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(54) **MOYENS ET METHODES POUR TRANSFORMER LES
MITOCHONDRIES DES VEGETAUX**

(54) **MEANS AND METHODS FOR TRANSFORMATION OF PLANT
MITOCHONDRIA**

(57) A method is provided for introducing a foreign, exogenously added DNA fragment into mitochondria of plants. Preferably the foreign DNA comprises a DNA fragment allowing replication in mitochondria of plants. The method for producing a transgenic plant or plant cell comprising a foreign DNA fragment in the mitochondria of its cells comprises the steps of a) contacting an untransformed plant cell, preferably derived from a multicellular plant, with the foreign DNA, under conditions allowing uptake of DNA into the plant cell to generate a transgenic plant cell; and b) optionally regenerating a transgenic plant from the transgenic plant cell wherein the foreign DNA comprises an origin of replication for mitochondria and preferably further comprises a gene of interest. Preferably, the gene of interest comprises a promoter region which can direct expression in mitochondria of a plant cell, operably linked to a coding region, and optionally, further operably linked to a transcription terminator region functional in mitochondria. The coding region may comprise one, two or more cistrons.



ABSTRACT

A method is provided for introducing a foreign, exogenously added DNA fragment into mitochondria of plants. Preferably the foreign DNA comprises a DNA fragment allowing replication in mitochondria of plants. The method for producing a transgenic plant or plant cell comprising a foreign DNA fragment in the mitochondria of its cells comprises the steps of a) contacting an untransformed plant cell, preferably derived from a multicellular plant, with the foreign DNA, under conditions allowing uptake of DNA into the plant cell to generate a transgenic plant cell; and b) optionally regenerating a transgenic plant from the transgenic plant cell wherein the foreign DNA comprises an origin of replication for mitochondria and preferably further comprises a gene of interest. Preferably, the gene of interest comprises a promoter region which can direct expression in mitochondria of a plant cell, operably linked to a coding region, and optionally, further operably linked to a transcription terminator region functional in mitochondria. The coding region may comprise one, two or more cistrons.

MEANS AND METHODS FOR TRANSFORMATION OF PLANT MITOCHONDRIA**FIELD OF THE INVENTION**

The present invention relates to the field of genetic transformation of mitochondria of multicellular plants, particularly higher plants such as the monocotyledoneae or the dicotyledoneae. The invention provides methods and means for introducing a foreign, exogenously added DNA fragment into mitochondria of plants. Preferably the foreign DNA comprises a DNA fragment allowing replication in mitochondria of plants.

10 BACKGROUND ART

In plants, like in all organisms, mitochondria provide the principal energy source for the cell in the form of ATP. Furthermore, they deliver numerous substrates via specific carriers for biosynthetic reactions in the cytoplasm.

15 Mitochondria are the third compartment in plants, containing DNA beside nucleus and chloroplasts. Higher plant mitochondrial genomes are much larger and more complex than those of other organisms. Nevertheless, plants like other eukaryotes, encode only a small number of their mitochondrial proteins. These proteins are mainly constituents of the inner mitochondrial membrane, where they are components of the electron transport chain or the F0-F1 ATPase complex. The majority of the mitochondrial proteins are encoded by nuclear genes, synthesized on cytosolic ribosomes and imported
20 posttranslationally into the mitochondria.

Agronomically useful traits such as cytoplasmic male sterility (cms) are also encoded by the mitochondrial genome. Some cms appear to be caused by novel mitochondrial genes not typical of mitochondrial genomes, whereas others seem to be due
30 to mutations of common mitochondrial genes.

Plant mitochondria often harbor several extrachromosomal elements in addition to the genomic DNA. These extrachromosomal elements or plasmids have been classified in three categories: circular DNA plasmids, linear DNA plasmids and RNA plasmids. The circular DNA plasmids are
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very small and lack homology to known genes.

Whereas transformation of nuclear genomes in plants has now become routine using a variety of methods for the introduction of foreign exogenously added donor DNA, and recently stable transformation of chloroplasts has also been achieved (O'Neill et al., 1993; Maliga, 1993), the manipulation of mitochondrial genomes in plants, however has been limited to production of so-called cybrids via protoplast fusion.

