provide tissue specific regulation to the AdoMetase enzyme in transgenic plants. In addition, the results obtained with the two different E8 promoters (lower E8 and SE8) suggest the use of these promoters for similar tissue specific expression of any desired gene product. A tissue or stage specific promoter is a region of DNA that regulates transcription of the immediately adjacent (downstream) gene to a specific plant tissue or developmental stage of the plant or plant tissue. Other gene products which may be useful to express using these promoters include genes encoding (i) flavor or color modification proteins, and (ii) enzymes, such as is encoded by the taumatin gene, that modify lycopene synthesis. Further, it is useful to restrict expression of some genes to specific tissues, such as the fruit -- for example, any gene that would be deleterious to the plant if it were expressed constitutively. Such genes would include genes which encoded degradative enzymes that deplete necessary metabolites. As can be seen from the results described above, derivatives of the E8 promoter region can be used as on/off switches for the tissue and stage specific expression of genes whose expression is under their control.

The present method is applicable to all higher plants. Regulatable promoters other than the E8 promoter can also be used in the practice of the present invention include, but are not limited to the following: the E4 gene promoter from tomatoes; and, the promoter for ethylene forming enzyme (EFE) from tomatoes. Further, the two regions of the E8 promoter (lower E8 and upper E8, Figure 4) can be

used as hybridization probes against libraries of DNA representative of the genomes of other plant species. Homologous sequences to the E8 promoter are then tested for tissue specific expression in the plant species from which they were isolated. Such promoters, as well as the E8 promoter itself, can be tested for regulatable expression in heterologous plant systems using the methods described herein. A reporter gene, such as GUS ($\beta$-glucuronidase), can be used to test tissue specific regulatable expression from these promoters. Expression of GUS protein can be easily measured by fluorometric, spectrophotometric or histochemical assays (Jefferson, 1987).

Variants of the E8 promoter may be isolated from different tomato cultivars by standard recombinant manipulations such as primer specific amplification (Mullis; Mullis, *et al.*) or oligonucleotide hybridization (Ausubel, *et al.*; Sambrook, *et al.*).

Another gene whose promoter may be used for AdoMetase expression is the polygalacturonase gene promoter from tomato.

The following examples illustrate, but in no way are intended to limit the present invention.

## Materials and Methods

Tomato seed (*Lycopersicon esculentum* Mill. var. *cerasiforme* (Dunal) Alef. cv. Large Red Cherry) were obtained from Peto Seed, Inc. (Saticoy, CA) and were grown under standard greenhouse conditions. Harvested fruit were stored at room temperature (22°C).

## EXAMPLE 1

### Cloning of the AdoMetase Gene

#### A. Isolation of the AdoMetase Gene.

The AdoMetase gene was identified on an *AluI-HaeIII* restriction fragment from purified T3 DNA (Hughes, *et al.*, 1987a). Bacteriophage T3 is available under ATCC No. 11303-B3 (American Type Culture Collection, 12301 Parklawn Dr., Rockville MD 20852). The DNA fragment was first cloned into the bacteriophage M13 MP8 vector (Pharmacia LKB Biotechnology, Inc., Piscataway, NJ). A MaeIII to BamHI fragment was subcloned into the pUC19 plasmid vector (Pharmacia) to produce pUC19-AdoMetase (pUC19-SAMase; Figure 2). The generation of the pUC19-AdoMetase vector was described in co-owned PCT Publication No. WO 91/09112 published 27 June 1991. This vector was transformed into *E. coli* and used as a source of DNA for further construction experiments and for DNA sequence determination.

B. Modification of the Amino-Terminal Sequence of the Cloned AdoMetase Gene.

The cloned AdoMetase gene was further engineered to contain a consensus eukaryotic translation initiation site (Kozak; Lutcke, *et al.*) by altering the nucleotide sequence surrounding the SAMase ATG start-codon using a synthetic double-stranded oligonucleotide.

The plasmid pUC19-AdoMetase was digested with *XmaI* and *BamH1* and the 1.9 kb and 1.3 kb fragments were purified by electro-elution after agarose gel electrophoresis. A double stranded synthetic oligonucleotide linker having the sequence indicated in Figure 3 was ligated to the 1.9 kb fragment and this ligated DNA subjected to *XmaI* digestion to remove excess linkers.

The linkered 1.9 kg fragment was then repurified by electrophoresis on low melting temperature agarose and ligated to the 1.3 kb fragment to form the plasmid pUC19-SAM-K. The altered gene

region was subjected to DNA sequence analysis. The gene sequence is given in Figure 11. This gene was designated SAM-K and used to construct the following plant expression vectors. This plasmid DNA can also be used to directly transform the plant host via electroporation, microinjection, or microprojectile bombardment.

C. Vector Constructions using the Tomato E8 Promoter.

Two different forms of the E8 promoter were used to construct SAM-K-containing vectors. The first (-1124 bp) was isolated from tomato (*Lycopersicon esculentum* var. *cerasiform*) DNA using polymerase chain reaction (PCR) (Mullis; Mullis, *et al.*; Perkin-Elmer Cetus, Norwalk CT). The primers used in the PCR reaction were based on the sequence described by Deikman, *et al.* (1988). The sequences of the oligonucleotide primers are given in Figure 3. The oligonucleotides were designed to incorporate restriction endonuclease sites (*XbaI* and *NcoI*) at the 5′ and 3′ ends, respectively, of the amplified E8-promoter fragment. These restriction endonuclease cleavage sites facilitated subcloning into the pUC19-SAM-K vector (see Figure 2): an *NcoI* site is present near the ATG start codon region in the synthetic oligonucleotide.

Figure 12A outlines the generation of the vector pESKN starting from vector pNCN (Pharmacia, Inc., Piscataway, NJ) and pUC-SAM-K (described above). The sequence of the E8 promoter (the lower E8 promoter) is similar to the sequence presented as bases 1090 to 2214 in Figure 13.

Figure 12B outlines one approach to the generation of Agrobacterium vectors for use in the present invention. However, the E8/AdoMetase cassette, present in, for example, pESKN, can be incorporated

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in a number of vectors useful for plant transformation.

Agrobacterium binary vectors were developed from pGA482 (An, *et al.*, 1985), a pBIN19 derivative (Clontech Laboratories) containing the neomycin phosphotransferase II gene fused to the nopaline synthesis gene promoter (An, *et al.*, 1988). The resulting vector, designated pGA-ESKN is shown in Figure 5A.

The second E8 promoter (-2254 bp) was isolated from a lambda EMBL-3 clone that contained the entire E8 gene. The E8 gene clone was selected from a tomato (*Lycopersicon esculentum* var. VFN8) genomic library obtained from Clontech Laboratories (Palo Alto, CA) using the PCR-derived E8 promoter fragment (described above) as a hybridization probe in plaque-lift filter hybridizations. The lambda clone carrying the E8 gene was identified by a positive hybridization signal. The E8-bearing phage was plaque purified and the lambda phage DNA isolated.

The lambda E8 genomic clone was used as a source of the *HindIII* to *XbaI* fragment that is the approximately -2254 to -1124 bp upstream region of the E8 promoter. This fragment was inserted 5′ of the approximately -1124 bp E8 promoter in pGA-ESKN at the *HindIII* and *XbaI* sites (Figure 4). The resulting plasmid was named pGA-SESKN. Figure 13 shows the nucleotide sequence of the -2216 bp region from one cultivar (Deikman, *et al.*, 1988, 1992). The *HindIII* to *XbaI* fragment (used for construction of the approximately -2254 promoter) contains additional sequences 5′ to the end of this -2216 bp sequence.

Figure 4 shows the relationship of the two portions of the E8 promoter that are present in pGA-SESKN.

Standard recombinant DNA techniques were employed in all constructions (Adams, *et al.*; Ausubel, *et al.*). Another lambda vector, pGEM7Zf(+)SAM-K, was constructed by cloning the *BamHI* to *KpnI* AdoMetase fragment from pUC19:SAM-K into the same sites of pGEM7Xf(+) (Promega, Inc., Madison, WI).

Other plant cloning vectors, such as pBI121 (Clontech Laboratories, Inc., Palo Alto, CA), can also be used to practice the present invention. The plant promoter upstream of the AdoMetase gene sequence can be varied to obtain tissue specific expression, temperature dependent expression, or light dependent expression in the transgenic plants. Another useful plant promoter, in addition to the E8 promoter described above, is the constitutive Cauliflower Mosaic Virus (CaMV) promoter (Pharmacia).

## EXAMPLE 2

### Plant Transformation

The pGA-ESKN and pGA-SESKN AdoMetase plasmids were separately introduced into *Agrobacterium* using a direct transformation method.

*Agrobacterium tumefaciens* strain EHA101 (Hood, *et al.*), a disarmed derivative of *Agrobacterium tumefaciens* strain C58, was used to introduce coding sequences into plants. This strain contains a T-DNA-less Ti plasmid. The pGA-ESKN and pGA-SESKN AdoMetase plasmids were transferred into EHA101 using electroporation essentially as described by Nagel, *et al*. Briefly, an *Agrobacterium tumefaciens* culture was grown to mid-log phase (OD 600 0.5 to 1.0) in YEP media (10 g yeast extract, 10 g peptone, and 5 g NaCl per liter). After chilling on ice, 50 mls of these cells were pelleted, resuspended in 1

ml of ice cold 20 mM $CaCl_2$ and split into 1 ml aliquots.

Typically, one $\mu$g of plasmid DNA was added to an aliquots and incubated on ice for 30 minutes. The aliquot was then frozen in liquid nitrogen and thawed at 37°C for 5 minutes. One ml of YEP media was added and incubated at 28°C for 2 hours. The cells were pelleted, resuspended in 50 $\mu$l of YEP, and plated on YEP agar plates containing 20 $\mu$g/ml kanamycin. Kanamycin-resistant transformed colonies appear within 2 days.

Tomato cotyledon tissue explants were excised from both the tip and base of the cotyledon. Cotyledon explants were pre-conditioned for 2 days on tobacco feeder plates (Fillatti, *et al.*). The pre-conditioned explants were inoculated with EHA101 containing the pGA-ESKN or pGA-SESKN AdoMetase plasmid of interest and finally placed in a 10 ml overnight culture of EHA101/[pGA-ESKN or pGA-SESKN] for 5 minutes. The explants were then co-cultivated with the EHA101 strains for 2 days on tobacco feeder plates as described by Fillatti, *et al.*

The explants were grown in tissue culture media (Fillatti, *et al.*) containing 2Z media, MS salts, Nitsch and Nitsch vitamins, 3% sucrose, 2 mg/l seatin, 500 mg/l carbenicillin, 100 mg/l kanamycin and 0.7% agar. The explants were grown in tissue culture for 8 to 10 weeks. The carbenicillin treatments were kept in place for 2 to 3 months in all media. The explants and plants were kept on carbenicillin until they were potted in soil as a counter-selection to rid the plants of viable *Agrobacterium tumefaciens* cells.

## EXAMPLE 3

### RNAase Protection Assays for the Detection of SAMase mRNA

Tomato fruits at various stages of development from transgenic plants and wild-type plants were used as mRNA sources. mRNA was extracted from tomato cells and purified using the "QUICK PREP RNA" kit from Pharmacia, Inc. RNAse Protection Assays (RPA) were performed following the manufacturer's instructions using an "RPAII" kit from Ambion, Inc. (Hialeah, FL). This method has been previously described by Lee, *et al*.

pGEM7Zf(+)SAM-K was used to generate $^{32}$P-UTP-labeled RNA probe using bacteriophage T7 RNA polymerase as contained in the "RIBOPROBE IT T7 RNA POLYMERASE SYSTEM" from Promega, Inc. The radiolabeled probe was purified on a preparative polyacrylamide gel and used for up to one week.

One microgram of isolated mRNA was hybridized to approximately 10,000 CPM of the RNA probe and further processed as per the instructions in the "RPA II" kit. Briefly, one microgram of the purified mRNA was mixed with 10,000 CPM of the RNA probe in a total volume of 15 $\mu$l. 20 $\mu$l of a hybridization buffer that allows hybridization of complementary sequences (Ausubel, *et al*.; Maniatis, *et al*.; Sambrook, *et al*.) is then added. The hybridization solution is provided in the "RPAII" kit from Ambion. The solution was heated to 90°C for 3-4 minutes to denature all the RNA and incubated at 45°C overnight to allow hybridization of complementary sequences. The solution was cooled to 37°C and RNase (provided in the Ambion kit) was added which degrades all unhybridized probe.

Protected probe was resolved on a denaturing polyacrylamide gel, dried, and exposed to film for up to 16 hours. Quantitative analysis of the RPA signals was accomplished by excising each band from the gel, dissolving the band in a liquid fluor, and determining the radioactivity present in the sample

using liquid scintillation counting. A standard curve was generated using various amounts of unlabeled RNA synthesized from a AdoMetase fragment cloned into pGEM5Z(+) in the sense orientation. The linear range of the assay was dependent on the amount of input $^{32}P$-labeled RNA probe in the RNAase protection assay but typically ranged from 10 pg to 1 ng of mRNA.

## EXAMPLE 4

### Ethylene Measurements

The assay for tomato ethylene evolution is performed over a 0.5 to 1.0 hour period by sealing glass jars containing individual fruit and sampling 2 ml aliquots for gas chromatographic analysis. A Hewlett Packard 5890 (Palo Alto, CA) gas chromatograph with a flame ionization detector and a 6ft Porapak N column was used for ethylene measurements (Adams, *et al.*). This system combined with an HP Vectra computer and the current version of "CHEMSTATION" (Hewlett Packard) allows measurement of ethylene concentrations as low as 0.2 nl of ethylene in a 2 ml sample (0.1 ppm). After measurement of the ethylene in the headspace, the values are converted to nl of ethylene per gram of tissue per hour.

