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(54) Title: PHOSPHOPANTETHEINYL TRANSFERASES FROM BACTERIA

(57) **Abrégé/Abstract:**

The invention relates generally to phosphopantetheinyl transferases that are required for activation of a polyketide synthase complex to synthesize long chain poly-unsaturated fatty acids (LC-PUF A's) such as docosahexaenoic acid and eicosapentaenoic acid. In particular, the invention relates to bacterial phosphopantetheinyl transferases, DNA constructs for their expression in host cells, and seed, oil and meal when the host cells comprise a plant. Also provided is a method for making a plant oil containing docosahexaenoic acid and/or eicosapentaenoic acid.

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(54) Title: PHOSPHOPANTETHEINYL TRANSFERASES FROM BACTERIA

(57) Abstract: The invention relates generally to phosphopantetheinyl transferases that are required for activation of a polyketide synthase complex to synthesize long chain poly-unsaturated fatty acids (LC-PUFA's) such as docosahexaenoic acid and eicosapentaenoic acid. In particular, the invention relates to bacterial phosphopantetheinyl transferases, DNA constructs for their expression in host cells, and seed, oil and meal when the host cells comprise a plant. Also provided is a method for making a plant oil containing docosahexaenoic acid and/or eicosapentaenoic acid.

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DESCRIPTION

PHOSPHOPANTETHEINYL TRANSFERASES FROM BACTERIA

BACKGROUND OF THE INVENTION

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Field of the Invention

The invention relates generally to phosphopantetheinyl transferases that are involved
 10 in the activation of a polyketide synthase to synthesize long chain polyunsaturated fatty acids
 (such as docosahexaenoic acid and eicosapentaenoic acid).

Description of the Related Art

The primary products of fatty acid biosynthesis in most organisms are 16- and 18-
 carbon compounds. The relative ratio of chain lengths and degree of unsaturation of these
 15 fatty acids vary widely among species. Mammals, for example, produce primarily saturated
 and monounsaturated fatty acids, while most higher plants produce fatty acids with one, two,
 or three double bonds, the latter two comprising polyunsaturated fatty acids (PUFA's). Very
 long chain PUFAs such docosahexaenoic acid (DHA, 22:6) and eicosapentaenoic acid (EPA,
 20:5) have been reported from several species of marine bacteria, including *Moritella* (*Vibrio*)
 20 *marina* and *Shewanella* sp. (US Patent 6,140,486) and from marine algae such as
Schizochytrium sp. and *Thraustochytrium* sp. (US Patent Publication 20040235127).

Two main families of PUFAs are the omega-3 fatty acids (also represented as "n-3"
 fatty acids), exemplified by docosahexaenoic acid and the omega-6 fatty acids (also
 represented as "n-6" fatty acids), exemplified by arachidonic acid (ARA, 20:4). PUFAs are
 25 important components of the plasma membrane of the cell and adipose tissue, where they may
 be found in phospholipids and triglycerides, respectively. PUFAs are necessary for proper
 development in mammals, particularly in the developing infant brain, and for tissue formation
 and repair.

Several disorders respond to treatment with PUFAs. Supplementation with PUFAs
 30 has been shown to reduce the rate of restenosis after angioplasty. The health benefits of
 certain dietary omega-3 fatty acids for cardiovascular disease and rheumatoid arthritis also

have been well documented (Simopoulos, 1997; James *et al.*, 2000). Further, PUFAs have been suggested for use in treatments for asthma and psoriasis. Evidence indicates that PUFAs may be involved in calcium metabolism, suggesting that PUFAs may be useful in the treatment or prevention of osteoporosis and of kidney or urinary tract stones.

5 The majority of evidence for health benefits applies to the long chain omega-3 fats, EPA and DHA, which are in fish and fish oil. With this base of evidence, health authorities and nutritionists in Canada (Scientific Review Committee, 1990, Nutrition Recommendations, Minister of National Health and Welfare, Canada, Ottawa), Europe (de
10 Deckerer *et al.*, 1998), the United Kingdom (The British Nutrition Foundation, 1992, Unsaturated fatty-acids - nutritional and physiological significance: The report of the British Nutrition Foundation's Task Force, Chapman and Hall, London), and the United States (Simopoulos *et al.*, 1999) have recommended increased dietary consumption of these PUFAs.

 Major long chain PUFAs of importance include DHA and EPA, which are primarily found in different types of fish oil, and ARA, found in filamentous fungi such as *Mortierella*.
15 For DHA, a number of sources exist for commercial production including a variety of marine organisms, oils obtained from cold water marine fish, and egg yolk fractions. However, there are several disadvantages associated with commercial production of PUFAs from natural sources. Natural sources of PUFAs, such as animals and fungi, tend to have highly heterogeneous oil compositions. The oils obtained from these sources therefore can require
20 extensive purification to separate out one or more desired PUFAs or to produce an oil which is enriched in one or more PUFAs.

 Natural sources of PUFAs also are subject to uncontrollable fluctuations in availability. Fish stocks may undergo natural variation or may be depleted by overfishing. In addition, even with overwhelming evidence of their therapeutic benefits, dietary
25 recommendations regarding omega-3 fatty acids are not heeded. Fish oils have unpleasant tastes and odors, which may be impossible to economically separate from the desired product, and can render such products unacceptable as food supplements. Animal oils, and particularly fish oils, can accumulate environmental pollutants. Foods may be enriched with fish oils, but again, such enrichment is problematic because of cost and declining fish stocks
30 worldwide. This problem is also an impediment to consumption and intake of whole fish. Nonetheless, if the health messages to increase fish intake were embraced by communities, there would likely be a problem in meeting demand for fish. Furthermore, there are problems with sustainability of this industry, which relies heavily on wild fish stocks for aquaculture feed (Naylor *et al.*, 2000).

Other natural limitations favor a novel approach for the production of omega-3 fatty acids. Weather and disease can cause fluctuation in yields from fish. Large scale fermentation of organisms such as *Mortierella* is expensive. Natural animal tissues contain low amounts of ARA and are difficult to process. Microorganisms such as *Porphyridium* and
5 *Mortierella* are difficult to cultivate on a commercial scale.

A number of marine microorganisms produce very long-chain PUFAs such as DHA and EPA by a polyketide synthase (PKS) mechanism. PKSs are enzyme complexes composed of multifunctional polypeptides that catalyze the synthesis of complex molecules from simple substrates in an iterative fashion. PKSs are well known in the art and numerous
10 examples of such sequences can be found in the literature. In *Moritella marina*, a PKS synthesizes DHA from malonyl-CoA and acetyl-CoA. To activate this PKS, a phosphopantetheinyl transferase is required.

Phosphopantetheinyl transferases (Ppts) catalyze the post-translational activation of carrier proteins, fatty acid synthases, polyketide synthases, and non-ribosomal polypeptide
15 synthetases by the covalent attachment of the 4'-phosphopantetheine moiety of coenzyme A to a conserved serine residue, a reaction required for the biosynthesis of natural products including fatty acids, polyketides, and nonribosomal peptides. Ppts have been classified according to their carrier protein specificity. In organisms containing multiple phosphopantetheine-requiring pathways, it has been suggested that each pathway has its own
20 Ppt. While the *M. marina* PKS has been cloned (US Patent No. 6,140,486 (Facciotti *et al.*)), the Ppt was not found. Allen and Bartlett (2002) stated that they were unable to clone a Ppt gene from *Moritella*.

A number of approaches have been attempted for production of DHA and EPA in plants (WO05103253A1 (Singh *et al.*), WO04071467A2 (Kinney *et al.*)). These approaches
25 had in common the use of desaturases/elongases in a stepwise fashion. This approach has the disadvantage of using 6-8 genes and leads to the accumulation of intermediates, a potentially undesirable outcome. Using a PKS/Ppt approach, the number of transgenes required would be smaller (4-5) and the accumulation of intermediates is not expected.

Therefore, it would be advantageous to obtain genetic material involved in long-chain
30 PUFA biosynthesis and to express the isolated material in a plant system, in particular, a land-based terrestrial crop plant system, which can be manipulated to provide production of commercial quantities of one or more PUFA's. There is also a need to increase omega-3 fatty acid intake in humans and animals. Thus there is a need to provide a wide range of omega 3-enriched foods and food supplements so that subjects can choose feed, feed ingredients, food

and food ingredients which suit their usual dietary habits. Particularly advantageous would be seed oils with increased DHA or EPA.

Currently there is only one omega-3 fatty acid, ALA, available in vegetable oils. However, there is poor conversion of ingested ALA to the longer-chain omega-3 fatty acids such as EPA and DHA. It has been demonstrated in copending U.S. Publication. No. 20040039058 for "Treatment And Prevention Of Inflammatory Disorders," that elevating ALA intake from the community average of 1 g/day to 14 g/day by use of flaxseed oil only modestly increased plasma phospholipid EPA levels. A 14-fold increase in ALA intake resulted in a 2-fold increase in plasma phospholipid EPA (Manzioris *et al.*, 1994). Thus, to that end, there is a need for efficient and commercially viable production of PUFAs using a polyketide synthesis complex and the Ppts that activate the complex, genes encoding the Ppt, and recombinant methods of producing them. A need also exists for oils containing higher relative proportions of DHA or EPA, and food compositions and supplements containing them. A need also exists for reliable and economical methods of producing specific PUFA's. Oils derived from oilseed crops such as canola, soybean, corn, sunflower, or flax, that express a bacterial PKS complex are enriched in a long chain PUFA, DHA or EPA. Such oils can be utilized to produce foods and food supplements enriched in omega-3 fatty acids and consumption of such foods effectively increases tissue levels of EPA and DHA. Foods and foodstuffs, such as milk, margarine and sausages, all made or prepared with omega-3 enriched oils, will result in therapeutic benefits. Thus, there exists a strong need for novel nucleic acids of phosphopantetheinyl transferases capable of activating PKS for use in transgenic crop plants with oils enriched in PUFAs, as well as the improved oils produced thereby.

SUMMARY OF THE INVENTION

In one aspect, the invention provides isolated nucleic acids encoding a polypeptide with phosphopantetheinyl transferase activity. These may be used to transform cells or modify the fatty acid composition of a plant or the oil produced by a plant. One embodiment of the invention is an isolated polynucleotide sequence selected from the group consisting of (a) a polynucleotide hybridizing to SEQ ID NO:6 or SEQ ID NO:8, or a complement thereof, under conditions of 5X SSC, 50% formamide and 42°C; (b) a polynucleotide encoding the polypeptide sequence of SEQ ID NO:5 or SEQ ID NO:7; and (c) a polynucleotide encoding a polypeptide with at least 75% sequence identity to a polypeptide sequence of SEQ ID NO:5 or SEQ ID NO:7. In certain further embodiments of the invention, the polynucleotides encode a polypeptide having at least 80%, 85% or 90% sequence identity to the polypeptide sequence of SEQ ID NO:5 or SEQ ID NO:7, including at least about 82%, 87%, 89%, 92%,

95%, 98% and 99% identity to these sequences. Those of skill in the art will recognize that, as these sequences are related, a given polypeptide may simultaneously share 90% or greater homology to more than one of these polypeptide sequences. In a further embodiment, the encoded polypeptide has phosphopantetheinyl transferase activity.

5 In yet another aspect, the invention provides a DNA construct comprising a heterologous promoter operably linked to a DNA molecule encoding a polypeptide having phosphopantetheinyl transferase activity, wherein the DNA molecule is selected from the group consisting of: (a) a polynucleotide encoding the polypeptide sequence of SEQ ID NO:5 or SEQ ID NO:7; (b) a polynucleotide hybridizing to SEQ ID NO:6 or SEQ ID NO:8,
10 or a complement thereof, under conditions of 5X SSC, 50% formamide and 42°C; and (c) a polynucleotide encoding a polypeptide with at least 75% sequence identity to a polypeptide sequence of SEQ ID NO:5 or SEQ ID NO:7. In other embodiments, the promoter is functional in a prokaryotic cell or a eukaryotic cell. In certain embodiments, the eukaryotic cell in which the promoter is functional is a plant cell. In a further embodiment, the promoter
15 is a seed-enhanced promoter.

 In still yet another aspect, the invention provides a host cell transformed with a DNA construct comprising a heterologous promoter operably linked to a DNA molecule encoding a polypeptide having phosphopantetheinyl transferase activity provided by the invention. In another embodiment, the host cell further comprises a heterologous promoter operably linked
20 to a DNA molecule encoding a polyketide synthase polypeptide comprising a phosphopantetheine attachment site. In a further embodiment, the DNA molecule encoding a polyketide synthase polypeptide comprising a phosphopantetheine attachment site is from *Moritella marina*. In yet another embodiment, the DNA molecule encodes a polyketide synthase polypeptide with at least 70% sequence identity to SEQ ID NO:19, or any known
25 polyketide synthase as described herein below. The host cell may be a plant, fungal or bacterial cell.

 In still yet another aspect, the invention provides a plant and its progeny comprised of the host cells transformed with a DNA construct comprising a heterologous promoter operably linked to a DNA molecule encoding a polypeptide having phosphopantetheinyl
30 transferase activity provided herein. Such a plant may be defined as comprising altered fatty acid metabolism relative to a plant of the same genotype lacking the DNA construct. In one embodiment, the plant is selected from the group consisting of canola, *Brassica campestris*, oilseed rape, rapeseed, soybean, crambe, mustard, castor bean, peanut, sesame, cottonseed, linseed, safflower, oil palm, flax, sunflower, corn, rice, barley, millet, rye, wheat, oat, alfalfa
35 and sorghum. The invention also provides seed, oil and meal produced from the plant, which

is defined as comprising a detectable DNA molecule or polypeptide provided by the invention. Additionally, the invention provides animal feed and human food compositions.

5 In still yet another aspect, the invention provides a method of making a plant oil containing docosahexaenoic acid and/or eicosapentaenoic acid comprising the steps of (a) growing a plant comprising the host cell of the invention further comprising a polyketide synthase; (b) producing seed; (c) and processing the seed to obtain oil.

BRIEF DESCRIPTION OF THE FIGURES

10 The following drawings form part of the present specification and are included to further demonstrate certain aspects of the present invention. The invention may be better understood by reference to one or more of these drawings in combination with the detailed description of specific embodiments presented herein.

FIG.. 1 shows a map of vector pMON68081.

FIG.. 2 shows a map of vector pMON68080.

FIG.. 3 shows a map of vector pMON94547.

15 FIG.. 4 shows a map of vector pMON94544.

FIG.. 5 shows a map of vector pMON94534.

FIG.. 6 shows a map of vector pMON68084.

FIG.. 7 shows a map of vector pMON68085.

FIG.. 8 shows a map of vector pMON97063.

20 FIG.. 9 shows a map of vector pMON94563.

FIG.. 10 shows a map of vector pMON97066.

FIG.. 11 shows a map of vector pMON96401.

FIG.. 12 shows a map of vector pMON78528.

DETAILED DESCRIPTION OF THE INVENTION

25 The invention overcomes the limitations of the prior art by providing methods and compositions for creation of plants with improved DHA and/or EPA content. The modification of fatty acid content of an organism such as a plant presents many advantages, including improved nutrition and health benefits. Modification of fatty acid content can be used to achieve beneficial levels of DHA and/or EPA in plants, plant parts, and plant
30 products, including plant seed oils as well as bacteria and fungi. For example, when DHA is

produced in the seed tissue of a plant, the oil may be isolated from the seeds, typically resulting in an oil containing DHA, which may in turn be used to provide beneficial characteristics in foodstuffs and other products.

Various aspects of the invention include methods and compositions for modification
5 of PUFA content of a cell, for example, modification of the PUFA content of a plant cell(s). Compositions related to the invention include novel isolated polynucleotide sequences, DNA constructs and plants and/or plant parts transformed by polynucleotides of the invention. Host cells may be manipulated to express a polynucleotide encoding a phosphopantetheinyl transferase polypeptide which catalyzes the pantetheinylation of a phosphopantetheine
10 attachment site of another polypeptide.

The following definitions are provided as an aid to understanding this invention. The phrases "DNA sequence," "nucleic acid sequence," "nucleic acid molecule," and "nucleic acid segment" refer to a physical structure comprising an orderly arrangement of nucleotides. The DNA segment, sequence, or nucleotide sequence may be contained within a larger
15 nucleotide molecule, vector, or the like. In addition, the orderly arrangement of nucleic acids in these sequences may be depicted in the form of a sequence listing, figure, table, electronic medium, or the like.