Kemble et al. (1988) described *Brassica napus* cybrid plants which were constructed via protoplast fusion. Such fusions resulted either in mitochondrial DNA plasmids being lost, or being transferred from mitochondria of one protoplast population to mitochondria of the other population.

Direct manipulation of mitochondrial genomes by introduction of foreign DNA has been achieved by high-velocity microprojectile bombardment in yeast and *Chlamydomonas*. Johnston et al. (1988) described transformation of respiratory yeast mutants by corresponding wild-type genes which integrated at the corresponding genelocus in the mitochondrial genome through homologous recombination. Fox et al. (1988) described stable transformation of yeast mitochondria lacking endogenous mtDNA by recombinant plasmids comprising the origin of replication of the 2 μ plasmid. Randolph-Anderson et al. (1993) used a respiratory-deficient mutant of *Chlamydomonas reinhardtii* as a recipient for stable mitochondrial transformation by donor DNA from *C. smithii*.

The respiratory mutations from *Chlamydomonas* and yeast, which are required to detect the infrequent event of transformation of mitochondria by rescue of the inactivated function, can be maintained on special media. However, this is less obvious in plants which has hitherto hampered the development of efficient methods for the stable introduction of foreign, exogenously added DNA, such as *in vitro* manipulated DNA, into mitochondria of higher plants.

The art is thus deficient in the provision of methods for the stable introduction of foreign, exogenously added DNA into mitochondria of plants.

EP 0 223 247 entitled "Insertion of DNA into plastids and mitochondria" suggests that direct transfer of useful genes into plastids and mitochondria and stable maintenance therein may be useful, but provides no examples of how to achieve the
5 stable introduction of foreign DNA into mitochondria of plants.

SUMMARY OF THE INVENTION

The invention provides a method for producing a transgenic plant or plant cell comprising a foreign DNA fragment in the mitochondria of its cells, the method
10 comprising the steps of a) contacting an untransformed plant cell, preferably derived from a multicellular plant, with said foreign DNA, under conditions allowing uptake of DNA into said plant cell to generate a transgenic plant cell; and b)
optionally regenerating a transgenic plant from the transgenic
15 plant cell wherein said foreign DNA comprises an origin of replication for mitochondria and preferably further comprises a gene of interest. Preferably, the gene of interest comprises a promoter region which can direct expression in mitochondria of a plant cell, operably linked to a coding region, and
20 optionally, further operably linked to a transcription terminator region functional in mitochondria. The coding region may comprise one, two or more cistrons.

The invention further provides a method for producing a transgenic plant or plant cell comprising a foreign DNA
25 fragment in the mitochondria of its cells, the method comprising the steps of a) contacting an untransformed plant cell, with the foreign DNA, preferably by treating plant protoplasts with a polyalcohol in the presence of the foreign DNA or by electroporation of the plant cells, particularly
30 plant protoplasts, in the presence of the foreign DNA or by bombardment of the plant cells with microprojectiles coated with the foreign DNA; and b) optionally regenerating a transgenic plant from the transgenic plant cell, wherein the foreign DNA comprises an origin of replication for
35 mitochondria.

It is another objective of the invention to provide a method for producing a transgenic plant or plant cell comprising a foreign DNA fragment in the mitochondria of its cells, the method comprising the steps of a) contacting an
5 untransformed plant cell, under conditions allowing uptake of DNA into the plant cell to generate a transgenic plant cell; and b) optionally regenerating a transgenic plant from the transgenic plant cell, wherein the foreign DNA comprises an origin of replication for mitochondria derived from a
10 mitochondrial plasmid, preferably derived from the mitochondrial plasmid mp1 of *Chenopodium album*, mt-plasmid 1 from *Vicia faba*, mt-plasmid 2 from *Vicia faba* or mt-plasmid 3 from *Vicia faba*, particularly comprising a nucleotide sequence selected from the group of the nucleotide sequence of
15 SEQ ID NO 1 from the nucleotide at position 628 to the nucleotide at position 781, the nucleotide sequence of SEQ ID NO 1 from the nucleotide at position 411 to the nucleotide at position 781 and the nucleotide sequence of SEQ ID NO 1 from the nucleotide at position 308 to the
20 nucleotide at position 781.

