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CA 2581257 C 2014/04/15

(11)(21) 2 581 257

(12) BREVET CANADIEN
CANADIAN PATENT

(13) C

(86) Date de dépôt PCT/PCT Filing Date: 2005/09/16
(87) Date publication PCT/PCT Publication Date: 2006/03/30
(45) Date de délivrance/Issue Date: 2014/04/15
(85) Entrée phase nationale/National Entry: 2007/03/21
(86) N° demande PCT/PCT Application No.: EP 2005/010168
(87) N° publication PCT/PCT Publication No.: 2006/032469
(30) Priorités/Priorities: 2004/09/24 (EP04077624.7);
2004/11/17 (US60/628,826)

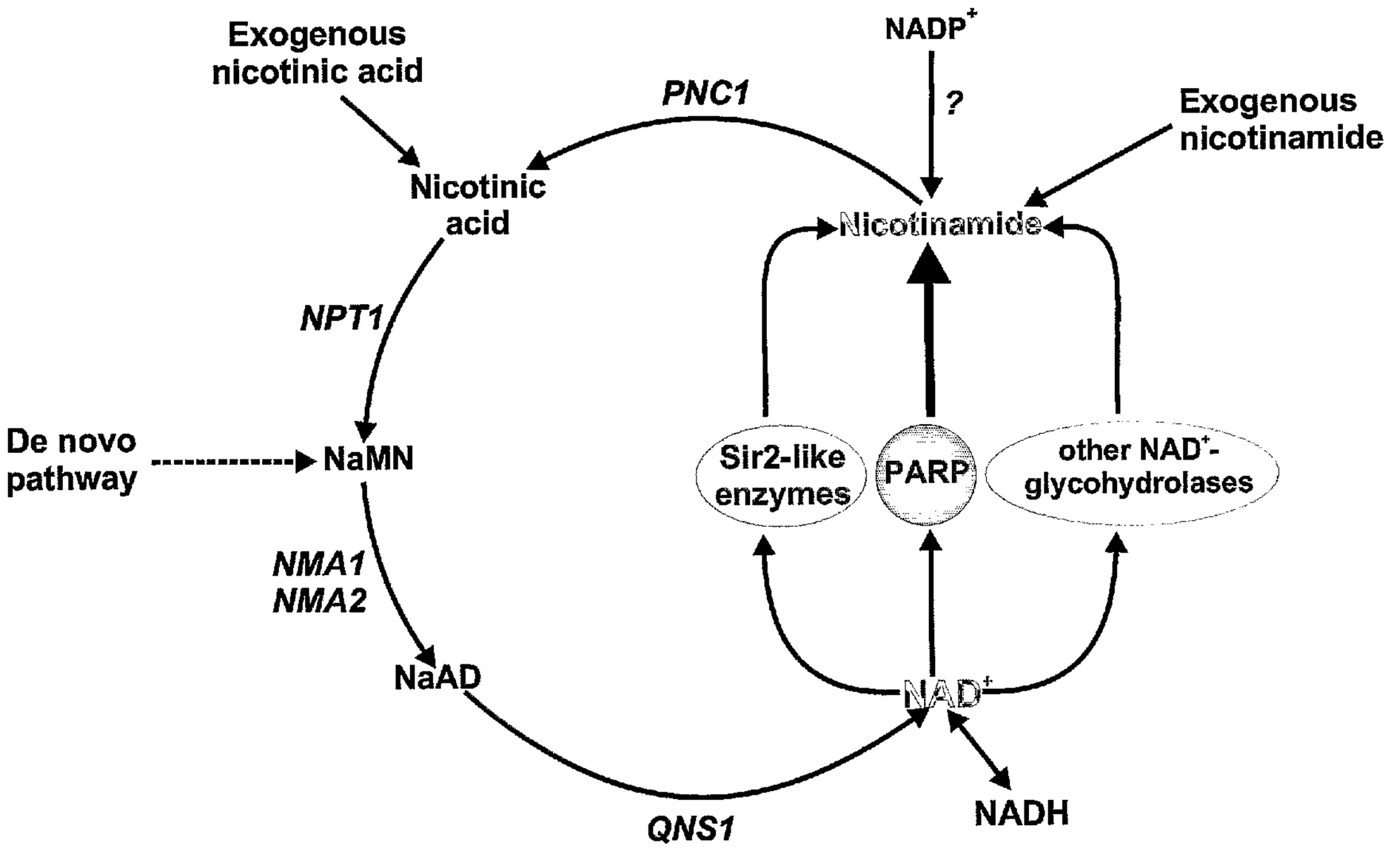
(51) Cl.Int./Int.Cl. *C12N 15/82* (2006.01),
A01H 5/00 (2006.01), *C12N 9/10* (2006.01),
C12N 9/12 (2006.01), *C12N 9/80* (2006.01)

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(54) Titre : VEGETAUX RESISTANT AU STRESS
(54) Title: STRESS RESISTANT PLANTS



NaMN: nicotinic acid mononucleotide
NaAD: desamino-NAD+

***PNC1*: nicotinamidase**
***NPT1*: nicotinate phosphoribosyl transferase**
***NMA*: NAD+ pyrophosphorylase**
***QNS1*: NAD+ synthetase**

(57) Abrégé/Abstract:
Stress tolerance in plants and plant cells is achieved by using nucleotide sequences encoding enzymes involved in the NAD salvage synthesis pathway and/or the NAD de novo synthesis pathway e.g. for overexpression in plants.

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
30 March 2006 (30.03.2006)

PCT

(10) International Publication Number
WO 2006/032469 A3

(51) International Patent Classification:
C12N 15/82 (2006.01) *C12N 9/12* (2006.01)
C12N 9/80 (2006.01) *A01H 5/00* (2006.01)
C12N 9/10 (2006.01)

(21) International Application Number:
PCT/EP2005/010168

(22) International Filing Date:
16 September 2005 (16.09.2005)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
04077624.7 24 September 2004 (24.09.2004) EP
60/628,826 17 November 2004 (17.11.2004) US

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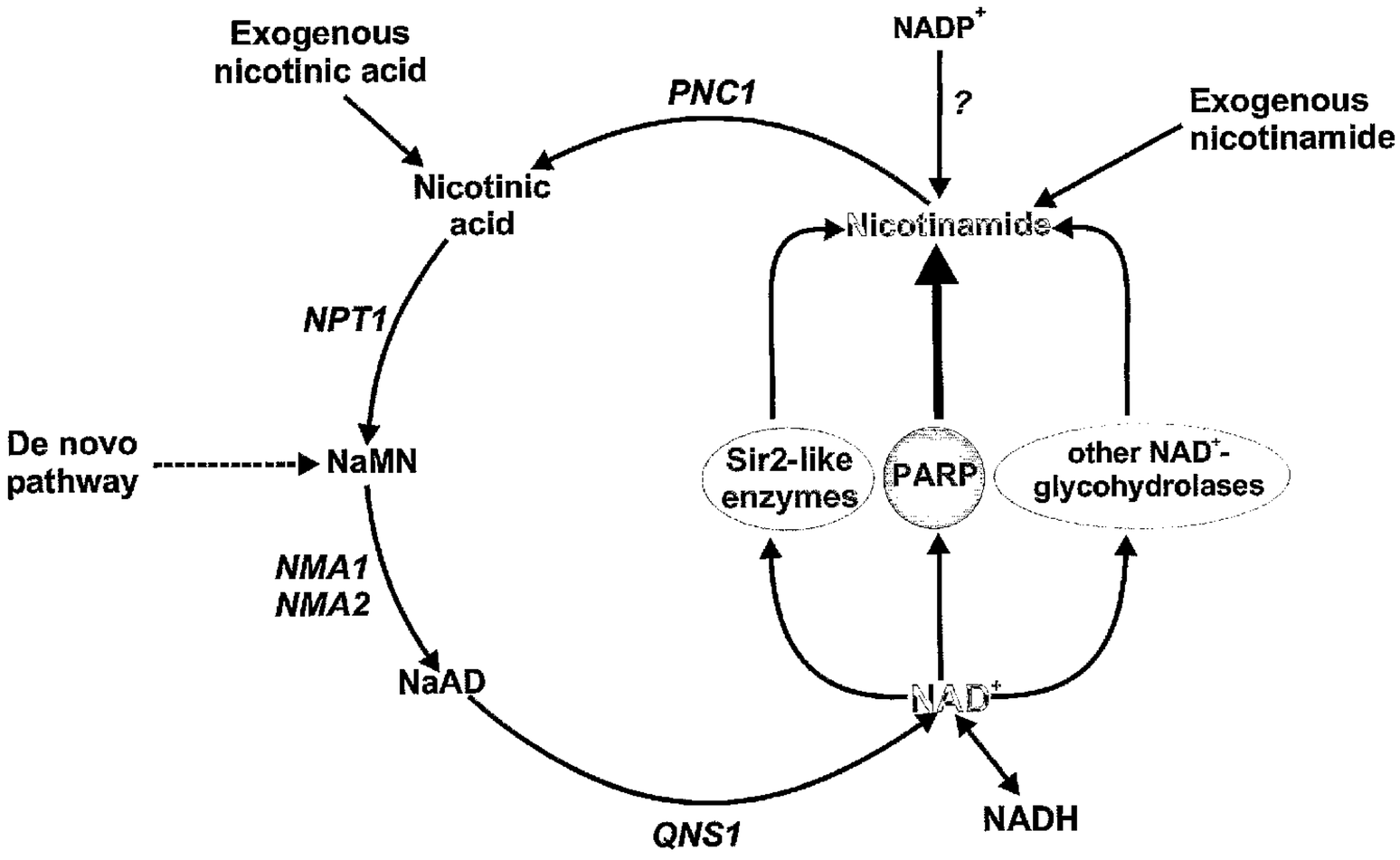
(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BW, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KM, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, LY, MA, MD, MG, MK, MN, MW, MX, MZ, NA, NG, NI, NO, NZ, OM, PG, PH, PL, PT, RO, RU, SC, SD, SE, SG, SK, SL, SM, SY, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, YU, ZA, ZM, ZW.

(84) Designated States *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HU, IE, IS, IT, LT, LU, LV, MC, NL, PL, PT, RO, SE, SI, SK, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Declarations under Rule 4.17:
— *as to the identity of the inventor (Rule 4.17(i))*
— *as to applicant's entitlement to apply for and be granted a patent (Rule 4.17(ii))*

[Continued on next page]

(54) Title: STRESS RESISTANT PLANTS



(57) Abstract: Stress tolerance in plants and plant cells is achieved by using nucleotide sequences encoding enzymes involved in the NAD salvage synthesis pathway and/or the NAD *de novo* synthesis pathway e.g. for overexpression in plants.

WO 2006/032469 A3

WO 2006/032469 A3

— *as to the applicant's entitlement to claim the priority of the earlier application (Rule 4.17(iii))*
— *of inventorship (Rule 4.17(iv))*

Published:
— *with international search report*
— *before the expiration of the time limit for amending the claims and to be republished in the event of receipt of amendments*

(88) Date of publication of the international search report:
6 July 2006

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

DEMANDE OU BREVET VOLUMINEUX

LA PRÉSENTE PARTIE DE CETTE DEMANDE OU CE BREVET COMPREND PLUS D'UN TOME.

CECI EST LE TOME 1 DE 2
CONTENANT LES PAGES 1 À 41

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NOM DU FICHIER / FILE NAME :

NOTE POUR LE TOME / VOLUME NOTE:

STRESS RESISTANT PLANTS

Methods are provided for increasing the stress resistance in plants and plant cells whereby enzymes involved in the NAD salvage synthesis pathway and/or the NAD *de novo* synthesis pathway are expressed in plants.

Background art

Tolerance of plants to adverse growing conditions, including drought, high light intensities, high temperatures, nutrient limitations, saline growing conditions and the like, is a very desired property for crop plants, in view of the never-ending quest to ultimately increase the actual yield of these plants.

Various ways of achieving that goal of improving what is commonly known as the stress resistance or stress tolerance of plants have been described. Since different abiotic stress conditions frequently result in the generation of harmfull reactive oxygen species ("ROS") such as superoxides or hydrogen peroxides, initial attempts to improve stress resistance in plants focused on prevention of the generation of the ROS or the removal thereof. Examples of these approaches are overexpression of ROS scavenging enzymes such as catalases, peroxidases, superoxide dismutases etc. or even increasing the amount of ROS scavenging molecules such as ascorbic acid, glutathione etc. These approaches and other attempts to engineer stress tolerant plants are reviewed e.g. in Wang et al. 2003, Planta 218:1-14.

Stress tolerance in plant cells and plants can also be achieved by reducing the activity or the level of the endogenous poly-ADP-ribose polymerases (ParP) or poly(ADP-ribose) glycohydrolases (ParG) as described in WO00/04173 and PCT/EP2004/003995, respectively. It is thought that in this way, fatal NAD and ATP depletion in plant cells subject to stress conditions, resulting in traumatic cell death, can be avoided or sufficiently postponed for the stressed cells to survive and acclimate to the stress conditions.

Uchimiya et al. (2002) et al. describe the isolation of a rice gene denoted YK1, as well as use of a chimeric YK1 gene to increase the tolerance of transgenic rice plants harboring that gene to rice blast and several abiotic stresses such as

NaCl, UV-C, submergence, and hydrogen peroxide. (Uchimiya et al., 2002, Molecular breeding 9: 25-31).

Uchimiya et al. further published a poster abstract describing that overexpression of a NAD dependent reductase gene (YK1) in rice cells also promoted the level of NAD(P)(H) through up-regulating NAD synthetase activities, and concluded that this modification in turn generated a pool of redox substances needed for ROS stress resistance (Uchimiya et al. 2003 Keystone symposium on Plant biology: Functions and control of cell death, Snowbird Utah April 10-15, 2003).

NAD synthetase from yeast has been well characterized and is the last enzyme in both the NAD *de novo* synthesis pathway and the NAD salvage pathway (see Figure 1). In the *de novo* pathway, quinolate is the precursor for NAD synthesis and is generated as a product of tryptophan degradation. In the salvage pathway, nicotinamide (which is a degradation product of NAD, generated through the action of various enzymes such as PARP, NAD-dependent deacetylases or other NAD glycohydrolases) is the precursor molecule. In a first step, nicotinamide is deamidated to nicotinic cid by a nicotinamidase. The nicotinic acid is transferred to 5-phosphoribosyl-1-pyrophosphate by the enzyme nicotinate phosphoribosyl transferase to yield nicotinic acid mononucleotide. This compound is shared between the *de novo* and the salvage pathway. Hence, further conversion of this compound by NAD+ pyrophosphorylase and NAD synthetase is achieved as in the *de novo* pathway.

In yeast, overexpression of PNC1 (encoding nicotinamidase) has been correlated with life span extension by calorie restriction and low-intensity stress (Anderson et al., 2003 Nature 423: p181-185; Gallo et al., 2004, Molecular and Cellular Biology 24: 1301-1312).

Little is known about the respective enzymes of the NAD biosynthesis pathways in plants. Hunt et al., 2004 describe the use of the available genomic information from Arabidopsis to identify the plant homologues of these enzymes (Hunt et al. , 2004, New Phytologist163(1): 31-44). The identified DNA sequences have the following Accession numbers: for nicotinamidase: At5g23220; At5g23230 and At3g16190; for nicotinate phosphoribosyltransferase: At4g36940, At2g23420, for

nicotinic acid mononucleotide adenyltransferase: At5g55810 and for NAD synthetase: At1g55090.