The phrases "coding sequence," "coding region," "structural sequence," and "structural nucleic acid sequence" refer to all or a segment of a DNA sequence, nucleic acid
20 sequence, nucleic acid molecule in which the nucleotides are arranged in a series of triplets that each form a codon. Each codon encodes a specific amino acid. Thus, the coding sequence, coding region, structural sequence, and structural nucleic acid sequence encode a series of amino acids forming a protein, polypeptide, or peptide sequence. The coding sequence, coding region, structural sequence, and structural nucleic acid sequence may be
25 contained within a larger nucleic acid molecule, vector, or the like. In addition, the arrangement of nucleotides in these sequences may be depicted in the form of a sequence listing, figure, table, electronic medium, or the like.

The term "cDNA" refers to a double-stranded DNA that is complementary to and derived from mRNA.

30 "Expression" refers to the process by which a gene's coded information is converted into structures present and operating in the cell. Expressed genes include those that are transcribed into RNA and then translated into protein and those that are transcribed into RNA but not translated into protein (*e.g.*, transfer RNA and ribosomal RNA).

As used herein, "gene" refers to a nucleic acid fragment that expresses a specific
35 protein, including regulatory sequences preceding (5' non-coding sequences) and following

(3' non-coding sequences) the coding sequence. "Native gene" refers to a gene as found in nature with its own regulatory sequences. "Chimeric gene" refers to any gene that is not a native gene, comprising regulatory and coding sequences that are not found together in nature. Accordingly, a chimeric gene may comprise regulatory sequences and coding sequences that are derived from different sources, or regulatory sequences and coding sequences derived from the same source, but arranged in a manner different than that found in nature. "Endogenous gene" refers to a native gene in its natural location in the genome of an organism. An "exogenous" gene or "transgene" refer to a gene that has been introduced into the genome by a transformation procedure. A transgene includes genomic DNA introduced by a transformation procedure (*e.g.*, a genomic DNA linked to its active promoter).

"Heterologous" refers to the relationship between 2 or more nucleic acid or protein sequences that are derived from different sources. For example, a promoter is heterologous with respect to a coding sequence if such a combination is not normally found in nature. In addition, a particular nucleic acid sequence may be "heterologous" with respect to a cell or organism into which it is inserted if it does not naturally occur in that particular cell or organism.

"Sequence homology" refers to the level of similarity between 2 or more nucleic acid or amino acid sequences in terms of percent of positional identity. The term homology is also used to refer to the concept of similar functional properties among different nucleic acids or proteins.

"Hybridization" refers to the ability of a first strand of nucleic acid to join with a second strand *via* hydrogen bond base pairing when the nucleic acid strands have sufficient sequence complementarity. As used herein, a nucleic acid molecule is said to be the "complement" of another nucleic acid molecule if they exhibit complete complementarity. As used herein, molecules are said to exhibit "complete complementarity" when every nucleotide of one of the molecules is complementary to a nucleotide of the other. Thus 2 nucleic acid strands are said to have sufficient complementarity when they can hybridize to one another with sufficient stability to permit them to remain annealed to one another under appropriate conditions.

As used herein, the term "homology" refers to the level of similarity or percent identity between polynucleotide sequences in terms of percent nucleotide positional identity, *i.e.*, sequence similarity or identity. As used herein, the term homology also refers to the concept of similar functional properties among different polynucleotide molecules. Polynucleotide molecules are homologous when under certain conditions they specifically hybridize to form a duplex molecule. Under these conditions, referred to as stringency

conditions, one polynucleotide molecule can be used as a probe or primer to identify other polynucleotide molecules that share homology. The phrase "stringent conditions" is functionally defined with regard to the hybridization of a nucleic-acid probe to a target nucleic acid (i.e., to a particular nucleic-acid sequence of interest) by the specific hybridization procedure, for example, discussed in Molecular Cloning: A Laboratory Manual, 3rd edition Volumes 1, 2, and 3. J. F. Sambrook, D. W. Russell, and N. Irwin, Cold Spring Harbor Laboratory Press, 2000 (Sambrook *et al.*). Accordingly, nucleotide sequences provided by the invention may be used for their ability to selectively form duplex molecules with complementary stretches of polynucleotide molecule fragments. Depending on the application envisioned one would desire to employ varying conditions of hybridization to achieve varying degrees of selectivity of probe towards target sequence. For applications requiring high selectivity, one will typically desire to employ relatively high stringent conditions to form the hybrids, e.g., one will select relatively low salt and/or high temperature conditions, such as provided by about 0.02 M to about 0.15 M NaCl at temperatures of about 50°C to about 70°C. A high stringent condition, for example, is to wash the hybridization filter at least twice with high-stringency wash buffer (0.2 x SSC, 0.1% SDS, 65°C). Additionally, formamide may be used to increase stringency. High stringency conditions therefore also include 5X SSC, 50% formamide and 42°C. Detection of polynucleotide molecules via hybridization is well known to those of skill in the art, and the teachings of U.S. Pat. Nos. 4,965,188 and 5,176,995 are exemplary of the methods of hybridization analyses.

The phrase "isolated" means having been removed from its natural environment, regardless of its eventual disposition. For example, a nucleic acid sequence "isolated" from rice, such as by cloning from a rice cell, remains "isolated" when it is inserted into the genome of a corn cell.

The phrase "operably linked" refers to the spatial arrangement of two or more nucleic acid regions or nucleic acid sequences so that they exert their appropriate effects with respect to each other. For example, a promoter region may be positioned relative to a nucleic acid sequence such that transcription of the nucleic acid sequence is directed by the promoter region. The promoter region and the nucleic acid sequence are "operably linked."

The term "phosphopantetheinyl transferase or PPT" refers to an enzyme that catalyzes the post-translational activation of carrier proteins, for example, a polypeptide of a polyketide synthase, by the covalent attachment of the 4'-phosphopantetheine moiety of coenzyme A to a conserved serine residue.

The term “polyketide synthase” refers to an enzyme complex composed of multifunctional polypeptides that catalyze the synthesis of complex molecules from simple substrates in an iterative fashion. In *Moritella marina*, a PKS complex synthesizes DHA from malonyl-CoA and acetyl-CoA. For example, in *M. marina*, the PKS contains 4
5 polypeptides encoded by the open reading frames Orf5, Orf6, Orf7 and Orf8 (Metz *et al.*, 2001), which are described as Orf6, Orf7, Orf8, and Orf9 in U.S. Patent 6,140,486, respectively. To activate this complex, a phosphopantetheinyl transferase is required to pantethenylate the polypeptide encoded by Orf5. The PKS complex of *Shewanella* sp. SCRC2738 synthesizes EPA (Metz *et al.*, 2001).

10 “Upstream” and “downstream” are positional terms used with reference to the location of a nucleotide sequence and the direction of transcription or translation of coding sequences, which normally proceeds in the 5’ to 3’ direction.

The terms “promoter” or “promoter region” refer to a nucleic acid sequence, usually found upstream (5’) to a coding sequence, capable of directing transcription of a nucleic acid
15 sequence into an RNA molecule. The promoter or promoter region typically provides a recognition site for RNA polymerase and the other factors necessary for proper initiation of transcription. As contemplated herein, a promoter or promoter region includes variations of promoters derived by inserting or deleting regulatory regions, subjecting the promoter to random or site-directed mutagenesis, and the like. The activity or strength of a promoter may
20 be measured in terms of the amounts of RNA it produces, or the amount of protein accumulation in a cell or tissue, relative to a second promoter that is similarly measured.

The phrase “3’ non-coding sequences” refers to nucleotide sequences located downstream of a coding sequence and include polyadenylation recognition sequences and other sequences encoding regulatory signals capable of affecting mRNA processing or gene
25 expression. These are commonly referred to as 3’-untranslated regions or 3’-UTRs. The polyadenylation signal is usually characterized by affecting the addition of polyadenylic acid tracts to the 3’ end of the mRNA precursor. The use of different 3’ non-coding sequences is exemplified by Ingelbrecht *et al.* (1989).

“Translation leader sequence” or “5’-untranslated region” or “5’-UTR” all refer to a
30 nucleotide sequence located between the promoter sequence of a gene and the coding sequence. The 5’-UTR is present in the fully processed mRNA upstream of the translation start sequence. The 5’-UTR may affect processing of the primary transcript to mRNA, mRNA stability or translation efficiency. Examples of translation leader sequences have been described (Turner and Foster, 1995).

“RNA transcript” refers to the product resulting from RNA polymerase-catalyzed transcription of a DNA sequence. When the RNA transcript is a perfect complementary copy of the DNA sequence, it is referred to as the primary transcript. An RNA sequence derived from posttranscriptional processing of the primary transcript is referred to as the mature RNA.

5 “Messenger RNA” (mRNA) refers to the RNA that is without introns and that can be translated into polypeptide by the cell.

“DNA construct” refers to the heterologous genetic elements operably linked to each other making up a recombinant DNA molecule and may comprise elements that provide expression of a DNA polynucleotide molecule in a host cell and elements that provide
10 maintenance of the construct. A plant expression cassette comprises the operable linkage of genetic elements that when transferred into a plant cell provides expression of a desirable gene product.

“Recombinant vector” refers to any agent by or in which a nucleic acid of interest is amplified, expressed, or stored, such as a plasmid, cosmid, virus, autonomously replicating
15 sequence, phage, or linear single-stranded, circular single-stranded, linear double-stranded, or circular double-stranded DNA or RNA nucleotide sequence. The recombinant vector may be synthesized or derived from any source and is capable of genomic integration or autonomous replication.

“Regulatory sequence” refers to a nucleotide sequence located upstream (5'), within,
20 or downstream (3') with respect to a coding sequence, or an intron, whose presence or absence affects transcription and expression of the coding sequence

“Substantially homologous” refers to two sequences that are at least about 90% identical in sequence, as measured by the CLUSTAL W algorithm in, for example DNASTar (Madison, WI).

25 “Substantially purified” refers to a molecule separated from substantially all other molecules normally associated with it in its native state. More preferably, a substantially purified molecule is the predominant species present in a preparation. A substantially purified molecule may be greater than about 60% free, preferably about 75% free, more preferably about 90% free, and most preferably about 95% free from the other molecules
30 (exclusive of solvent) present in the natural mixture. The phrase “substantially purified” is not intended to encompass molecules present in their native state. Preferably, the nucleic acid molecules and polypeptides of this invention are substantially purified.

The term “transformation” refers to the introduction of nucleic acid into a recipient host. The term “host” refers to bacteria cells, fungi, animals or animal cells, plants or seeds,

or any plant parts or tissues including plant cells, protoplasts, calli, roots, tubers, seeds, stems, leaves, seedlings, embryos, and pollen.

As used herein, a "transgenic plant" is a plant having stably introduced into its genome, for example, the nuclear or plastid genomes, an exogenous nucleic acid.

5 The term "isogenic" as a comparative term between plants or plant lines having or lacking a transgene means plants or lines having the same or similar genetic backgrounds, with the exception of the transgene in question. For example, so-called sister lines representing phenotypically similar or identical selections from the same parent F2 population are considered to be "isogenic." When the progeny of a stable transformant plant are crossed
10 and backcrossed with the plants of the untransformed parent line for 3 to 6 generations (or more) using the untransformed parent as the recurrent parent while selecting for type (genotype by molecular marker analysis, phenotype by field observation, or both) and for the transgene, the resulting transgenic line is considered to be highly "isogenic" to its untransformed parent line.

15 The terms "seeds" "kernels" and "grain" are understood to be equivalent in meaning. The term kernel is frequently used in describing the seed of a corn or rice plant. In all plants the seed is the mature ovule consisting of a seed coat, embryo, aleurone, and an endosperm.

Nucleic acids encoding phosphopantetheinyl transferase

20 The invention provides, in one embodiment, novel nucleic acids encoding phosphopantetheinyl transferases from *Moritella marina*. In certain embodiments, the nucleic acids comprise SEQ ID NOs:2, 4, 6 or 8. The invention also provides methods of using such nucleic acids, including SEQ ID NOs:2, 4, 6 and 8. In one embodiment, these nucleic acid molecules are used in the context of this invention for altering the oil composition of a seed from a plant.

25 Such nucleic acid can be amplified using cDNA, mRNA or genomic DNA as a template and appropriate oligonucleotide primers according to standard PCR™ amplification techniques. Alternatively, they can be synthesized using standard synthetic techniques, such as an automated DNA synthesizer. Polynucleotides encoding desired phosphopantetheinyl transferases can be identified in a variety of ways. As an example, a source of the desired
30 phosphopantetheinyl transferase, for example a library from *Moritella*, is screened with detectable enzymatically- or chemically-synthesized probes, which can be made from DNA, RNA, or non-naturally occurring nucleotides, or mixtures thereof. Probes may be enzymatically synthesized from polynucleotides of known phosphopantetheinyl transferases for normal or reduced-stringency hybridization methods. Oligonucleotide probes also can be
35 used to screen sources and can be based on sequences of known phosphopantetheinyl

transferases, including sequences conserved among known phosphopantetheinyl transferases, or on peptide sequences obtained from the desired purified protein. Oligonucleotide probes based on amino acid sequences can be degenerate to encompass the degeneracy of the genetic code, or can be biased in favor of the preferred codons of the source organism.

5 Oligonucleotides also can be used as primers for PCRTM from reverse transcribed mRNA from a known or suspected source; the PCRTM product can be the full length cDNA or can be used to generate a probe to obtain the desired full length cDNA. Alternatively, a desired protein can be entirely sequenced and total synthesis of a DNA encoding that polypeptide performed.

10 Once the desired genomic or cDNA has been isolated, it can be sequenced by known methods. It is recognized in the art that such methods are subject to errors, such that multiple sequencing of the same region is routine and is still expected to lead to measurable rates of mistakes in the resulting deduced sequence, particularly in regions having repeated domains, extensive secondary structure, or unusual base compositions, such as regions with high GC
15 base content. When discrepancies arise, resequencing can be done and can employ special methods. Special methods can include altering sequencing conditions by using: different temperatures; different enzymes; proteins which alter the ability of oligonucleotides to form higher order structures; altered nucleotides such as ITP or methylated dGTP; different gel compositions, for example adding formamide; different primers or primers located at different
20 distances from the problem region; or different templates such as single stranded DNAs. Sequencing of mRNA also can be employed.

If desired, the sequences of nucleic acids that code for phosphopantetheinyl transferases can be modified without changing the resulting amino acid sequence of the expressed protein so that the sequences are more amenable to expression in plant hosts. A
25 coding sequence can be an artificial DNA. An artificial DNA, as used herein means a DNA polynucleotide molecule that is non-naturally occurring. Artificial DNA molecules can be designed by a variety of methods, such as, methods known in the art that are based upon substituting the codon(s) of a first polynucleotide to create an equivalent, or even an improved, second-generation artificial polynucleotide, where this new artificial
30 polynucleotide is useful for enhanced expression in transgenic plants. The design aspect often employs a codon usage table produced by compiling the frequency of occurrence of codons in a collection of coding sequences isolated from a plant, plant type, family or genus. Other design aspects include reducing the occurrence of polyadenylation signals, intron splice sites, or long AT or GC stretches of sequence (U.S. Patent 5,500,365). Full length coding
35 sequences or fragments thereof can be made of artificial DNA using methods known to those skilled in the art. Modifications of the nucleotide sequences or regulatory elements disclosed

herein which maintain the functions contemplated herein are within the scope of this invention. Such modifications include insertions, substitutions and deletions, and specifically substitutions which reflect the degeneracy of the genetic code.

5 The inventors have isolated DNA sequences from *Moritella marina* that produce polypeptides with phosphopantetheinyl transferase activity. The sequences encoding the phosphopantetheinyl transferases may be expressed in transgenic plants, microorganisms or animals to effect activation of a polyketide synthase. Other polynucleotides which are substantially identical to the phosphopantetheinyl transferase polynucleotides provided herein, or which encode polypeptides which are substantially identical to the
10 phosphopantetheinyl transferase polypeptides, also can be used. "Substantially identical" refers to an amino acid sequence or nucleic acid sequence exhibiting in order of increasing preference at least 75%, 80%, 85%, 90%, 95%, 98 or 99% identity to the phosphopantetheinyl transferase polypeptide sequence in SEQ ID NO:5, SEQ ID NO:7 or sequences encoding these polypeptides. Polypeptide or polynucleotide comparisons may be carried out using
15 sequence analysis software, for example, the Sequence Analysis software package of the GCG Wisconsin Package (Accelrys, San Diego, CA) and MEGAlign (DNASTar, Inc., 1228 S. Park St., Madison, Wis. 53715). Such software matches similar sequences by assigning degrees of similarity or identity.

DNA Constructs

20 The invention provides DNA constructs comprising a heterologous promoter operably linked to a nucleic acid described herein. The selection of promoters, *e.g.*, promoters that may be described as strongly expressed, weakly expressed, inducibly expressed, tissue-enhanced expressed (*i.e.*, specifically or preferentially expressed in a tissue), organ-enhanced expressed (*i.e.*, specifically or preferentially expressed in an organ) and
25 developmentally-enhanced expressed (*i.e.*, specifically or preferentially expressed during a particular stage(s) of development), is within the skill in the art. Similarly, the combining of a nucleic acid molecule as described above with a promoter is also within the skill in the art (see, *e.g.*, Sambrook *et al.*, 1989).