It is yet another objective of the present invention to provide a method for producing a transgenic plant or plant cell comprising a foreign DNA fragment in the mitochondria of its cells, the method comprising the steps of a) contacting an
25 untransformed plant cell, under conditions allowing uptake of DNA into the plant cell to generate a transgenic plant cell; and b) optionally regenerating a transgenic plant from the transgenic plant cell, wherein the foreign DNA comprises an origin of replication for mitochondria, preferably wherein the
30 foreign DNA is a chimeric mitochondrial plasmid, and particularly further comprises a chimeric gene capable of expression in mitochondria comprising:

(a) a promoter region which can direct expression in mitochondria of a plant cell, preferably a promoter
35 region which is selected from the group consisting of the promoter region upstream of the cox2 gene, the promoter region upstream of the rpl5 gene in pea, the

about 0.6 kb promoter region upstream of the atp9-1 encoding ORF and the promoter region with the nucleotide sequence of SEQ ID NO 2 from the nucleotide at position 1 to the nucleotide at position 650; and

- (b) a coding region, preferably a coding region, which when expressed, results in the production of a marker polypeptide or protein, or a coding region encoding inhibitory RNA or a coding region when expressed, results in cytoplasmic male sterility.

It is another objective of the invention to provide a method for producing a transgenic plant comprising a foreign DNA fragment in the mitochondria of its cells, the method comprising the steps of a) contacting an untransformed plant cell, under conditions allowing uptake of DNA into the plant cell to generate a transgenic plant cell; and b) regenerating a transgenic plant from the transgenic plant cell, wherein the foreign DNA comprises an origin of replication for mitochondria and crossing the transgenic plant with another plant to obtain progeny plants comprising the foreign DNA in their mitochondria.

The invention also provides a plant cell or plant comprising transgenic mitochondria, preferably a plant cell or plant with mitochondria comprising a chimeric mitochondrial plasmid, particularly plant cells and plants wherein chimeric mitochondrial plasmid further comprises a gene of interest, said gene of interest comprising a promoter which can be expressed in mitochondria of a plant cell; operably linked to a coding region and optionally further operably linked to a transcription termination region functional in mitochondria.

It is another object of the invention to provide seed and propagules, preferably propagules obtainable by vegetative propagation of the plants obtainable by the methods of the invention, wherein the cells of the seed or propagules comprise transgenic mitochondria.

It is yet another objective of the invention to

provide an isolated DNA molecule comprising an origin of replication for mitochondria, preferably an origin of replication derived from a circular mitochondrial plasmid, preferably an origin of replication derived from mp1, particularly an origin of replication comprising a nucleotide sequence selected from the group of the nucleotide sequence of SEQ ID NO 1 from the nucleotide at position 628 to the nucleotide at position 781, the nucleotide sequence of SEQ ID NO 1 from the nucleotide at position 411 to the nucleotide at position 781, the nucleotide sequence of SEQ ID NO 1 from the nucleotide at position 308 to the nucleotide at position 781 and the nucleotide sequence of SEQ ID NO 1, and a gene of interest, the gene of interest comprising the following operably linked DNA fragments:

- (a) a promoter region which can direct expression in mitochondria of a plant cell, preferably a promoter selected from the group consisting of the promoter region upstream of the cox2 gene, the promoter region upstream of the rpl5 gene in pea, the about 0.6 kb promoter region upstream of the atp9-1 encoding ORF and the promoter region with the nucleotide sequence of SEQ ID NO 2 from the nucleotide at position 1 to the nucleotide at position 650;
- (b) a coding region; and
- (c) optionally, a transcription terminator region functional in mitochondria.