## EXAMPLE 5

### Characterization of Transgenic Tomatoes

A. Promoter Effect on SAMase mRNA Levels in Ripening Transgenic Fruit.

Transgenic fruit were selected from two transgenic plants, ESKN #18 and SESKN #22A, at three stages of ripening, breaker (Br), Orange (Or) and Ripe (Ri). Transgenic plant ESKN #18 contained the lower E8 promoter (Figure 4) adjacent the Sam-K AdoMetase gene. Transgenic plant SESKN #22A contained the entire SE8 promoter (Figure 4) adjacent

the Sam-K AdoMetase gene. The AdoMetase mRNA level in ripening transgenic fruit was determined as described in Example 3.

The products of the RNA protection assay were resolved on polyacrylamide gels and exposed to X-ray film. A representative autoradiogram of the RNA protection assay is presented in Figure 6. As can be seen in the figure, AdoMetase mRNA was present in both transgenic plants at the breaker stage of fruit ripening. However, the levels of AdoMetase mRNA drop in the ESKN transgenic plant, relative to the SESKN transgenic plant, at the orange and ripe stages of fruit ripening.

The level of AdoMetase mRNA was quantitated as described in Example 3 by liquid scintillation counting and determination of mRNA concentrations relative to a standard curve. Figure 7 presents the results of this analysis. The results are consistent with those shown in Figure 6. AdoMetase mRNA was present in both transgenic plants at the breaker stage of fruit ripening with the concentrations lower in ESKN #18. At the orange and ripe stages of fruit ripening the levels of AdoMetase mRNA drop in the ESKN transgenic plant, relative to the level at breaker stage and the levels in the fruit from the SESKN transgenic plant. The AdoMetase mRNA levels stay relatively constant in the SESKN transgenic plant.

B. <u>Relative Levels of SAMase Activity in Ripening Transgenic Tomatoes</u>.

To determine whether the presence of AdoMetase enzyme activity correlated with the level of AdoMetase mRNA, a 14C-SAM-based AdoMetase assay was performed using extracts from four different fruit stages from a single pGA-ESKN transgenic plant.

Plant tissues to be assayed for AdoMetase enzyme activity were frozen and ground to a powder

in liquid nitrogen. The ground tissue was then suspended in 1.5 volumes of 200 mM Tris-HCl (pH 7.5), 10 mM DTT, and 10 mM EDTA. The suspension was vortexed vigorously then subjected to centrifugation at 40,000 × g at 4°C for 20 minutes. The following was added to 50 μl of extract: 5 μl of $^{14}$C-SAM (DuPont-New England Nuclear, NEC-363) at 20 μCi/ml and a specific activity of 58.0 mCi/mmol. The reaction was incubated at 37°C for 1 hour then 40 μl of the reaction was spotted on a cellulose think layer chromatography (TLC) plate (J.T. Baker, Inc., Phillipsburg, N.J., Baker-Flex Cellulose F) and resolved for 3 hours in 70:70:20:40, butanol:-acetone:acetic acid:water. The MTA and MTR spots were identified using autoradiography, excised, and counted using liquid scintillation.

Figure 8 shows the level of AdoMetase activity in mature green, breaker, orange, and ripe fruit. The level of AdoMetase activity is defined as the percent conversion of SAM (S-adenosylmethionine) to MTA (5'-Methylthioadenosine) and MTR (5'-Methyl-thioribose). The decreasing level of AdoMetase activity from breaker to ripe fruit in the ESKN transgenic plant is consistent with the AdoMetase mRNA levels shown in Figure 7.

Untransformed tomato fruit extracts do not degrade SAM to MTA or MTR at any stage of ripening when used in this assay.

C. <u>Ethylene Production in Ripening Transgenic Fruit</u>.

Ethylene produced from transgenic tomatoes carrying the AdoMetase gene under the regulation of the SE8 promoter (Figure 4) was determined as described in Example 4. Greenhouse grown tomatoes from 4 transgenic lines were tested. The results of the analysis are presented in Figures 9A to 9D. Each of the four graphs shown in Figure 9 represent

the comparison of fruit from one pGA-SESKN transgenic line (Es 19-2, LS 4-2, ES 35-1 and ES 22A-1) with the fruit from untransformed controls. The control values (open squares) are the same in each of the four graphs and represent the average of six fruit from two different plants. The values from each transgenic line (closed symbols) are the average of ethylene determinations for three fruit. Error bars represent one standard deviation of the data.

The data represent a time period of ten days after the breaker stage of fruit ripening (post-breaker). These data demonstrate a reduction in the amount of ethylene production in transgenic tomatoes versus normal fruit over the ten day period.

D. Post-Harvest Shelf-life of SESKN Tomatoes. Tomatoes from the SESKN transgenic plants that synthesized less ethylene were assessed for their shelf life properties when stored at 22°C. Three fruit from each from SESKN lines 35-1, 22A-1 and LS4-2 were compared with tomatoes from two untransformed, normal plants (M16 and M15). Senescence was determined each day by visual examination of the fruit for the occurrence of contraction and wrinkles on the tomato skin. The results of these senescence assessments are shown in Figure 10.

As can be seen from the results in the figure, the bar graph shows the time for the fruit to achieve each stage: all fruit were picked at the breaker stage. For instance, line 35-1 took 18 days to ripen (Ripe stage) but then senescence developed at day 27. Line 22A-1 took 7 days to turn orange, 13 days to turn red, then 52 days to senescence. Even at 55 days post-breaker, the 22A-1 tomatoes remained firm and appeared to be suffering more from

dehydration than from the softening-induced senescence of the normal tomatoes.

Firmness was not measured for the tomatoes from the five plants described above, however, the firmness was noted to be much greater in the fruit from the transgenic lines.

While the invention has been described with reference to specific methods and embodiments, it will be appreciated that various modifications and changes may be made without departing from the invention.

**IT IS CLAIMED:**

1. An expression vector for use in transforming plant cells, comprising

(i) a tomato E8 gene promoter characterized by its ability to confer high level expression in fruit through the fully ripe stage of fruit development, and

(ii) a DNA sequence encoding S-adenosylmethionine hydrolase,

where said promoter is operably linked to said DNA sequence to enable expression of S-adenosylmethionine hydrolase in transformed plant cells.

2. The vector of claim 1, further comprising a second DNA sequence encoding a selectable marker functional in plant cells, where said second DNA sequence is flanked by regulatory elements effective to allow expression of said second sequence in a plant host.

3. The vector of claim 2, wherein said selectable marker confers kanamycin resistance.

4. The vector of any of claims 1-3, where the E8 promoter consists essentially of the sequence presented in Figure 13.

5. The vector of any of claims 1-3, wherein the sequence of said promoter includes a polynucleotide region corresponding to nucleotides 1090 to 2214 of the sequence presented as Fig. 13.

6. The vector of any of claims 1-3, wherein the sequence of said promoter includes a polynucleotide region corresponding to nucleotides 1 to 1089 of the sequence presented as Fig. 13.

7. The vector of any of claims 1-6, wherein said S-adenosylmethionine hydrolase coding sequence is derived from a bacteriophage selected from the group

consisting of *Escherichia coli* bacteriophage T3, coliphage BA14, Klebsiella phage K11, and Seratti phage IV.

8. The vector of any of claims 1-6, wherein said S-adenosylmethionine hydrolase sequence encodes the protein whose sequence is presented in Figure 11.

9. The vector of any of claims 1-6, wherein said DNA coding sequence comprises a contiguous series of nucleotides contained within the sequence presented as Fig. 11.

10. The vector of any of claims 1-9, wherein the promoter is isolated by the steps of:

(i) selecting a probe DNA molecule from a tomato plant E8 gene,

(ii) contacting the probe with a plurality of target DNA molecules obtained from the genome of a tomato-plant under specific hybridization conditions,

(iii) identifying a target molecule which specifically hybridizes to the probe under said conditions, and

(iv) isolating promoter sequences associated with the target molecule having the ability to confer high level expression in fruit through the fully ripe stage of fruit development.

11. A method for producing a transgenic fruit-bearing plant, where fruit produced by said plant has a modified ripening phenotype, comprising

(i) introducing into progenitor cells of the plant a vector of any of claims 1 to 10, and

(ii) growing progenitor cells containing said vector to produce a transgenic plant bearing fruit,

wherein the level of ethylene biosynthesis of fruit produced by said plant is reduced in comparison to wild-type fruit, resulting in fruit having a modified ripening phenotype in which the time course of ripening is delayed over that of non-transformed fruit.

12. The method of claim 11, wherein said introducing is carried out by a direct transformation methodology selected from the group consisting of *Agrobacterium*-mediated transformation, electroporation, microinjection, and microprojectile bombardment.

13. A method for reducing ethylene biosynthesis in fruit cells of a plant through the fully ripe stage of fruit development, comprising:

(i) providing a vector of any of claims 1-10

(ii) transforming plant host cells with said vector, and

(iii) growing the transformed host cells to produce a transgenic plant bearing fruit, wherein fruit cells of said fruit are capable of expressing S-adenosylmethionine hydrolase enzyme.

14. A method for modifying ripening of fruit of a fruit bearing plant, comprising,

(i) providing a vector of any of claims 1-l0,

(ii) transforming plant host cells with said vector, and

(iii) growing the transformed host cells to produce a transgenic plant bearing fruit, wherein the level of ethylene biosynthesis of fruit produced by said plant is reduced in comparison to wild-type fruit, resulting in fruit having a modified ripening phenotype in which the time course of ripening is delayed over that of non-transformed fruit.

15. A method of extending the post-harvest shelf life of a fruit, comprising,

(i) providing a vector of any of claims 1-10,

(ii) transforming plant host cells with said vector, and

(iii) growing the transformed host cells to produce a transgenic plant bearing fruit, wherein the level of ethylene biosynthesis of fruit produced by said plant is reduced in comparison to wild-type fruit, resulting in fruit having a modified ripening

phenotype in which the time course of ripening is delayed over that of non-transformed fruit to thereby extend its post-harvest shelf life.

16. A DNA fragment comprising:

(i) a tomato E8 gene promoter characterized by its ability to confer high level expression in fruit through the fully ripe stage of fruit development, and

(ii) a DNA sequence encoding S-adenosylmethionine hydrolase, where said promoter is operably linked to said DNA sequence to enable expression of S-adenosylmethionine hydrolase in transformed plant cells.

17. The DNA fragment of claim 16, where the E8 promoter consists essentially of the sequence presented in Figure 13.

18. The DNA fragment of claim 16, wherein the sequence of said promoter includes a polynucleotide region corresponding to nucleotides 1090 to 2214 of the sequence presented as Fig. 13.

19. The DNA fragment of claim 16, wherein the sequence of said promoter includes a polynucleotide region corresponding to nucleotides 1 to 1089 of the sequence presented as Fig. 13.

20. The DNA fragment of any of claims 16-19, wherein said S-adenosylmethionine hydrolase coding sequence is derived from a bacteriophage selected from the group consisting of *Escherichia coli* bacteriophage T3, coliphage BA14, Klebsiella phage KII, and Seratti phage IV.

21. The DNA fragment of any of claims 16-19, wherein said S-adenosylmethionine hydrolase sequence encodes the protein whose sequence is presented in Figure 11.

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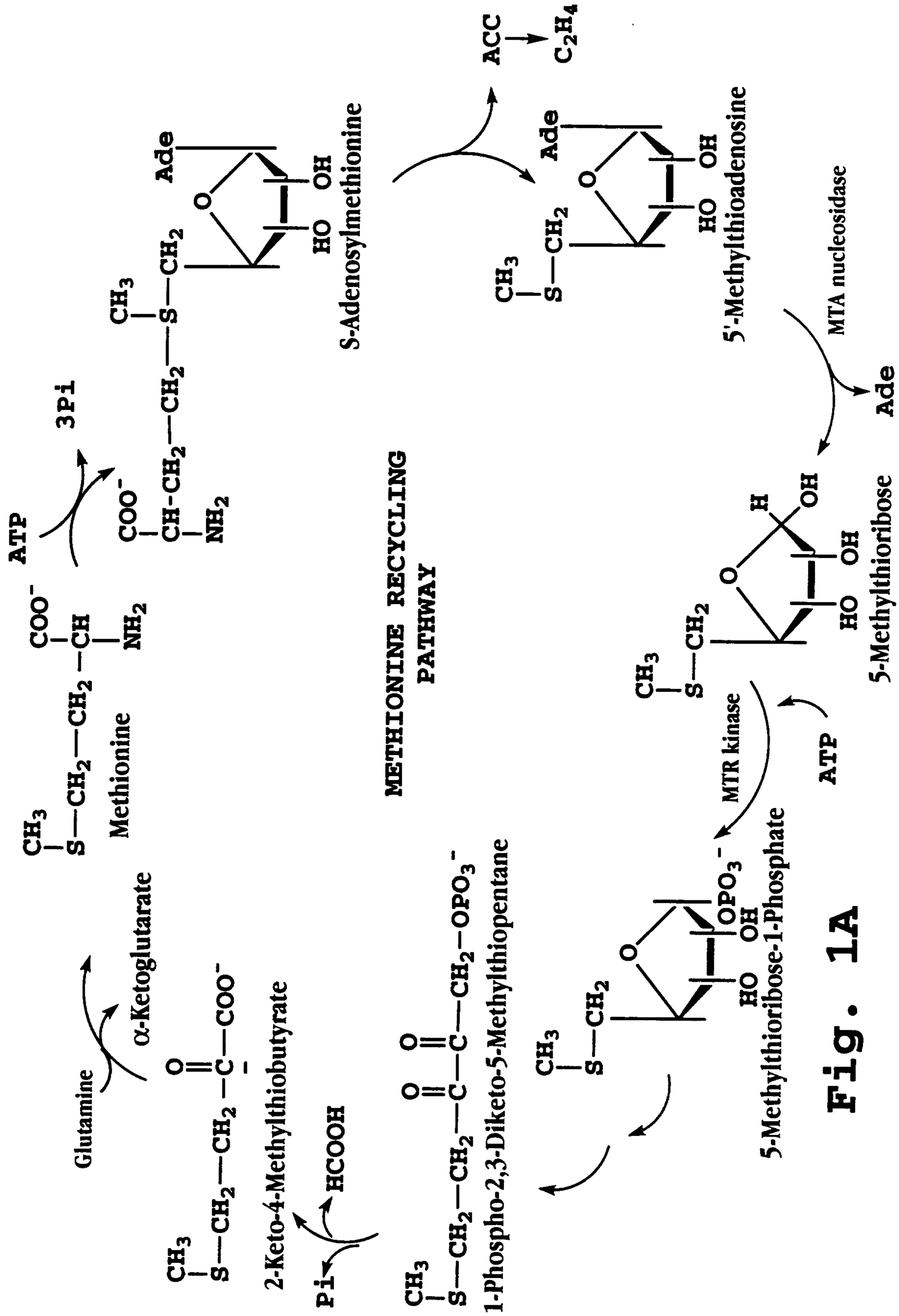
22. The DNA fragment of any of claims 16-19, wherein said DNA coding sequence comprises a contiguous series of nucleotides contained within the sequence presented as Fig. 11.