Promoters for use with the invention include, but are not limited to, promoters that
30 function in bacteria, bacteriophages, fungi or plant cells. Useful promoters for bacterial expression are the lacZ, Sp6, T7, T5 or E. coli glgC promoters. Useful promoters for fungi include *Saccharomyces cerevisiae* Gal1 (West, *et al.* (1984)), *Saccharomyces pombe* nmt1 (Maundrell, K. (1990)), *Neurospora crassa* ccg-1 (Freitag M and Selker EU (2005)) and *Pichia methanolica* AUG1 (Invitrogen). Useful promoters for plants cells include the gamma
35 zein Z27 promoter (see, for example, Lopes *et al.* (1995), L3 oleosin promoter (U.S. Patent No. 6,433,252), barley PER1 promoter (Stacey *et al.*, 1996), CaMV 35S promoter (Odell *et*

al. 1985), the CaMV 19S (Lawton *et al.*, 1987), nos (Ebert *et al.*, 1987), Adh (Walker *et al.*, 1987), sucrose synthase (Yang *et al.*, 1990), actin (Wang *et al.*, 1992), cab (Sullivan *et al.*, 1989), PEPCase promoter (Hudspeth *et al.*, 1989), or those associated with the R gene complex (Chandler *et al.*, 1989). The Figwort Mosaic Virus (FMV) promoter (Richins *et al.*, 1987), arcelin, tomato E8, patatin, ubiquitin, mannopine synthase (mas) and tubulin promoters are other examples of useful promoters.

There are a wide variety of plant promoter sequences which may be used to drive tissue-specific expression of polynucleotides encoding phosphopantetheinyl transferases in transgenic plants. Indeed, in particular embodiments of the invention, the promoter used is a seed specific promoter. Examples of such promoters include the 5' regulatory regions from such genes as napin (Kridl *et al.*, 1991), phaseolin (Bustos, *et al.*, 1989), soybean trypsin inhibitor (Riggs, *et al.*, 1989), ACP (Baerson *et al.*, 1993), stearyl-ACP desaturase (Slocombe *et al.*, 1994), soybean a' subunit of β -conglycinin (P-Gm7S alpha', see for example, Chen *et al.*, 1986), *Vicia faba* USP (P-Vf.Usp, see for example, SEQ ID NO:1, 2, and 3, U.S. Patent Application 10/429,516), the globulin promoter (see for example Belanger and Kriz, (1991), soybean alpha subunit of β -conglycinin (7S alpha) (U.S. Patent Application 10/235,618, incorporated by reference) and *Zea mays* L3 oleosin promoter (P-Zm.L3, see, for example, Hong *et al.*, 1997).

Promoters expressed in maize include promoters from genes encoding zeins, which are a group of storage proteins found in maize endosperm. Genomic clones for zein genes have been isolated (Pedersen *et al.*, 1982; Russell *et al.*, 1997) and the promoters from these clones, including the 15 kD, 16 kD, 19 kD, 22 kD and 27 kD genes, can be used. Other seed-expression enhanced promoters known to function in maize and in other plants include the promoters for the following genes: Waxy (granule bound starch synthase), Brittle and Shrunken 2 (ADP glucose pyrophosphorylase), Shrunken 1 (sucrose synthase), branching enzymes I and II, starch synthases, debranching enzymes, oleosins, glutelins, and Bet11 (basal endosperm transfer layer). Other promoters useful in the practice of the invention that are known by one of skill in the art are also contemplated by the invention.

Moreover, transcription enhancers or duplications of enhancers can be used to increase expression from a particular promoter. Examples of such enhancers include, but are not limited to the Adh intron1 (Callis *et al.*, 1987), a rice actin intron (McElroy *et al.*, 1991; U.S. Patent No. 5,641,876), sucrose synthase intron (Vasil *et al.*, 1989), a maize HSP70 intron (also referred to as Zm.DnaK) (U.S. Patent 5,424,412, Brown *et al.*) a TMV omega element (Gallie *et al.*, 1999), the CaMV 35S enhancer (U.S. Patents 5,359,142 & 5,196,525, McPherson *et al.*) or an octopine synthase enhancer (U.S. Patent 5,290,924, Last *et al.*). As the DNA sequence between the transcription initiation site and the start of the coding

sequence, *i.e.*, the untranslated leader sequence, can influence gene expression, one may also wish to employ a particular leader sequence. Any leader sequence available to one of skill in the art may be employed. Preferred leader sequences direct optimum levels of expression of the attached gene, for example, by increasing or maintaining mRNA stability and/or by preventing inappropriate initiation of translation (Joshi, 1987). The choice of such sequences is at the discretion of those of skill in the art.

DNA constructs of the invention may include a sequence near the 3' end of the cassette that acts as a signal to terminate transcription from a heterologous nucleic acid and that directs polyadenylation of the resultant mRNA. These are commonly referred to as 3' untranslated regions or 3' UTRs. Some 3' elements that can act as transcription termination signals include those from the nopaline synthase gene (*nos*) of *Agrobacterium tumefaciens* (Bevan *et al.*, 1983), a napin 3' untranslated region (Kridl *et al.*, 1991), a globulin 3' untranslated region (Belanger and Kriz, 1991), 3' untranslated region from the *Adr12* gene of soybean (auxin down regulated) (Wang *et al.*, PCT Publication WO200250295) or one from a zein gene, such as Z27 (Lopes *et al.*, 1995). Other 3' regulatory elements known to the art also can be used in the vectors of the invention.

A nucleic acid molecule as described herein can be cloned into any suitable vector and can be used to transform or transfect any suitable host. The selection of vectors and methods to construct them are commonly known to the art and are described in general technical references (see, in general, "Recombinant DNA Part D" (1987)). The vector will preferably comprise regulatory sequences, such as transcription and translation initiation and termination codons, which are specific to the type of host (*e.g.*, bacterium, fungus, or plant) into which the vector is to be introduced, as appropriate and taking into consideration whether the vector is DNA or RNA.

Vectors that are circular or linear can be prepared to contain an entire nucleic acid sequence as described above or a portion thereof ligated to a replication system functional in a prokaryotic or eukaryotic host cell. Replication systems can be derived from ColE1, 2 μ plasmid, λ phage, ϕ 1 filamentous phage, *Agrobacterium* species (*e.g.*, *A. tumefaciens* and *A. rhizogenes*), and the like.

In addition to the replication system and the inserted nucleic acid sequence, the vector can include one or more marker genes that allow for selection of transformed or transfected hosts. Marker genes include biocide resistance, such as resistance to antibiotics, heavy metals, herbicides, etc., complementation in an auxotrophic host to provide prototrophy, and the like.

The invention provides host cells comprising a nucleic acid molecule described herein, optionally in the form of a vector. Suitable hosts include plant, bacterial and fungal cells, including *Escherichia coli*, *Bacillus subtilis*, *Agrobacterium tumefaciens*, *Saccharomyces cerevisiae* and *Neurospora crassa*. *E. coli* hosts include TB-1, TG-2, DH5 α ,
5 XL-Blue MRF' (Stratagene, Austin, TX), SA2821, Y1090 and TG02. Plant cells include, but not limited to, soybean, *Brassica campestris*, canola, oilseed rape, rapeseed, crambe, mustard, castor bean, peanut, sesame, cottonseed, linseed, safflower, oil palm, flax, sunflower, alfalfa, corn, wheat, barley, oats, rye, millet, sorghum, and rice.

Expression in a host cell can be accomplished in a transient or stable fashion.
10 Transient expression can occur from introduced constructs which contain expression signals functional in the host cell, but which constructs do not replicate and rarely integrate in the host cell, or where the host cell is not proliferating. Transient expression also can be accomplished by inducing the activity of a regulatable promoter operably linked to the gene of interest, although such inducible systems frequently exhibit a low basal level of expression.
15 Stable expression can be achieved by introduction of a construct that can integrate into the host genome or that autonomously replicates in the host cell. Stable expression of the gene of interest can be selected for through the use of a selectable marker located on or transfected with the expression construct, followed by selection for cells expressing the marker. When stable expression results from integration, integration of constructs can occur randomly within
20 the host genome or can be targeted through the use of constructs containing regions of homology with the host genome sufficient to target recombination with the host locus. Where constructs are targeted to an endogenous locus, all or some of the transcriptional and translational regulatory regions can be provided by the endogenous locus.

Expression in a host cell may involve fermentation techniques known to one skilled
25 in the art. The fermented host cell may be a prokaryote, such as *Escherichia coli*, or a eukaryote, such as the yeast *Saccharomyces cerevisiae*, or *Neurospora crassa*, a filamentous fungi. Examples of production of PUFA by fermentation include *Mortierella* (U.S. Patent 6,319,698) and *Thraustochytriales* (U.S. Patent 6,451,567).

It is contemplated that more than one gene may be introduced and propagated in a
30 host cell through the use of episomal or integrated expression vectors. Where two or more genes are expressed from separate replicating vectors, it is desirable that each vector has a different means of replication. Each introduced construct, whether integrated or not, should have a different means of selection and should lack homology to the other constructs to maintain stable expression and prevent reassortment of elements among constructs. Judicious
35 choices of regulatory regions, selection means and method of propagation of the introduced

construct can be experimentally determined so that all introduced polynucleotides are expressed at the necessary levels to provide for synthesis of the desired products.

Polypeptides

The invention provides phosphopantetheinyl transferases encoded by nucleic acid molecules described herein. Polyketide synthases are enzyme complexes composed of multifunctional polypeptides that catalyze the synthesis of complex molecules from simple substrates in an iterative fashion. In *Moritella marina*, a PKS complex synthesizes DHA from malonyl-CoA and acetyl-CoA. To activate this complex, a phosphopantetheinyl transferase is required. The polypeptide preferably comprises an amino end and a carboxyl end. The polypeptide can comprise D-amino acids, L-amino acids or a mixture of D- and L-amino acids.

Alterations of the native amino acid sequence to produce variant polypeptides can be prepared by a variety of means known to those ordinarily skilled in the art. For instance, amino acid substitutions can be conveniently introduced into the polypeptides by changing the sequence of the nucleic acid molecule at the time of synthesis. Site-specific mutations can also be introduced by ligating into an expression vector a synthesized oligonucleotide comprising the modified sequence. Alternately, oligonucleotide-directed, site-specific mutagenesis procedures can be used, such as disclosed in Walder *et al.* (1986); Bauer *et al.* (1985); and U.S. Patents 4,518,584 and 4,737,462.

It is within the skill of the ordinary artisan to select synthetic and naturally-occurring amino acids that effect conservative or neutral substitutions for any particular naturally-occurring amino acids. The ordinarily skilled artisan desirably will consider the context in which any particular amino acid substitution is made, in addition to considering the hydrophobicity or polarity of the side-chain, the general size of the side chain and the pK value of side-chains with acidic or basic character under physiological conditions. For example, lysine, arginine, and histidine are often suitably substituted for each other, and more often arginine and histidine. As is known in the art, this is because all three amino acids have basic side chains, whereas the pK value for the side-chains of lysine and arginine are much closer to each other (about 10 and 12) than to histidine (about 6). Similarly, glycine, alanine, valine, leucine, and isoleucine are often suitably substituted for each other, with the proviso that glycine is frequently not suitably substituted for the other members of the group. This is because each of these amino acids is relatively hydrophobic when incorporated into a polypeptide, but glycines lack of an α -carbon allows the phi and psi angles of rotation (around the α -carbon) so much conformational freedom that glycyl residues can trigger changes in conformation or secondary structure that do not often occur when the other amino acids are substituted for each other. Other groups of amino acids frequently suitably

substituted for each other include, but are not limited to, the group consisting of glutamic and aspartic acids; the group consisting of phenylalanine, tyrosine and tryptophan; and the group consisting of serine, threonine and, optionally, tyrosine. Additionally, the ordinarily skilled artisan can readily group synthetic amino acids with naturally-occurring amino acids.

5 If desired, the polypeptides can be modified, for instance, by glycosylation, amidation, carboxylation, or phosphorylation, or by the creation of acid addition salts, amides, esters, in particular C-terminal esters, and N-acyl derivatives of the polypeptides of the invention. The polypeptides also can be modified to create protein derivatives by forming covalent or noncovalent complexes with other moieties in accordance with methods known in
10 the art. Covalently-bound complexes can be prepared by linking the chemical moieties to functional groups on the side chains of amino acids comprising the polypeptides, or at the N- or C-terminus. Desirably, such modifications and conjugations do not adversely affect the activity of the polypeptides (and variants thereof). While such modifications and conjugations can have greater or lesser activity, the activity desirably is not negated and is
15 characteristic of the unaltered polypeptide.

The polypeptides (and fragments, variants and fusion proteins) can be prepared by any of a number of conventional techniques. The polypeptide can be isolated or substantially purified from a naturally occurring source or from a recombinant source. For instance, in the case of recombinant proteins, a DNA fragment encoding a desired protein can be subcloned
20 into an appropriate vector using well-known molecular genetic techniques (see, *e.g.*, Maniatis *et al.*, 1989) and other references cited herein under "EXAMPLES"). The fragment can be transcribed and the protein subsequently translated in vitro. Commercially available kits also can be employed (*e.g.*, such as manufactured by Clontech, Mountain View, CA; Amersham Life Sciences, Inc., Arlington Heights, IL; Invitrogen, Carlsbad, CA and the like). The
25 polymerase chain reaction optionally can be employed in the manipulation of nucleic acids.

Polypeptides can be synthesized using an automated peptide synthesizer in accordance with methods known in the art. Alternately, the polypeptide (and fragments, variants, and fusion proteins) can be synthesized using standard peptide synthesizing techniques well-known to those of ordinary skill in the art (*e.g.*, as summarized in Bodanszky
30 (1984)). In particular, the polypeptide can be synthesized using the procedure of solid-phase synthesis (see, *e.g.*, Merrifield, 1963; Barany *et al.*, 1987; and U.S. Patent 5,424,398). If desired, this can be done using an automated peptide synthesizer. Removal of the t-butyloxycarbonyl (t-BOC) or 9-fluorenylmethyloxycarbonyl (Fmoc) amino acid blocking groups and separation of the protein from the resin can be accomplished by, for example, acid
35 treatment at reduced temperature. The polypeptide-containing mixture then can be extracted, for instance, with diethyl ether, to remove non-peptidic organic compounds, and the

synthesized protein can be extracted from the resin powder (*e.g.*, with about 25% w/v acetic acid). Following the synthesis of the polypeptide, further purification (*e.g.*, using HPLC) optionally can be done in order to eliminate any incomplete proteins, polypeptides, peptides or free amino acids. Amino acid and/or HPLC analysis can be performed on the synthesized polypeptide to validate its identity. For other applications according to the invention, it may be preferable to produce the polypeptide as part of a larger fusion protein, either by chemical conjugation, or through genetic means known to the art. In this regard, this invention also provides a fusion protein comprising the polypeptide (or fragment thereof) or variant thereof and one or more other polypeptides/protein(s) having any desired properties or effector functions.

Assays for the production and identification of specific proteins are based on various physical-chemical, structural, functional, or other properties of the proteins. Unique physical-chemical or structural properties allow the proteins to be separated and identified by electrophoretic procedures, such as native or denaturing gel electrophoresis or isoelectric focusing, or by chromatographic techniques such as ion exchange or gel exclusion chromatography. The unique structures of individual proteins offer opportunities for use of specific antibodies to detect their presence in formats such as an ELISA assay. Combinations of approaches can be used to achieve even greater specificity such as western blotting in which antibodies are used to locate individual gene products that have been separated by electrophoretic techniques. Additional techniques can be used to absolutely confirm the identity of the product of interest such as evaluation by amino acid sequencing following purification. Although these are among the most common, other procedures can also be used.

Assay procedures can identify the expression of proteins by their functionality, particularly where the expressed protein is an enzyme capable of catalyzing chemical reactions involving specific substrates and products. For example, in plant extracts these reactions can be measured by providing and quantifying the loss of substrates or the generation of products of the reactions by physical and/or chemical procedures.

In many cases, the expression of a gene product is determined by evaluating the phenotypic results of its expression. Such evaluations may be simply as visual observations, or may involve assays. Such assays can take many forms, such as analyzing changes in the chemical composition, morphology, or physiological properties of the plant. Chemical composition may be altered by expression of genes encoding enzymes or storage proteins that change amino acid composition and these changes can be detected by amino acid analysis, or by enzymes that change starch quantity, which can be analyzed by near infrared reflectance spectrometry or by enzymes that change oil composition, which can be detected by gas chromatography. Morphological changes may include greater stature or thicker stalks.