The isolated DNA molecule may further comprises a mitochondrial marker gene.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1. Upper panel. Detection of mp1-specific fragments obtained after PCR amplification using mp1-specific primers and total DNA of tobacco cells, calli and plants which developed from protoplasts transformed with the shuttle chimeric

mitochondrial plasmid pMP2. Total DNA was isolated from the different explants. For control of the template quality, the same DNA samples were subjected to a PCR using chloroplast specific primers (lower panel). Wt 3d: total DNA obtained from wild type untransformed tobacco protoplasts, isolated 3 days after initiation of the experiment; Wt 6d: total DNA obtained from wild type untransformed tobacco protoplasts, isolated 6 days after initiation of the experiment; Plasmid control: template DNA is pMP2 plasmid; 35S-GFP: total DNA obtained from calli, derived from tobacco protoplasts transformed by a 35S-GFP construct; pMP2 plant: total DNA obtained from a plant regenerated from tobacco protoplasts transformed by pMP2; pMP2 26d: total DNA obtained from tobacco protoplasts, transformed by pMP2 isolated 26 days after initiation of the experiment (transformation); pMP2 16d: total DNA obtained from tobacco protoplasts, transformed by pMP2 isolated 16 days after initiation of the experiment (transformation); pMP2 13d: total DNA obtained from tobacco protoplasts, transformed by pMP2 isolated 13 days after initiation of the experiment (transformation); pMP2 12d: total DNA obtained from tobacco protoplasts, transformed by pMP2 isolated 12 days after initiation of the experiment (transformation); Wt 26d: total DNA obtained from wild type untransformed tobacco protoplasts, isolated 26 days after initiation of the experiment; Wt: total DNA obtained from wt untransformed tobacco plants; 1kb: size reference DNA.

Figure 2. Southern blot analysis of total DNA of plants transformed by the shuttle chimeric mitochondrial plasmid pMP2. Total DNA was isolated from different shoots which were regenerated from pMP2 treated protoplasts, and digested by XbaI. In two plants (1a/5 and 1b/3) a fragment of about 4.5 kb was detected by hybridization with pMP2 specific probe. In wild type plants (wt) no fragments hybridizing in an unspecific manner were found.

Figure 3. Southern analysis of nuclear, mitochondrial (mt-DNA) and chloroplast DNA (cp-DNA). The DNA of either nucleus, mitochondria or chloroplasts was separately isolated from

either non-transformed tobacco plants (wt) or plants which were regenerated from pMP2 treated protoplasts (MP2-1, MP2-4). The DNA was digested with XbaI and hybridized against a mp1 specific probe. As controls, 10 pg of non-digested (pMP2) or
5 XbaI digested vector DNA (pMP2 XbaI) were included. 1 kb: size reference DNA.

Figure 4. Southern blot analysis using a pMP2 specific probe of mitochondrial DNA isolated from progeny of pMP2 containing plants. Wt: wild-type plants; MP2-1 plant regenerated from
10 pMP2 treated protoplasts; MP2x wt: progeny derived from pollination of pMP2 transformed plants with wild type pollen; wt xMP2-1: progeny derived from pollination of wild type plants with pollen from pMP2 transformed plants. 1 kb: size reference DNA.

15 **Figure 5.** Southern blot analysis using a pMP2 specific probe of mitochondrial DNA isolated from different progeny plants. Mitochondrial DNA was isolated from wild type plants (wt) and progeny plants derived from crosses of different plants regenerated from pMP2 treated protoplasts (Mp1.7, Mp8, Mp1,
20 Mp1.7, Mp12.5, Mp4.3) with wild type plants. Mp x wt refers to crosses wherein the indicated Mp plant was pollinated with wild type pollen; Wt xMp refers to crosses wherein the wild type plant was pollinated with pollen derived from the indicated Mp plant.

25 **Figure 6.** RT-PCR analysis of transcription of the GFP gene under control of a mitochondrial promoter. RNA was isolated from 2 months old calli which were regenerated from PEG-treated protoplasts transformed with either MP-GFP mitochondrial plasmid (MP-GFP) for mitochondria specific expression, or with
30 the control constructs pCAT-GFP or p35S-GFP for nuclear expression. In calli transformed with MP-GFP, pCAT-GFP and p35S-GFP, GFP specific PCR products were observed (upper gel). In the lower gel, PCR amplification was performed using an RNA template without or with (indicated by *) DNase treatment.