23. A plant cell containing the DNA fragment of any of claims 16-22.

B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Glutamine
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Pi
HCOOH
1-Phospho-2,3-Diketo-5-Methylthiopentane
5-Methylthioribose-1-Phosphate
MTR kinase
ATP
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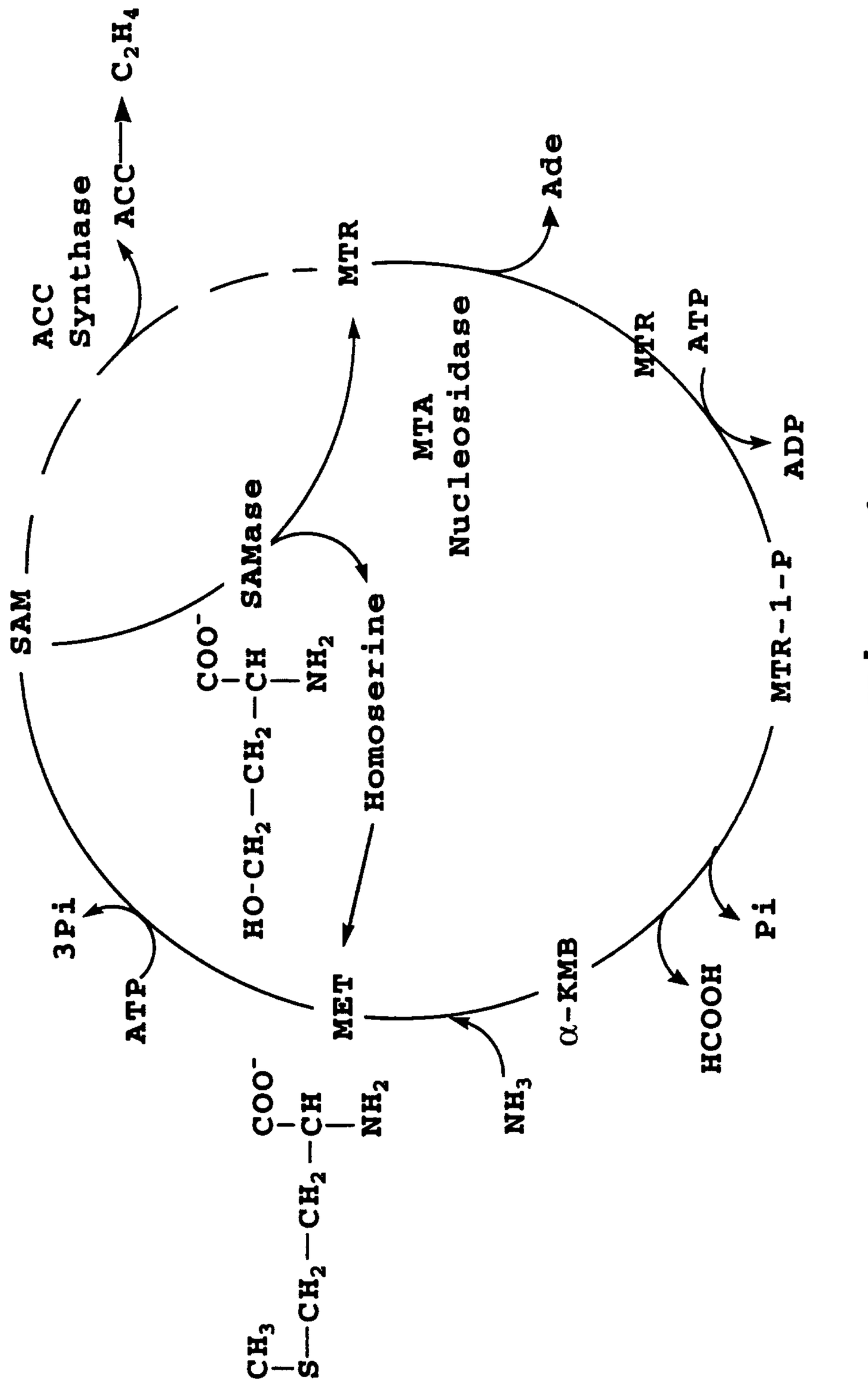
MTA nucleosidase
5'-Methylthioadenosine
Ade

Fig. 1A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ACC
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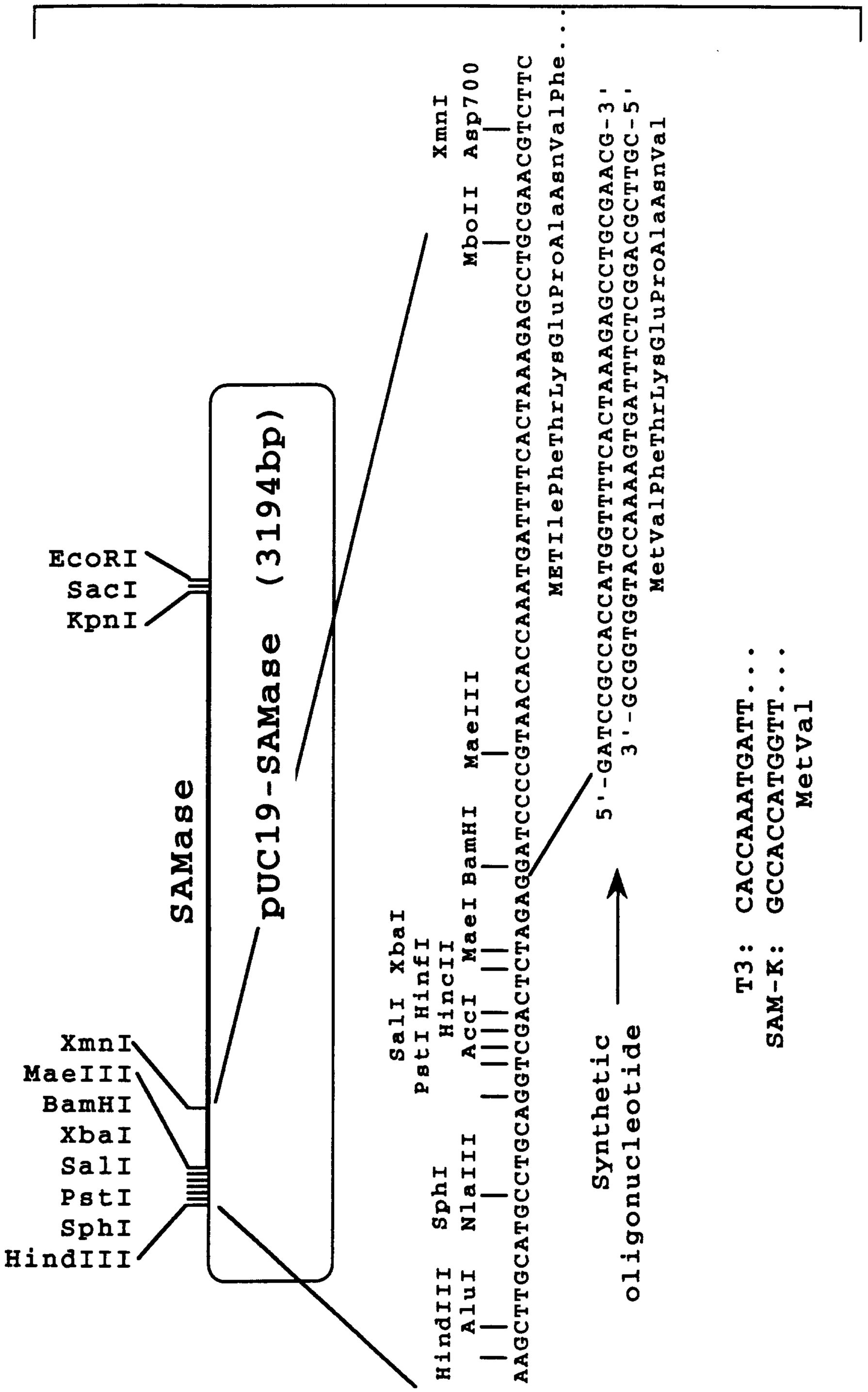
Fig. 1B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HindIII
XmnI
Asp700
MboII
METIlePheThrLysGluProAlaAsnVal
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HindIII SphI
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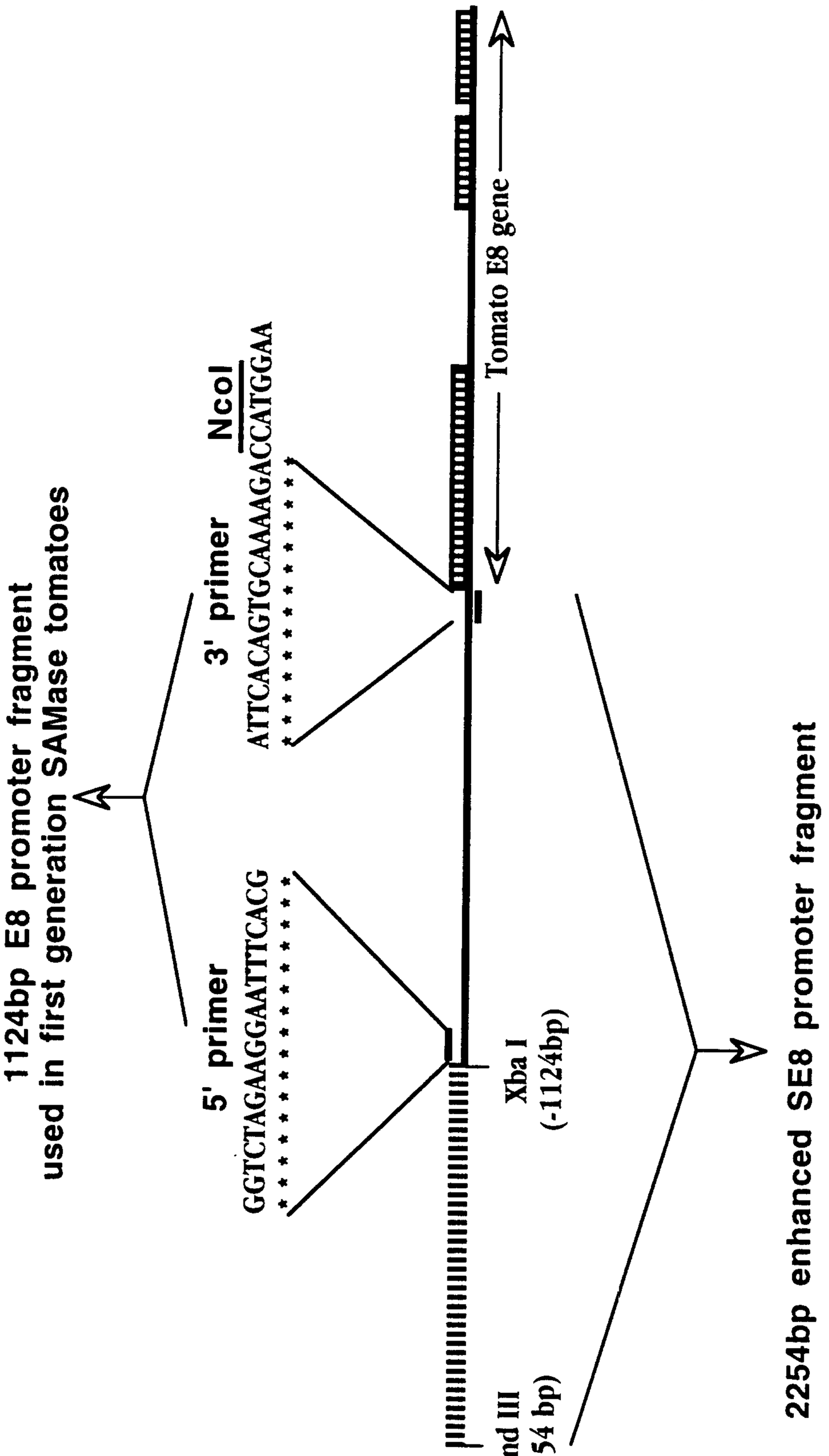
Fig. 2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used in first generation SAMase tomatoes
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3' primer
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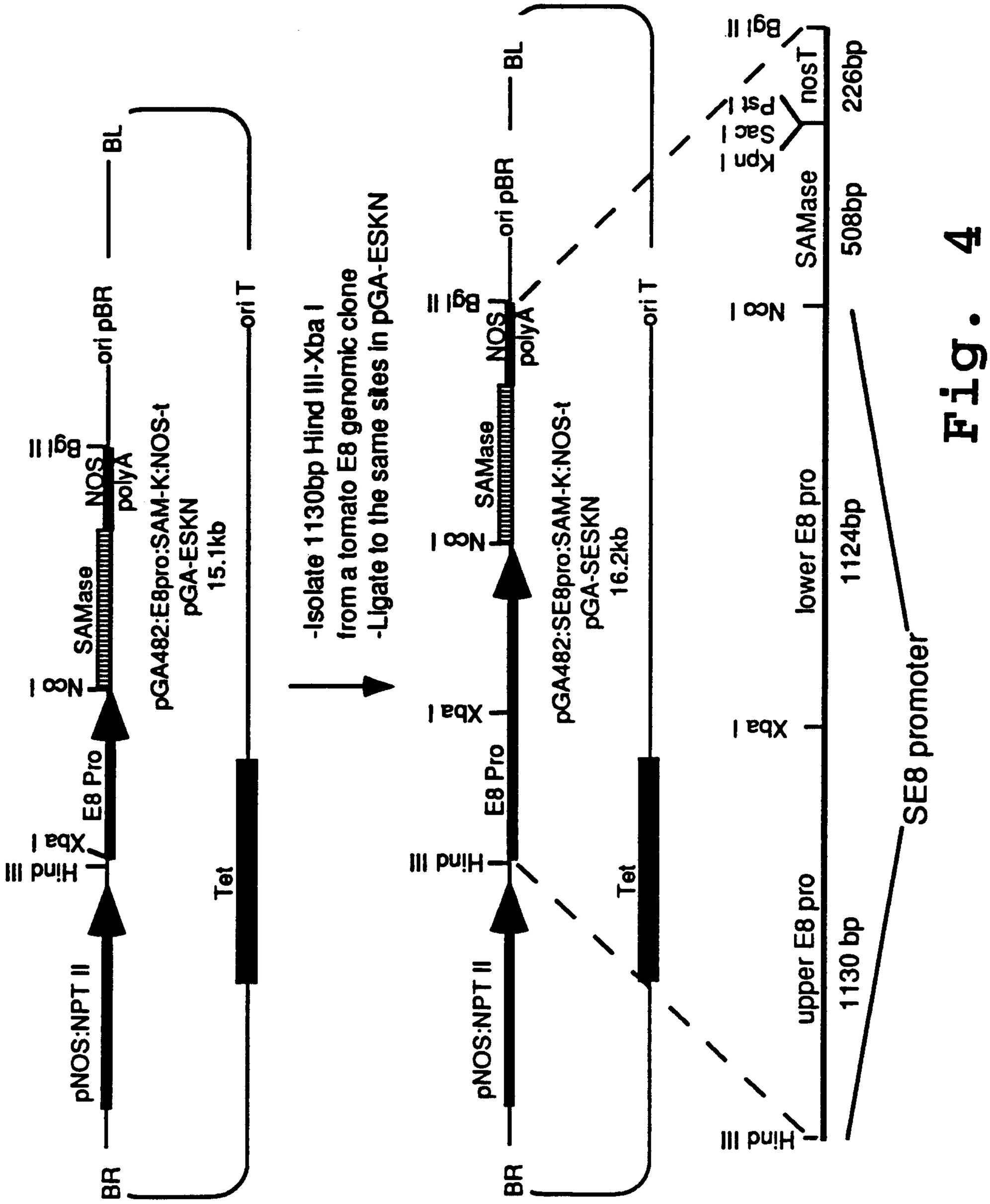
Tomato E8 gene
Xba I
(-1124bp)
Hind III
(-2254 bp)
2254bp enhanced SE8 promoter fragment