Alternative methods for increasing stress tolerance in plants are still required and the embodiments described hereinafter, including the claims, provide such methods and means.

Summary of the invention

In one embodiment of the invention, a method is provided for obtaining a plant with increased stress resistance comprising introducing a chimeric gene into a cells of a plant to obtain transgenic cells whereby the chimeric gene comprises the following operably linked DNA fragments:

i. A plant-expressible promoter;
ii. A DNA region coding for a plant-functional enzyme of the nicotinamide adenine dinucleotide salvage synthesis pathway selected from nicotinamidase, nicotinate phosphoribosyltransferase, nicotinic acid mononucleotide adenyl transferase or nicotinamide adenine dinucleotide synthetase;
iii. A 3'end region involved in transcription termination and polyadenylation,

followed by regenerating the transgenic cells to obtain a population of transgenic plants; and selecting a plant from the population of transgenic plants which exhibits increased stress resistance or selecting a plant which exhibits a reduced level of reactive oxygen species or maintains a high level of NADH under stress conditions when compared to a similar non-transgenic plant. The DNA region may code for a protein comprising an amino acid sequence selected from the aminoacid sequence of SEQ ID No.:2, SEQ ID No.:4, SEQ ID No.:6; SEQ ID No.:8, SEQ ID No.:10, SEQ ID No.:12; SEQ ID

No.:14; SEQ ID No.:16, SEQ ID No.:18, SEQ ID No.:20, SEQ ID No.: 22, SEQ ID No.:24 or a protein having about 60% sequence identity and having the enzymatic activity of nicotinamide adenine dinucleotide salvage synthesis pathway such as the nucleotide sequences of SEQ ID No.:1, SEQ ID No.:3, SEQ ID No.:5; SEQ ID No.:7, SEQ ID No.:9, SEQ ID No.:11; SEQ ID No.:13; SEQ ID No.:15, SEQ ID No.:17, SEQ ID No.:19, SEQ ID No.: 21 or SEQ ID No.:23.

In another embodiment, the invention relates to the chimeric genes as described herein, plant cells comprising these chimeric genes, and plants consisting essentially of plant cells comprising these chimeric genes, and seeds of such plants. These plants and plant cells may be characterized in that they have a lower level of reactive oxygen species under stress conditions than a similar plant not comprising such a chimeric gene.

In yet another embodiment, the invention relates to the use of the described chimeric genes to increase the stress resistance of a plant or to decrease the level of reactive oxygen species in a plant or a plant cell under stress conditions.

The invention further provides the use of a DNA sequence encoding a plant functional enzyme of the nicotinamide adenine dinucleotide salvage synthesis pathway selected from nicotinamidase, nicotinate phosphoribosyltransferase, nicotinic acid mononucleotide adenyl transferase or nicotinamide adenine dinucleotide synthetase, such as a a DNA sequence encoding a protein comprising an amino acid sequence selected from the aminoacid sequence of SEQ ID No.:2, SEQ ID No.:4, SEQ ID No.:6; SEQ ID No.:8, SEQ ID No.:10, SEQ ID No.:12; SEQ ID No.:14; SEQ ID No.:16, SEQ ID No.:18, SEQ ID No.:20, SEQ ID No.: 22, SEQ ID No.:24 or a protein having about 60% sequence identity and having the enzymatic activity of nicotinamide adenine dinucleotide salvage synthesis pathway, including a DNA sequence

comprising an nucleotide sequence selected from the nucleotide sequence of SEQ ID No.:1, SEQ ID No.:3, SEQ ID No.:5; SEQ ID No.:7, SEQ ID No.:9, SEQ ID No.:11; SEQ ID No.:13; SEQ ID No.:15, SEQ ID No.:17, SEQ ID No.:19, SEQ ID No.:21 or SEQ ID No.:23, to increase the stress resistance of a plant or to decrease the level of reactive oxygen species or maintain the level of NADH in a plant or a plant cell under stress conditions.

Specific aspects of the invention include:

- a method for obtaining a plant with increased stress resistance comprising a. introducing a chimeric gene into cells of a plant to obtain transgenic cells, said chimeric gene comprising the following operably linked DNA fragments: i. a plant-expressible promoter; ii. a DNA region coding for an enzyme of the nicotinamide adenine dinucleotide salvage synthesis pathway from yeast or fungi, said enzyme being selected from nicotinamidase, nicotinatephosphoribosyltransferase, nicotinic acid mononucleotide adenyl transferase or nicotinamide adenine dinucleotide synthetase; and iii. a 3' end region involved in transcription termination and polyadenylation; b. regenerating said transgenic cells to obtain a population of transgenic plants; and c. selecting a plant from said population of transgenic plants which exhibits increased stress resistance or selecting a plant which exhibits a reduced level of reactive oxygen species or maintains a high level of NADH under stress conditions when compared to a similar non-transgenic plant;

- use of a DNA molecule encoding an enzyme of the nicotinamide adenine dinucleotide salvage synthesis pathway from yeast or fungi, said enzyme being selected from nicotinamidase, nicotinatephosphoribosyltransferase, nicotinic acid mononucleotide adenyl transferase or nicotinamide adenine dinucleotide synthetase, to increase the stress resistance of a plant; and

- use of a DNA molecule encoding an enzyme of the nicotinamide adenine dinucleotide salvage synthesis pathway from yeast or fungi, said enzyme being

selected from nicotinamidase, nicotinatephosphoribosyltransferase, nicotinic acid mononucleotide adenyl transferase or nicotinamide adenine dinucleotide synthetase, to decrease the level of reactive oxygen species in a plant or a plant cell under stress conditions or to maintain the level of NADH in a plant or plant cell under stress conditions.

Brief description of the Figures

Figure 1 is a schematic representation of the NAD salvage pathway and the *de novo* NAD synthesis pathway as known in baker's yeast (*Saccharomyces cerevisea*).

Figures 2 to 11 are schematic representations of the various T-DNA vectors comprising DNA regions encoding enzymes from the NAD salvage pathway or the NAD *de novo* synthesis pathway under control of plant-expressible control elements. Abbreviations used are: RB: right T-DNA border; 3'35S: transcription termination and polyadenylation signal from CaMV 35S transcript; Cab22L: untranslated leader sequence of the Cab22L transcript; P35S2: CaMV 35S promoter; 3'g7: transcription termination and polyadenylation signal from Agrobacterium tumefaciens T-DNA gene 7; bar: phosphinotricin acetyltransferase coding region; pSSUAra promoter of the Rubisco small subunit transcript from Arabidopsis; LB; left T-DNA border; Sm/Sp: Spectinomycin and streptomycin resistance gene; pVS1ori; origin of VS1 suitable for replication in Agrobacterium; ColE1: origin of replication; NLS: nuclear localization signal; PNC1: DNA region coding for nicotinamidase from *Saccharomyces cereviseae*; npt1: the nicotinate phosphoribosyltransferase from *Saccharomyces cereviseae*; nma1: nicotinic acid mononucleotide adenyl transferase 1 from *Saccharomyces cereviseae*; nma2: nicotinic acid mononucleotide adenyl transferase 2 from *Saccharomyces cereviseae*; qns1: NAD synthetase (QNS1) from *Saccharomyces cereviseae*.

Detailed description

The current invention is based on the finding that DNA sequences encoding plant-functional enzymes from the NAD salvage pathway in yeasts could be used to obtain transgenic plants which were more resistant to stress, particularly abiotic stress, than plants not comprising these DNA sequences. The transgenic plants also exhibited a significantly reduced level of reactive oxygen species ("ROS") and maintained a high level of NADH, when put under stress conditions, compared to control plants

Thus in one embodiment of the invention, a method is provided to obtain a plant with increased stress resistance, whereby the method comprises the steps of

- introducing a stress resistant chimeric gene as herein described into cells of a plant to obtain cells comprising the stress resistant chimeric gene;
- regenerating these cells comprising the stress resistant chimeric gene to obtain a population of plants comprising the stress resistant chimeric gene; and
- selecting a plant from the population of these plants which exhibits increased stress resistance and/or decreased ROS level under stress conditions and/or maintains a high level of NADH, when compared to a similar non-transgenic plant.

The stress resistant chimeric gene thereby comprises a plant-expressible promoter operably linked to a DNA region coding for a plant-functional enzyme of the nicotinamide adenine dinucleotide salvage synthesis pathway selected from nicotinamidase, nicotinate phosphoribosyltransferase, nicotinic acid mononucleotide adenyl transferase or nicotinamide adenine dinucleotide synthetase and a 3'end region involved in transcription termination and polyadenylation.

As used herein, "a plant-functional enzyme of the nicotinamide adenine dinucleotide salvage synthesis pathway" is an enzyme which when introduced into plants, linked to appropriate control elements such as plant expressible promoter and terminator region, can be transcribed and translated to yield a enzyme of the NAD salvage synthesis pathway functional in plant cells. Included are the enzymes (and encoding genes) from the NAD salvage synthesis, which are obtained from a plant source, but also the enzymes obtained from yeast (Saccharomyces cereviseae) or from other yeasts or fungi. It is thought that the latter proteins may be even more suitable for the methods according to the invention, since these are less likely to be subject to the enzymatic feedback regulation etc. to which similar plant-derived enzymes may be subject.

Enzymes involved in the NAD salvage synthesis pathway comprise the following

- Nicotinamidase (EC 3.5.1.19) catalyzing the hydrolysis of the amide group of nicotinamide, thereby releasing nicotinate and NH3. The enzyme is also known as nicotinamide deaminase, nicotinamide amidase, YNDase or nicotinamide amidohydrolase
- Nicotinate phophoribosyltransferase (EC 2.4.2.11) also known as niacin ribonucleotidase, nicotinic acid mononucleotide glycohydrolase; nicotinic acid mononucleotide pyrophosphorylase; nicotinic acid phosphoribosyltransferase catalyzing the following reaction

  Nicotinate-D-ribonucleotide + diphosphate = nicotinate + 5-phospho-α-D-ribose 1-diphosphate
- Nicotinate-nucleotide adenylyltransferase, (EC 2.7.7.18) also known as deamido-NAD+ pyrophosphorylase; nicotinate mononucleotide adenylyltransferase; deamindonicotinamide adenine dinucleotide pyrophsophorylase; NaMT-ATase; nicotinic acid mononucleotide adenylyltransferase catalyzing the following reaction

  ATP+nicotinate ribonucleotide = diphosphate + deamido-NAD^+

- NAD-synthase (EC 6.3.1.5) also known as NAD synthetase; NAD^+synthase; nicotinamide adenine dinucleotide synthetase; diphosphopyridine nucleotide synthetase, catalyzing the following reaction

  Deamido-NAD^++ATP+NH3 = AMP+ diphosphate + NAD^+

In one embodiment of the invention, the coding regions encoding the different enzymes of the NAD salvage pathway comprise a nucleotide sequence encoding proteins with the amino acid sequences as set forth in SEQ ID Nos 2, 4, 6, 8 or 10, such as the nucleotide sequences of SEQ ID Nos 1, 3, 5, 7 or 9.

However, it will be clear that variants of these nucleotide sequences, including insertions, deletions and substitutions thereof may be also be used to the same effect. Equally, homologues to the mentioned nucleotide sequences from species different from *Saccharomyces cerevisea* can be used. These include but are not limited to nucleotide sequences from plants, and nucleotide sequences encoding proteins with the same amino acid sequences, as well as variants of such nucleotide sequences. Examples of the latter are nucleotide sequences encoding a protein with an amino acid sequence as set forth in SEQ ID Nos 12, 14, 16, 18, 20, 22 or 24 such as the nucleotide sequences of SEQ ID Nos 11, 13, 15, 17, 19, 21 or 23.

Variants of the described nucleotide sequence will have a sequence identity which is preferably at least about 80%, or 85 or 90% or 95% with identified nucleotide sequences encoding enzymes from the NAD salvage pathway, such as the ones identified in the sequence listing. Preferably, these variants will encode functional proteins with the same enzymatic activity as the enzymes from the NAD salvage pathway. For the purpose of this invention, the "sequence identity" of two related nucleotide or amino acid sequences, expressed as a percentage, refers to the number of positions in the two optimally aligned sequences which have identical residues (x100) divided by the number of positions compared. A gap, i.e. a position in an alignment where a residue is

present in one sequence but not in the other, is regarded as a position with non-identical residues. The alignment of the two sequences is performed by the Needleman and Wunsch algorithm (Needleman and Wunsch 1970). The computer-assisted sequence alignment above, can be conveniently performed using standard software program such as GAP which is part of the Wisconsin Package Version 10.1 (Genetics Computer Group, Madision, Wisconsin, USA) using the default scoring matrix with a gap creation penalty of 50 and a gap extension penalty of 3.

Nucleotide sequences homologous to the nucleotide sequences encoding an enzyme from the NAD salvage pathway in yeast, or encoding a homologous enzyme from an organism different than yeast may be identified by in silico analysis of genomic data, as described by Hunt et al. (vide supra).

Homologous nucleotide sequence may also be identified and isolated by hybridization under stringent conditions using as probes identified nucleotide sequences encoding enzymes from the NAD salvage pathway, such as the ones identified in the sequence listing.

"Stringent hybridization conditions" as used herein means that hybridization will generally occur if there is at least 95% and preferably at least 97% sequence identity between the probe and the target sequence. Examples of stringent hybridization conditions are overnight incubation in a solution comprising 50% formamide, 5 x SSC (150 mM NaCl, 15 mM trisodium citrate), 50 mM sodium phosphate (pH 7.6), 5x Denhardt's solution, 10% dextran sulfate, and 20 µg/ml denatured, sheared carrier DNA such as salmon sperm DNA, followed by washing the hybridization support in 0.1 x SSC at approximately 65 °C, preferably twice for about 10 minutes. Other hybridization and wash conditions are well known and are exemplified in Sambrook et al, Molecular Cloning: A Laboratory Manual, Second Edition, Cold Spring Harbor, NY (1989), particularly chapter 11.

Such variant sequences may also be obtained by DNA amplification using oligonucleotides specific for genes encoding enzymes from the NAD salvage pathway as primers, such as but not limited to oligonucleotides comprising about 20 to about 50 consecutive nucleotides selected from the nucleotide sequences of SEQ ID Nos 1, 3, 5, 7, 9, 11, 13, 15, 17, 19, 21, 23 or their complement.

The methods of the invention can be used to obtain plants tolerant to different kinds of stress-inducing conditions, particularly abiotic stress conditions including submergence, high light conditions, high UV radiation levels, increased hydrogen peroxide levels, drought conditions, high or low temperatures, increased salinity conditions. The methods of the invention can also be used to reduce the level of ROS in the cells of plants growing under adverse conditions, particularly abiotic stress conditions including submergence, high light conditions, high UV radiation levels, increased hydrogen peroxide levels, drought conditions, high or low temperatures, increased salinity conditions etc. The level of ROS or the level of NADH can be determined using the methods known in the art, including those described in Example 3.