The nucleic acid molecules, DNA constructs and polypeptides of this invention can be used in agricultural methods and various screening assays. For example, a nucleic acid molecule can be used to express phosphopantetheinyl transferase via a vector in a host cell, to detect mRNA transcripts encoding phosphopantetheinyl transferase in a biological sample, to
5 detect a genetic alteration in a gene encoding phosphopantetheinyl transferase *via* a Southern blot, to suppress phosphopantetheinyl transferase, or to up-regulate phosphopantetheinyl transferase. The polypeptides can be used to compensate for deficiencies in phosphopantetheinyl transferase or for the presence of a mutated phosphopantetheinyl transferase having reduced or no activity in a plant, or to treat excessive levels of substrates,
10 whether direct or indirect, for phosphopantetheinyl transferase in a plant. Alternatively, the polypeptides can be used to screen agents for the ability to modulate their activity. The antibodies can be used to detect and isolate the respective polypeptides as well as decrease the availability of such polypeptides *in vivo*.

Plant transformation

15 In a preferred embodiment of the invention, a transgenic plant expressing the desired protein or proteins is produced. Various methods for the introduction of a desired polynucleotide sequence encoding the desired protein into plant cells are known to the art, including: (1) physical methods such as microinjection, electroporation, and microparticle-mediated delivery (biolistics or gene gun technology); (2) virus-mediated delivery; and (3)
20 *Agrobacterium*-mediated transformation.

The most commonly used methods for transformation of plant cells are the *Agrobacterium*-mediated DNA transfer process and the biolistics or microprojectile microparticle bombardment mediated process. Typically, nuclear transformation is desired but where it is desirable to specifically transform plastids, such as chloroplasts or amyloplasts,
25 plant plastids may be transformed utilizing a microparticle-mediated delivery of the desired polynucleotide.

Agrobacterium-mediated transformation is achieved through the use of a genetically engineered soil bacterium belonging to the genus *Agrobacterium*. A number of wild-type and disarmed strains of *Agrobacterium tumefaciens* and *Agrobacterium rhizogenes* harboring Ti
30 or Ri plasmids can be used for gene transfer into plants. Gene transfer is done *via* the transfer of a specific DNA known as "T-DNA" that can be genetically engineered to carry any desired piece of DNA into many plant species, as further elaborated, for example, in U.S. Patent 6,265,638 to Bidney *et al.*, the disclosures of which are hereby incorporated herein by reference.

Agrobacterium-mediated genetic transformation of plants involves several steps. The first step, in which the virulent *Agrobacterium* and plant cells are first brought into contact with each other, is generally called "inoculation". Inoculation is preferably accompanied by some method of injury to some of the plant cells, which releases plant cellular constituents, such as coumaryl alcohol, sinapinate (which is reduced to acetosyringone), sinapyl alcohol, and coniferyl alcohol, that activate virulence factors in the *Agrobacterium*. Following the inoculation, the *Agrobacterium* and plant cells/tissues are permitted to grow together for a period of several hours to several days or more under conditions suitable for growth and T-DNA transfer. This step is termed "co-culture". Following co-culture and T-DNA delivery, the plant cells are treated with bactericidal or bacteriostatic agents to kill the *Agrobacterium* remaining in contact with the explant and/or in the vessel containing the explant. If this is done in the absence of any selective agents to promote preferential growth of transgenic versus non-transgenic plant cells, then this is typically referred to as the "delay" step. If done in the presence of selective pressure favoring transgenic plant cells, then it is referred to as a "selection" step. When a "delay" is used, it is typically followed by one or more "selection" steps.

With respect to microparticle bombardment (U.S. Patent 5,550,318 (Adams *et al.*); U.S. Patent 5,538,880 (Lundquist *et al.*), U.S. Patent 5,610,042 (Chang *et al.*); and PCT WO 95/06128 (Adams *et al.*); each of which is specifically incorporated herein by reference in its entirety), microscopic particles are coated with nucleic acids and delivered into cells by a propelling force. Exemplary particles include those comprised of tungsten, platinum, and preferably, gold.

An illustrative embodiment of a method for delivering DNA into plant cells by acceleration is the Biolistics Particle Delivery System (BioRad, Hercules, CA), which can be used to propel particles coated with DNA or cells through a screen, such as a stainless steel or Nytex screen, onto a filter surface covered with monocot plant cells cultured in suspension.

Microparticle bombardment techniques are widely applicable, and may be used to transform virtually any plant species. Examples of species that have been transformed by microparticle bombardment include monocot species such as maize (International Publication No. WO 95/06128 (Adams *et al.*)), barley, wheat (U.S. Patent 5,563,055 (Townsend *et al.*) incorporated herein by reference in its entirety), rice, oat, rye, sugarcane, and sorghum; as well as a number of dicots including tobacco, soybean (U.S. Patent 5,322,783 (Tomes *et al.*), incorporated herein by reference in its entirety), sunflower, peanut, cotton, tomato, and legumes in general (U.S. Patent 5,563,055 (Townsend *et al.*) incorporated herein by reference in its entirety).

To select or score for transformed plant cells regardless of transformation methodology, the DNA introduced into the cell contains a gene that functions in a regenerable plant tissue to produce a compound that confers upon the plant tissue resistance to an otherwise toxic compound. Genes of interest for use as a selectable, screenable, or scorable marker would include but are not limited to β -glucuronidase (GUS), green fluorescent protein (GFP), luciferase (LUX), antibiotic or herbicide tolerance genes. Examples of antibiotic resistance genes include the penicillins, kanamycin (and neomycin, G418, bleomycin); methotrexate (and trimethoprim); chloramphenicol; kanamycin and tetracycline. Polynucleotide molecules encoding proteins involved in herbicide tolerance are known in the art, and include, but are not limited to a polynucleotide molecule encoding 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) described in U.S. Patent 5,627,061 (Barry, *et al.*), U.S. Patent 5,633,435 (Barry, *et al.*), and U.S. Patent 6,040,497 (Spencer, *et al.*) and *aroA* described in U.S. Patent 5,094,945 (Comai) for glyphosate tolerance; a polynucleotide molecule encoding bromoxynil nitrilase (Bxn) described in U.S. Patent 4,810,648 (Duerrschnabel, *et al.*) for Bromoxynil tolerance; a polynucleotide molecule encoding phytoene desaturase (CrtI) described in Misawa *et al.*, (1993); Misawa *et al.*, (1994) for norflurazon tolerance; a polynucleotide molecule encoding acetohydroxyacid synthase (AHAS, aka ALS) described in Sathasiivan *et al.* (1990) for tolerance to sulfonylurea herbicides; and both the *pat* gene described in Wohlleben, *et al.*, (1988) and *bar* gene described in DeBlock, *et al.* (1987), each of which provides glufosinate and bialaphos tolerance.

The regeneration, development, and cultivation of plants from various transformed explants are well documented in the art. This regeneration and growth process typically includes the steps of selecting transformed cells and culturing those individualized cells through the usual stages of embryonic development through the rooted plantlet stage. Transgenic embryos and seeds are similarly regenerated. The resulting transgenic rooted shoots are thereafter planted in an appropriate plant growth medium such as soil. Cells that survive the exposure to the selective agent, or cells that have been scored positive in a screening assay, may be cultured in media that supports regeneration of plants. Developing plantlets are transferred to soil less plant growth mix, and hardened off, prior to transfer to a greenhouse or growth chamber for maturation.

This invention can be used with any transformable cell or tissue. By transformable as used herein is meant a cell or tissue that is capable of further propagation to give rise to a plant. Those of skill in the art recognize that a number of plant cells or tissues are transformable in which after insertion of exogenous DNA and appropriate culture conditions

the plant cells or tissues can form into a differentiated plant. Tissue suitable for these purposes can include but is not limited to immature embryos, scutellar tissue, suspension cell cultures, immature inflorescence, shoot meristem, nodal explants, callus tissue, hypocotyl tissue, cotyledons, roots, and leaves. The Tomes *et al.* '783 patent, cited above, describes a method of treatment with a cytokinin followed by incubation for a period sufficient to permit undifferentiated cells in cotyledonary node tissue to differentiate into meristematic cells and to permit the cells to enter the phases between the G1 and division phases of development, which is stated to improve susceptibility for transformation.

Any suitable plant culture medium can be used. Suitable media include but are not limited to MS-based media (Murashige and Skoog, 1962) or N6-based media (Chu *et al.*, 1975) supplemented with additional plant growth regulators including but not limited to auxins, cytokinins, ABA, and gibberellins. Those of skill in the art are familiar with the variety of tissue culture media, which when supplemented appropriately, support plant tissue growth and development and are suitable for plant transformation and regeneration. These tissue culture media can either be purchased as a commercial preparation, or custom prepared and modified. Those of skill in the art are aware that media and media supplements such as nutrients and growth regulators for use in transformation and regeneration and other culture conditions such as light intensity during incubation, pH, and incubation temperatures that can be optimized for the particular variety of interest.

After a DNA construct is stably incorporated in transgenic plants and confirmed to be operable, it can be introduced into other plants of the same or another sexually compatible species by sexual crossing. Any of a number of standard breeding techniques can be used, depending upon the species to be crossed. Therefore, the current invention not only encompasses a plant directly transformed or regenerated from cells which have been transformed in accordance with the current invention, but also the progeny of such plants. As used herein the term "progeny" denotes the offspring of any generation of a parent plant prepared in accordance with the instant invention, wherein the progeny comprises a selected DNA construct prepared in accordance with the invention. "Crossing" a plant to provide a plant line having one or more added transgenes or alleles relative to a starting plant line, as disclosed herein, is defined as the techniques that result in a particular sequence being introduced into a plant line by crossing a starting line with a donor plant line that comprises a transgene or allele of the invention. To achieve this one could, for example, perform the following steps: (a) plant seeds of the first (starting line) and second (donor plant line that comprises a desired transgene or allele) parent plants; (b) grow the seeds of the first and second parent plants into plants that bear flowers; (c) pollinate a flower from the first parent

plant with pollen from the second parent plant; and (d) harvest seeds produced on the parent plant bearing the fertilized flower.

Backcrossing is herein defined as the process including the steps of: (a) crossing a plant of a first genotype containing a desired gene, DNA sequence or element to a plant of a second genotype lacking said desired gene, DNA sequence or element; (b) selecting one or more progeny plant containing the desired gene, DNA sequence or element; (c) crossing the progeny plant to a plant of the second genotype; and (d) repeating steps (b) and (c) for the purpose of transferring a desired DNA sequence from a plant of a first genotype to a plant of a second genotype.

Introgression of a DNA element into a plant genotype is defined as the result of the process of backcross conversion. A plant genotype into which a DNA sequence has been introgressed may be referred to as a backcross converted genotype, line, inbred, or hybrid. Similarly a plant genotype lacking the desired DNA sequence may be referred to as an unconverted genotype, line, inbred, or hybrid.

Seeds, Meal, Oil and Products Comprising Seeds, Meal and Oil

This invention also provides a container of over about 1000, more preferably about 20,000, and even more preferably about 40,000 seeds where over about 10%, more preferably about 25%, more preferably about 50%, and even more preferably about 75% or more preferably about 90% of the seeds are seeds derived from a plant of this invention.

This invention also provides a container of over about 10 kg, more preferably about 25 kg, and even more preferably about 50 kg seeds where over about 10%, more preferably about 25%, more preferably about 50%, and even more preferably about 75% or more preferably about 90% of the seeds are seeds derived from a plant of this invention.

Any of the plants or parts thereof of this invention may be harvested and, optionally, processed to produce a feed, meal, or oil preparation. A particularly preferred plant part for this purpose is harvested grain, but other plant parts can be harvested and used for stover or silage. Methods to produce feed, meal, and oil preparations are known in the art. See, for example, U.S. Patents 4,957,748; 5,100,679; 5,219,596; 5,936,069; 6,005,076; 6,146,669; and 6,156,227. The grain or meal of this invention may be blended with other grains or meals.

Methods

The present invention provides a method for providing transgenic plants with an increased content of DHA or EPA. This method may include, for example, introducing DNA

encoding phosphopantetheinyl transferase as well as a PKS complex into plant cells and regenerating plants with increased DHA or EPA content from the transgenic cells.

More specifically, the invention provides a method of making a plant oil containing DHA or EPA comprising the steps of (a) growing a plant comprising the host cell transformed with a DNA construct comprising a heterologous promoter operably linked to a DNA molecule encoding a polypeptide having phosphopantetheinyl transferase activity, wherein the DNA molecule is selected from the group consisting of: a polynucleotide hybridizing to SEQ ID NO:6 or SEQ ID NO:8, or a complement thereof, under conditions of 5X SSC, 50% formamide and 42°C; a polynucleotide encoding the polypeptide sequence of SEQ ID NO:5 or SEQ ID NO:7; a polynucleotide encoding a polypeptide with at least 75% sequence identity to a polypeptide sequence of SEQ ID NO:5 or SEQ ID NO:7; a polynucleotide encoding the polypeptide of SEQ ID NO:1; and a polynucleotide encoding the polypeptide of SEQ ID NO:3 wherein the host cell further comprises a polyketide synthase; (b) producing seed; and (c) processing the seed to obtain oil.

The present invention further provides a method for providing transgenic plants which may contain elevated levels of DHA or EPA, wherein said elevated levels are greater than levels found in non-transformed plants.

For dietary supplementation, the purified PUFAs, transformed plants or plant parts, or derivatives thereof, may be incorporated into cooking oils, fats or margarines formulated so that in normal use the recipient would receive the desired amount. The PUFAs may also be incorporated into infant formulas, nutritional supplements or other food products, and may find use as anti-inflammatory or cholesterol lowering agents.

As used herein, "edible composition" is defined as compositions which may be ingested by a mammal such as foodstuffs, nutritional substances and pharmaceutical compositions. As used herein "foodstuffs" refer to substances that can be used or prepared for use as food for a mammal and include substances that may be used in the preparation of food (such as frying oils) or food additives. For example, foodstuffs include animals used for human consumption or any product therefrom, such as, for example, eggs. Typical foodstuffs include but are not limited to beverages, (e.g., soft drinks, carbonated beverages, ready to mix beverages), infused foods (e.g. fruits and vegetables), sauces, condiments, salad dressings, fruit juices, syrups, desserts (e.g., puddings, gelatin, icings and fillings, baked goods and frozen desserts such as ice creams and sherbets), soft frozen products (e.g., soft frozen creams, soft frozen ice creams and yogurts, soft frozen toppings such as dairy or non-dairy whipped toppings), oils and emulsified products (e.g., shortening, margarine, mayonnaise,

butter, cooking oil, and salad dressings) and intermediate moisture foods (e.g., rice and dog foods).

Furthermore, edible compositions described herein can also be ingested as an additive or supplement contained in foods and drinks. These can be formulated together with a nutritional substance such as various vitamins and minerals and incorporated into substantially liquid compositions such as nutrient drinks, soymilks and soups; substantially solid compositions; and gelatins or used in the form of a powder to be incorporated into various foods. The content of the effective ingredient in such a functional or health food can be similar to the dose contained in a typical pharmaceutical agent.

The purified PUFAs, transformed plants or plant parts may also be incorporated into animal, particularly livestock, feed. In this way, the animals themselves may benefit from a PUFA rich diet, while human consumers of food products produced from such livestock may benefit as well.

For pharmaceutical use (human or veterinary), the compositions may generally be administered orally but can be administered by any route by which they may be successfully absorbed, e.g., parenterally (i.e. subcutaneously, intramuscularly or intravenously), rectally, vaginally or topically, for example, as a skin ointment or lotion. The PUFAs, transformed plants or plant parts of the present invention may be administered alone or in combination with a pharmaceutically acceptable carrier or excipient. Where available, gelatin capsules are the preferred form of oral administration. Dietary supplementation as set forth above can also provide an oral route of administration. The unsaturated acids of the present invention may be administered in conjugated forms, or as salts, esters, amides or prodrugs of the fatty acids. Any pharmaceutically acceptable salt is encompassed by the present invention; especially preferred are the sodium, potassium or lithium salts. Also encompassed are the N-alkylpolyhydroxamine salts, such as N-methyl glucamine, found in PCT publication WO 96/33155. The preferred esters are the ethyl esters. As solid salts, the PUFAs also can be administered in tablet form. For intravenous administration, the PUFAs or derivatives thereof may be incorporated into commercial formulations such as Intralipids.