Fig. 3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15.1kb
Tet
ori pBR
BL
ori T
BR
-Isolate 1130bp Hind III-Xba I
from a tomato E8 genomic clone
-Ligate to the same sites in pGA-ESKN
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pGA-SESKN
16.2kb
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SE8 promoter
upper E8 pro
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226bp
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Fig. 4

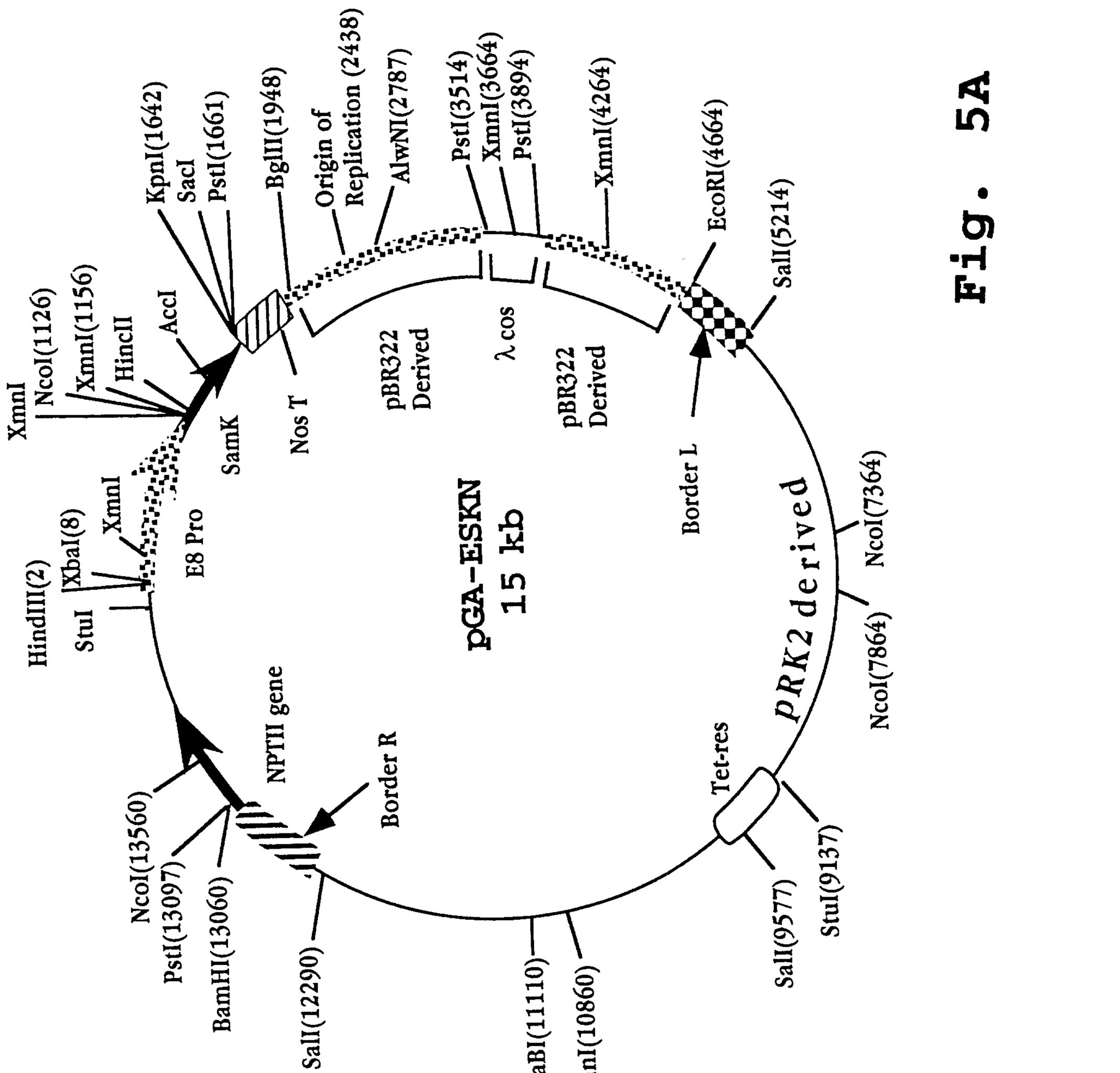
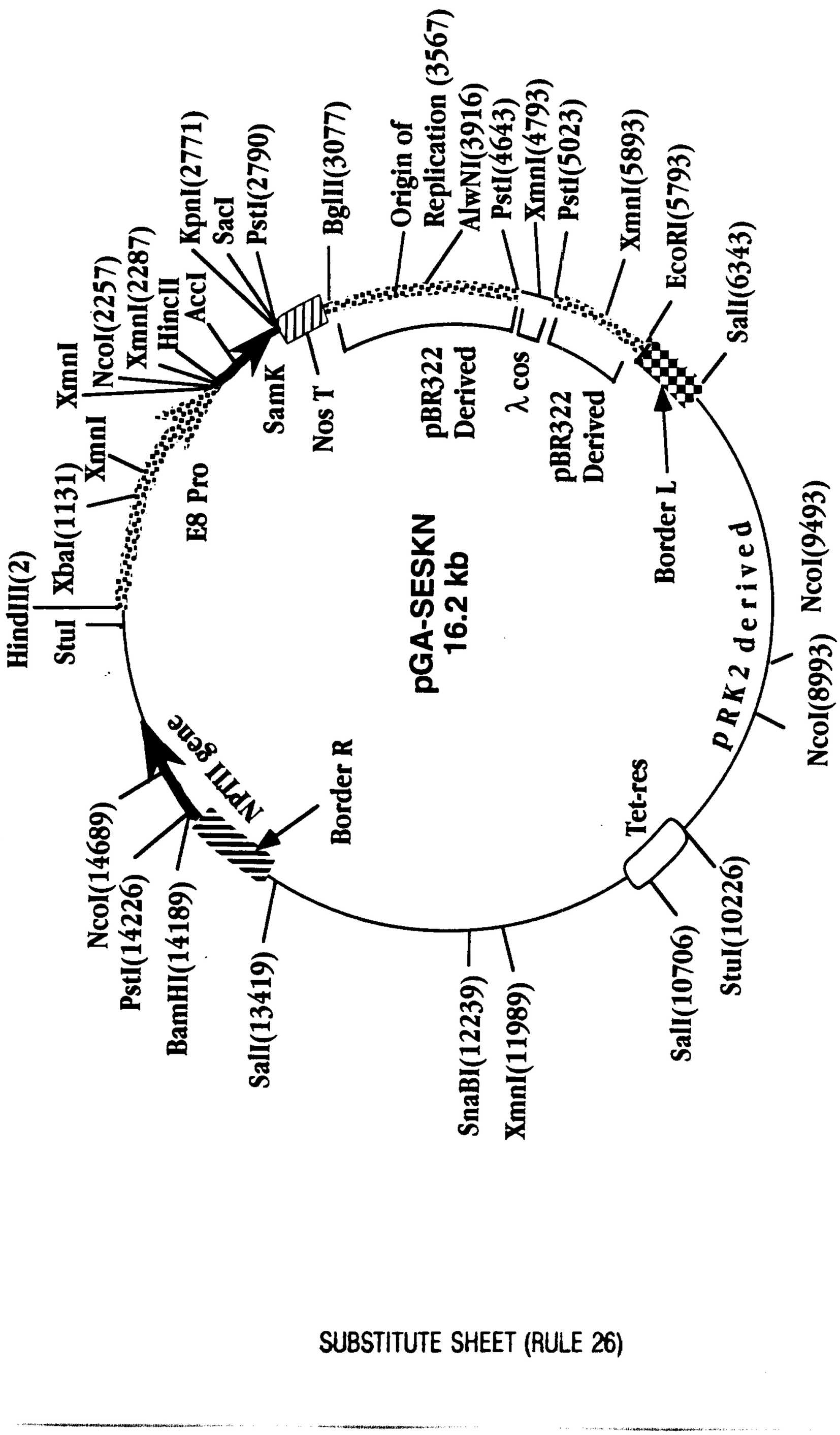



Fig. 5A

Gowling, Strathy & Henderson

A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Origin of Replication (35
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Border R
Tet-res
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XmnI(2287)
HincII
AccI
KpnI(2771)
SacI
PstI(2790)
BglII(3077)
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XmnI(11989)
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BamHI(
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NcoI(14689)
NcoI(14226)
HindIII(14189)
HindIII(2)
StuI
XbaI(11
XmnI
NcoI(1131)

Fig. 5B

| ESKN #18 | | | SESKN #22A | | |
|---|---|---|---|---|---|
| Br | Or | Ri | Br | Or | Ri |