Using the methods described herein, plants may be obtained wherein the level of ROS is equal to or lower than in control plants under non-stressed conditions, such as but not limited to low light. In these plants, under non-stressed conditions, the level of ROS may range from 50% to 100% of the level of control plants under low light conditions, more particularly from about 60% to about 85%. The level of the ROS in these plants under stress conditions is about 50% to 80% of the level of ROS in control plants under stress conditions, corresponding to about 60 to 80% of the level of ROS in control plants under non-stressed conditions. Similarly, the NADH level in these plants is equal to or higher than in control plants under non-stressed conditions, such as but not limited to low light. In these plants, under non-stressed conditions, the level of NADH may range from 100% to 160% of the level of NADH in control plants under low light

conditions, more particularly from about 120% to about 140%. The level of NADH in these plants under stress conditions is about 200 to 300% of the level of NADH in control plants under stress conditions, corresponding to about 100 to 160% of the level of ROS in control plants under non-stressed conditions.

Methods to obtain transgenic plants are not deemed critical for the current invention and any transformation method and regeneration suitable for a particular plant species can be used. Such methods are well known in the art and include *Agrobacterium*-mediated transformation, particle gun delivery, microinjection, electroporation of intact cells, polyethyleneglycol-mediated protoplast transformation, electroporation of protoplasts, liposome-mediated transformation, silicon-whiskers mediated transformation etc. The transformed cells obtained in this way may then be regenerated into mature fertile plants.

The obtained transformed plant can be used in a conventional breeding scheme to produce more transformed plants with the same characteristics or to introduce the chimeric gene according to the invention in other varieties of the same or related plant species, or in hybrid plants. Seeds obtained from the transformed plants contain the chimeric genes of the invention as a stable genomic insert and are also encompassed by the invention.

It will be clear that the different stress resistant chimeric genes described herein, with DNA regions encoding different enzymes from the NAD salvage pathway can be combined within one plant cell or plant, to further enhance the stress tolerance of the plants comprising the chimeric genes. Thus, in one embodiment of the invention, plant cells and plants are provided which comprise at least two stress resistant chimeric genes each comprising a different coding region.

The transgenic plant cells and plant lines according to the invention may further comprise chimeric genes which will reduce the expression of endogenous PARP and/or PARG genes as described in WO 00/04173 and PCT/EP2004/003995 .

These further chimeric genes may be introduced e.g. by crossing the transgenic plant lines of the current invention with transgenic plants containing PARP and/or PARG gene expression reducing chimeric genes. Transgenic plant cells or plant lines may also be obtained by introducing or transforming the chimeric genes of the invention into transgenic plant cells comprising the PARP or PARG gene expression reducing chimeric genes or vice versa.

For the purpose of the invention, the promoter is a plant-expressible promoter. As used herein, the term "plant-expressible promoter" means a DNA sequence which is capable of controlling (initiating) transcription in a plant cell. This includes any promoter of plant origin, but also any promoter of non-plant origin which is capable of directing transcription in a plant cell, i.e., certain promoters of viral or bacterial origin such as the CaMV35S (Harpster et al., 1988 *Mol. Gen. Genet.* **212**, 182-190), the subterranean clover virus promoter No 4 or No 7 (WO9606932), or T-DNA gene promoters but also tissue-specific or organ-specific promoters including but not limited to seed-specific promoters (e.g., WO89/03887), organ-primordia specific promoters (An et al., 1996, *The Plant Cell* **8**, 15-30), stem-specific promoters (Keller et al., 1988, *EMBO J.* **7**, 3625-3633), leaf specific promoters (Hudspeth et al., 1989, *Plant Mol Biol* **12**, 579-589), mesophyl-specific promoters (such as the light-inducible Rubisco promoters), root-specific promoters (Keller et al.,1989, *Genes Devel.* **3**, 1639-1646), tuber-specific promoters (Keil et al., 1989, *EMBO J.* **8**, 1323-1330), vascular tissue specific promoters (Peleman et al., 1989, *Gene* **84**, 359-369), stamen-selective promoters (WO 89/10396, WO 92/13956), dehiscence zone specific promoters (WO 97/13865) and the like.

The chimeric genes of the inventions may also be equipped with a nuclear localization signal ("NLS") functional in plants, operably linked to the DNA region encoding an enzyme of the NAD salvage pathway such as the SV40 NLS.

Having read this document, a person skilled in the art will immediately realize that similar effects with regard to increased stress resistance can be obtained whenever natural variants of plants are obtained wherein the endogenous genes coding for NAD salvage pathway enzymes are more active or expressed at a higher level. Such variant plants can be obtained by subjecting a population of plants to mutagenesis, such as, but not limited to EMS mutagenesis, followed by a screening for an increased activity of any one of the NAD salvage pathway enzymes, or a combination thereof.

It will also be immediately clear that a population of different varieties or cultivars can be screened for increased tolerance to the above mentioned stress conditions in general or particular selected abiotic stresses, followed by a correlation of the increased tolerance to stress conditions with the presence of a particular allele of any of the endogenous genes encoding an enzyme of the NAD salvage pathway enzyme. Such alleles can than be introduced into a plant of interest by crossing, if the species are sexually compatible, or they may be identified using conventional techniques as described herein (including hybridization or PCR amplification) and introduced using recombinant DNA technology. Introduction of particularly desired alleles using breeding techniques may be followed using molecular markers specific for the alleles of interest.

The methods and means described herein are believed to be suitable for all plant cells and plants, both dicotyledonous and monocotyledonous plant cells and plants including but not limited to cotton, Brassica vegetables, oilseed rape, wheat, corn or maize, barley, sunflowers, rice, oats, sugarcane, soybean, vegetables (including chicory, lettuce, tomato), tobacco, potato, sugarbeet, papaya, pineapple, mango, *Arabidopsis thaliana*, but also plants used in horticulture, floriculture or forestry.

As used herein “comprising” is to be interpreted as specifying the presence of the stated features, integers, steps or components as referred to, but does not

preclude the presence or addition of one or more features, integers, steps or components, or groups thereof. Thus, e.g., a nucleic acid or protein comprising a sequence of nucleotides or amino acids, may comprise more nucleotides or amino acids than the actually cited ones, i.e., be embedded in a larger nucleic acid or protein. A chimeric gene comprising a DNA region which is functionally or structurally defined, may comprise additional DNA regions etc.

The following non-limiting Examples describe the construction of chimeric genes to increase stress resistance in plant cells and plants and the use of such genes.

Unless stated otherwise in the Examples, all recombinant DNA techniques are carried out according to standard protocols as described in Sambrook et al. (1989) Molecular Cloning: A Laboratory Manual, Second Edition, Cold Spring Harbor Laboratory Press, NY and in Volumes 1 and 2 of Ausubel et al. (1994) Current Protocols in Molecular Biology, Current Protocols, USA. Standard materials and methods for plant molecular work are described in Plant Molecular Biology Labfax (1993) by R.D.D. Croy, jointly published by BIOS Scientific Publications Ltd (UK) and Blackwell Scientific Publications, UK. Other references for standard molecular biology techniques include Sambrook and Russell (2001) Molecular Cloning: A Laboratory Manual, Third Edition, Cold Spring Harbor Laboratory Press, NY, Volumes I and II of Brown (1998) Molecular Biology LabFax, Second Edition, Academic Press (UK). Standard materials and methods for polymerase chain reactions can be found in Dieffenbach and Dveksler (1995) PCR Primer: A Laboratory Manual, Cold Spring Harbor Laboratory Press, and in McPherson at al. (2000) PCR - Basics: From Background to Bench, First Edition, Springer Verlag, Germany.

Throughout the description and Examples, reference is made to the following sequences:

SEQ ID No. 1: nucleotide sequence of the nicotinamidase from *Saccharomyces cereviseae (PNC1).*

SEQ ID No. 2:. amino acid sequence of the nicotinamidase from *Saccharomyces cereviseae (PNC1).*

SEQ ID No. 3:. nucleotide sequence of the nicotinate phosphoribosyltransferase from *Saccharomyces cereviseae (NPT1)* (complement)

SEQ ID No. 4:. amino acid sequence of the nicotinate phosphoribosyltransferase from *Saccharomyces cereviseae (NPT1)*

SEQ ID No. 5: nucleotide sequence of the nicotinic acid mononucleotide adenyl transferase 1 (NMA1) from *Saccharomyces cereviseae.*

SEQ ID No. 6: amino acid sequence of the nicotinic acid mononucleotide adenyl transferase 1 (NMA1) from *Saccharomyces cereviseae*

SEQ ID No. 7: nucleotide sequence of the nicotinic acid mononucleotide adenyl transferase 2 (NMA2) from *Saccharomyces cereviseae.*

SEQ ID No. 8: amino acid sequence of the nicotinic acid mononucleotide adenyl transferase 2 (NMA2) from *Saccharomyces cereviseae.*

SEQ ID No. 9: nucleotide sequence of the NAD synthetase (QNS1) from *Saccharomyces cereviseae..*

SEQ ID No. 10: amino acid sequence of the NAD synthetase (QNS1) from *Saccharomyces cereviseae.*

SEQ ID No. 11:. nucleotide sequence of the nicotinamidase from *Arabidopsis thaliana (isoform 1).*

SEQ ID No. 12:. Amino acid sequence of the nicotinamidase from *Arabidopsis thaliana (isoform 1).*

SEQ ID No. 13:. nucleotide sequence of the nicotinamidase from *Arabidopsis thaliana (isoform 2)*

SEQ ID No. 14: Amino acid sequence of the nicotinamidase from *Arabidopsis thaliana (isoform 2).*

SEQ ID No. 15:. nucleotide sequence of the nicotinamidase from *Arabidopsis thaliana (isoform 3)*

SEQ ID No. 16: Amino acid sequence of the nicotinamidase from *Arabidopsis thaliana (isoform 3).*

SEQ ID No. 17: nucleotide sequence of the nicotinate phosphoribosyltransferase from *Arabidopsis thaliana (isoform 1).*

SEQ ID No. 18: amino acid sequence of the nicotinate phosphoribosyltransferase from *Arabidopsis thaliana (isoform 1).*

SEQ ID No. 19: nucleotide sequence of the nicotinate phosphoribosyltransferase from *Arabidopsis thaliana (isoform 2).*

SEQ ID No. 20: amino acid sequence of the nicotinate phosphoribosyltransferase from *Arabidopsis thaliana (isoform 2).*

SEQ ID No. 21: nucleotide sequence of the nicotinic acid mononucleotide adenyl transferase from *Arabidopsis thaliana.*

SEQ ID No. 22: amino acid sequence of the nicotinic acid mononucleotide adenyl transferase from *Arabidopsis thaliana.*

SEQ ID No. 23: nucleotide sequence of the NAD synthetase from *Arabidopsis thaliana.*

SEQ ID No. 24: amino acid sequence of the NAD synthetase from *Arabidopsis thaliana.*

SEQ ID No. 25: nucleotide sequence of T-DNA vector pTVE 467

SEQ ID No. 26: nucleotide sequence of T-DNA vector pTVE 468

SEQ ID No. 27: nucleotide sequence of T-DNA vector pTVE 469

SEQ ID No. 28: nucleotide sequence of T-DNA vector pTVE 470

SEQ ID No. 29: nucleotide sequence of T-DNA vector pTVE 496

SEQ ID No. 30: nucleotide sequence of T-DNA vector pTVE 497

SEQ ID No. 31: nucleotide sequence of T-DNA vector pTVE 500

SEQ ID No. 32: nucleotide sequence of T-DNA vector pTVE 501

SEQ ID No. 33: nucleotide sequence of T-DNA vector pTVE 502

SEQ ID No. 34: nucleotide sequence of T-DNA vector pTVE 503

Examples

Example 1: Assembly of stress resistant chimeric genes and introduction into plants.

pTVE467

To increase the stress resistance in plants, a chimeric gene was constructed using conventional techniques comprising the following DNA fragments in order:

- A promoter region from Cauliflower Mosaic Virus (CaMV 35S);
- A DNA fragment of about 60 bp corresponding to the untranslated leader Cab22L;
- A DNA fragment encoding nicotinamidase from Saccharomyces cereviseae (SEQ ID NO 1);
- A fragment of the 3' untranslated end from the 35 S transcript of CaMV (3' 35S)

This chimeric gene was introduced in a T-DNA vector, between the left and right border sequences from the T-DNA, together with a selectable marker gene providing resistance to the herbicide phosphinotricin, to yield pTVE467 (SEQ ID 25). T-DNA vector pTVE467 is schematically represented in Figure 2.

T-DNA vector pTVE467 comprises the following molecule features:

(C) indicates complementary strand.

Start (nt)	End (nt)	
198	222	RB: right T-DNA border
521	300 (C)	3'35S: transcription termination signal
1181	534 (C)	PNC1 coding region
1250	1191(C)	cab22 leader
1781	1251(C)	P35S2 promoter
2293	2082 (C)	3'g7 transcription termination signal
2866	2315 (C)	bar coding region

4592	2867 (C)	PSSuAra promoter
4760	4784	Left T-DNA border
6352	5352 (C)	Sm/Sp resistance gene
6875	10645	pVS1origin of replication
10646	11709	ColE1 origin of replication

pTVE468

A similar chimeric gene as present in pTVE467 was constructed, wherein the nicotinamidase was equipped with a conventional nuclear localization signal. The chimeric gene thus comprises the following operably linked DNA fragments:

- A promoter region from Cauliflower mosaic Virus (CaMV 35S);
- A DNA fragment of about 60 nt corresponding to the untranslated leader Cab22L;
- A DNA fragment of about 20 nt encoding a peptide comprising a nuclear localization signal (NLS),
- A DNA fragment encoding nicotinamidase from Saccharomyces cereviseae (SEQ ID NO 1); whereby the NLS signal is fused in frame;
- A fragment of the 3' untranslated end from the 35 S transcript of CaMV (3' 35S)

This chimeric gene was introduced in a T-DNA vector, between the left and right border sequences from the T-DNA, together with a selectable marker gene providing resistance to the herbicide phosphinotricin, to yield pTVE468 (SEQ ID 26). T-DNA vector pTVE468 is schematically represented in Figure 3.

T-DNA vector pTVE468 comprises the following molecule features:

(C) indicates complementary strand.

Start (nt)	End (nt)	
198	222	RB: right T-DNA border

521	300 (C)	3'35S: transcription termination signal
1169	534 (C)	PNC1 coding region
1187	1167 (C)	Nuclear localization signal
1268	1209(C)	cab22 leader
1799	1269(C)	P35S2 promoter
2311	2100 (C)	3'g7 transcription termination signal
2884	2333 (C)	bar coding region
4610	2885 (C)	PSSuAra promoter
4778	4802	Left T-DNA border
6370	5370 (C)	Sm/Sp resistance gene
6893	10663	pVS1origin of replication
10664	11727	ColE1 origin of replication

pTVE469

To increase stress resistance in plants, a chimeric gene was constructed using conventional techniques comprising the following DNA fragments in order:

- A promoter region from Cauliflower mosaic Virus (CaMV 35S);
- A DNA fragment of about 60 bp corresponding to the untranslated leader Cab22L;
- A DNA fragment encoding nicotinate phosphoribosyltransferase from *Saccharomyces cereviseae* (NPT1; SEQ ID NO 3);
- A fragment of the 3' untranslated end from the 35 S transcript of CaMV (3' 35S)

This chimeric gene was introduced in a T-DNA vector, between the left and right border sequences from the T-DNA, together with a selectable marker gene providing resistance to the herbicide phosphinotricin, to yield pTVE469 (SEQ ID 27). T-DNA vector pTVE469 is schematically represented in Figure 4.

T-DNA vector pTVE469 comprises the following molecule features:

(C) indicates complementary strand.