EXAMPLES

The following examples are included to illustrate embodiments of the invention. It should be appreciated by those of skill in the art that the techniques disclosed in the examples that follow represent techniques discovered by the inventor to function well in the practice of the invention. However, those of skill in the art should, in light of the present disclosure, appreciate that many changes can be made in the specific embodiments which are disclosed and still obtain a like or similar result. More specifically, it will be apparent that certain agents which are both chemically and physiologically related may be substituted for the agents described herein while the same or similar results would be achieved. The scope of the claims should not be limited by the preferred

embodiments set forth herein, but should be given the broadest interpretation consistent with the description as a whole.

Example 1

Cloning of Phosphopantetheinyl Transferase Sequences

5 Three bacterial phosphopantetheinyl transferases were cloned. The amino acid sequence of the phosphopantetheinyl transferase (Ppt) from *Shewanella* SCRC-2738 (SEQ ID NO:17) was used to search public databases for novel ppts that function in EPA or DHA biosynthesis. This search yielded putative ppts from *Shewanella* oneidensis MR-1 (SEQ ID NO:1) (So-ppt) and *Colwellia* psychrerythraea (SEQ ID NO:3) (Cp-ppt). The nucleic acid sequences of ppts from *Shewanella* oneidensis MR-1 (SEQ ID NO:2) and *Colwellia* psychrerythraea (SEQ ID NO:4) were cloned using the Expand High Fidelity PCR system (Roche, Applied Science, Indianapolis, IN) with the following primer pairs:

Shew new 5': tcgagctcgcatatgaagattgagcttttttataacc (SEQ ID NO:9)

Shew 3': tcttaattaattagtcagccaaactagccgc (SEQ ID NO:10)

15 Colwe new 5': tcgagctcgcatatgacttcttttctcaatctg (SEQ ID NO:11)

Colwe 3': tcttaattaattagatttctgataaccaagtag (SEQ ID NO:12).

The genes were amplified for 25 cycles with the melting temperature set at 55°C and 52°C for the *Shewanella* and *Colwellia* ppts, respectively. The PCR products were digested with NdeI and PacI and ligated into NdeI- and PacI-digested Novagen pACYC-Duet-1 (EMD Biosciences, Darmstadt, Germany) resulting in the formation of pMON68081 (FIG. 1) and pMON68080 (FIG. 2) respectively.

To clone the *Moritella marina* phosphopantetheinyl transferase (Mm-ppt), the nucleotide sequences of the *Shewanella* SCRC-2738 ppt (SEQ ID NO:18), the *C. psychrerythraea* ppt, and the *S. oneidensis* MR-1 ppt were aligned to identify the most conserved region of these sequences. A region of conserved nucleotide sequence in So-ppt (bps 425 –635 of SEQ ID NO: 2) and Cp-ppt (bps 389 –596 of SEQ ID NO: 4) was identified by this alignment and sequence in this region was chosen to generate probes using genomic DNA from *C. psychrerythraea* and *S. oneidensis* MR-1 as template DNAs and the primers below:

Shewanella F1 taggtgtcgatattgagcggg (SEQ ID NO:13)

Shewanella R1 tcaaaggcaaaggattttaac (SEQ ID NO:14)

Colwellia F1 tcggttgatgttgaaaatac (SEQ ID NO:15)

Colwellia R1 ttaaaactaaaatcagcgagt (SEQ ID NO:16).

5

Digoxigenin-labeled probes were generated using PCR DIG Probe Synthesis Kit (Roche) according to the manufacturers protocol for 30 cycles of 30 s each at 94°C, 55 °C, and 65°C followed by a 7 min incubation at 65°C and subsequent incubation at 4°C. The digoxigenin-labeled probes were used in a Southern hybridization to probe for homologous sequences in *M. marina* total genomic DNA with *S. oneidensis* MR-1 and *C. psychrerythraea* as positive controls. The hybridization was performed at 30°C using DIG Easy Hyb (Roche) according to the manufacturer's protocol. The filter was washed twice using 0.5 x SSC, 0.1% SDS at room temperature. Dig-labeled probes were visualized using the Anti-Digoxigenin-AP, Fab fragments and Dig Wash and Block Buffer Set (Roche) according to the manufacturers protocol.

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The strongest signals from the *M. marina* DNA were obtained using the *Colwellia* probe. In some cases these signals coincided with weak signals obtained from *M. marina* DNA using the *Shewanella* probe.

Based on the Southern hybridization experiment, *Bgl*III- and *Pst*I-digests of *M. marina* DNA were chosen to clone the hybridizing fragments. Total genomic DNA was digested with *Bgl*III or *Pst*I, size-fractionated on an agarose gel and the appropriately sized fragment excised. The DNA fragments were purified using the Qiagen Gel Extraction kit (Qiagen, Valencia, CA). Aliquots of fractionated DNA were run on an agarose gel and blotted onto a nylon membrane (Roche, Mannheim, Germany) using a Turboblotter (Schleicher & Schuell, Keene, NH), according to the manufacturers protocol. The target fragment was identified by a Southern hybridization using the *Colwellia* probe.

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The *Bgl*III fraction 5 and *Pst*I fraction 4 were chosen to generate a partial library in pSP72 (Promega, Madison, WI). These libraries were transformed into *Escherichia coli* DH5α and pools of clones were aliquoted into the wells of a 96-well plate for over night growth. Culture aliquots were spun down, the supernatant was discarded, and the cell pellets were resuspended in 10 μl 10% SDS solution. The cell pellets were heated for 1 min to 100°C and spotted on nylon membranes (Roche). DNA was denatured by 5 min incubation in 0.5 M NaOH, containing 1.5 M NaCl, neutralized by 5 min incubation in 0.5 M Tris/HCl, pH 7.6 containing 1.5 M NaCl, washed for 5 min in 2 x SSC, and fixed by 1 min UV incubation in a Stratagene UV-Stratalinker 2400 (Stratagene, La Jolla, CA) according to the

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manufacturers protocol. The blot was probed with the *Colwellia ppt* probe. Positive signals were traced back to the well of origin and an aliquot from those wells was plated in order to obtain single colonies. Those single colonies were inoculated into 250 µl LB containing 100 mg/l carbampicillin. Cells were grown and the hybridization procedure described above was repeated to identify wells containing single positive clones. Positive clones were grown and plasmid DNA was isolated and digested with *Bgl*II, *Pvu*II, *Pst*I, or *Sal*I. These digests were used in a Southern hybridization to confirm positive clones. At this point all remaining clones were found to be positive.

Three of the final clones (two *Bgl*II clones and one *Pst*I clone) were chosen for DNA sequence analysis. Bioinformatics analysis of the complete sequence revealed that the *Pst*I clone contained only a partial *Mm-ppt*, while the *Bgl*II clones contained the complete open reading frame. The complete DNA sequence of all three clones was assembled in one contig. One *Bgl*II clone was chosen for further cloning experiments (pMON96400). The putative amino acid sequence of the *Mm-ppt* is shown in SEQ ID NO:5 if the start codon is TTG (referred to as *Mm-ppt long*). An alternative start codon using Met is found at amino acid 43 of SEQ ID NO:5 (yielding a polypeptide referred to as *Mm-ppt short* SEQ ID NO:7). The nucleic acid sequence of *Mm-ppt long* is SEQ ID NO:6. The nucleic acid sequence of *Mm-ppt short* is shown in SEQ ID NO:8. A comparison of the amino acid relatedness of the Ppts of the invention is shown in Table 1.

Table.1: Amino acid sequence identity of phosphopantetheinyl transferases

	<i>Colwellia psychrerythraea</i>	<i>Shewanella</i> SCRC 2738	<i>Schewanella oneidensis</i> MRI
<i>Moritella marina (long)</i>	60.9 %	31.5 %	30.0 %
<i>Colwellia psychrerythraea</i>		32.4 %	33.5 %
<i>Shewanella</i> SCRC 2738			46.6 %

Example 2

Expression of Phosphopantetheinyl Transferase Sequences in *Escherichia coli*

To demonstrate functionality of the *ppts* described in Example 1, the *Moritella marina* polyketide synthase (PKS) genes were cloned into the Novagen pDUET vectors (EMD, Biosciences, Darmstadt, Germany), a set of 4 compatible *E. coli* expression vectors. This PKS consists of 4 polypeptides encoded by the nucleic acids *orf5* (SEQ ID NO:20), *orf6* (SEQ ID NO:22), *orf7* (SEQ ID NO:24) and *orf8* (SEQ ID NO:26), which are described as *orf6*, *orf7*, *orf8*, and *orf9* in US 6,140,486, respectively. The expression vectors pMON94547

(Orf5 and Orf6) (FIG. 3), pMON94544 (Orf7) (FIG. 4) and pMON94534 (Orf8) (FIG. 5) were constructed using 3 of the pDUET vectors. The fourth pDUET vector was used for *ppt* expression.

To obtain an enzymatically active PKS, the Orf5 expression product requires pantethenylation, which is catalyzed by the Ppt. Each of the bacterial *ppts* was cloned into pACYC-DUET-1. The construction of pMON68081 and pMON68080 is described in Example 1. Similarly the two different putative *M. marina* *ppts*, *Mm-ppt short* or *Mm-ppt long*, were cloned into the same base vector as the *Colwellia* and the *Shewanella* *ppts* to yield pMON68084 (FIG. 6) and pMON68085 (FIG. 7), respectively. The *Mm-ppt long* PCRTM primer (SEQ ID NO:27) changed the putative TTG start to an ATG. Each *ppt* is then expressed together with the *M. marina* PKS genes in *E. coli*, incubated for 24h, and the lyophilized *E. coli* cells are methylated directly with sulfuric acid/methanol, and the fatty acid methyl esters are analyzed for EPA and DHA content by gas chromatography. The results are shown below in Table 2.

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Table 2

Gene combination	DHA Produced
PKS only	No
PKS + Mm-ppt long	Yes
PKS + Mm-ppt short	Yes
PKS + So-ppt	Yes
PKS + Cp-ppt	Yes
PKS - Orf8 + Cp-ppt	No

Co-expression of the complete *Moritella marina* PKS with any of the tested phosphopantetheinyl transferases in *E. coli* resulted in the accumulation of DHA, while expression of the *M. marina* PKS without co-expression of a Ppt did not result in DHA accumulation. Coexpression of the Cp-ppt with an incomplete PKS (missing Orf8) also did not result in DHA accumulation. These results demonstrate that all PPTs tested pantothenylated the *M. marina* PKS resulting in the formation of an active multi enzyme complex.

It has been demonstrated that Orf7 (Orf8 in U.S. Patent 6,140,486) controls the chain length in the final product of PUFA-producing PKSs. The PKS of *Shewanella putrefaciens* produces EPA. In experiments in *E. coli* containing the *S. putrefaciens* PKS cluster, an *orf7* deletion mutant produced DHA when complemented with *Moritella marina orf7*. The Ppt used to activate the PKS does not change the product, therefore the Ppts of this invention are

used to produce EPA when combined with an EPA-producing PKS and DHA when combined with a DHA-producing PKS.

Example 3

Expression of Phosphopantetheinyl Transferase Sequences in Plants

5 To demonstrate the ability of the *M. marina* PKS, including the *M. marina ppt*, to synthesize DHA in plants, several plant expression cassettes were generated. The genes for *orf5-8* were modified for expression in dicotyledonous plants. It is known that non-endogenous protein-encoding sequences may not express well in plants (U.S. Patent 5,880,275, herein incorporated by reference). Therefore, using the native PKS polypeptide sequences for Orfs5-8 (SEQ ID NOs: 19, 21, 23, and 25), artificial protein-encoding polynucleotide sequences were designed and constructed by 1) using a codon usage bias similar to that of highly expressed soybean proteins, and by 2) removal of RNA destabilizing elements previously characterized and known to affect mRNA stability *in planta* (U.S. Patent 5,880,275) and by introducing a Kozak sequence prior to the ATG start codon (Joshi *et al.*, 15 1997). The resulting modified polynucleotide sequences encode polypeptides identical in sequence to the native polypeptides.

The binary vector pMON97063 (FIG. 8) contains the expression cassettes for *orf5* (codon-modified, SEQ ID NO:28) (under the control of the FMV.35S-enh promoter with the L-Ph.DnaK leader) and *Mm-ppt short* (SEQ ID NO:8) (under the control of the CaMV35S-enh promoter and the L-CaMV35S leader). This vector carries the Bar gene as selectable marker. The binary vector pMON94563 (FIG. 9) was generated by cloning of the expression cassettes for *orf6* (codon-modified, SEQ ID NO:29) (under the control of the CaMV35S-enh promoter with the L-CaMV35S leader) *orf7* (codon-modified, SEQ ID NO:30) (under the control of the FMV35S-enh promoter with the L-Ph.DnaK leader), and *orf8* (codon-modified, 25 SEQ ID NO:31) (under the control of the CaMV35S-enh promoter with the L-CaMV35S leader). pMON94563 carries the CP4 marker which provides glyphosate resistance. The binary vector pMON97066 (FIG. 10) contains the same expression cassettes as pMON94563, but with the *orf7* cassette preceding the *orf6* cassette instead of following it. All constructs were sequence verified by DNA sequencing.

30 The binary vector pairs pMON97063 and pMON94563 or pMON97063 and pMON97066 are cotransformed into *Arabidopsis thaliana* using *Agrobacterium*-mediated transformation. Plants are regenerated and leaf material of the transformed R1 *Arabidopsis* plants and R2 seed of these plants is analyzed for fatty acid content and composition.

To generate a single multi gene binary vector harboring all 4 PKS genes and the *ppt* the low copy number binary vector pMON83934 was digested with *HindIII* and *NotI* and ligated with a polylinker consisting of the DNA oligomers MCS-3 (SEQ ID NO:32) and MCS-4 (SEQ ID NO:33). The resulting vector was designated pMON68091. The expression
 5 cassettes for *orf6*, *orf7*, *orf8* and the CP4 selectable marker were excised by *HindIII/BsiWI* digest from pMON94563 and ligated into *HindIII/BsiWI*-digested pMON68091. The resulting binary vector is digested with *AscI* and *BsiWI* and ligated with the expression cassettes containing *orf5* and *Mm-ppt* from pMON97063 excised by *BsiWI/AscI* digest. The resulting binary vector, pMON96401 (FIG. 11), is transformed via *Agrobacterium*-mediated
 10 transformation into *Arabidopsis thaliana* and soybean. Plants are regenerated and leaf and seed material from these plants are analyzed for fatty acid content and composition.

48 R1 events containing pMON96401 were generated in *Arabidopsis*. Mature R2 seed from this study was analyzed by gas chromatography. 9 of the 48 events analyzed produced DHA (Table 3).

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Table 3 DHA content of pMON96401-containing seed

Event	Construct	Generation	DHA
AT_G3764	pMON96401	R2	0.07
AT_G3756	pMON96401	R2	0.05
AT_G3732	pMON96401	R2	0.04
AT_G3737	pMON96401	R2	0.03
AT_G3730	pMON96401	R2	0.03
AT_G3740	pMON96401	R2	0.03
AT_G3728	pMON96401	R2	0.02
AT_G3750	pMON96401	R2	0.02
AT_G3748	pMON96401	R2	0.02
Control		VARIETY	0

Molecular characterization of 4 DHA-containing events of R2 *Arabidopsis* transformed with pMON96401 seed are represented in Table 4. The data demonstrates that events that produced DHA were positive for the presence of the 5 transgenes as determined
 20 by TaqMan ® (Applied Biosystems, Foster City, CA) endpoint assay.

Table 4 Arabidopsis pMON96401 gene presence

Sample	DHA	PKS5	PKS-Ppt	PKS6	PKS7	PKS8
Control	0	neg	neg	neg	neg	neg
At G3748	0.02	POS	POS	POS	POS	POS
At G3756	0.04	POS	POS	POS	POS	POS
At G3764	0.04	POS	POS	POS	POS	POS
At G3764	0.07	POS	POS	POS	POS	POS

In the R3 generation of pMON96401 *Arabidopsis* seed, the phenotype persisted with a range of 0.025-0.1% DHA by gas chromatography. The gas chromatography peak was confirmed as DHA by gas chromatography/time of flight mass spectrometry with fish oil as a standard.

For seed-specific expression of the *Moritella marina* PKS, the native or codon-modified genes are cloned as single gene expression cassettes using seed-specific promoters such as p7Sa, p7Sa', Arcelin-5, USP88, pNapin, pFAE or pOleosin. Subsequently these expression cassettes are assembled to combine all five genes in a single binary vector using a low copy number binary vector such as pMON83934 as base vector. The resulting five-gene vectors (each of them harboring all four PKS genes plus the ppt expression cassette) may contain the expression cassettes in varying order or orientation relative to each other. These vectors are transformed into soybean and the resulting soybean seed are analyzed for fatty acid content and composition.