35 DETAILED DESCRIPTION OF PREFERRED EMBODIMENTS

As will be explained in detail hereafter, it has been

found by the inventors that introduction into plant cells, such as plant protoplasts, of exogenously added foreign DNA, capable of replication in plant mitochondria resulted in transgenic plant cells and regenerated plants comprising mitochondria wherein the exogenous, foreign DNA was incorporated. Moreover, the exogenously added DNA incorporated in the transgenic mitochondria could be transmitted to progeny plants. This method of introducing foreign DNA into mitochondria also allowed to introduce a chimeric gene capable of mitochondrial expression into mitochondria of a plant.

In one embodiment, the invention thus relates to a method for the production of a plant cell or plant comprising a foreign DNA fragment in the mitochondria of its cell, comprising the step of contacting an untransformed plant cell with exogenously added DNA under conditions allowing uptake of the DNA into the plant cell to generate a transgenic plant cell, optionally followed by regeneration of a plant from the transgenic plant cell. The exogenously added DNA fragment should preferably be capable of replication in mitochondria, particularly it should be capable of autonomous replication in mitochondria of a plant cell. In other words, the foreign DNA should comprise at least an origin of replication, preferably autonomous replication for mitochondria.

As used herein, "exogenously added DNA" refers to DNA, which has been isolated in a relatively pure form *in vitro*, prior to the contacting with plant cells in order to be introduced into these plant cells, preferably into the mitochondria. Although the DNA is preferably in a naked form when contacted with the plant cell, it may still be incorporated in particular structures, e.g. liposomes.

The term "untransformed plant cell" which is contacted with the exogenously added or foreign DNA, is used herein solely to refer to a plant cell which does not yet comprise that foreign DNA. However, it is clear that the plant cell may have been transformed previously, and may thus contain transgenes either integrated into the nuclear or chloroplastic genome or even replicating in its mitochondria.

As used herein, "an origin of replication for mitochondria" refers to a DNA fragment which allows multiplication of that DNA fragment and of any DNA which is operably linked to such an origin of replication for mitochondria, when present in a functional mitochondrion or an *in vitro* equivalent thereof. Multiplication of a DNA molecule comprising an origin of replication for mitochondria may proceed via DNA or RNA intermediates. Preferably, an origin of replication for mitochondria is derived from a mitochondrial plasmid, particularly from a circular mitochondrial plasmid. Methods for assaying *in vitro* whether a DNA fragment comprises an origin of replication for mitochondria have been described e.g. Backert *et al.* (1998) (incorporated by reference). "Autonomous replication" as used herein refers to the fact that the DNA molecule which replicates does not have to be operably linked to any other DNA molecule, and preferably is not dependent for its replication upon factors encoded by another replicating DNA molecule.

In preferred embodiments of the invention, the origin of replication of the exogenously added DNA molecule is derived from the mt-plasmid mp1, originally isolated from *Chenopodium album*, particularly the origin of replication comprises an about 148 bp BamHI/EcoRI fragment of mp1 (comprises the nucleotide sequence of SEQ ID NO 1 from the nucleotide at position 628 to the nucleotide at position 781 or the complement thereof), more particularly the origin of replication comprises an about 363 bp BamHI/SmaI fragment or an about 464 bp BamHI/KpnI fragment from mp1 (respectively comprising the nucleotide sequence of SEQ ID NO 1 from the nucleotide at position 411 to the nucleotide at position 781 or the nucleotide sequence of SEQ ID NO 1 from the nucleotide at position 308 to the nucleotide at position 781 or the complements thereof). In a particularly preferred embodiment of the invention, the exogenously added DNA comprises mp1 (the nucleotide sequence of SEQ ID NO 1 or the complement thereof). It goes without saying that also variants, such as deletion or substitution mutants, of the naturally isolated origins of

replication for mitochondria may be used to similar effect, provided they are still functional.