Start (nt)	End (nt)	
198	222	RB: right T-DNA border
521	300 (C)	3'35S: transcription termination signal
1765	534 (C)	NPT1 coding region
1832	1773(C)	cab22 leader
2363	1833(C)	P35S2 promoter
2875	2664(C)	3'g7 transcription termination signal
3448	2897 (C)	bar coding region
5175	3449 (C)	PSSuAra promoter
5342	5366	Left T-DNA border
6934	5934(C)	Sm/Sp resistance gene
7457	11227	pVS1origin of replication
11228	12291	ColE1 origin of replication

pTVE470

A similar chimeric gene as present in pTVE469 was constructed, wherein the nicotinate phosphoribosyltransferase from *Saccharomyces cereviseae* was equipped with a conventional nuclear localization signal The chimeric gene thus comprises the following operably linked DNA fragments:

- A promoter region from Cauliflower mosaic Virus (CaMV 35S);
- A DNA fragment of about 60 nt corresponding to the untranslated leader Cab22L;
- A DNA fragment of about 20 nt encoding a peptide comprising a nuclear localization signal (NLS),
- A DNA fragment encoding nicotinate phosphoribosyltransferase from *Saccharomyces cereviseae* (NPT1; SEQ ID NO 3); whereby the NLS signal is fused in frame;
- A fragment of the 3' untranslated end from the 35 S transcript of CaMV (3' 35S)

This chimeric gene was introduced in a T-DNA vector, between the left and right border sequences from the T-DNA, together with a selectable marker gene providing resistance to the herbicide phosphinotricin, to yield pTVE470 (SEQ ID 28). T-DNA vector pTVE470 is schematically represented in Figure 5.

T-DNA vector pTVE470 comprises the following molecule features:

(C) indicates complementary strand.

Start (nt)	End (nt)	
198	222	RB: right T-DNA border
521	300 (C)	3'35S: transcription termination signal
1787	534 (C)	NPT1 coding region
1775	1755 (C)	Nuclear localization signal SV40
1853	1794(C)	cab22 leader
2384	1854(C)	P35S2 promoter
2896	2685 (C)	3'g7 transcription termination signal
3469	2918(C)	bar coding region
5195	3470 (C)	PSSuAra promoter
5363	5387	Left T-DNA border
6955	5955 (C)	Sm/Sp resistance gene
7478	11248	pVS1origin of replication
11249	12312	ColE1 origin of replication

pTVE496

To increase stress resistance in plants, a chimeric gene was constructed using conventional techniques comprising the following DNA fragments in order:

- A promoter region from Cauliflower mosaic Virus (CaMV 35S);
- A DNA fragment of about 60 bp corresponding to the untranslated leader Cab22L;

- A DNA fragment encoding nicotinic acid mononucleotide adenyl transferase 1 from *Saccharomyces cereviseae* (NMA1; SEQ ID NO 5);
- A fragment of the 3' untranslated end from the 35 S transcript of CaMV (3' 35S)

This chimeric gene was introduced in a T-DNA vector, between the left and right border sequences from the T-DNA, together with a selectable marker gene providing resistance to the herbicide phosphinotricin, to yield pTVE496 (SEQ ID 29). T-DNA vector pTVE496 is schematically represented in Figure 6.

T-DNA vector pTVE496 comprises the following molecule features:

(C) indicates complementary strand.

Start (nt)	End (nt)	
198	222	RB: right T-DNA border
521	300 (C)	3'35S: transcription termination signal
1739	534 (C)	NMA1 coding region
1805	1746(C)	cab22 leader
2336	1806(C)	P35S2 promoter
2848	2637(C)	3'g7 transcription termination signal
3421	2870 (C)	bar coding region
5147	3422 (C)	PSSuAra promoter
5315	5339	Left T-DNA border
6907	5907(C)	Sm/Sp resistance gene
7430	11200	pVS1origin of replication
11201	12264	ColE1 origin of replication

pTVE497

A similar chimeric gene as present in pTVE496 was constructed, wherein the nicotinic acid mononucleotide adenyl transferase 1 from *Saccharomyces cereviseae* was equipped with a conventional nuclear localization signal The chimeric gene thus comprises the following operably linked DNA fragments:

- A promoter region from Cauliflower mosaic Virus (CaMV 35S);
- A DNA fragment of about 60 nt corresponding to the untranslated leader Cab22L;
- A DNA fragment of about 20 nt encoding a peptide comprising a nuclear localization signal (NLS),
- A DNA fragment encoding nicotinic acid mononucleotide adenyl transferase 1 from *Saccharomyces cereviseae* (NMA1; SEQ ID NO 5); whereby the NLS signal is fused in frame;
- A fragment of the 3' untranslated end from the 35 S transcript of CaMV (3' 35S)

This chimeric gene was introduced in a T-DNA vector, between the left and right border sequences from the T-DNA, together with a selectable marker gene providing resistance to the herbicide phosphinotricin, to yield pTVE497 (SEQ ID 30). T-DNA vector pTVE497 is schematically represented in Figure 7.

T-DNA vector pTVE497 comprises the following molecule features:

(C) indicates complementary strand.

Start (nt)	End (nt)	
198	222	RB: right T-DNA border
521	300 (C)	3'35S: transcription termination signal
1757	534 (C)	NMA1 coding region
1748	1731 (C)	Nuclear localization signal SV40
1823	1764(C)	cab22 leader
2354	1824(C)	P35S2 promoter
2866	2655 (C)	3'g7 transcription termination signal
3439	2888(C)	bar coding region
5165	3440 (C)	PSSuAra promoter
5333	5357	Left T-DNA border
6925	5925 (C)	Sm/Sp resistance gene

7448	11218	pVS1origin of replication
11219	12282	ColE1 origin of replication

pTVE500

To increase stress resistance in plants, a chimeric gene was constructed using conventional techniques comprising the following DNA fragments in order:

- A promoter region from Cauliflower mosaic Virus (CaMV 35S);
- A DNA fragment of about 60 bp corresponding to the untranslated leader Cab22L;
- A DNA fragment encoding nicotinic acid mononucleotide adenyl transferase 2 from *Saccharomyces cereviseae* (NMA2; SEQ ID No. 7);
- A fragment of the 3' untranslated end from the 35S transcript of CaMV (3' 35S).

This chimeric gene was introduced in a T-DNA vector, between the left and right border sequences from the T-DNA, together with a selectable marker gene providing resistance to the herbicide phosphinotricin, to yield pTVE500 (SEQ ID 31). T-DNA vector pTVE500 is schematically represented in Figure 8.

T-DNA vector pTVE500 comprises the following molecule features:

(C) indicates complementary strand.

Start (nt)	End (nt)	
198	222	RB: right T-DNA border
521	300 (C)	3'35S: transcription termination signal
1721	534 (C)	NMA2 coding region
1787	1728(C)	cab22 leader
2318	1788(C)	P35S2 promoter
2830	2619(C)	3'g7 transcription termination signal
3403	2852 (C)	bar coding region
5129	3404 (C)	PSSuAra promoter

5297	5321	Left T-DNA border
6889	5889(C)	Sm/Sp resistance gene
7412	11182	pVS1origin of replication
11183	12246	ColE1 origin of replication

pTVE501

A similar chimeric gene as present in pTVE500 was constructed, wherein the nicotinic acid mononucleotide adenyl transferase 2 from *Saccharomyces cereviseae* was equipped with a conventional nuclear localization signal The chimeric gene thus comprises the following operably linked DNA fragments:

- A promoter region from Cauliflower mosaic Virus (CaMV 35S);
- A DNA fragment of about 60 nt corresponding to the untranslated leader Cab22L;
- A DNA fragment of about 20 nt encoding a peptide comprising a nuclear localization signal (NLS),
- A DNA fragment encoding nicotinic acid mononucleotide adenyl transferase 2 from *Saccharomyces cereviseae* (NMA2; SEQ ID No. 7); whereby the NLS signal is fused in frame;
- A fragment of the 3' untranslated end from the 35 S transcript of CaMV (3' 35S)

This chimeric gene was introduced in a T-DNA vector, between the left and right border sequences from the T-DNA, together with a selectable marker gene providing resistance to the herbicide phosphinotricin, to yield pTVE502 (SEQ ID 32). T-DNA vector pTVE501 is schematically represented in Figure 9.

T-DNA vector pTVE501 comprises the following molecule features:

(C) indicates complementary strand.

Start (nt)	End (nt)	
198	222	RB: right T-DNA border

521	300 (C)	3'35S: transcription termination signal
1739	534 (C)	NMA2 coding region
1733	1713 (C)	Nuclear localization signal SV40
1805	1746(C)	cab22 leader
2336	1806(C)	P35S2 promoter
2848	2637 (C)	3'g7 transcription termination signal
3421	2870(C)	bar coding region
5165	3440 (C)	PSSuAra promoter
5315	5339	Left T-DNA border
6907	5907 (C)	Sm/Sp resistance gene
7430	11200	pVS1origin of replication
11201	12264	ColE1 origin of replication

pTVE502

To increase stress resistance in plants, a chimeric gene was constructed using conventional techniques comprising the following DNA fragments in order:

- A promoter region from Cauliflower mosaic Virus (CaMV 35S);
- A DNA fragment of about 60 bp corresponding to the untranslated leader Cab22L;
- A DNA fragment encoding NAD synthase from *Saccharomyces cereviseae* (QNS1; SEQ ID No. 9);
- A fragment of the 3' untranslated end from the 35S transcript of CaMV (3' 35S).

This chimeric gene was introduced in a T-DNA vector, between the left and right border sequences from the T-DNA, together with a selectable marker gene providing resistance to the herbicide phosphinotricin, to yield pTVE502 (SEQ ID 33). T-DNA vector pTVE502 is schematically represented in Figure 10.

T-DNA vector pTVE502 comprises the following molecule features:

(C) indicates complementary strand.

Start (nt)	End (nt)	
198	222	RB: right T-DNA border
521	300 (C)	3'35S: transcription termination signal
2678	534 (C)	QNS1 coding region
2744	2685(C)	cab22 leader
3275	2745(C)	P35S2 promoter
3787	3576 (C)	3'g7 transcription termination signal
4360	3809 (C)	bar coding region
6086	4361 (C)	PSSuAra promoter
6254	6278	Left T-DNA border
7846	6846 (C)	Sm/Sp resistance gene
8369	12139	pVS1origin of replication
12140	13203	ColE1 origin of replication

pTVE503

A similar chimeric gene as present in pTVE502 was constructed, wherein the NAD synthase from *Saccharomyces cereviseae* was equipped with a conventional nuclear localization signal The chimeric gene thus comprises the following operably linked DNA fragments:

- A promoter region from Cauliflower mosaic Virus (CaMV 35S);
- A DNA fragment of about 60 nt corresponding to the untranslated leader Cab22L;
- A DNA fragment of about 20 nt encoding a peptide comprising a nuclear localization signal (NLS),
- A DNA fragment encoding NAD synthase from *Saccharomyces cereviseae* (QNS1; SEQ ID No. ç); whereby the NLS signal is fused in frame;
- A fragment of the 3' untranslated end from the 35 S transcript of CaMV (3' 35S)

This chimeric gene was introduced in a T-DNA vector, between the left and right border sequences from the T-DNA, together with a selectable marker gene providing resistance to the herbicide phosphinotricin, to yield pTVE503 (SEQ ID No. 34). T-DNA vector pTVE503 is schematically represented in Figure 11.

T-DNA vector pTVE503 comprises the following molecule features:

(C) indicates complementary strand.

Start (nt)	End (nt)	
198	222	RB: right T-DNA border
521	300 (C)	3'35S: transcription termination signal
2699	534 (C)	QNS1 coding region
2690	2670(C)	Nuclear localization signal SV40
2765	2706(C)	cab22 leader
3296	2766(C)	P35S2 promoter
3808	3597 (C)	3'g7 transcription termination signal
4381	3830 (C)	bar coding region
6107	4382 (C)	PSSuAra promoter
6275	6299	Left T-DNA border
7867	6867 (C)	Sm/Sp resistance gene
8390	12610	pVS1origin of replication
12161	13224	ColE1 origin of replication

The T-DNA vectors were introduced into Agrobacterium strains comprising a helper Ti-plasmid using conventional methods. The chimeric genes were introduced into Arabidopsis plants by Agrobacterium mediated transformation as described in the art.

Example 2. Analysis of transgenic Arabidopsis lines comprising the chimeric genes described in Example 1.

Seed of transgenic Arabidopsis lines (T1-generation) expressing the yeast genes of the NAD-salvage pathway, obtained as described in Example 1 were germinated and grown on medium containing 15 mg L^{-1} phosphinotricin (PPT). Arabidopsis thaliana cv Col-0 was used as a control.

All plants were subjected to high light stress. Two week old plants grown at 30 µEinstein m^{-2} sec^{-1} were transferred to 250 µEinstein m^{-2} sec^{-1} (high light) for 6 hours, followed by 8 hours in the dark and again 8 hours high light.

After this treatment, NADH content and superoxide radicals content were determined for all lines and compared to measurement of the same compounds in transgenic and control lines grown under low light conditions. The results are summarized in Table 1.

Transgenic plants exhibited a higher NADH content under high light than control plants, and produced less reactive oxygen species under high light than control plants. No difference was observed between constructs wherein the encoded NAD salvage pathway enzyme was equipped with a nuclear localization signal or not.

Transgenic plant lines were also phenotypically scored for tolerance to high light stress conditions. To this end, plants were grown in vitro at low light conditions (30 µEinstein m^{-2} sec^{-1}) for two weeks and transferred for 3 days to high light conditions (250 µEinstein m^{-2} sec^{-1}; 16 hrs light -8hrs dark). After the high light treatment the plants were returned to low light conditions and grown for another three days before scoring the phenotype.

Whereas control plants were small, and had started flowering (stress-induced), the plants of the transgenic lines comprising the chimeric genes as described in Example 1 were larger than the control plants and only had started to bolt.

Table 1. High light tolerance of transgenic Arabidopsis lines over-expressing the chimeric yeast genes as described in Example 1.

Chimeric genes	Segregation for PPT tolerance	% NADH versus low light control		% superoxides versus low light control	
		Low light	High light	Low light	High light
Control	-	100	68	100	145
PNC1 (NLS) line 1	3:1	108	128	80	73
PNC1 (NLS) line 2	3:1	139	128	82	76
NPT1 line 1	6:1	128	147	66	70
NPT1 line 2	6:1	122	135	82	76
NPT1 (NLS)	12:1	106	150	61	80

STANDARD ERROR OF MEAN < 10%

Example 3: protocols for measurement of NADH content and superoxide content.

Intracellular NAD(P)H quantification using a water-soluble tetrazolium salt

Reference

Jun Nakamura, Shoji Asakura, Susan D. Hester, Gilbert de Murcia, Keith W. Caldecott and James A. Swenberg (2003) Quantitation of intracellular NAD(P)H can monitor an imbalance of DNA single strand break repair in base excision repair deficient cells in real time. Nucleic Acids Research 31(17), e104.