Fig. 6

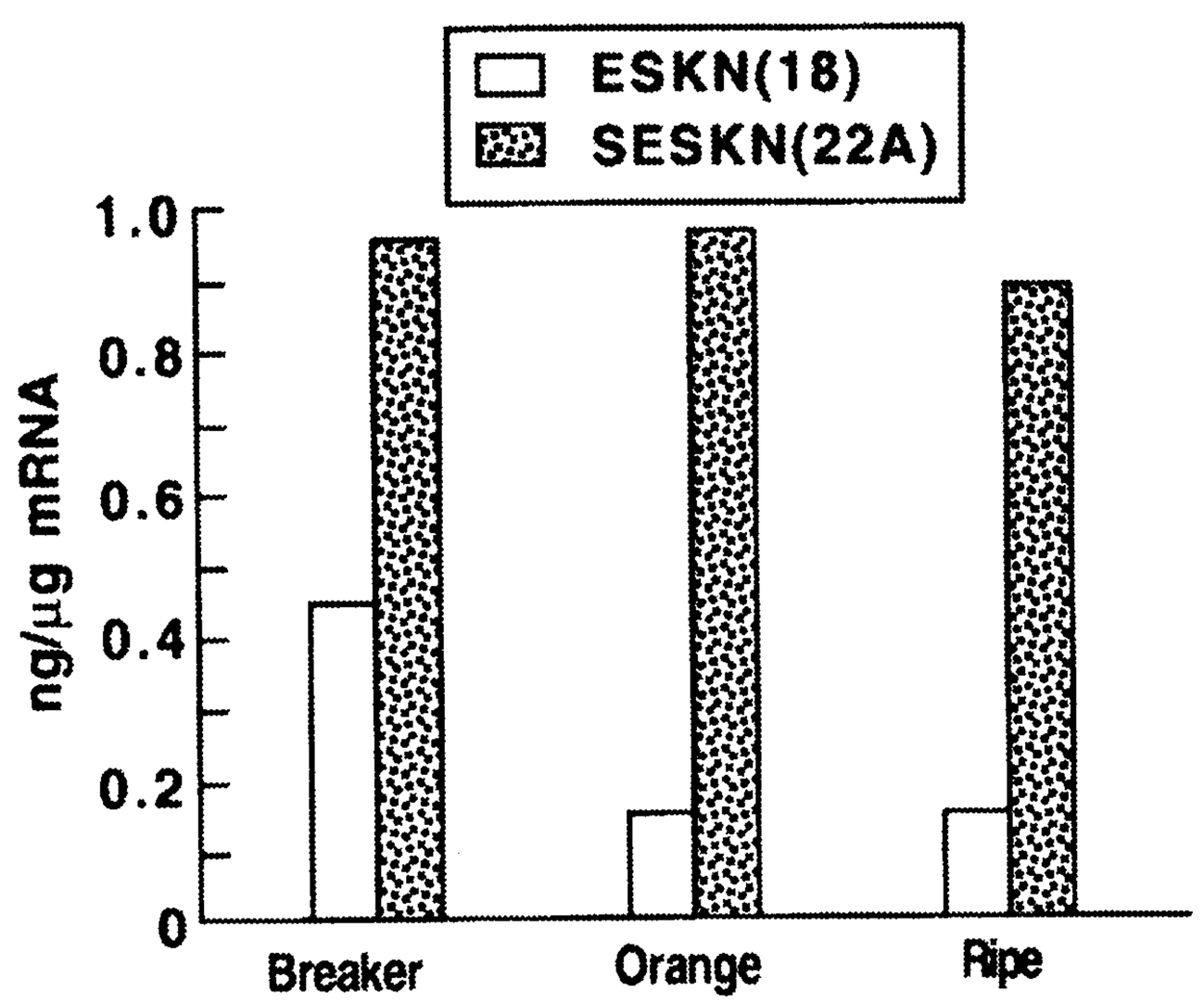


Fig. 7

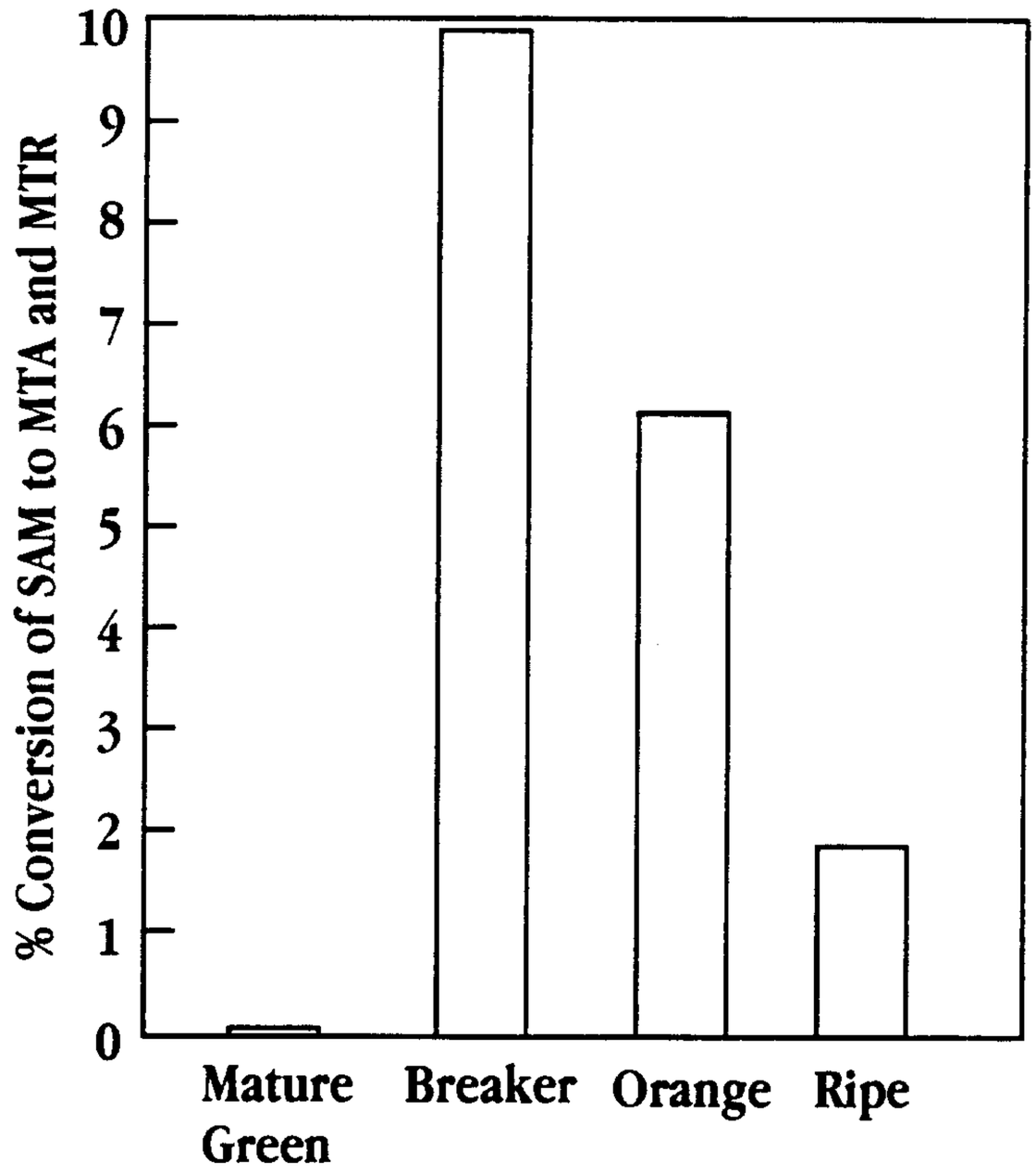
% Conversion of SAM to MTA and MTR
10
9
8
7
6
5
4
3
2
1
0
Mature Green
Breaker
Orange
Ripe

Fig.8

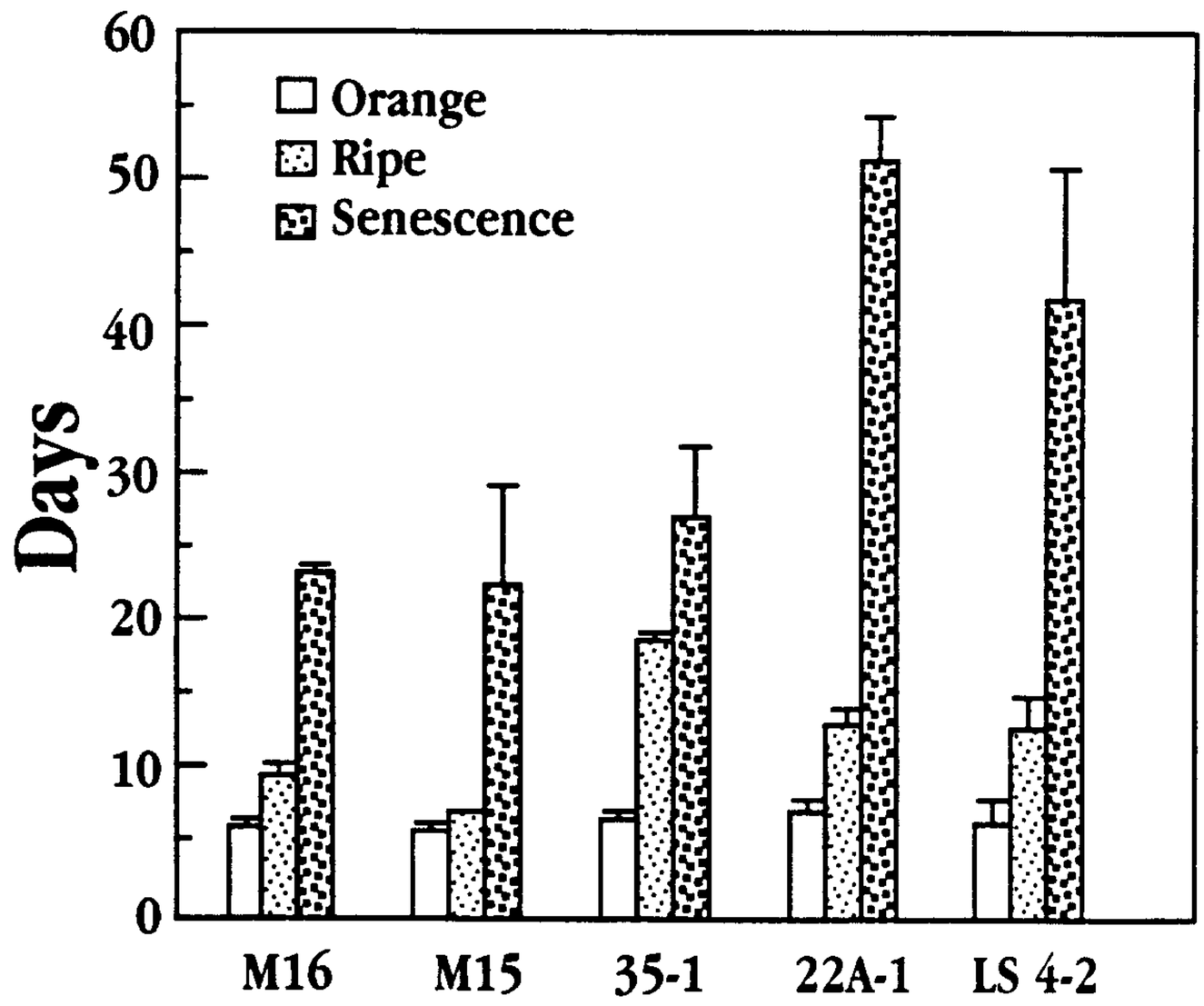
Days
60
50
40
30
20
10
0
Orange
Ripe
Senescence
M16
M15
35-1
22A-1
LS 4-2
Line

Fig.10

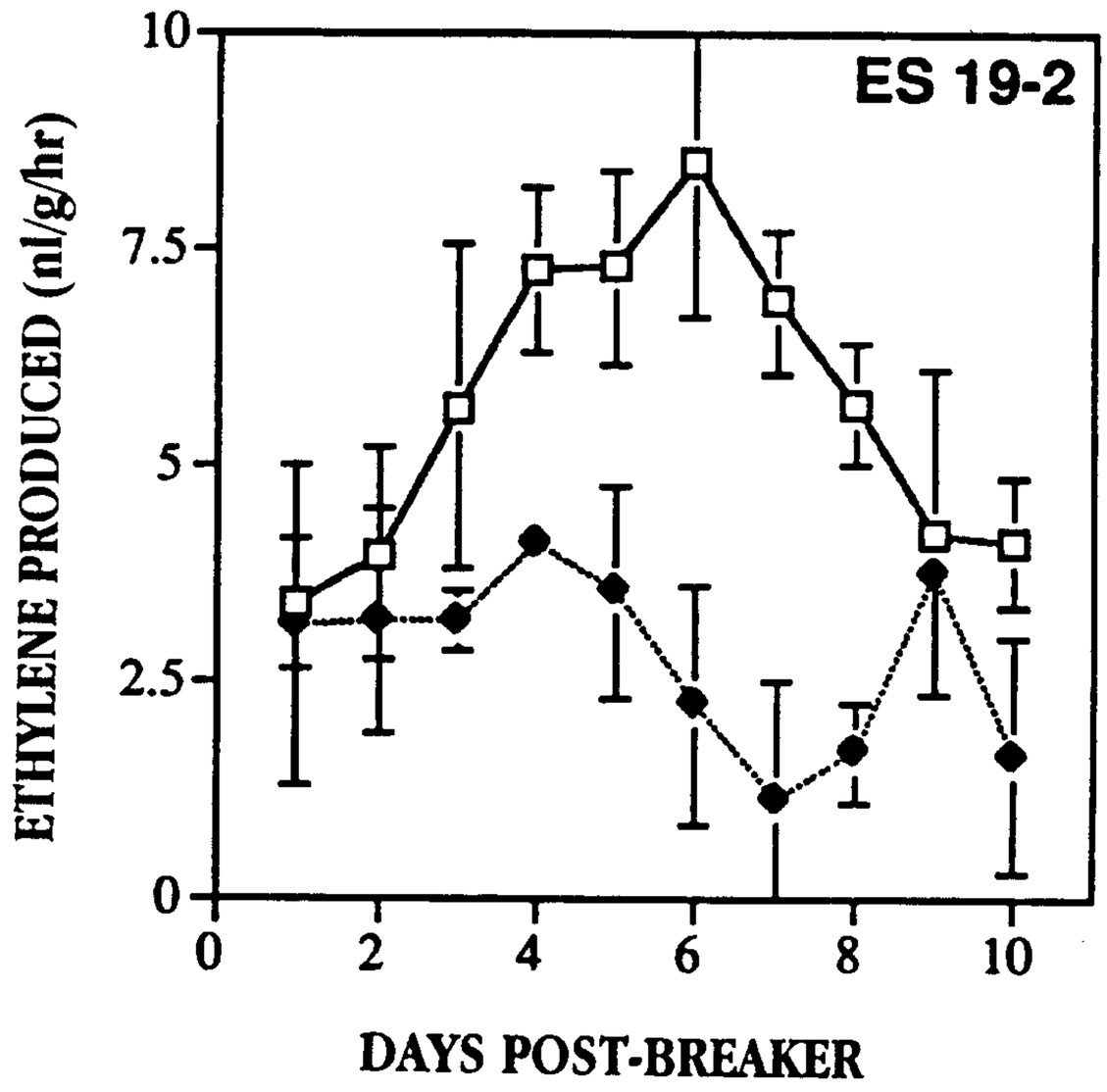
ES 19-2
ETHYLENE PRODUCED (nl/g/hr)
10
7.5
5
2.5
0
0
2
4
6
8
10
DAYS POST-BREAKER