An example of a multi-gene vector for seed-specific expression of the *M. marina* PKS and *M. marina* ppt follows. Expression cassettes for seed-specific expression of the dicot codon-enhanced PKS and ppt genes are assembled as described in Table 5. The expression cassettes are assembled in the head to tail orientation resulting in the formation of pMON78528 (FIG. 12). This binary vector is transformed into soybean and *Arabidopsis* using *Agrobacterium*-mediated transformation and the resulting seed are analyzed for fatty acid content and composition.

Table 5: Seed-specific expression cassettes for *M. marina* PKS.

PROMOTER	GOI	3' UTR
napin (SEQ ID NO:35)	<i>Mm-ppt short</i> (SEQ ID NO:34)	napin 3' (SEQ ID NO:36)
Arcelin 5	<i>Orf7</i> (SEQ ID NO:30)	Arcelin 5 3'
7Sa'	<i>Orf6</i> (SEQ ID NO:29)	7Sa' 3'
7Sa	<i>Orf8</i> (SEQ ID NO:31)	nos 3'
USP88	<i>Orf5</i> (SEQ ID NO:28)	Adr12

To demonstrate the ability of the *M. marina* PKS, together with the *M. marina ppt*, to synthesize DHA in corn, several plant expression cassettes are generated. The genes for *orfs* 5-8 and a *ppt* are modified for expression in monocotyledonous plants. It is known that non-endogenous protein-encoding sequences may not express well in plants (U.S. Patent 5,880,275, herein incorporated by reference). Therefore, using the native Orf and Ppt polypeptide sequences previously described, artificial protein-encoding polynucleotide sequences are designed and constructed by 1) using a codon usage bias similar to that of highly expressed corn proteins, and by 2) removal of RNA destabilizing elements previously characterized and known to affect mRNA stability *in planta* (U.S. Patent 5,880,275). The resulting modified polynucleotide sequences encode polypeptides identical in sequence to the native polypeptides. Transformed explants are obtained through *Agrobacterium tumefaciens*-mediated transformation for vectors containing the modified polynucleotide sequences. Plants are regenerated from transformed tissue. The greenhouse-grown plants are then analyzed for gene of interest expression levels as well as oil composition, including DHA or EPA.

Example 4

Cloning of Polyketide Synthase Sequences

Eight candidate polyketide synthase genes were cloned from 2 species. The deduced amino acid sequences of *M. marina* PKS genes (SEQ ID NOs: 19, 21, 23 and 25) were used to search available databases for novel polyketide synthase genes in *Shewanella oneidensis* (ATCC # 700550) and *Colwellia psychrerythrae* (ATCC # BAA-681). *S. oneidensis* accumulates EPA while *C. psychrerythrae* accumulates DHA. Based on this, it was believed that the PUFA production in these bacteria would result from a PKS mechanism. The search yielded a set of 4 candidate PKS genes from each bacterium. Using PCR cloning techniques, these genes were cloned into TOPO cloning vectors, the sequence-verified, and subcloned in Duet expression vectors (see Table 6). Expression of the *S. oneidensis orf5* together with the *M. marina orf6*, *orf7*, *orf8*, and *ppt* in *E. coli* was found to result in the formation of up to

0.2% DHA as determined by gas chromatography, confirming the predicted function of the *S. oneidensis orf5*. Similarly, the function of each gene listed in Table 6 is confirmed by expression with the *M. marina* PKS genes in *E. coli* or by expression of the complete PKS gene from *Shewanella* or *Colwellia* or a combination of the two species in *E. coli*.

5 Alternatively the function is demonstrated in plants.

Table 6: *E. coli* expression vectors for *Shewanella* and *Colwellia* PKS genes.

Source organism	Gene designation	<i>E. coli</i> expression vector
<i>Shewanella oneidensis</i>	<i>orf5</i> SEQ ID NO: 37	pMON108255
<i>Shewanella oneidensis</i>	<i>orf6</i> SEQ ID NO: 38	pMON108256
<i>Shewanella oneidensis</i>	<i>orf7</i> SEQ ID NO: 39	pMON108258
<i>Shewanella oneidensis</i>	<i>orf8</i> SEQ ID NO: 40	pMON108259
<i>Moritella marina</i>	<i>orf6</i> SEQ ID NO: 22	pMON108252
<i>Shewanella oneidensis</i>	<i>orf5</i> SEQ ID NO: 37	
<i>Colwellia psychrerythrae</i>	<i>orf5</i> SEQ ID NO: 41	pMON108267
<i>Colwellia psychrerythrae</i>	<i>orf7</i> SEQ ID NO: 43	pMON108269
<i>Colwellia psychrerythrae</i>	<i>orf8</i> SEQ ID NO: 44	pMON108270
<i>Colwellia psychrerythrae</i>	<i>orf5</i> SEQ ID NO: 41 <i>orf6</i> SEQ ID NO: 42	pMON108268

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The references listed below are referred to, to the extent that they supplement, explain, provide a background for, or teach methodology, techniques, and/or compositions employed herein.

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The embodiments of the present invention for which an exclusive property or privilege is claimed are defined as follows:

1. A recombinant DNA molecule comprising a sequence selected from the group consisting of:

(a) a polynucleotide hybridizing to the complement of SEQ ID NO:6 or SEQ ID NO:8, under conditions of 5X SSC, 50% formamide and 42°C,

wherein the polynucleotide encodes a polypeptide with phosphopantetheinyl transferase activity;

(b) a polynucleotide encoding the polypeptide sequence of SEQ ID NO:5 or SEQ ID NO:7; and

(c) a polynucleotide encoding a polypeptide with phosphopantetheinyl transferase activity and having at least 75% sequence identity to the polypeptide sequence of SEQ ID NO:5 or SEQ ID NO:7;

wherein said recombinant DNA molecule is operably linked to a heterologous promoter.

2. The DNA molecule of claim 1, wherein the promoter is functional in a prokaryotic cell.

3. The DNA molecule of claim 1, wherein the promoter is functional in a eukaryotic cell.

4. The DNA molecule of claim 3, wherein the promoter is functional in a plant cell.

5. The DNA molecule of claim 4, wherein the promoter is a seed-enhanced promoter.

6. A host cell transformed with the recombinant DNA molecule of claim 1.

7. The host cell of claim 6, wherein the host cell further comprises a DNA molecule encoding a polyketide synthase polypeptide comprising a phosphopantetheine attachment site, wherein the DNA molecule encoding a polyketide synthase polypeptide is operably linked to a heterologous promoter.

8. The host cell of claim 7, wherein the polyketide synthase polypeptide comprises a phosphopantetheine attachment site from *Moritella marina*.
9. The host cell of claim 7, wherein the DNA molecule encoding a polyketide synthase polypeptide has at least 75% sequence identity to the polypeptide sequence of SEQ ID NO:19.
10. The host cell of claim 6, wherein the host cell is a plant cell.
11. The host cell of claim 6, wherein the host cell is a fungal or bacterial cell.
12. The host cell of claim 6, defined as exhibiting altered fatty acid biosynthesis relative to a cell of the same genotype as said host cell but lacking the recombinant DNA molecule of claim 1, wherein the altered fatty acid biosynthesis results in synthesis of at least one of docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA).
13. A plant cell comprising the recombinant DNA molecule of claim 1.
14. The cell of claim 13, wherein the cell is selected from the group consisting of canola, *Brassica campestris*, oilseed rape, rapeseed, soybean, crambe, mustard, castor bean, peanut, sesame, cottonseed, linseed, safflower, oil palm, flax, sunflower, corn, rice, barley, millet, rye, wheat, oat, alfalfa and sorghum cells.
15. The cell of claim 13, further defined as comprising a DNA molecule encoding a polyketide synthase.
16. A seed cell comprising the DNA molecule of claim 1.
17. Use of a transgenic plant having the cell of claim 13 for producing food or feed.
18. The use of claim 17, wherein the food or feed is oil, silage, meal, grain, starch, flour, or protein.
19. A food or feed composition comprising a detectable nucleic acid molecule comprising the recombinant DNA molecule of claim 1.

20. A food or feed composition comprising docosahexaenoic acid or eicosapentaenoic acid and the cell of claim 14.
21. The food or feed composition of claim 20, wherein the cell is of a species that does not produce docosahexaenoic acid or eicosapentaenoic acid.
22. Use of a transgenic plant having the recombinant DNA molecule of claim 1 for producing docosahexaenoic acid or eicosapentaenoic acid.
23. A food or feed composition produced from a plant having the plant cell according to claim 13, comprising docosahexaenoic acid or eicosapentaenoic acid and the plant cell of claim 13.

FIG. 1.