Plant material

Most plant material can be used:

- In vitro grown Arabidopsis shoots 14-18 days old but NOT flowering
- Hypocotyl explants of oilseed rape

Cell Counting Kit-8 (CCK-8)

Sopachem n.v./Belgium

72A, Avenue du Laarbeeklaan - 1090 Brussels

Belgium

Contents:

5mL bottles containing 5mMol/L WST-8 (tetrazolium salt), 0.2mMol/L 1-Methoxy PMS, 150mMol/L NaCl

Reaction solution:

- 10mL 25mM K-phosphate buffer pH7.4
- 0.5mL CCK-8

- 0.1mM 1-Methoxy-5-methylphenazinium methyl sulfate (= 1-Methoxyphenazine methosulfate): 1µL/mL of 100mM stock (MW= 336.4; 100mg in 2.973mL water)
- 1 drop Tween20/25mL

Procedure

- Harvest plant material and put in 25mM K-phosphate buffer pH7.4
  e.g.: 150 oilseed rape hypocotyl explants
  1gr Arabidopsis shoots (without roots)

- Replace buffer with reaction solution
  15mL for 1gr Arabidopsis shoots
  15mL for 150 oilseed rape hypocotyl explants

- Incubate at 26°C in the dark for about 1/2 hour (follow reaction)

- Measure the absorbance of the reaction solution at 450nm

Measuring superoxide production by quantifying the reduction of XTT

Ref.: De Block, M., De Brouwer, D. (2002) A simple and robust in vitro assay to quantify the vigour of oilseed rape lines and hybrids. Plant Physiol. Biochem. 40, 845-852

A. BRASSICA NAPUS

Media and reaction buffers

Sowing medium (medium 201):

Half concentrated Murashige and Skoog salts

2% sucrose

pH 5.8

0.6% agar (Difco Bacto Agar)

250mg/l triacillin

Callus inducing medium A2S3:

MS medium, 0.5g/l Mes (pH 5.8), 3% sucrose, 40mg/l adenine-SO_4, 0.5% agarose, 1mg/l 2,4-D, 0.25mg/l NAA, 1mg/l BAP, 250mg/l triacillin

Reaction buffer:

25mM K-phosphate buffer pH 8

1mM sodium,3'-{1-[phenylamino-carbonyl]-3,4-tetrazolium}-bis(4-methoxy-6-nitro) = XTT (BioVectra, Canada) (MW 674.53)

Dissolve XTT by careful warming solution (± 37°C) (cool down to room temperature before use)

1 drop Tween20 for 25ml buffer

Sterilization of seeds - pregermination of seeds - growing of the seedlings

Seeds are soaked in 70% ethanol for 2 min, then surface-sterilized for 15 min in a sodium hypochlorite solution (with about 6% active chlorine) containing 0.1% Tween20. Finally, the seeds are rinsed with 1l of sterile tap water.

Incubate seeds for at least one hour in sterile tap water (to allow diffusion from seeds of components that may inhibit germination).

Seeds are put in 250ml erlenmeyer flasks containing 50ml of sterile tap water (+ 250mg/l triacillin). Shake for about 20 hours.

Seeds from which the radicle is protruded are put in Vitro Vent containers from Duchefa containing about 125ml of sowing medium (10 seeds/vessel, not too many to reduce loss of seed by contamination). The seeds are germinated at ±24°C and 10-30μEinstein $s^{-1}m^{-2}$ with a daylength of 16h.

P.S.: For calculating the amount of seeds that have to be sawn: 5 hypocytyl segments/seedling

Preculture of the hypocotyl explants and induction of stress

- 12-14 days after sowing, the hypocotyls are cut in about 7-10mm segments.
- The hypocotyl explants (25 hypocotyls/Optilux Petridish, Falcon S1005, Denmark) are cultured for 5 days on medium A2S3 at 25°C (at 10-30μEinstein $s^{-1}m^{-2}$).

P.S.: 150 hypocotyl explants are used per condition.

- Induction of stress:

  Transfer hypocotyl explants to A2S3 medium containing respectively 0, 25 and 50mg/l acetylsalicylic acid.

  Incubate for about 24 hours at 25°C and10-30μEinstein $s^{-1}m^{-2}$ with a

daylength of 16h.

XTT-assay

- Transfer 150 hypocotyl explants to a 50ml Falcon tube.
- Wash with reaction buffer (without XTT).
- Add 20mL reaction buffer + XTT.
  (explants have to be submerged, but do not vacuum infiltrate)
- Incubate in the dark at 26°C
- Follow the reaction by measuring the absorption of the reaction medium at 470nm

B. ARABIDOPSIS THALIANA

Media and reaction buffers

Plant medium:

Half concentrated Murashige and Skoog salts
B5 vitamins
1.5% sucrose
pH 5.8
0.7% Difco agar

Incubation medium:

1/2 concentrated MS-salts
1% sucrose
0.5g/L MES pH 5.8
1 drop Tween20 for 25ml medium

Reaction buffer:

25mM K-phosphate buffer pH 8
1mM sodium,3'-{1-[phenylamino-carbonyl]-3,4-tetrazolium}-bis(4-methoxy-6-nitro) = XTT (BioVectra, Canada) (MW 674.53)

Dissolve XTT by careful warming solution (± 37°C) (cool down to room temperature before use)

1 drop Tween20 for 25ml buffer

***Arabidopsis* plants**

- Arabidopsis lines: control (mother line from which tested lines were derived)
  lines to test

- Sterilization of *Arabidopsis* seeds:

2min. 70% ethanol

10 min. bleach (6% active chlorine) + 1drop Tween 20 for 20ml solution

wash 5 times with sterile tap water

P.S.: sterilization is done in 2ml eppendorf tubes *Arabidopsis* seeds sink to the bottom of the tube, allowing removal of the liquids by means of a 1ml pipetman

- Pregermination of seeds:

In 9cm Optilux Petridishes (Falcon) containing 12ml sterile tap water.

Low light overnight to 24 hours.

- Growing of *Arabidopsis* plants

Seeds are sown in Intergrid Tissue Culture disks of Falcon (nr. 3025) containing ±125ml of plant medium: 1 seed/grid.

Plants are grown at 24°C

30µEinstein $s^{-1}m^{-2}$

16hours light - 8hours dark

for about 18 days (before bolting)

P.S.: 1g of plant material (shoots without roots)/line/condition are needed to cary out the asssay. 1g shoots corresponds wilth 40-60 plants.

Induction of stress

Paraquat

- Harvest *Arabidopsis* shoots (without roots)
- Put 1g shoots in incubation medium (shoots have to be submerged, but do not vacuum infiltrate) containing respectively 0, 5 and 10µM paraquat

Incubation medium: ±150ml in Intergrid Tissue Culture disks of Falcon (nr. 3025)

- Incubate at 24°C in the dark for ±24 hours and 30-50µEinstein $s^{-1}m^{-2}$ with a daylength of 16h.

High light

- Transfer half of the plates to high light (250µEinstein $s^{-1}m^{-2}$) and incubate for 4 to 20 hours

XTT-assay

- Harvest shoots (without roots) from agar plates (high light stress) or from liquid incubation medium (paraquat stress) and put them in 50ml Falcon tubes containing reaction buffer (without XTT)
- Replace reaction buffer with buffer containing XTT (15mL/gr)
- Shoots have to be submerged, but do not vacuum infiltrate
- Incubate in the dark at 26°C
- Follow the reaction by measuring the absorption of the reaction medium at 470nm (about one hour)

Example 4: Increased ozone tolerance of Arabidopsis thaliana plants over-expressing the yeast nicotineamidase (Pnc1) gene.

The chimeric vector pTVE467 (Example 1) was used for transformation of *A. thaliana* ecotype Columbia. Primary transformants were analyzed by Southern-DNA- and Northern-RNA-blot analysis. One transgenic line was identified to carry a single copy of the Pnc1-transgene construct and to have a high steady state level of transgenic full-length Pnc1-mRNA (20 pg/5 µg total RNA).

6 weeks after germination 100 individual plants each of the single copy transgenic line and of wild-type Columbia as a control, were exposed to ozone in fumigation chambers. During 2 consecutive days the plants were treated for 5h/day with ozone concentrations of 250, 350 and 500 ppb respectively. After

treatment all plants were visually screened for ozone injury manifested as necrotic lesions. The results are summarized in Table 2. At 500 ppb ozone exposure nearly all plants showed necrotic lesions whereas at the 2 lower ozone concentrations a significantly lower percentage of transgenic plants were injured.

In addition, the evolution of the vitality performance index (PI) was determined for all plants of the transgenic line and of the wild-type plants under increasing ozone concentration. PI can be calculated by the formula: PI = (ABS/CS) x (TR/CS) x (ET/CS). (ABS = flux of photons absorbed by the antenna pigments Chl*; CS = cross section; TR = energy trapped by the reaction centre and converted into redox energy; ET = electron flux further downstream leading to CO_2 fixation) In the transgenic line, the vitality performance index PI significantly increased with increasing ozone concentrations whereas this index remains constant in wild-type plants treated with increasing ozone concentrations. This can be explained by a physiological compensation response within the transgenic line to counteract the ozone damage.

Table 2. Increased ozone tolerance of Arabidopsis thaliana plants over-expressing the yeast nicotineamidase (Pnc1) gene.

	250 ppb O_3	**350 ppb O_3**	**500 ppb O_3**
Wild-type	45%*	50%	100%
Pnc1	20%	25%	100%

* percentage of the plants exhibiting necrotic lesions

Furthermore, control plants, homozygous transgenic populations of plants comprising the chimeric Pnc1 gene as well as a heterozygous transgenic population, were subjected to ozone fumigations and scored for visible injury and various physiological responses compared to non-fumigated plants. The assessment included measurement of non-modulated fluorescence, modulated fluorescence, chlorophyll measurement and fresh weight determination.

Based on the visible injury and physiological responses, a ranking was made for each population indicating the degree of the ozone impact. The more negative the evaluation, the more sensitive the population's response to ozone.

Whereas the control non-transgenic population and the heterozygous transgenic population had a cumulative score of -13, the two homozygous transgenic populations had a score of -6 and -2 respectively. It is therefore clear that the homozygous transgenic populations performed statistically significantly better than the control plants.

DEMANDE OU BREVET VOLUMINEUX

LA PRÉSENTE PARTIE DE CETTE DEMANDE OU CE BREVET COMPREND PLUS D'UN TOME.

CECI EST LE TOME 1 DE 2
CONTENANT LES PAGES 1 À 41

NOTE : Pour les tomes additionels, veuillez contacter le Bureau canadien des brevets

JUMBO APPLICATIONS/PATENTS

THIS SECTION OF THE APPLICATION/PATENT CONTAINS MORE THAN ONE VOLUME

THIS IS VOLUME 1 OF 2
CONTAINING PAGES 1 TO 41

NOTE: For additional volumes, please contact the Canadian Patent Office

NOM DU FICHIER / FILE NAME :

NOTE POUR LE TOME / VOLUME NOTE:

CLAIMS:

1. A method for obtaining a plant with increased stress resistance comprising

a. introducing a chimeric gene into cells of a plant to obtain transgenic cells, said chimeric gene comprising the following operably linked DNA fragments:

i. a plant-expressible promoter;

ii. a DNA region coding for an enzyme of the nicotinamide adenine dinucleotide salvage synthesis pathway from yeast or fungi, said enzyme being selected from nicotinamidase, nicotinate phosphoribosyltransferase, nicotinic acid mononucleotide adenyl transferase or nicotinamide adenine dinucleotide synthetase; and

iii. a 3' end region involved in transcription termination and polyadenylation;

b. regenerating said transgenic cells to obtain a population of transgenic plants; and

c. selecting a plant from said population of transgenic plants which exhibits increased stress resistance or selecting a plant which exhibits a reduced level of reactive oxygen species or maintains a high level of NADH under stress conditions when compared to a similar non-transgenic plant.

2. The method according to claim 1, wherein said DNA region codes for an enzyme comprising an amino acid sequence selected from the amino acid sequence of SEQ ID No.:2, SEQ ID No.:4, SEQ ID No.:6; SEQ ID No.:8, or SEQ ID No.:10.

3. The method according to claim 2, wherein said DNA region comprises the nucleotide sequence of SEQ ID No.:1, SEQ ID No.:3, SEQ ID No.:5, SEQ ID No.:7, or SEQ ID No.:9.

4. The method according to any one of claims 1 to 3, further comprising the step of crossing said plant with another plant.

5. A chimeric gene as described in any one of claims 1 to 3.

6. A cell of a plant comprising the chimeric gene as described in claim 5.

7. The plant cell of claim 6 which is from a plant selected from cotton, Brassica vegetables, oilseed rape, wheat, corn or maize, barley, sunflower, rice, oats, sugarcane, soybean, vegetables, chicory, lettuce, tomato, tobacco, potato, sugarbeet, papaya, pineapple, mango or *Arabidopsis thaliana*.

8. The plant cell according to claim 6 or 7, further characterized in that said plant has a lower level of reactive oxygen species under stress conditions than a similar plant not comprising such a chimeric gene.

9. The plant cell of any one of claims 6-8, which is from a seed.

10. Use of the chimeric gene according to claim 5 to increase the stress resistance of a plant.

11. Use of a chimeric gene according to claim 5 to decrease the level of reactive oxygen species in a plant or a plant cell under stress conditions or to maintain the level of NADH in a plant or plant cell under stress conditions.

12. Use of a DNA molecule encoding an enzyme of the nicotinamide adenine dinucleotide salvage synthesis pathway from yeast or fungi, said enzyme being selected from nicotinamidase, nicotinate phosphoribosyltransferase, nicotinic acid mononucleotide adenyl transferase or nicotinamide adenine dinucleotide synthetase, to increase the stress resistance of a plant.

13. Use of a DNA molecule encoding an enzyme of the nicotinamide adenine dinucleotide salvage synthesis pathway from yeast or fungi, said enzyme being selected from nicotinamidase, nicotinate phosphoribosyltransferase, nicotinic acid mononucleotide adenyl transferase or nicotinamide adenine dinucleotide

synthetase, to decrease the level of reactive oxygen species in a plant or a plant cell under stress conditions or to maintain the level of NADH in a plant or plant cell under stress conditions.

14. Use of a DNA molecule encoding a protein comprising an amino acid sequence selected from the amino acid sequence of SEQ ID No.:2, SEQ ID No.:4, SEQ ID No.:6; SEQ ID No.:8, or SEQ ID No.:10, to increase the stress resistance of a plant.

15. Use of a DNA molecule encoding a protein comprising an amino acid sequence selected from the amino acid sequence of SEQ ID No.:2, SEQ ID No.:4, SEQ ID No.:6; SEQ ID No.:8, or SEQ ID No.:10, to decrease the level of reactive oxygen species in a plant or a plant cell under stress conditions or to maintain the level of NADH in a plant or plant cell under stress conditions.

16. Use of a DNA molecule comprising a nucleotide sequence selected from the nucleotide sequence of SEQ ID No.:1, SEQ ID No.:3, SEQ ID No.:5; SEQ ID No.:7, or SEQ ID No.:9, to increase the stress resistance of a plant.

17. Use of a DNA molecule comprising a nucleotide sequence selected from the nucleotide sequence of SEQ ID No.:1, SEQ ID No.:3, SEQ ID No.:5; SEQ ID No.:7, or SEQ ID No.:9, to increase the stress resistance of a plant, to decrease the level of reactive oxygen species in a plant or a plant cell under stress conditions or to maintain the level of NADH in a plant or plant cell under stress conditions.