Fig. 9A

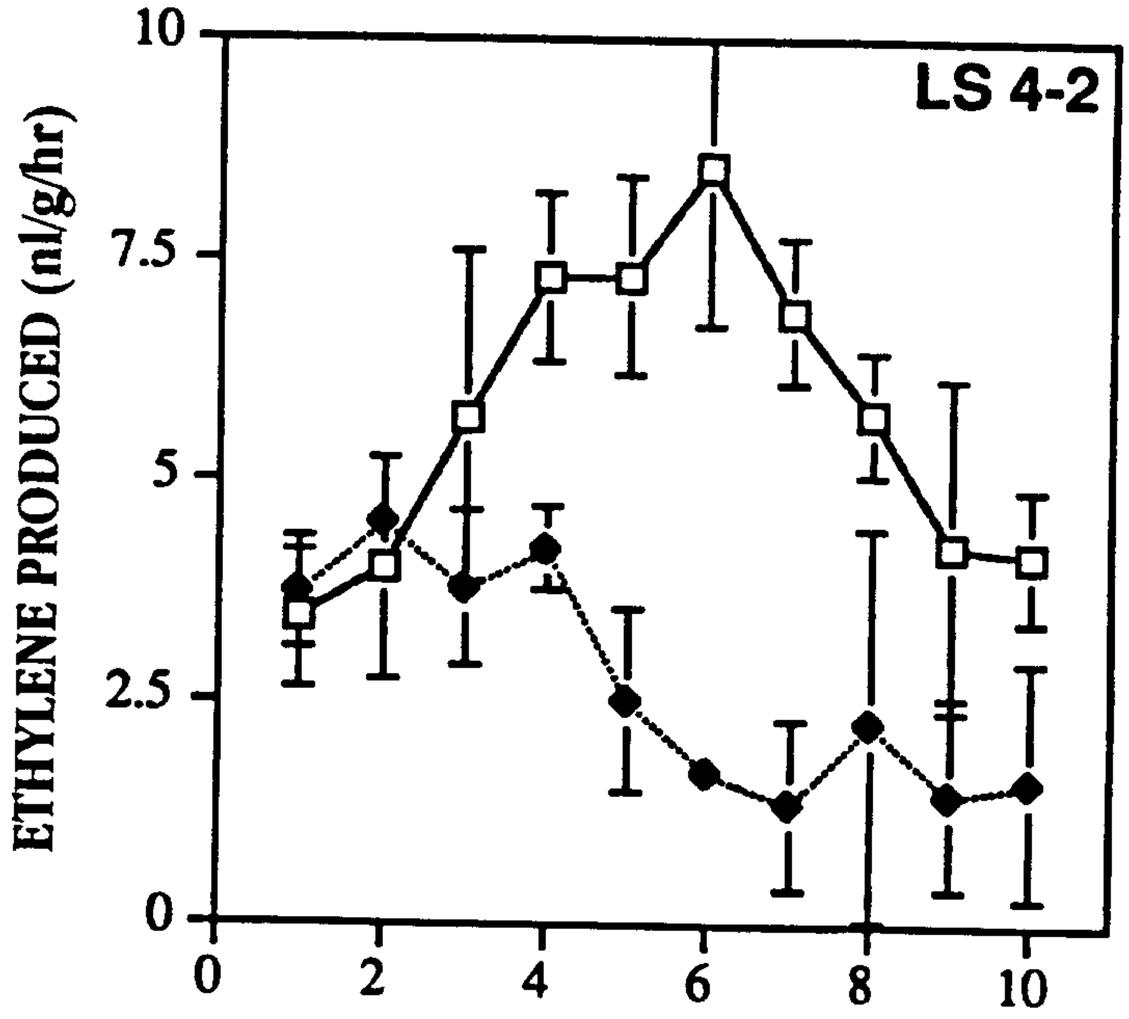
LS 4-2
ETHYLENE PRODUCED (nl/g/hr)
10
7.5
5
2.5
0
0
2
4
6
8
10
DAYS POST-BREAKER

Fig. 9B

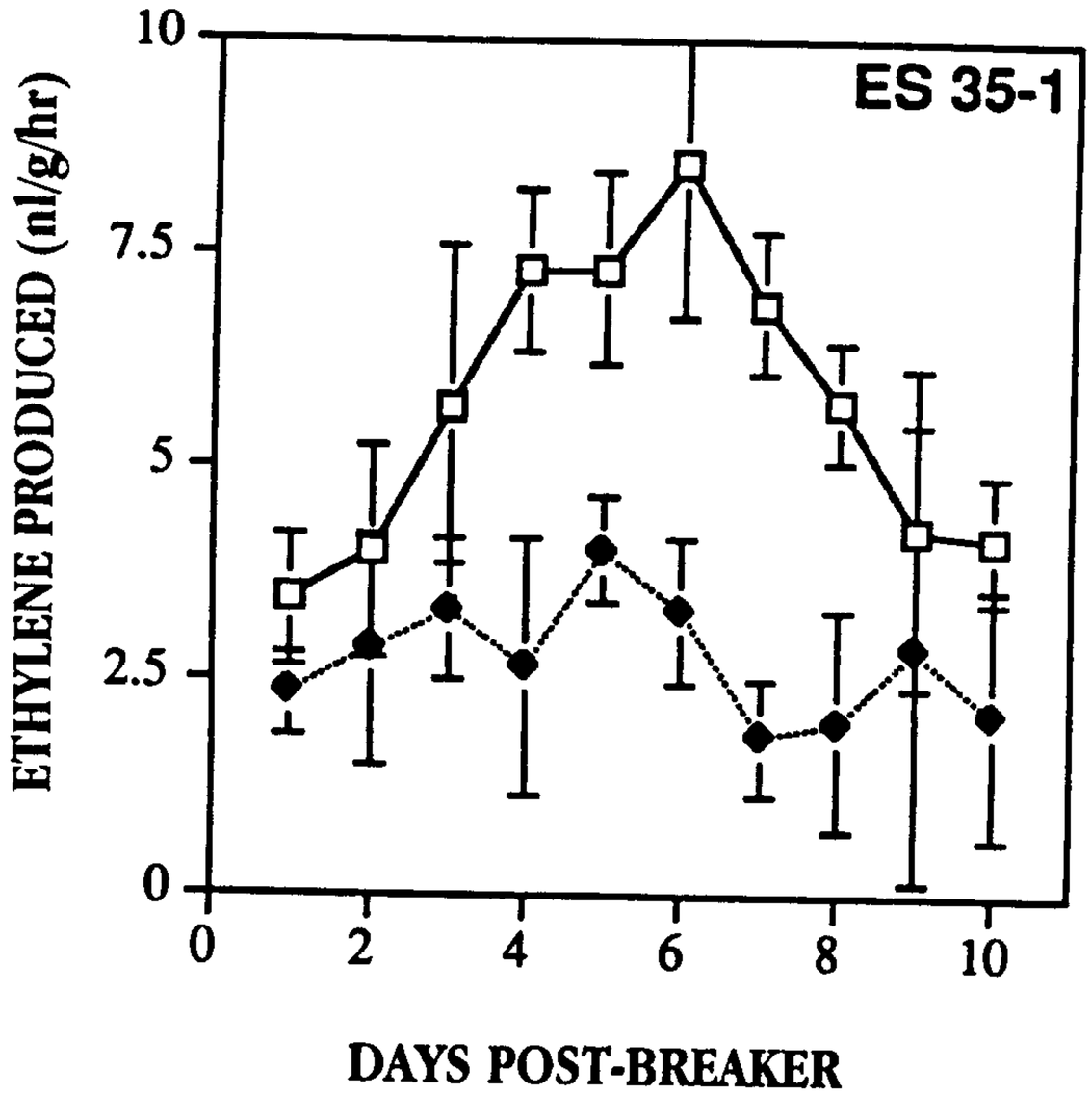
ES 35-1
ETHYLENE PRODUCED (nl/g/hr)
10
7.5
5
2.5
0
0
2
4
6
8
10
DAYS POST-BREAKER

Fig. 9C

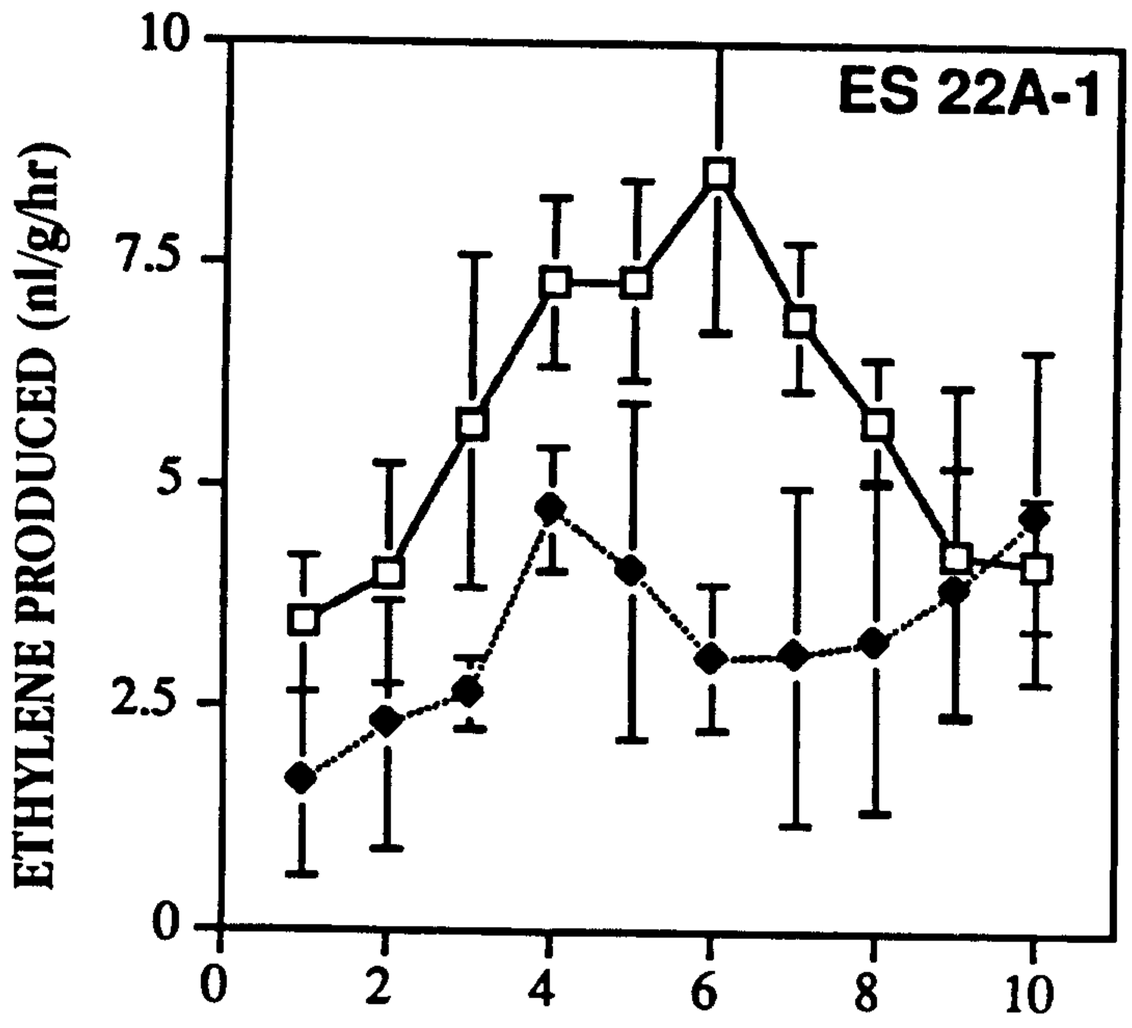
ES 22A-1
ETHYLENE PRODUCED (nl/g/hr)
10
7.5
5
2.5
0
0
2
4
6
8
10
DAYS POST-BREAKER

Fig. 9D

# Fig. 11(1)

```
                                                                  XhoII
                                                                  Sau3AI
                                                        TaqI        NlaIV
                                                       SalI  XbaI  NdeII
                                                         MnlI      MboI
                                        HindIII                PstI HinfI  CpfI
                                        EcoVIII   SphI           HincII    BstI
          AluI          NlaIII          AluI      NlaIII    AccI  MaeI  BamHI
           |             |             | |         |      | ||||  ||     | |
  1 ACAGCTATGACCATGATTACGCCAAGCTTGCATGCCTGCAGGTCGACTCTAGAGGATCC

                        MnlI     MnlI              HphI              NlaIII
                         |        |                 |                  |
115 CTTTCCGTTCTAACCTCTGCGATGAGGTGAATATGAGCAGACACCGCCACATGGTAAGC
    laPheArgSerAsnLeuCysAspGluValAsnMETSerArgHisArgHisMETValSer

                           MnlI
                        HinP1I
                          HhaI
                          CfoI    DdeI                        RsaI
                        |  ||      |                          |
235 ATCGTGAGGCAATCTCAAGCGCACCAACTGAGGAAAAAACTGTTCGTGTACGCTACAAG
    yrArgGluAlaIleSerSerAlaProThrGluGluLysThrValArgValArgTyrLys
```

# Fig. 11(2)

```
                                          XmnI
     NcoI                          MboII  Asp700     RsaI
     |                             |      |          |
GCCACCATGGTTTTCACTAAAGAGCCTGCGAACGTCTTCTATGTACTGGTTTCCG 114
   METValPheThrLysGluProAlaAsnValPheTyrValLeuValSerA

                  ScrFI
                  NciI                                ScrFI
                 MspI                                 NciI
                 HpaII                      HinfI     MspI
           Fnu4HI                NlaIV      HincII    HpaII MnlI
           |    ||                |     |    |        ||    |
ACTTTACGTGCCGCACCGGGTCTTTATGGCTCCGTTGAGTCAACCGATTTGACCGGGTGCT 234
ThrLeuArgAlaAlaProGlyLeuTyrGlySerValGluSerThrAspLeuThrGlyCysT

       HinP1I
         HhaI
          Fnu4HI                  MaeI
         CfoI          BbvI         AluI
        | ||             |          |  |
GACAAAGCGCAGGCACTCAATGTTGCACGCCTAGCTTGTAATGAGTGGGAGCAAGATTGCG 354
AspLysAlaGlnAlaLeuAsnValAlaArgLeuAlaCysAsnGluTrpGluGlnAspCysV
```