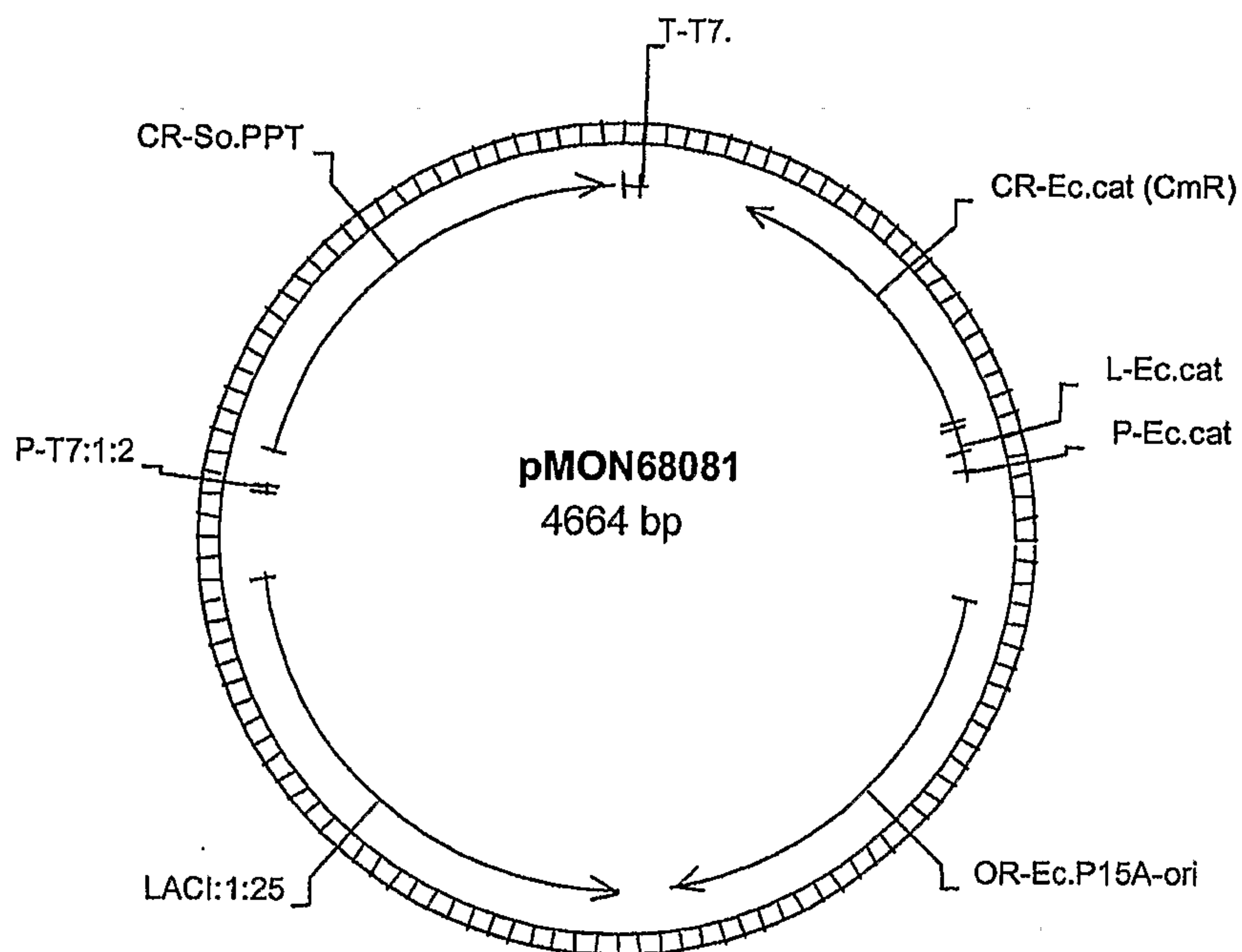


FIG. 2

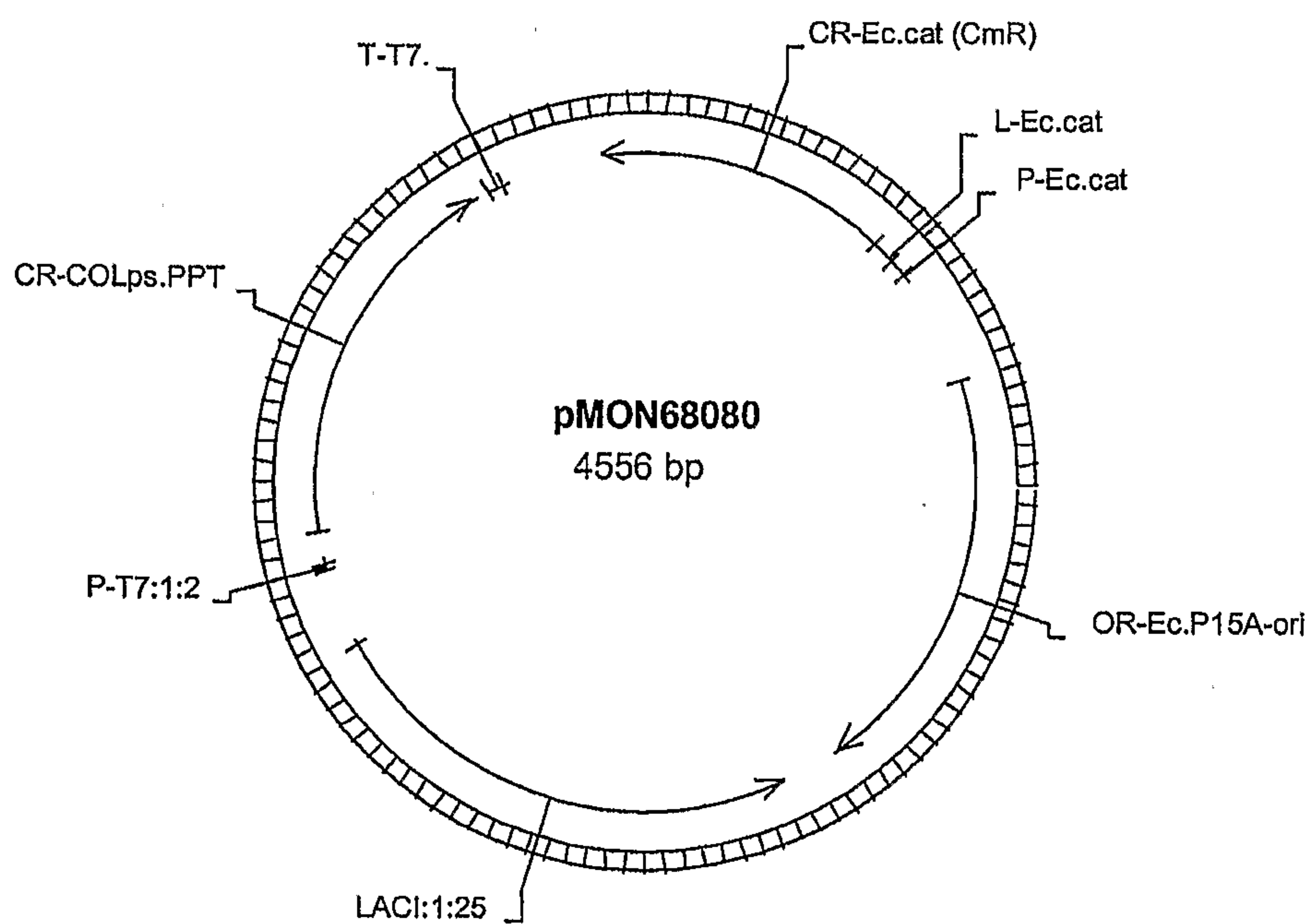


FIG. 3

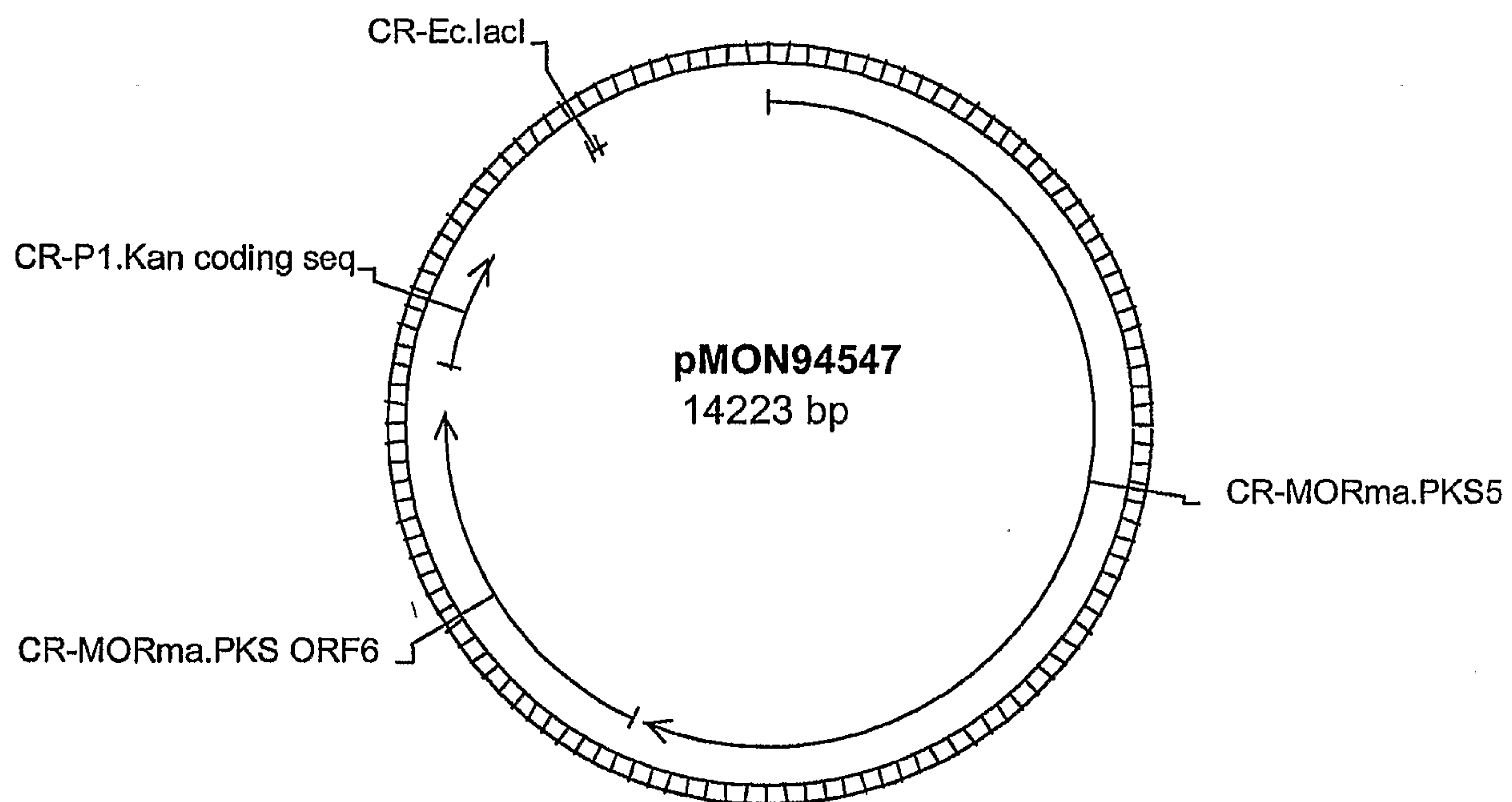


FIG. 4

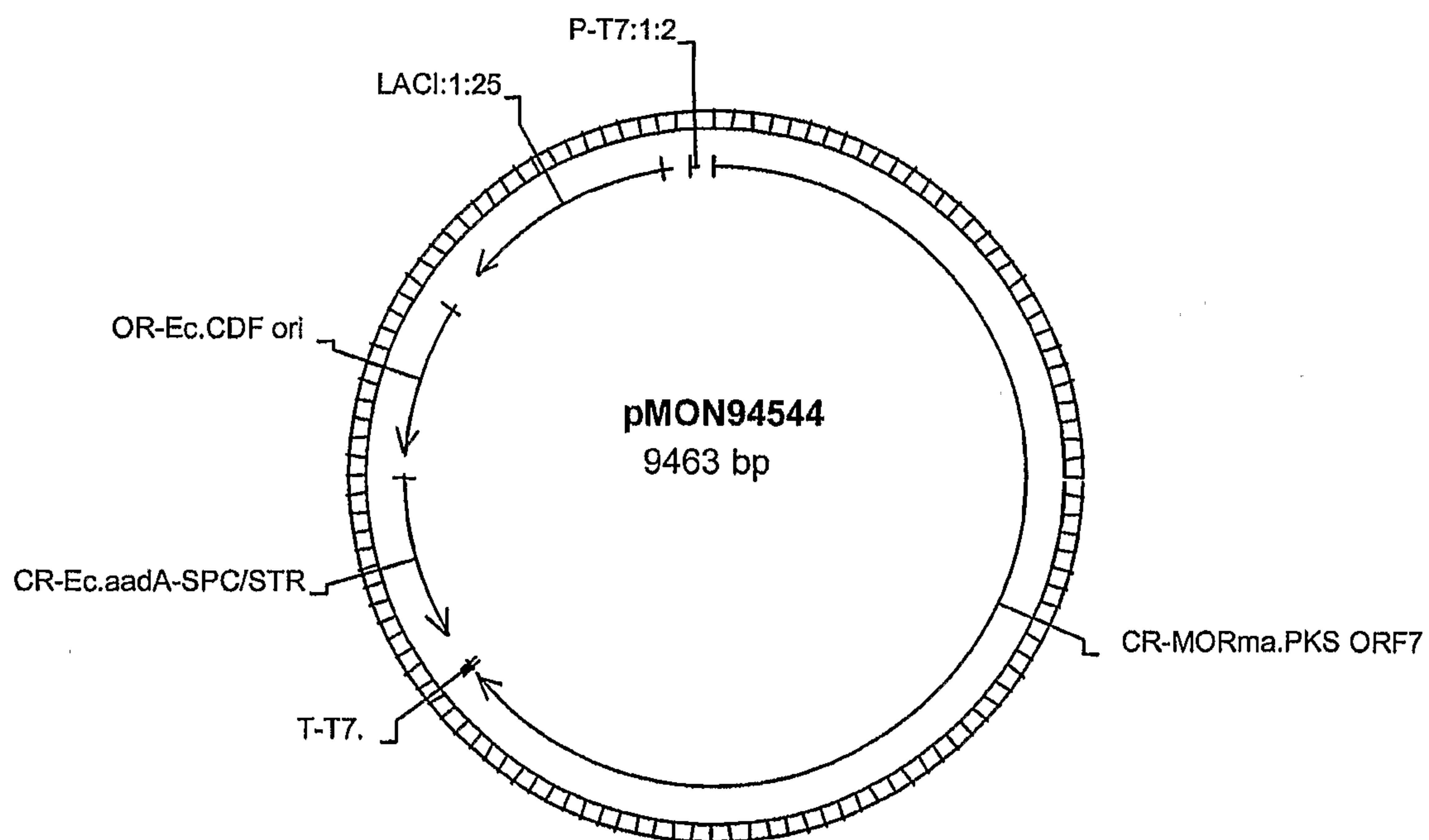


FIG. 5

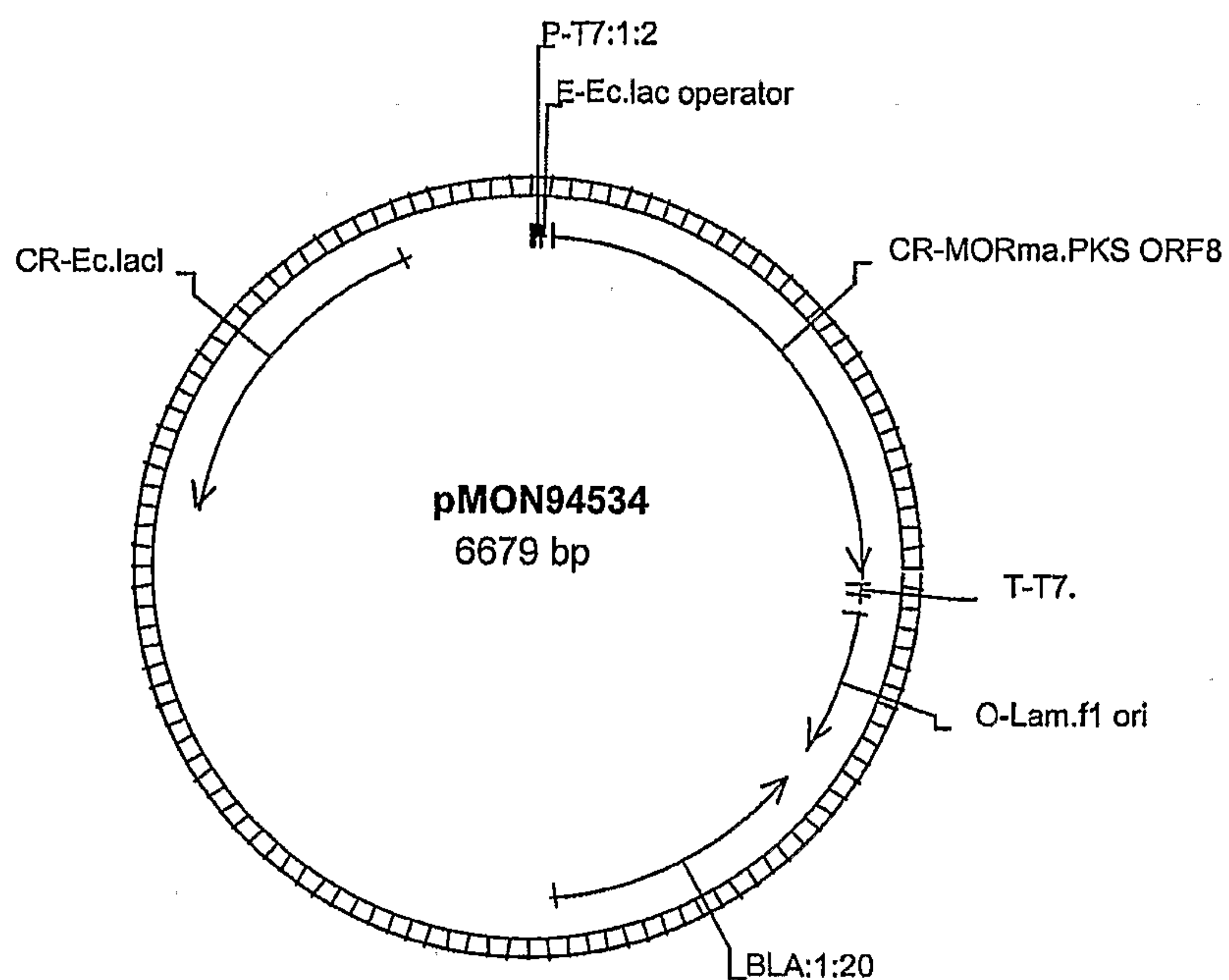


FIG. 6

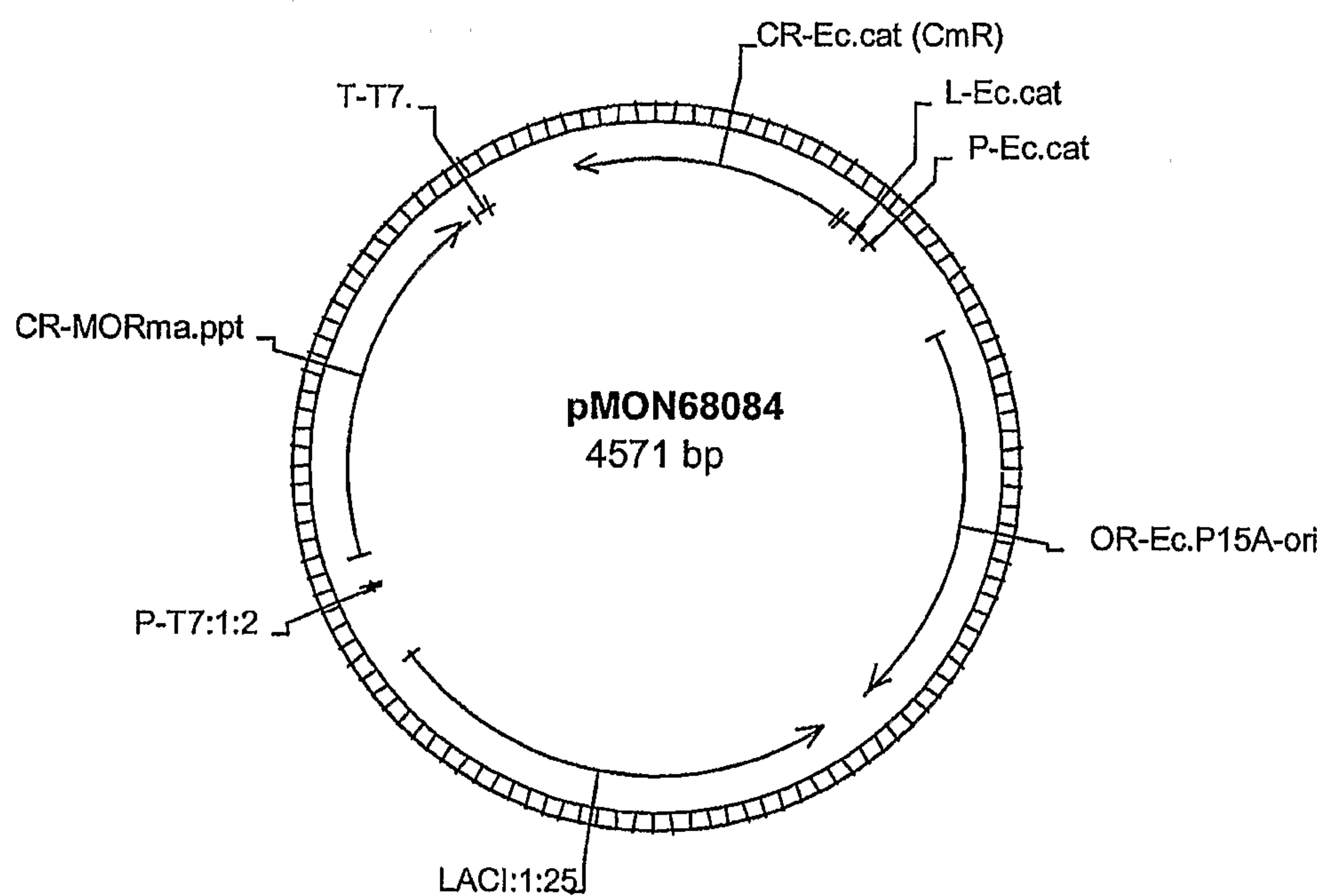