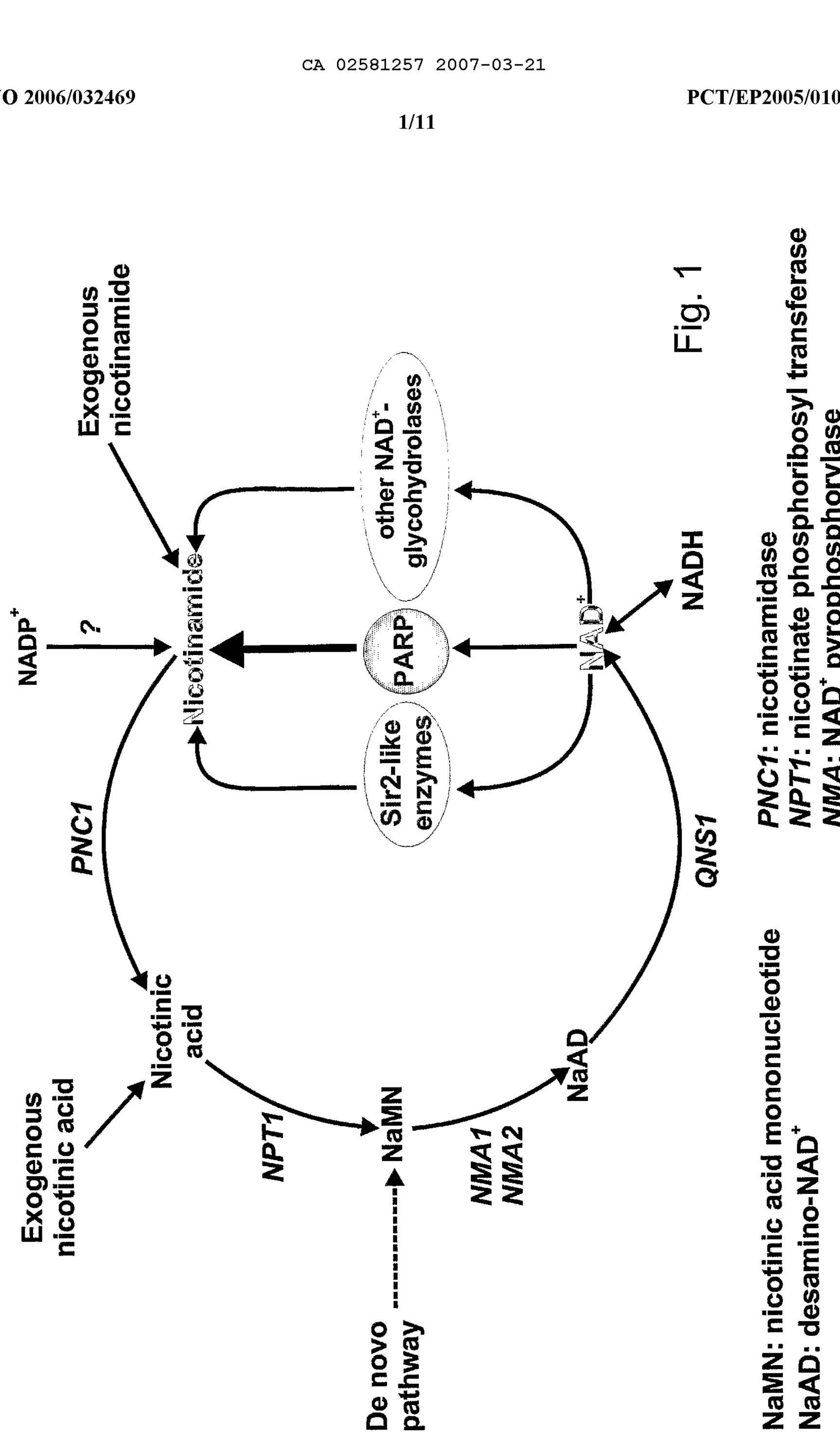


Fig. 1

PNC1: nicotinamidase
NPT1: nicotinate phosphoribosyl transferase
NMA: NAD^+ pyrophosphorylase
QNS1: NAD^+ synthetase

NaMN: nicotinic acid mononucleotide
NaAD: desamino-NAD^+

Fig. 2

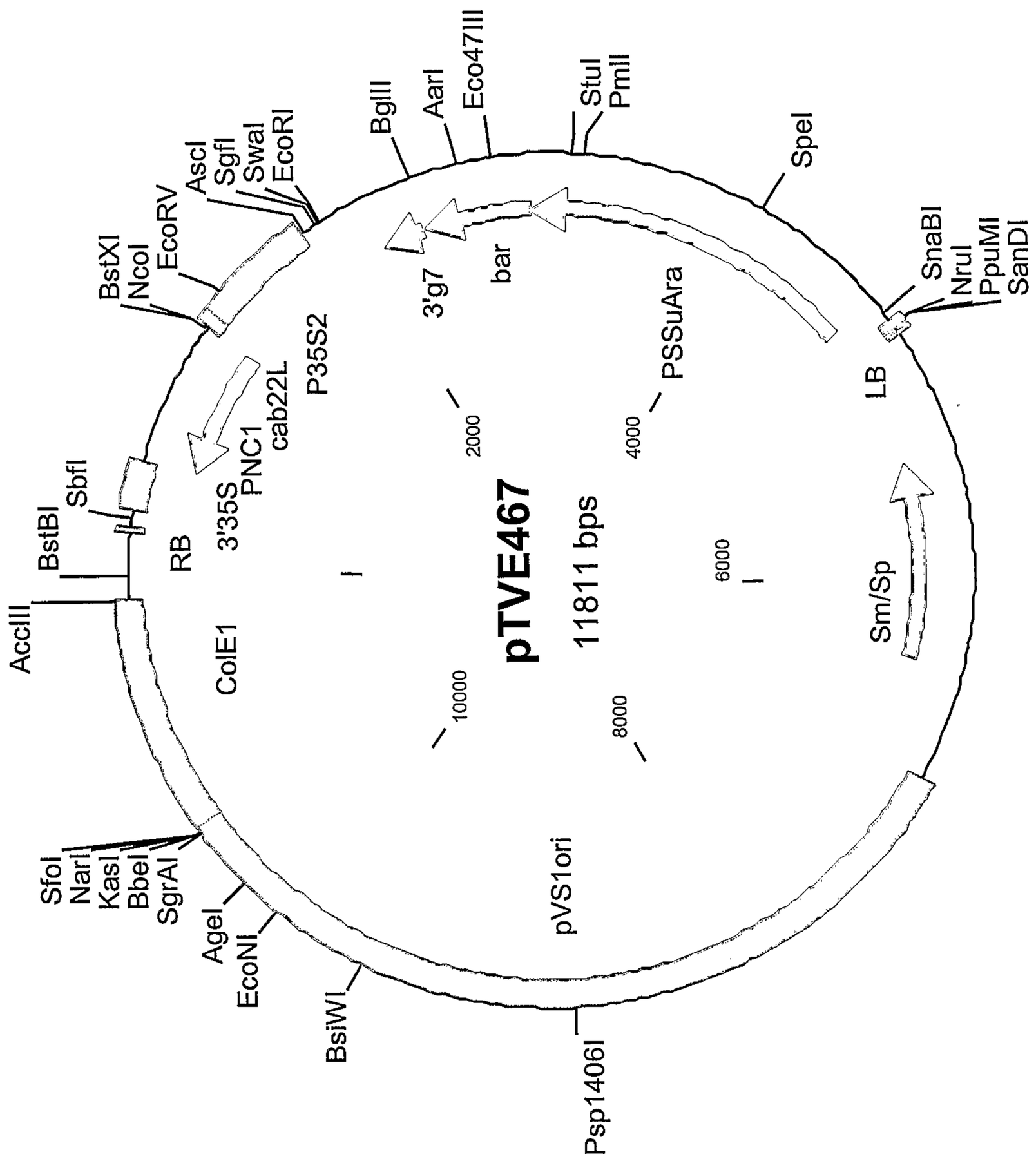

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Fig. 3

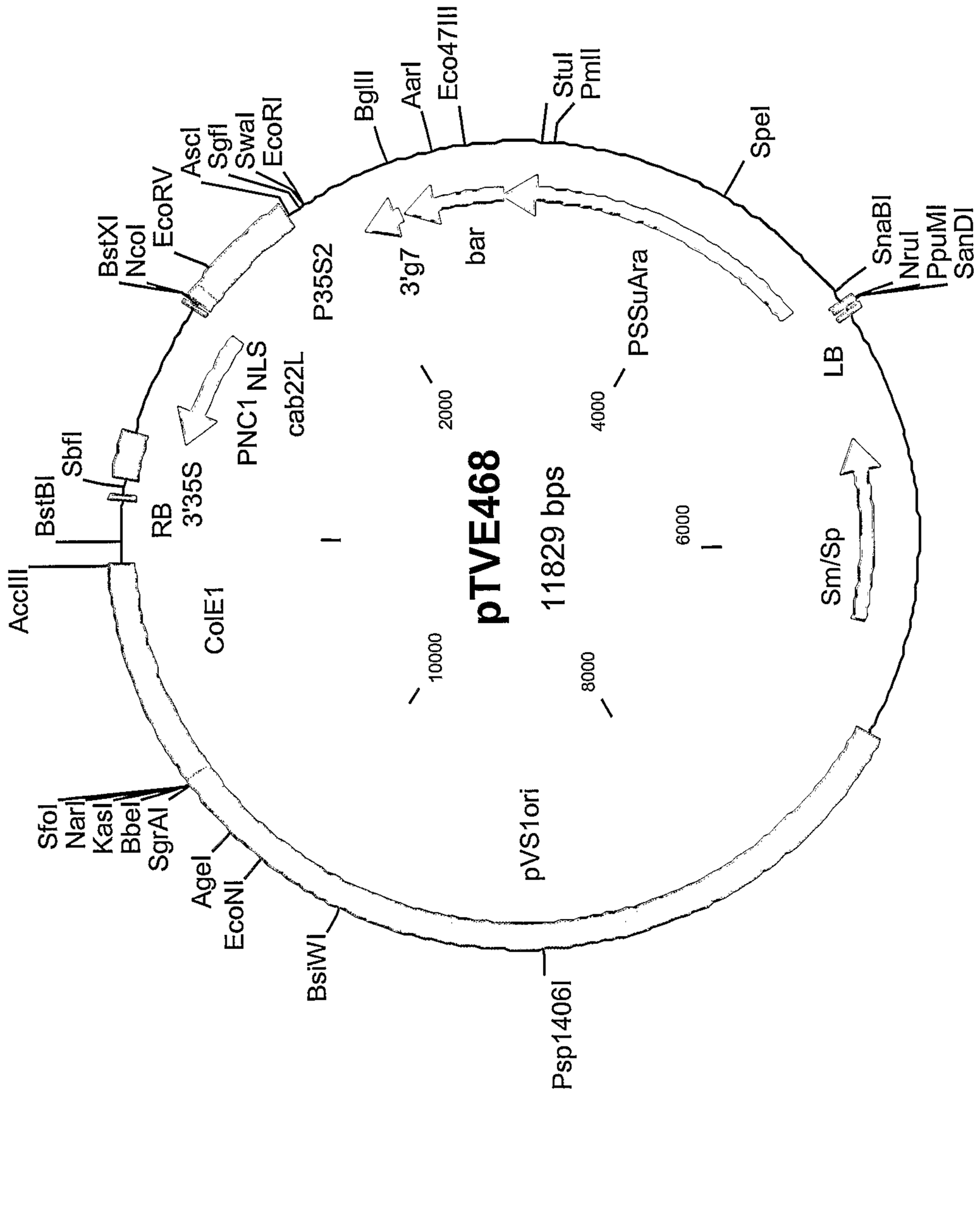

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Fig. 4

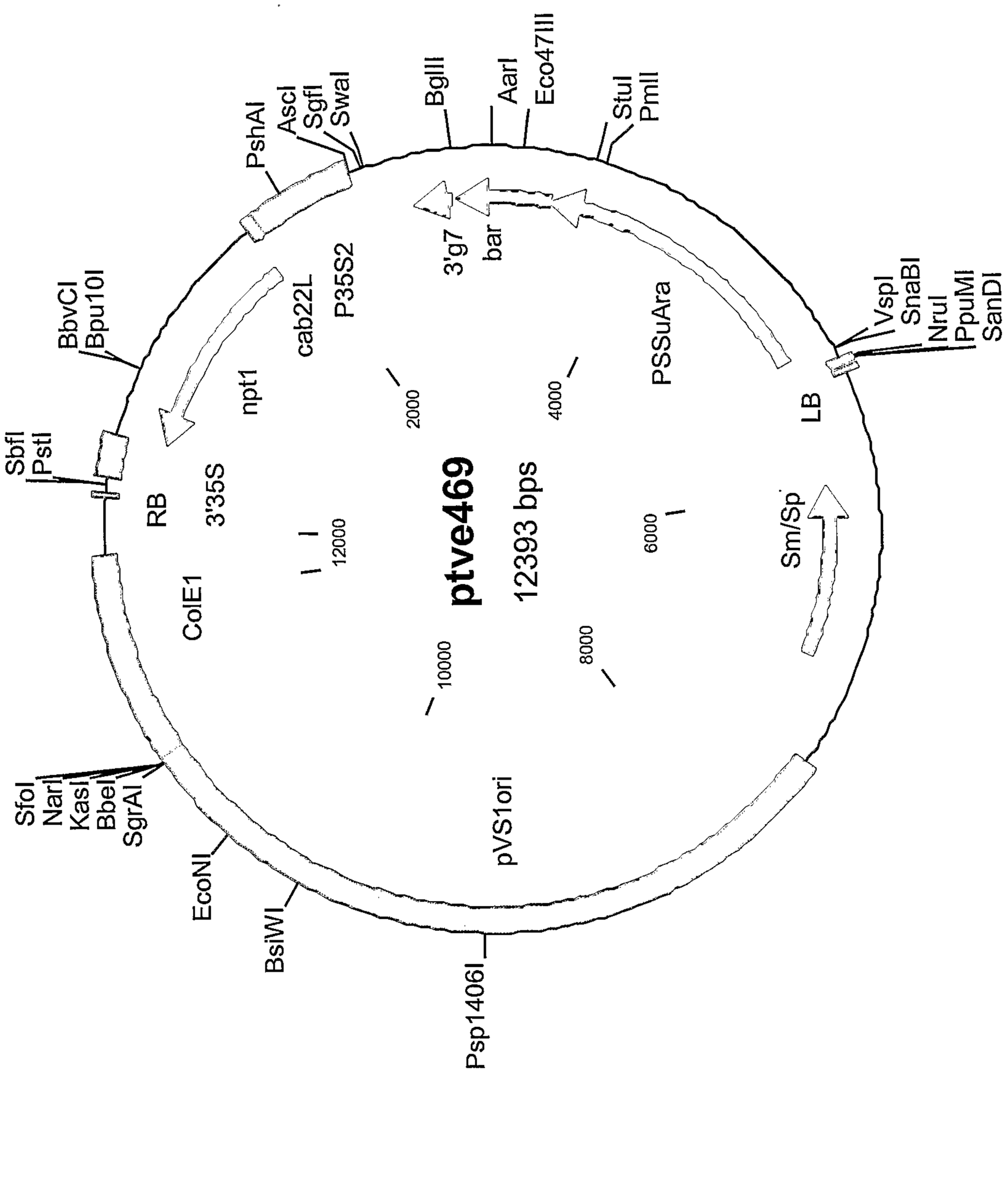

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PpuMI
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Fig. 5