2159101

PCT/US94/03886

```
          RsaI   AccI          HinfI                      RsaI          TaqI
           |      |             |                          |             |
355  TACTGGTATACAAATCACAGACTCACACGGCTGGTCTGGTGTACGCTAAAGGTATCGAC
     alLeuValTyrLysSerGlnThrHisThrAlaGlyLeuValTyrAlaLysGlyIleAsp

          Fnu4HI                                 Fnu4HI      RsaI
           |                                      |           |
474  AAGGCTGCTTCACTATTGATGAGTTCGGTCGCCGCTGGCAAGTACAATAAGTGTTAAAC
     lnGlyCysPheThrIleAspGluPheGlyArgArgTrpGlnValGln***
```

**Fig. 11(3)**



2159101

PCT/US94/03886

21591101

PCT/US94/03886



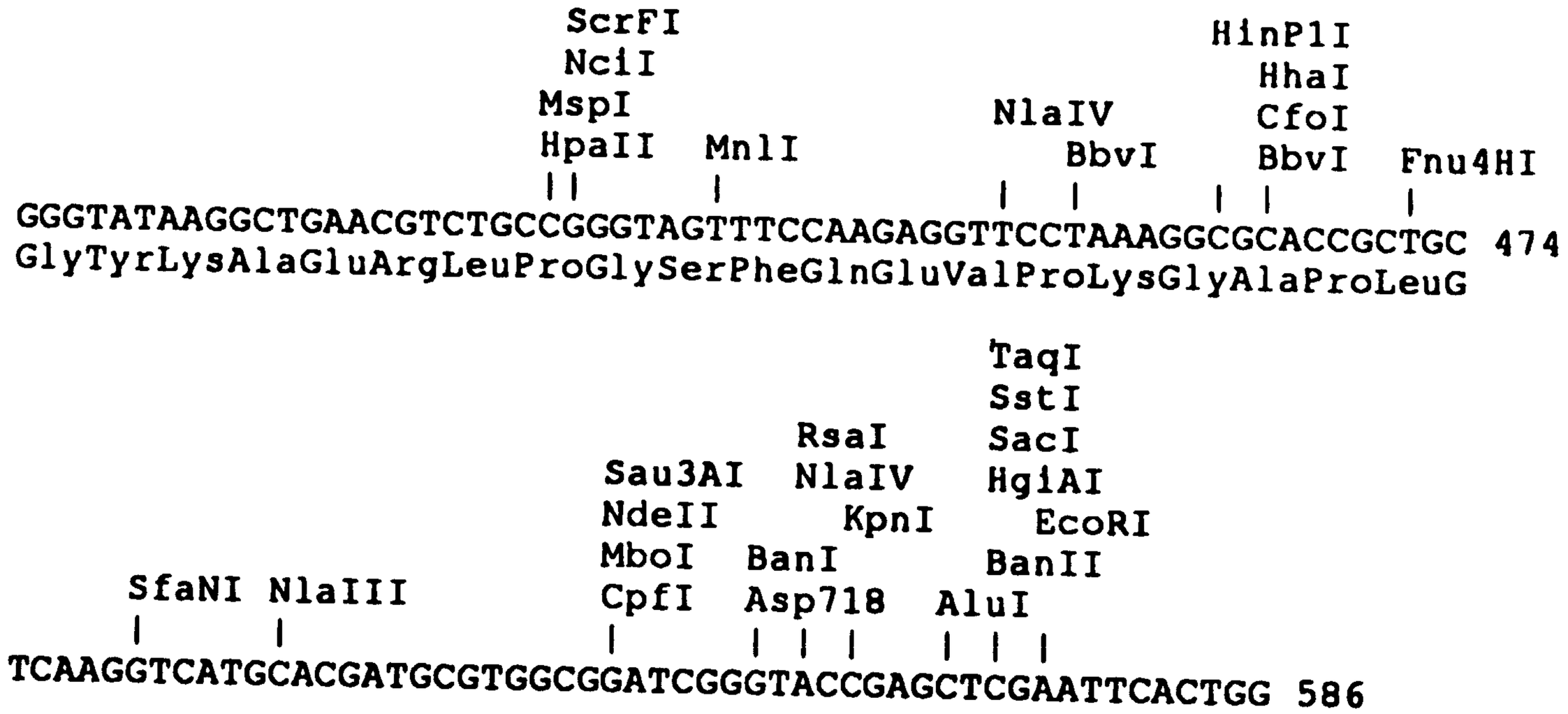


**Fig. 11(4)**

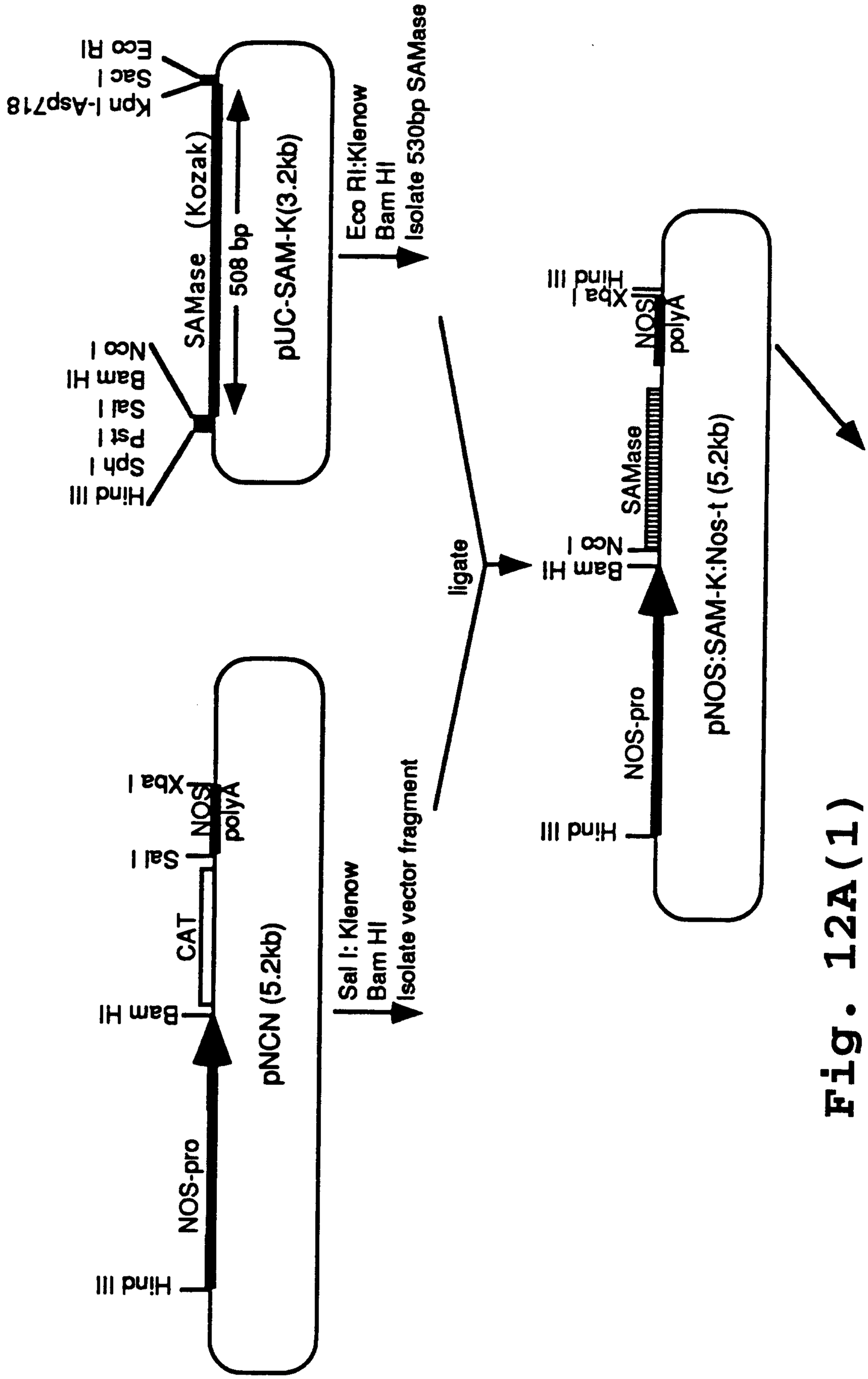
Kpn I-Asp718
Sac I
Eco RI
SAMase (Kozak)
508 bp
pUC-SAM-K(3.2kb)
Eco RI:Klenow
Bam HI
Isolate 530bp SAMase
Hind III
Sph I
Pst I
Sal I
Bam HI
Nco I
ligate
Xba I
NOS
polyA
SAMase
Bam HI
Nco I
pNOS:SAM-K:Nos-t (5.2kb)
Hind III
NOS-pro
Xba I
NOS
polyA
Sal I
CAT
Bam HI
pNCN (5.2kb)
Sal I: Klenow
Bam HI
Isolate vector fragment
Hind III
NOS-pro

Fig. 12A(1)

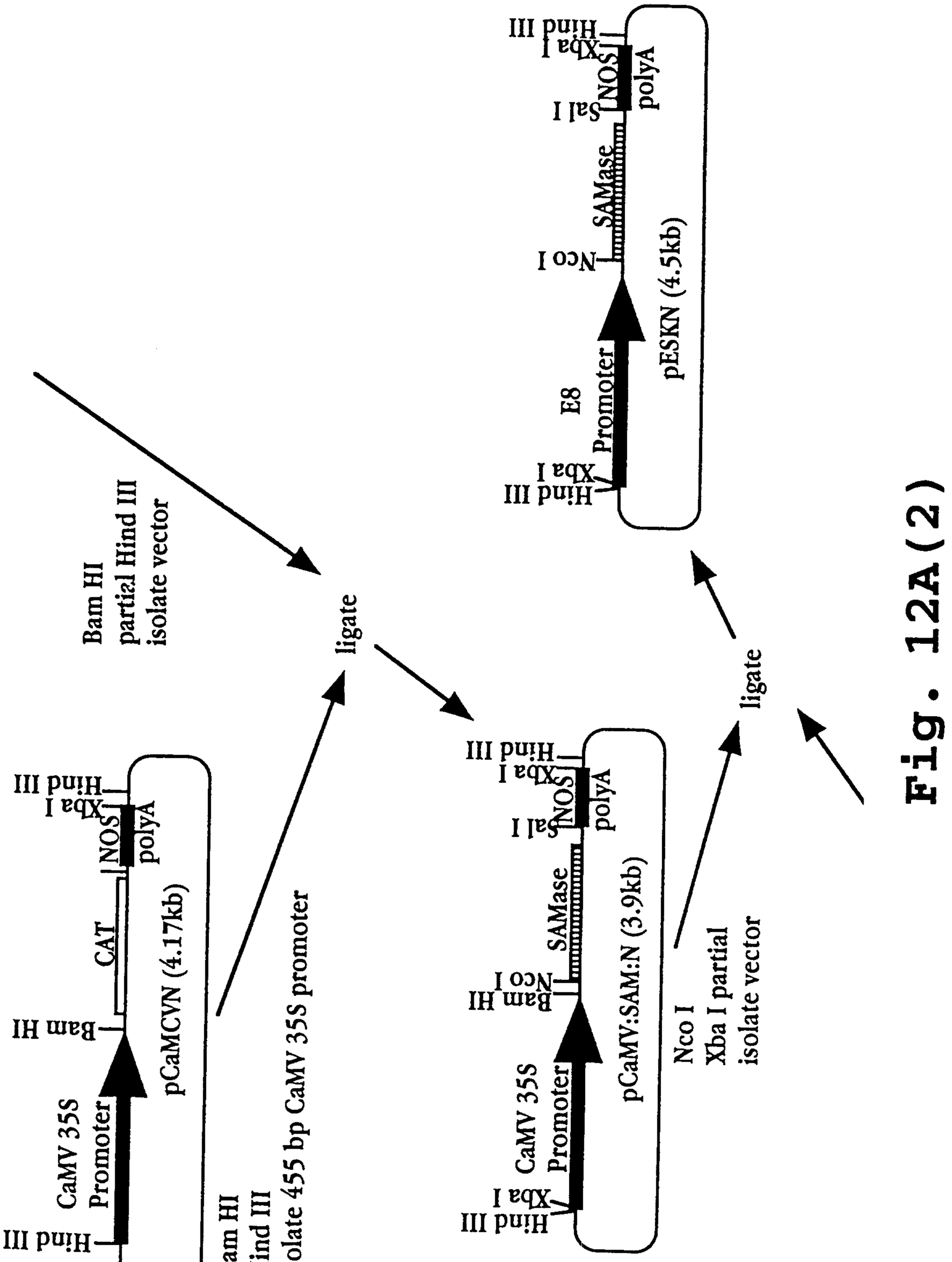
Bam HI
partial Hind III
isolate vector
ligate
pESKN (4.5kb)
Hind III
Xba I
E8
Promoter
Nco I
SAMase
Sal I
NOS
polyA
Xba I
Hind III
pCaMCVN (4.17kb)
Bam HI
CAT
CaMV 35S
Promoter
Hind III
Bam HI
Hind III
isolate 455 bp CaMV 35S promoter
pCaMV:SAM:N (3.9kb)
Bam HI
Nco I
SAMase
Sal I
NOS
polyA
Xba I
Hind III
CaMV 35S
Promoter
Hind III
Xba I
Nco I
Xba I partial
isolate vector
ligate

Fig. 12A(2)