FIG. 7

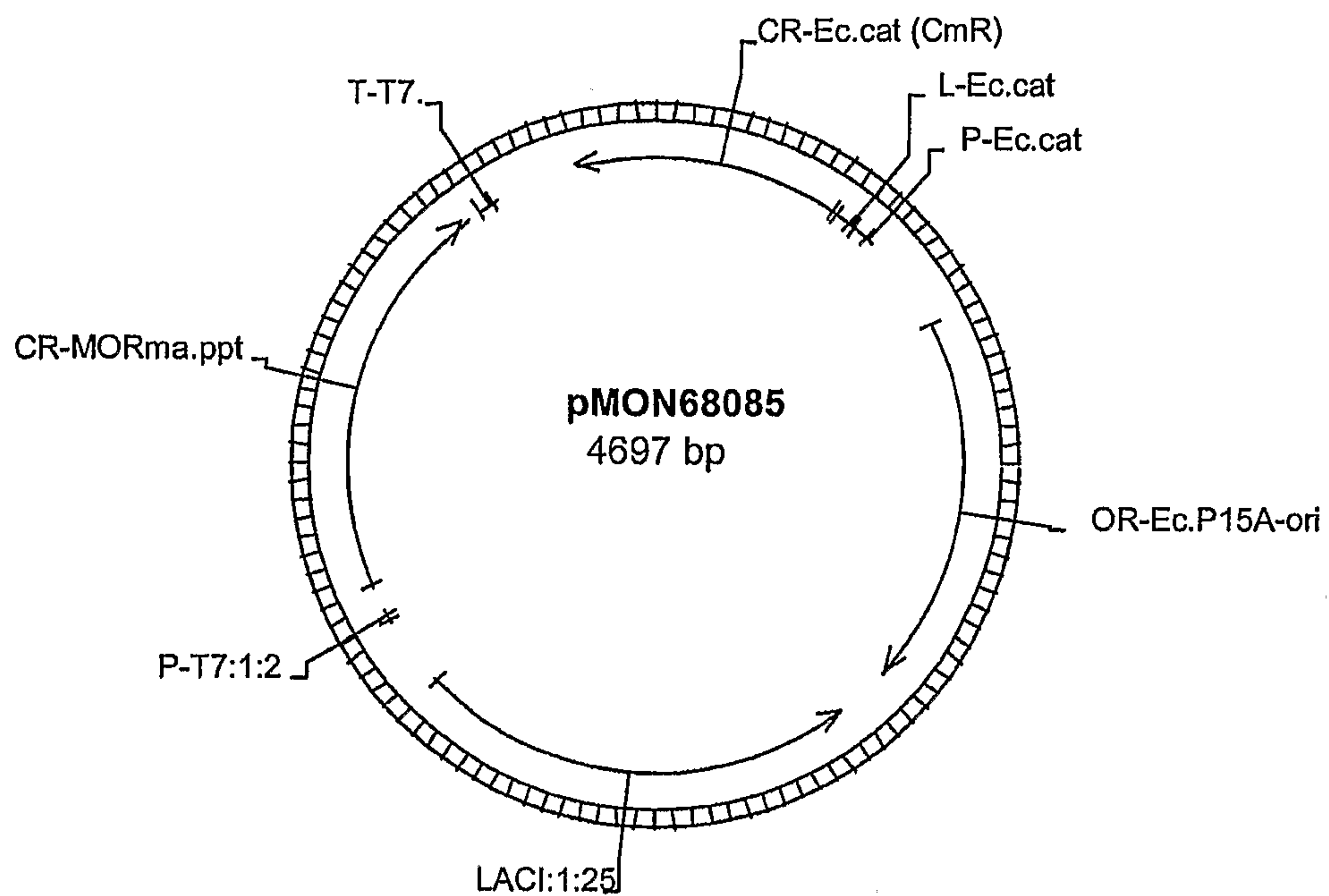


FIG. 8

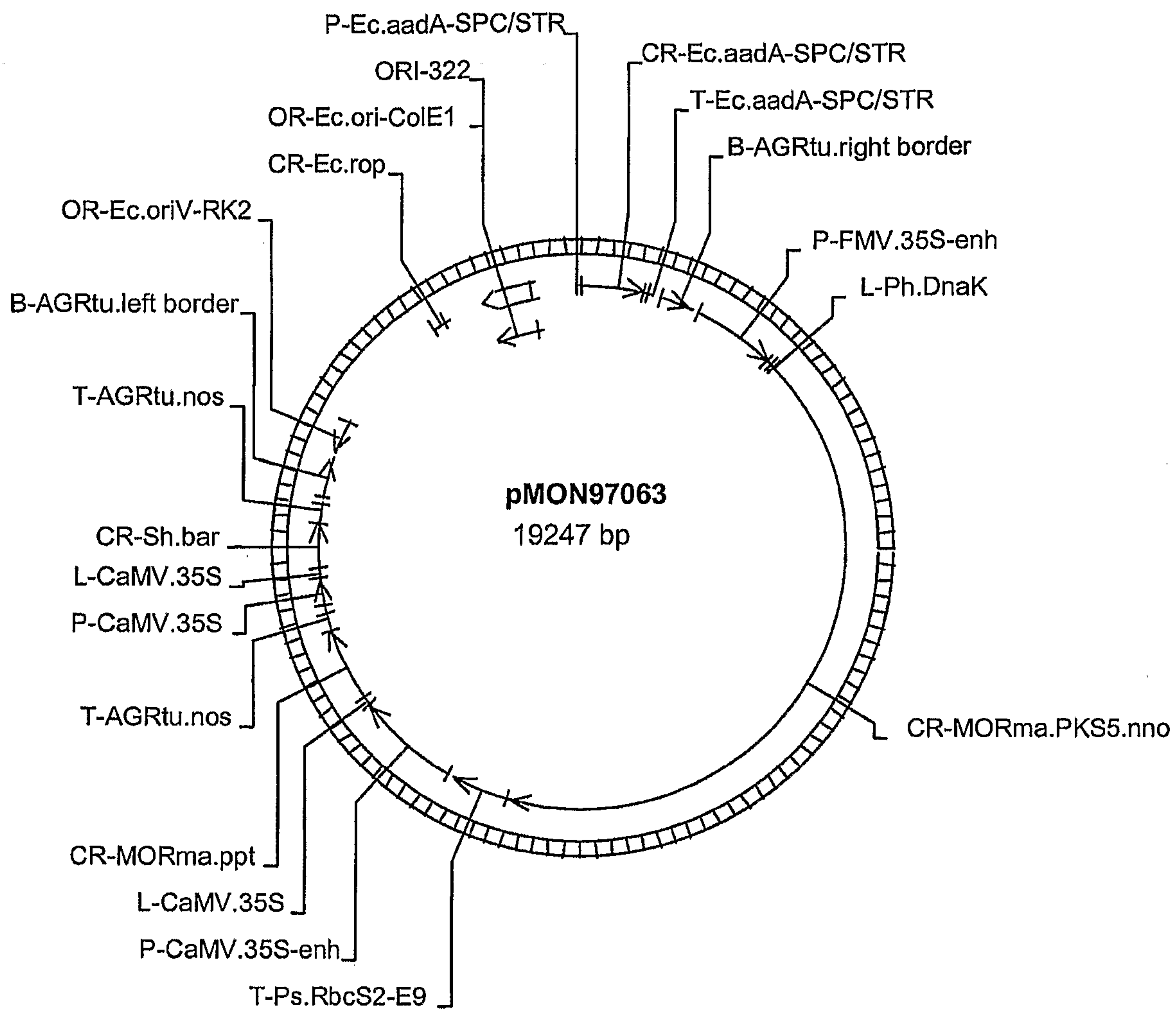


FIG. 9

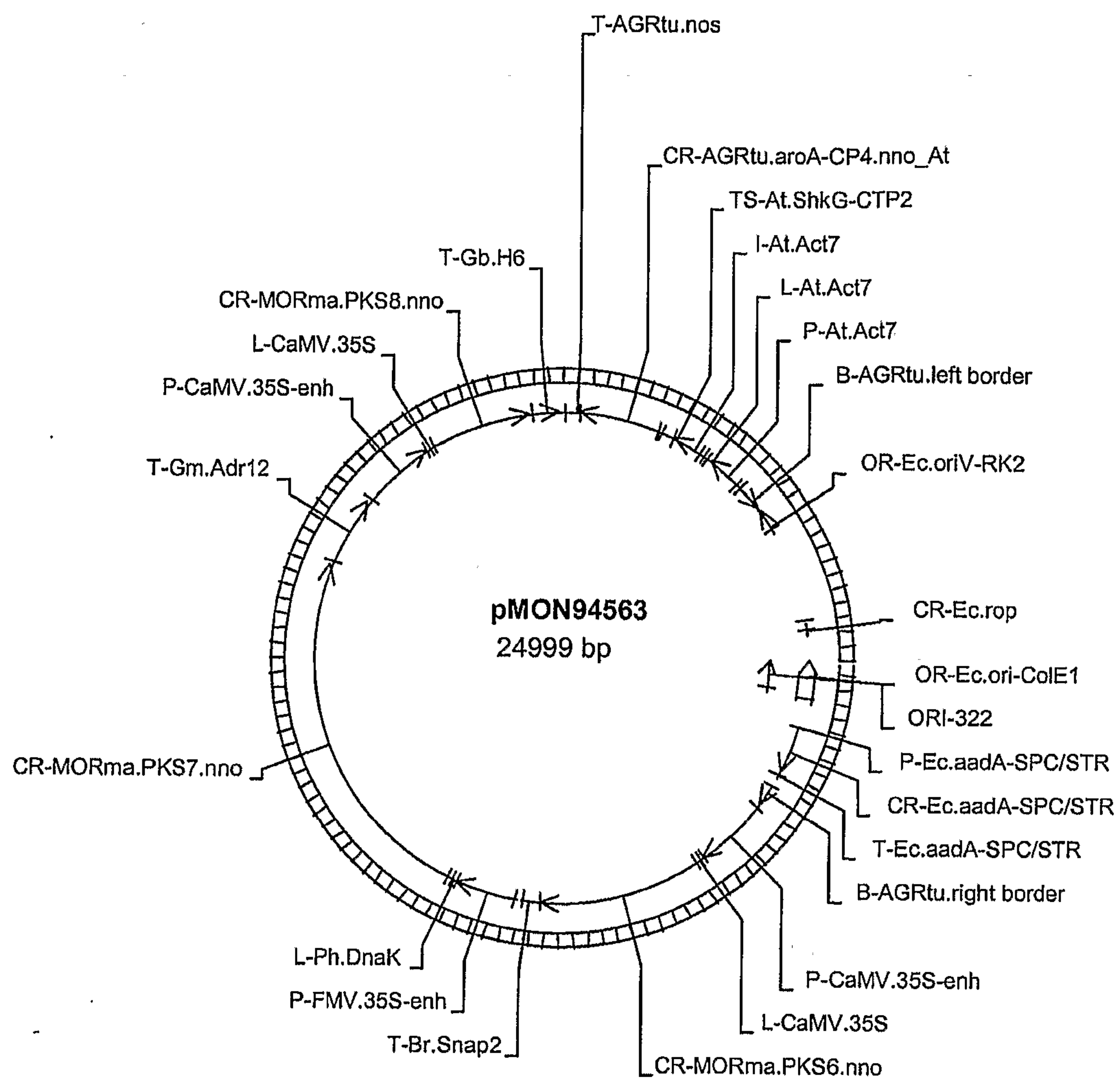


FIG. 10

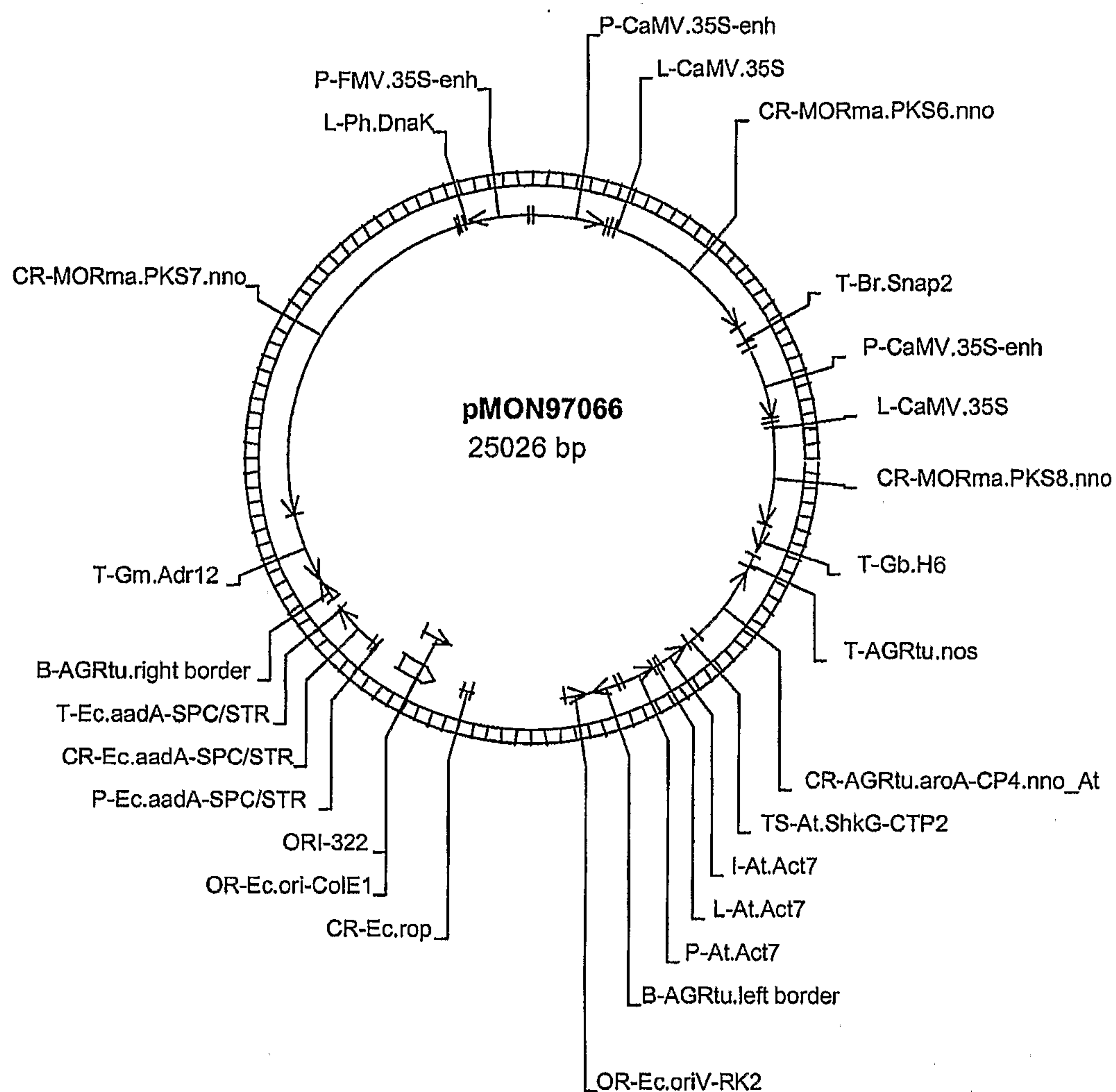


FIG. 11

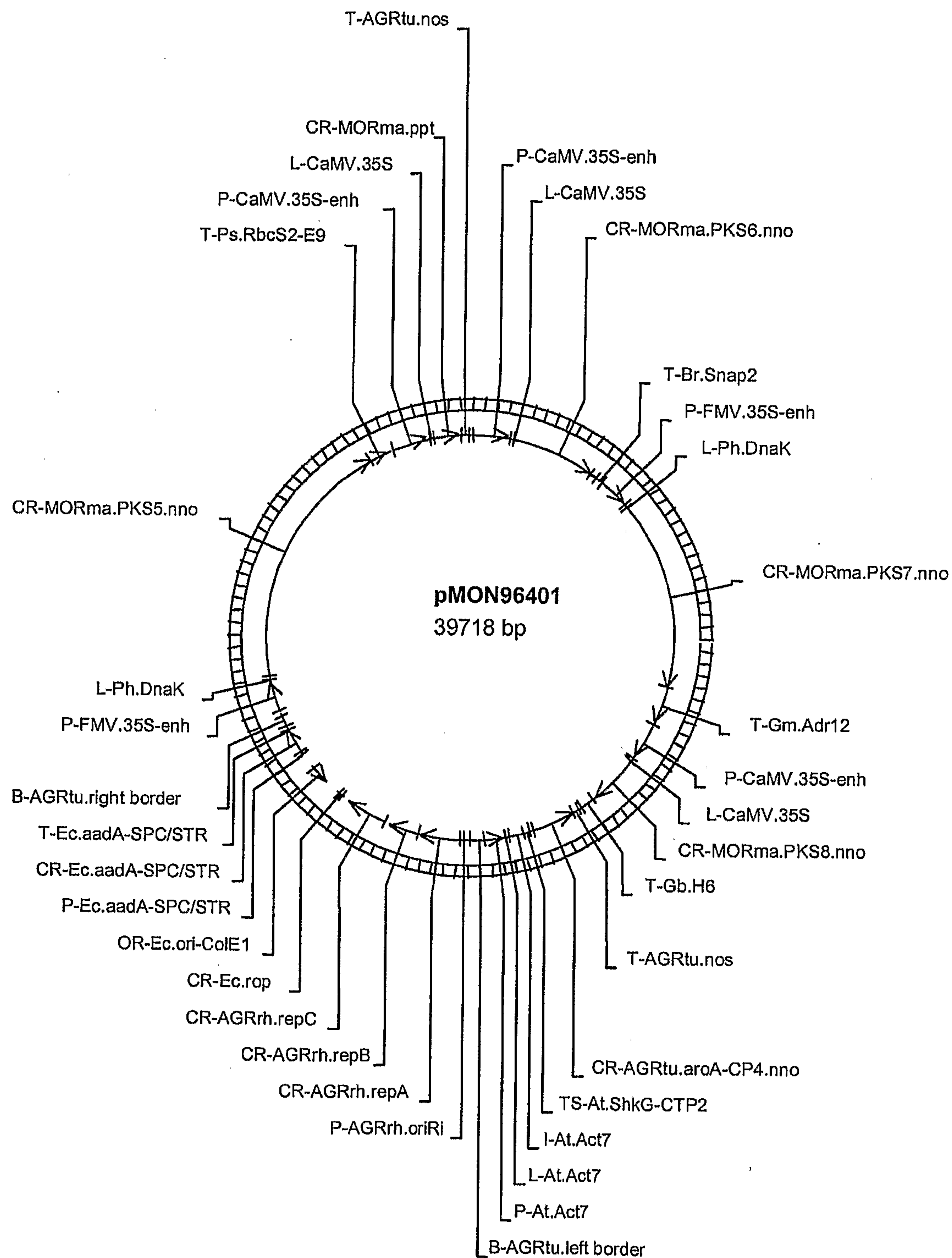


FIG. 12