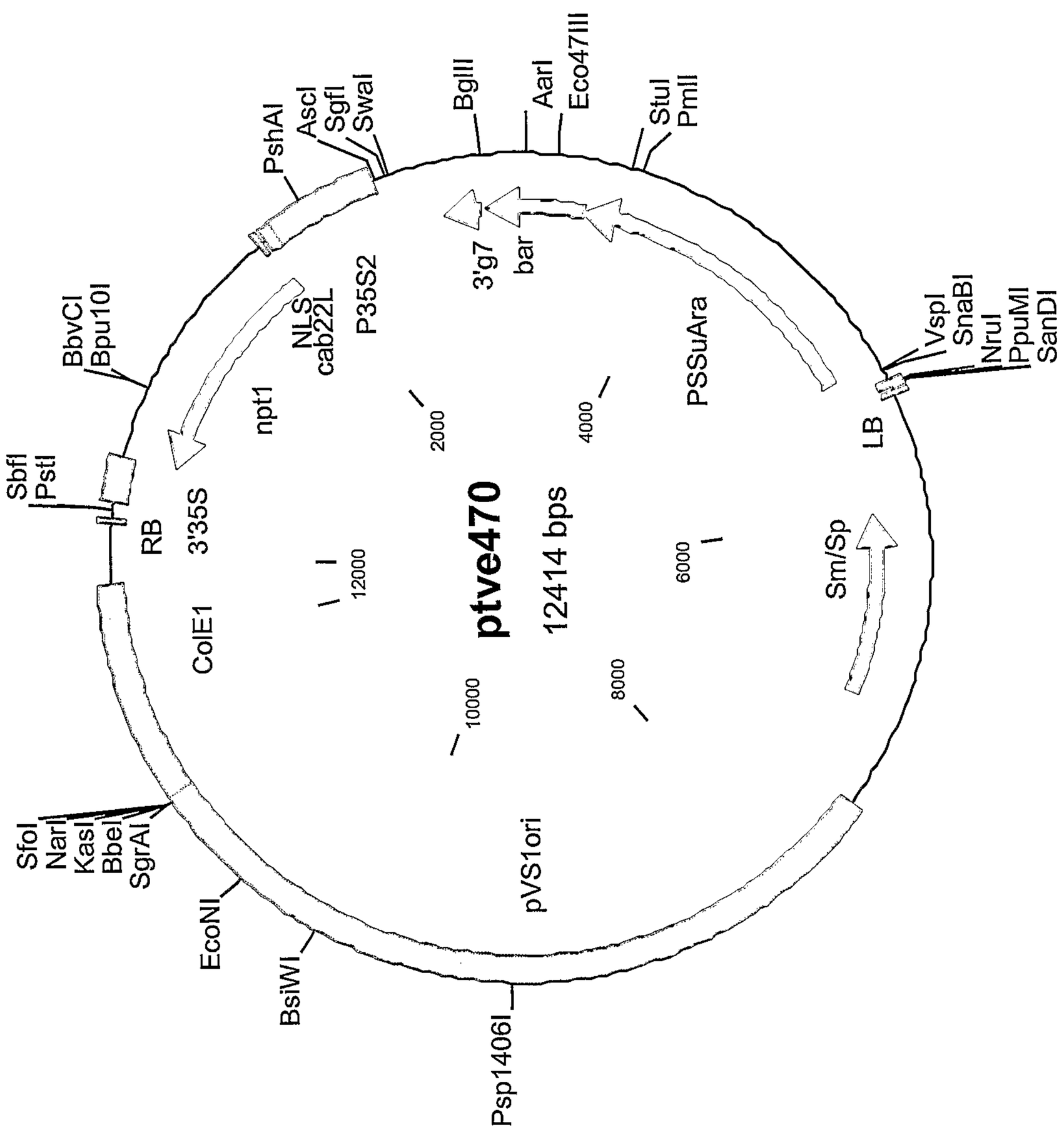

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Fig. 6

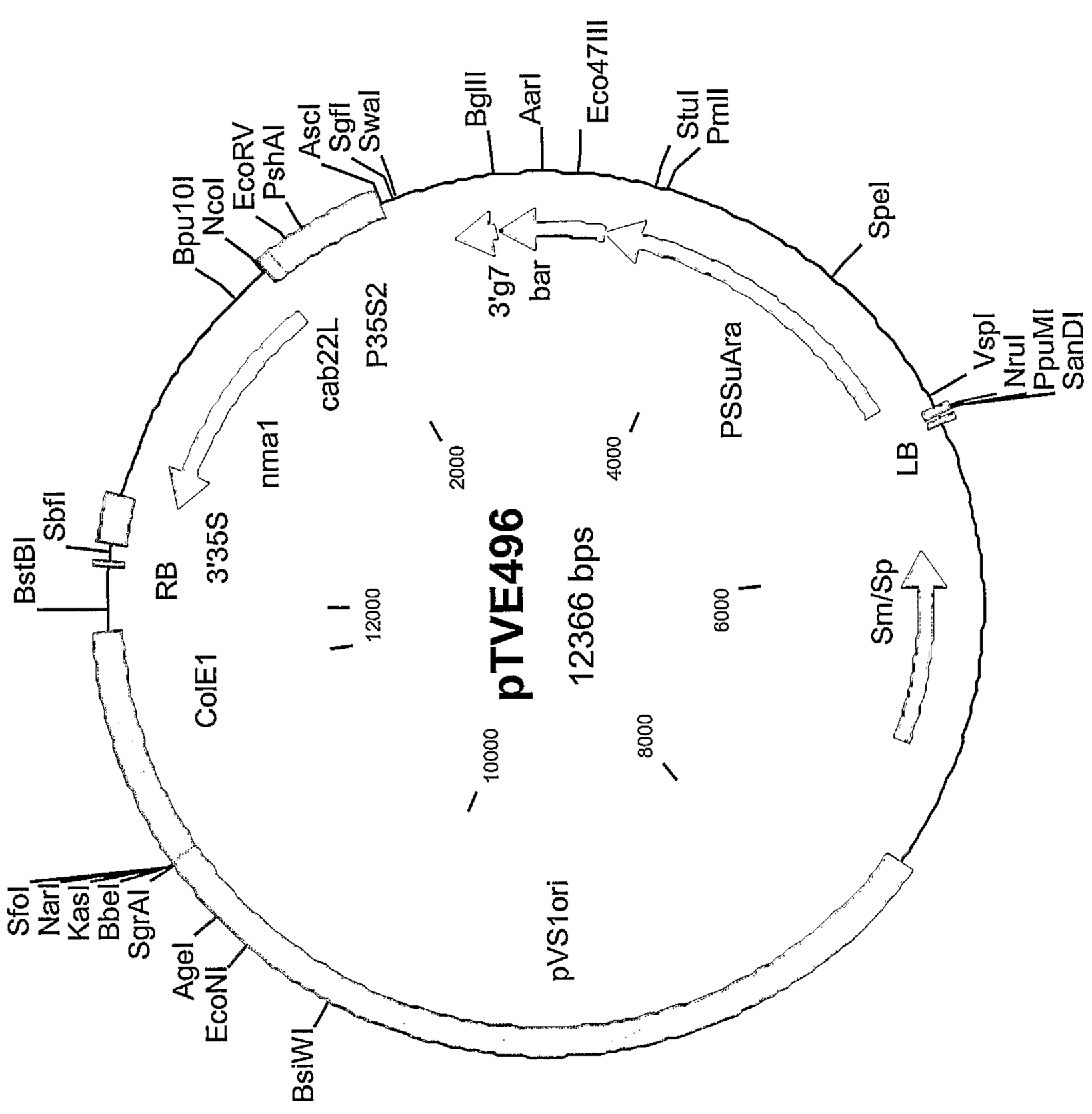

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Fig. 7

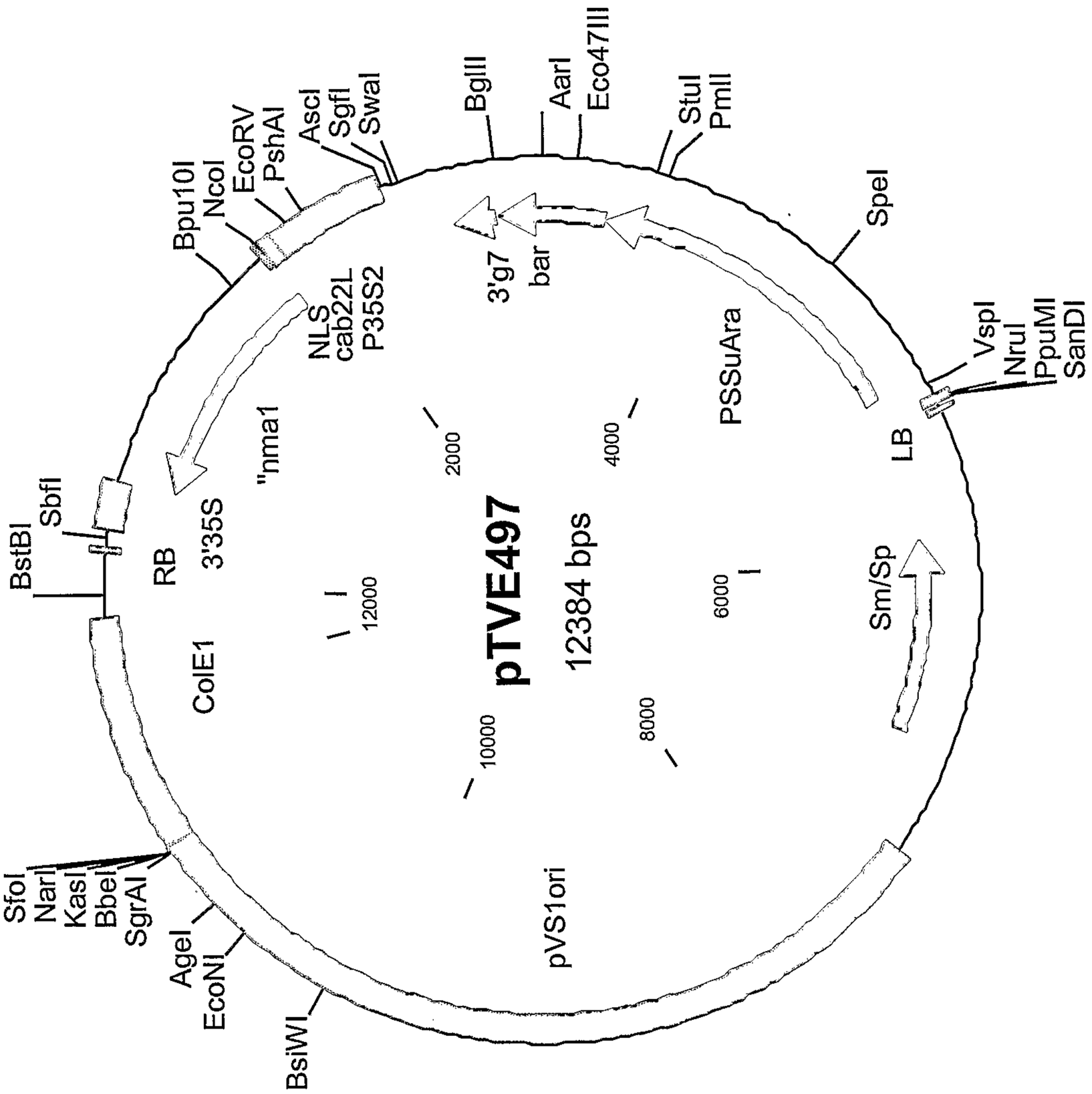

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Fig. 8

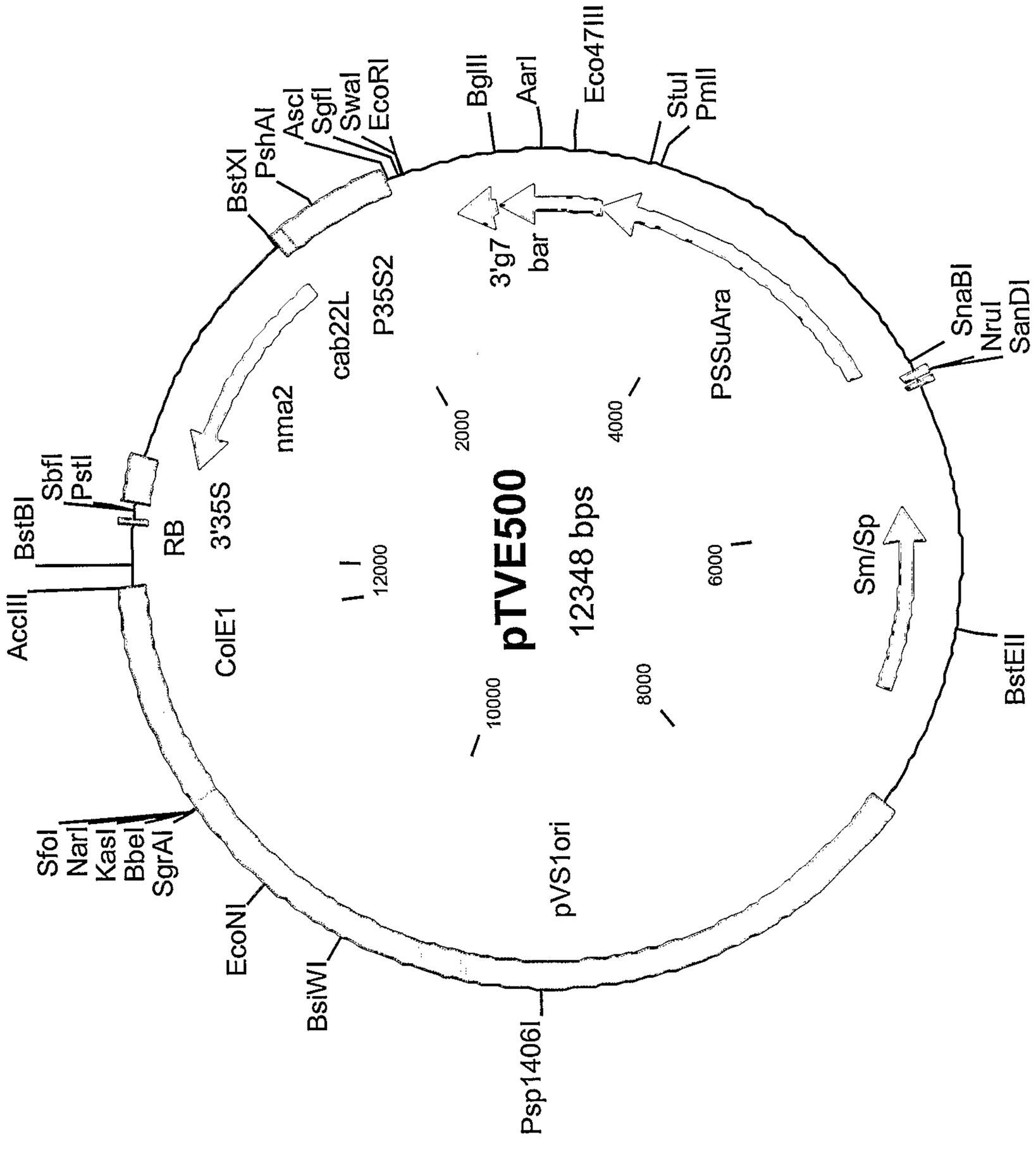

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Fig. 9

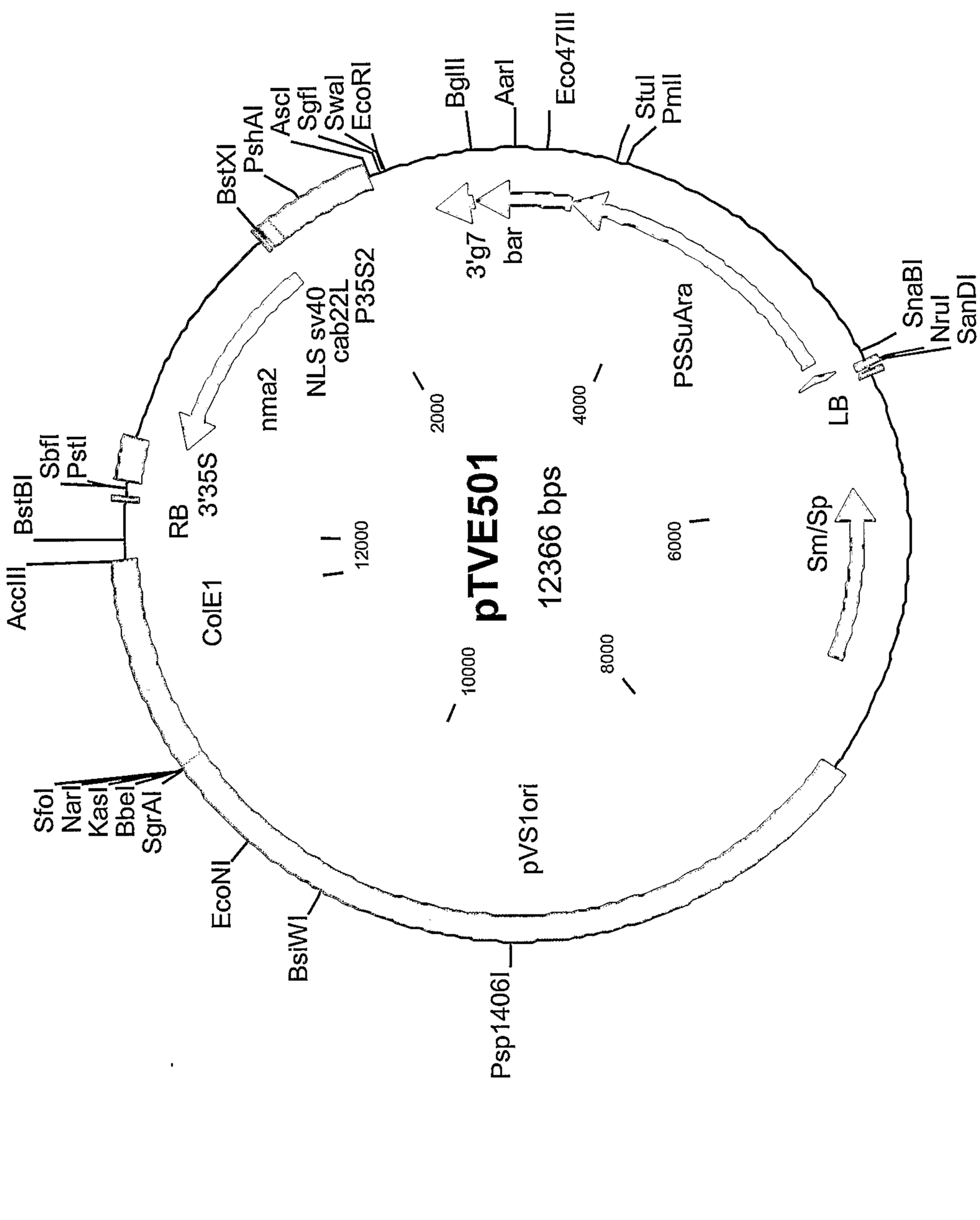