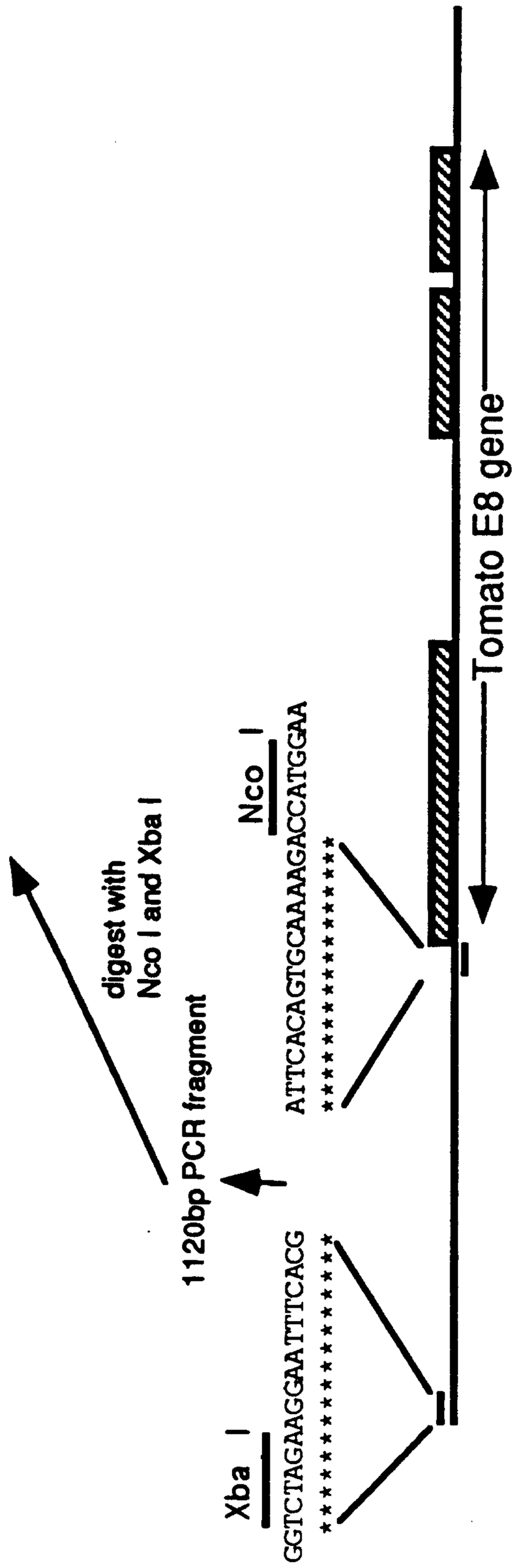
Tomato E8 gene
Nco I
ATTCACAGTGCAAAAGACCATGGAA
**********
digest with
Nco I and Xba I
1120bp PCR fragment
Xba I
GGTCTAGAAGGAATTTCACG
**********

Fig. 12A(3)

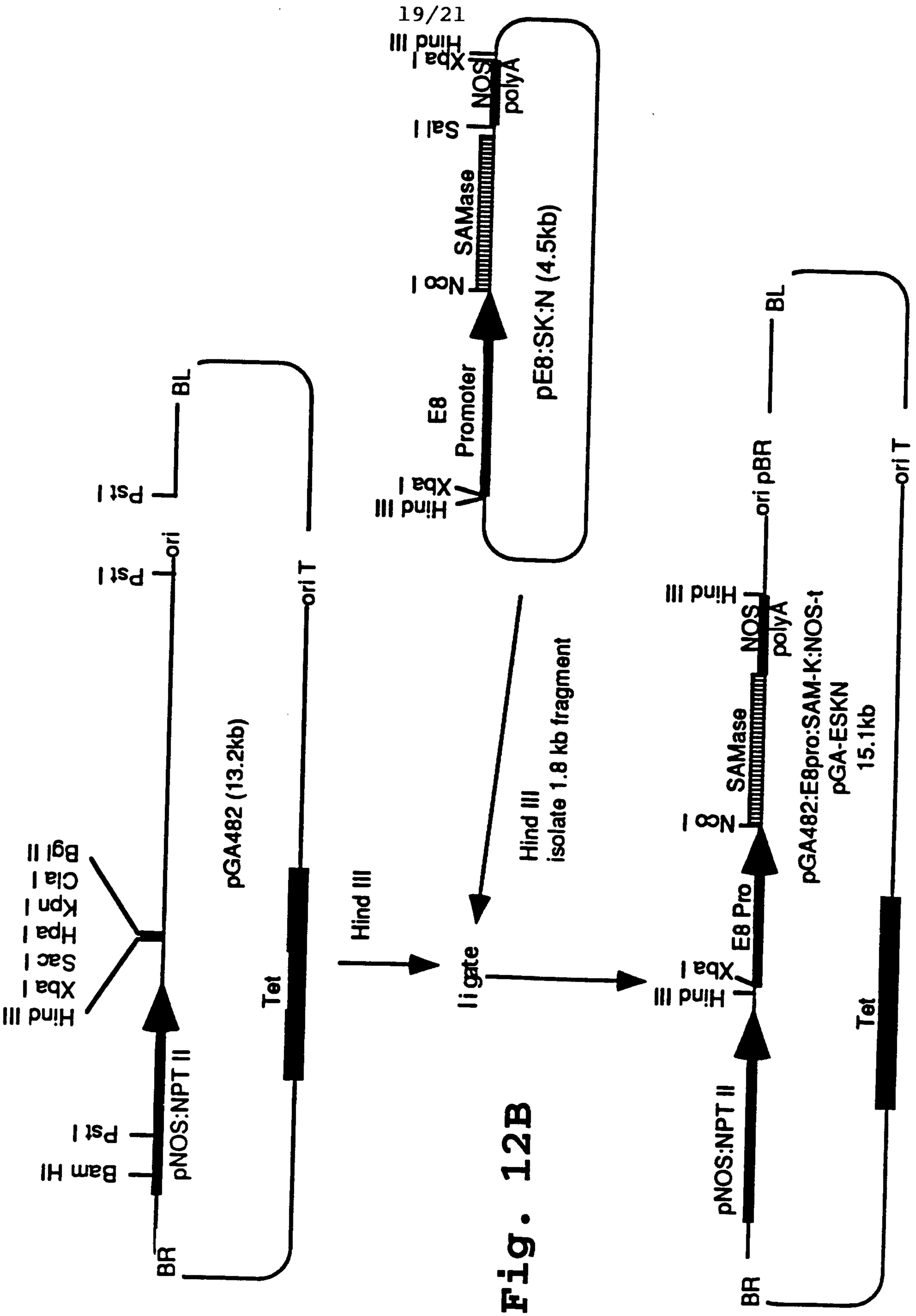
pE8:SK:N (4.5kb)
E8 Promoter
Hind III
Xba I
Nco I
SAMase
NOS
polyA
Sal I
Xba I
Hind III
pGA482 (13.2kb)
BL
ori
ori T
Pst I
Tet
pNOS:NPT II
BR
Bam HI
Pst I
Hind III
Xba I
Sac I
Hpa I
Kpn I
Cla I
Bgl II
Hind III
isolate 1.8 kb fragment
ligate
pGA482:E8pro:SAM-K:NOS-t
pGA-ESKN
15.1kb
E8 Pro
ori pBR

Fig. 12B

# Fig. 13

Sequence Range: 1 to 2216
>EcoR1

```
|
|       10          20          30          40          50          60          70
GAATTCATTT TTGACATCCC TAATGATATT GTTCACGTAA TTAAGTTTTG TGGAAGTGAG AGAGTCCAAT

        80          90         100         110         120         130         140
TTTGATAAGA AAAGAGTCAG AAAACGTAAT ATTTTAAAAG TCTAAATCTT TCTACAAATA AGAGCAAATT

       150         160         170         180         190         200         210
TATTTATTTT TTAATCCAAT AAATATTAAT GGAGGACAAA TTCAATTCAC TTGGTTGTAA AATAAACTTA

       220         230         240         250         260         270         280
AACCAATAAC CAAAGANCTA ATAAATCTGA AGTGGAATTA TTAAGGATAA TGTACATAGA CAATGAAGAA

       290         300         310         320         330         340         350
ATAATAGGTT CGATGAATTA ATAATAATTA AGGATGTTAC AATCATCATG TGCCAAGTAT ATACACAATA

       360         370         380         390         400         410         420
TTCTATGGGA TTTATAATTT CGTTACTTCA CTTAACTTTT GCGTAAATAA AACGAATTAT CTGATATTTT

       430         440         450         460         470         480         490
ATAATAAAAC AGTTAATTAA GAACCATCAT TTTTAACAAC ATAGATATAT TATTTCTAAT AGTTTAATGA

       500         510         520         530         540         550         560
TACTTTTAAA TCTTTTAAAT TTTATGTTTC TTTTAGAAAA TAAAAATTCA AAAAAATTAA ATATATTTAC

       570         580         590         600         610         620         630
AAAAACTACA ATCAAACACA ACTTCATATA TTAAAAGCAA AATATATTTT GAAAATTTCA AGTGTCCTAA

       640         650         660         670         680         690         700
CAAATAAGAC AAGAGGAAAA TGTACGATGA GAGACATAAA GAGAACTAAT AATTGAGGAG TCCTATAATA

       710         720         730         740         750         760         770
TATAATAAAG TTTATTAGTA AACTTAATTA TTAAGGACTC CTAAAATATA TGATAGGAGA AAATGAATGG

       780         790         800         810         820         830         840
TGAGAGATAT TGGAAAACTT AATAATTAAG GATNTTAAAA TATATGGTAA AAGATAGGCA AAGTATCCAT

       850         860         870         880         890         900         910
TATCCCCTTT TAACTTGAAG TCTACCTAGG CGCATGTGAA AGGTTGATTT TTTGTCACGT CATATAGCTA

       920         930         940         950         960         970         980
TAACGTAAAA AAAGAAAGTA AAATTTTTAA TTTTTTTTAA TATATGACAT ATTTTAAACG AAATATAGGA

       990        1000        1010        1020        1030        1040        1050
CAAAATGTAA ATGAATAGTA AAGGAAACAA AGATTAATAC TTACTTTGTA AGAATTTAAG ATAAATTTAA

                          >Xba1                   >Xba1
                          |                       |
      1060        1070    |   1080        1090        1100        1110        1120
AATTTAATAG ATCAACTTTA CGTCTAGAAA GACCCATATC TAGAAGGAAT TTCACGAAAT CGGCCCTTAT

      1130        1140        1150        1160        1170        1180        1190
TCAAAAATAA CTTTTAAATA ATGAATTTTA AATTTTAAGA AATAATATCC AATGAATAAA TGACATGTAG
```

```
      1200        1210        1220        1230        1240        1250        1260
CATTTTACCT AAATATTTCA ACTATTTTAA TCCAATATTA ATTTGTTTTA TTCCCAACAA TAGAAAGTCT

      1270        1280        1290        1300        1310    |   1320        1330
TGTGCAGACA TTTAATCTGA CTTTTCCAGT ACTAAATATT AATTTTCTGA AGATTTTCGG GTTTAGTCCA

      1340        1350        1360        1370        1380        1390        1400
CAAGTTTTAG TGAGAAGTTT TGCTCAAAAT TTTAGGTGAG AAGGTTTGAT ATTTATCTTT TGTTAAATTA

      1410        1420        1430        1440        1450        1460        1470
ATTTATCTAG GTGACTATTA TTTATTTAAG TAGAAATTCA TATCATTACT TTTGCCAACT TGTAGTCATA

      1480        1490        1500        1510        1520        1530        1540
ATAGGAGTAG GTGTATATGA TGAAGGAATA AACAAGTTCA GTGAAGTGAT TAAAATAAAA TATAATTTAG

      1550        1560        1570        1580        1590        1600        1610
GTGTACATCA AATAAAAACC TTAAAGTTTA GAAAGGCACC GAATAATTTT GCATAGAAGA TATTAGTAAA

      1620        1630        1640        1650        1660        1670        1680
TTTATAAAAA TAAAAGAAAT GTAGTTGTCA AGTTGTCTTC TTTTTTTTGG ATAAAAATAG CAGTTGGCTT

      1690        1700        1710        1720        1730        1740        1750
ATGTCATTCT TTTACAACCT CCATGCCACT TGTCCAATTG TTGACACTTA ACTAATTAGT TTGATTCATG

      1760        1770        1780        1790        1800        1810        1820
TATGAATACT AAATAATTTT TTAGGACTGA CTCAAATATT TTTATATTAT CATAGTAATA TTTATCTAAT

      1830        1840        1850        1860        1870        1880        1890
TTTTAGGACC ACTTATTACT AAATAATAAA TTAACTACTA CTATATTATT GTTGTGAAAC AACAACGTTT

      1900        1910        1920        1930        1940        1950        1960
TGGTTGTTAT GATGAAACGT ACACTATATC AGTATGAAAA ATTCAAAACG ATTAGTATAA ATTATATTGA

      1970        1980        1990        2000        2010        2020        2030
AAATTTGATA TTTTTCTATT CTTAATCAGA CGTATTGGGT TTCATATTTT AAAAAGGGAC TAAACTTAGA

      2040        2050        2060        2070        2080        2090        2100
AGAGAAGTTT GTTTGAAACT ACTTTTGTCT CTTTCTTGTT CCCATTTCTC TCTTAGATTT CAAAAAGTGA
|
      2110        2120        2130        2140        2150        2160    |   2170
        *           *           *           *           *           *       |       *
ACTACTTTAT CTCTTTCTTT GTTCACATTT TATTTTATTC TATTATAAAT ATGGCATCCT CATATTGAGA

            >Xmn1                                       >Nco1
            |                                       >E8_Start_codon
            |               |                         |   |
      2180  |     2190      |     2200          2210  |
          *   |         *   |         *               |*  |
TTTTTAGAAA TTATTCTAAT CATTCACAGT GCAAAAGACC ATGGAA
```

Fig. 13 (con't)