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Fig. 10

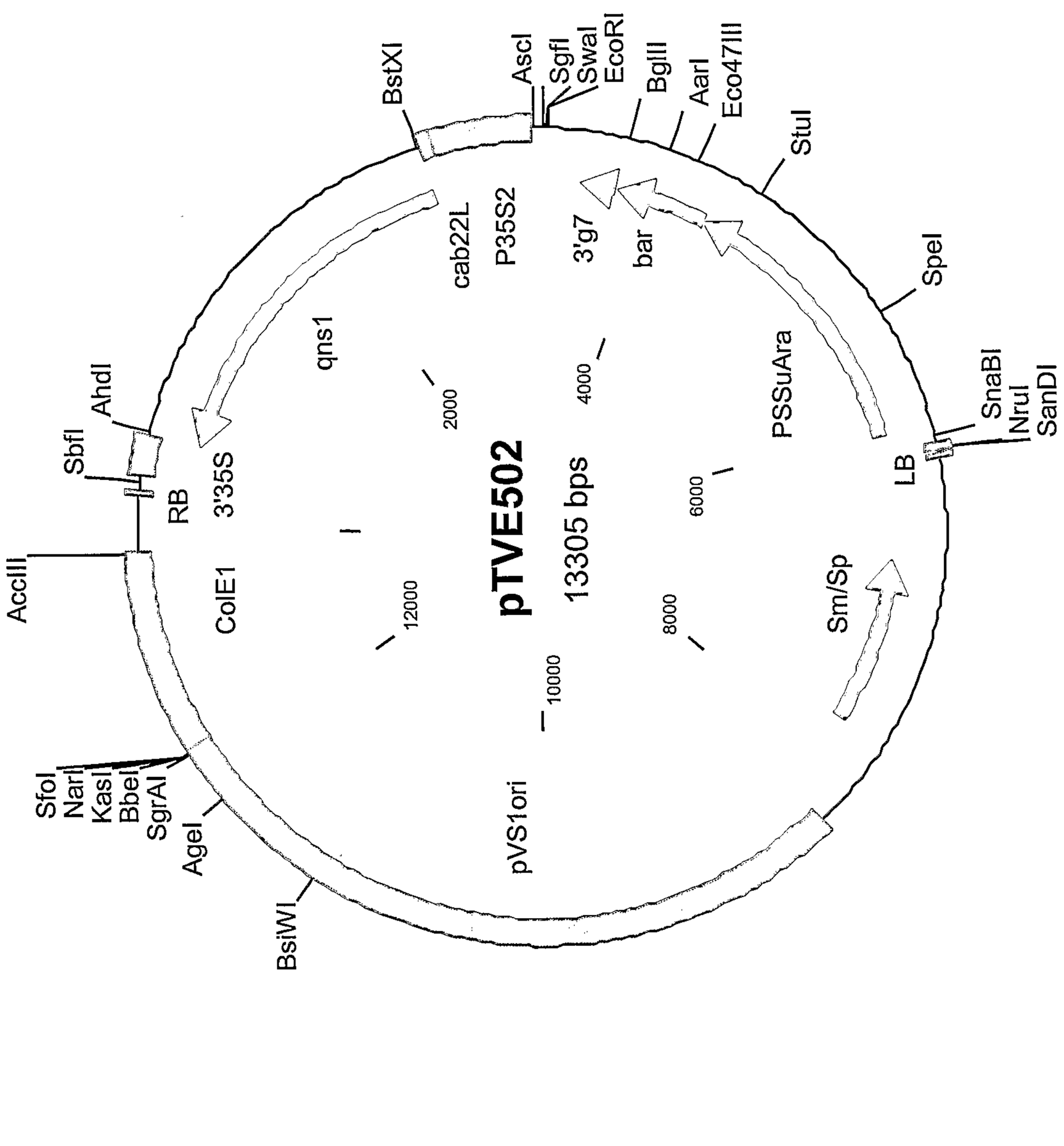
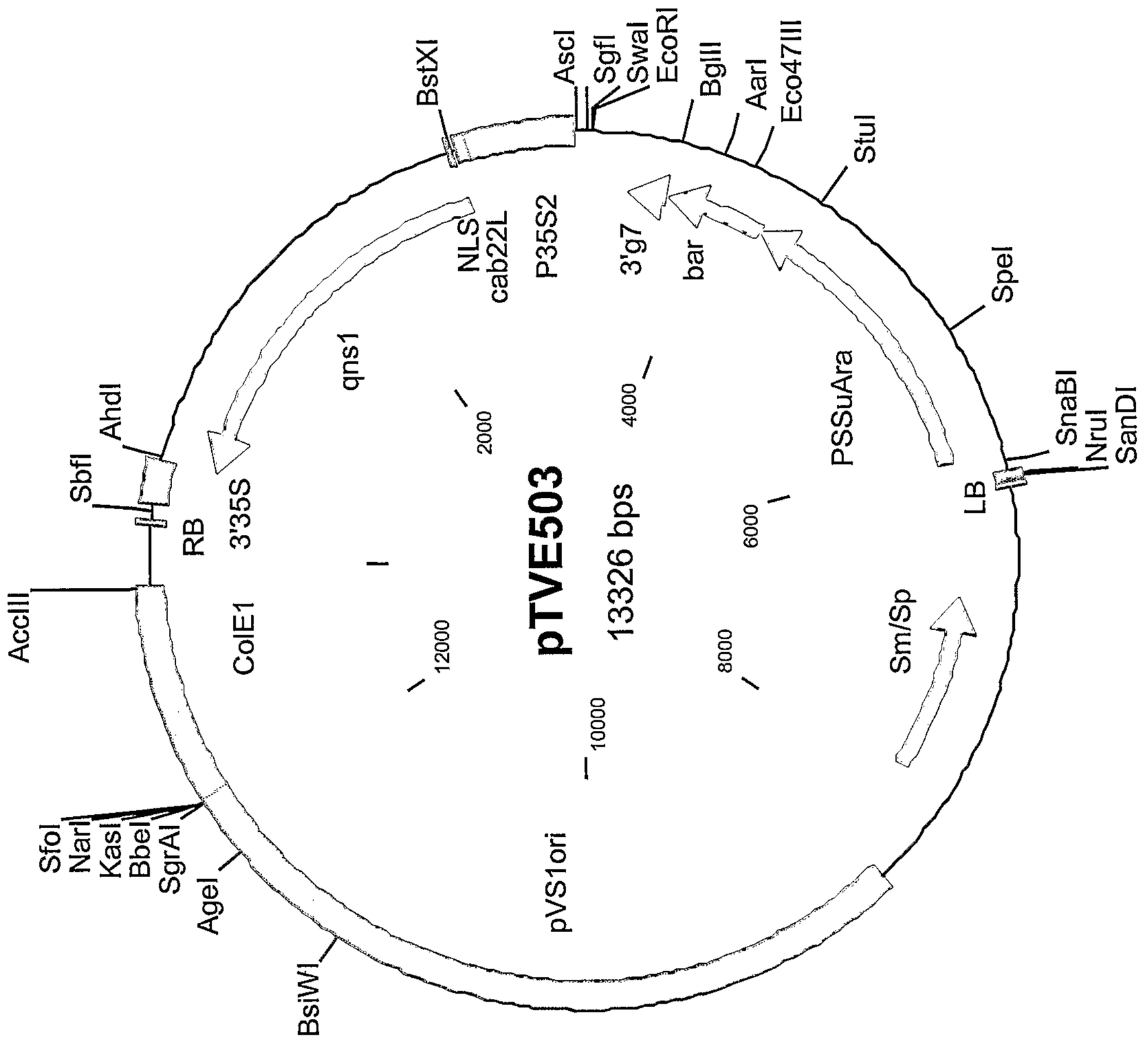
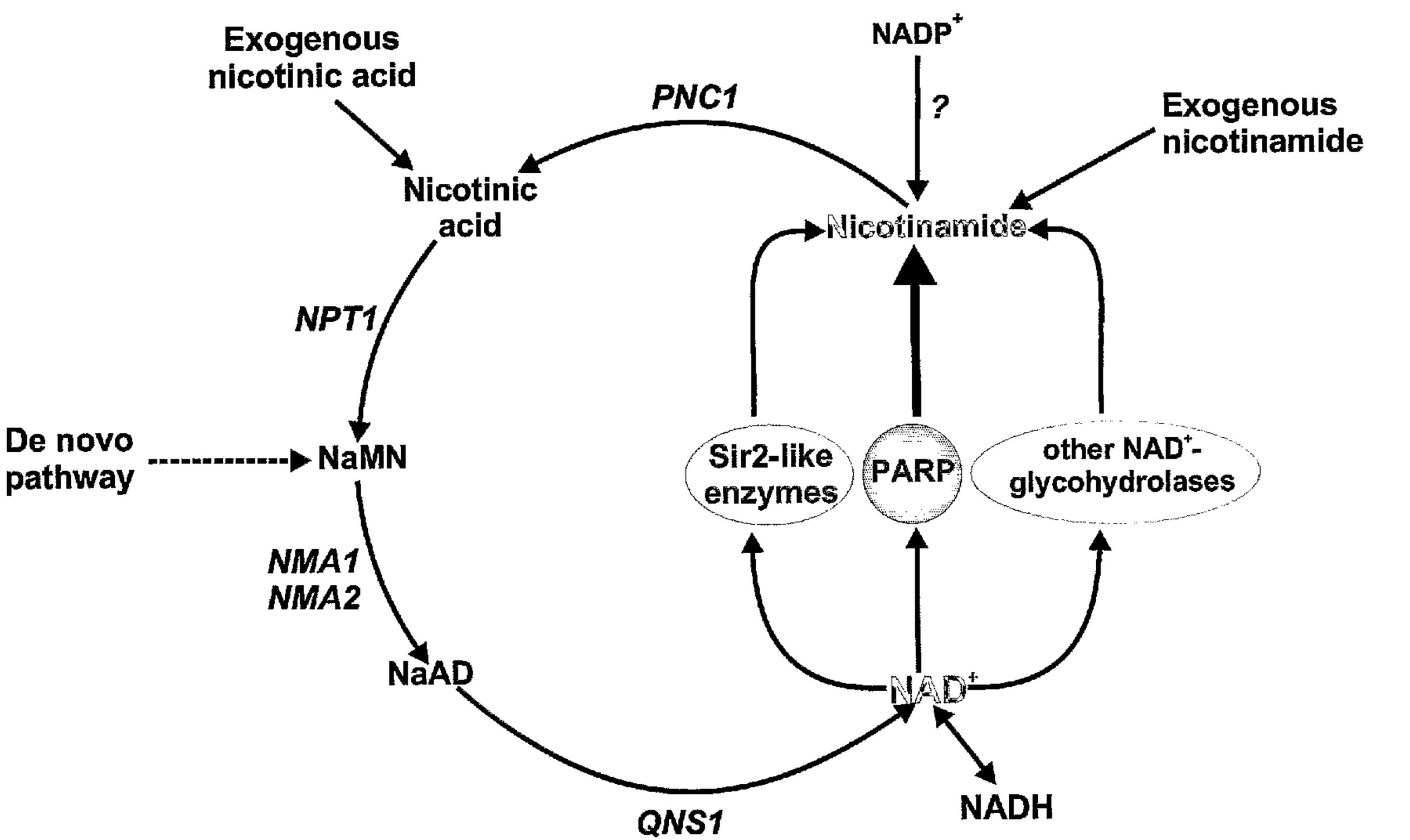

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Fig. 11


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NarI
SfoI



NaMN: nicotinic acid mononucleotide
NaAD: desamino-NAD^+

PNC1: nicotinamidase
NPT1: nicotinate phosphoribosyl transferase
NMA: NAD^+ pyrophosphorylase
QNS1: NAD^+ synthetase