It is further envisioned by the invention, to isolate and use origins of replication which can identified and subsequently isolated by conventional DNA identification methods using all or part, preferably at least 25 nucleotides, particularly at least 50 nucleotides, of the mentioned origins of replication for mitochondria, such as Southern type hybridization or PCR amplification under stringent conditions (e.g. Southern hybridization at 68°C in a buffer comprising 6 x SSC and Denhardt's solution, followed by at least one wash in a buffer comprising 0.1 SSC at 68°C or PCR amplification using e.g. 30-mer oligonucleotides and a 30 temperature cycles of 1 min at 92°C, 1 min at 65°C, 2 min at 72°C).

A "mitochondrial plasmid (mt-plasmid)" as used herein refers to an extrachromosomal element which can replicate in mitochondria of a eukaryotic cell, preferably in a plant cell, and which comprises an origin of replication. mt-Plasmids can be circular or linear. The term includes natural isolates, chimeric derivatives or synthetic, man-made plasmids. Different examples of mitochondrial plasmids include but are not limited to mp1 (originally isolated from *Chenopodium album*), mt-plasmid 1, mt-plasmid 2, mt-plasmid 3 (originally isolated from *Vicia faba*), plasmids B1 to B4 (originally isolated from *Oryza sativa*), plasmids K1 and K2 from *Lupinus albus*, plasmids P1 and P2 from *Helianthus annuus*, the 1.9 kb and the 1.4 kb plasmid from *Zea mays*. These and other mitochondrial plasmids are described in Brown and Zhang (1995) (incorporated by reference) or Lonsdale and Grienenberger (1992) and references therein.

As used herein, "a chimeric mitochondrial plasmid" is a mitochondrial plasmid which contains heterologous or foreign DNA. "Heterologous" or "foreign" as used in this context such a DNA is not found associated with the mitochondrial plasmid when it is naturally found in the mitochondria of a cell of a plant. In a preferred embodiment, the chimeric plasmid comprises mp2 (Backert et al. 1996; see also Examples).

In a more general context, "foreign" or "heterologous" with regard to a DNA sequence, such as a foreign or heterologous coding region, means that such a DNA is not in the same genomic environment (e.g. not operably linked to the same promoter region and/or 3' end) in a plant cell, transformed with such a DNA in accordance with this invention, as is such DNA when it is naturally found in a cell of the plant, bacteria, animal, fungus, virus, or the like from which such a DNA originates.

"Contacting of a plant cell with exogenously added DNA under conditions allowing uptake of the DNA" refers to the process of physically adding DNA to the plant cell in such a way that the DNA is introduced into the plant cell, particularly into the mitochondria of the plant cell. In a preferred embodiment the plant cells are devoid of the major part of their cell wall, (i.e. are protoplasts) and the DNA is introduced into the plant protoplast by treatment with a polyalcohol, such as polyethyleneglycol (PEG) according to methods available in the art (see e.g. Krens et al., 1982).

It is however expected that other methods for DNA transfer, particularly other methods referred to in the art as "direct gene transfer methods", including but not limited to: introduction of DNA via electroporation, particularly high-voltage electroporation of protoplasts or intact cells or tissues (e.g. callus tissue, immature embryos, etc.) in the presence of exogenously added DNA; micro-injection into plant cells; high-velocity microprojectile bombardment of plant cells or tissues and the like can be used to similar effect and these alternative methods are also preferred embodiments of the invention.

It goes without saying that if the ultimate goal of the method is to produce plants, the suitable recipient plant cells and tissue should be capable of regeneration into mature plants, and preferably fertile mature plants.

The methods of the invention can be used to express a gene of interest in mitochondria of plants or plant cells. In a preferred embodiment, the invention relates to a method for

producing a transgenic plant cell or plant comprising a foreign DNA fragment in the mitochondria of its cells, comprising the step of contacting an untransformed plant cell with exogenously added DNA under conditions allowing uptake of the DNA into the plant cell to generate a transgenic plant cell, optionally followed by regeneration of a plant from the transgenic plant cell, wherein the foreign DNA comprises an origin of replication for mitochondria and a gene of interest, particularly a chimeric gene of interest, capable of being expressed in the mitochondria of a plant cell.

Preferably, the gene of interest thus comprises a promoter region which can direct expression in mitochondria of a plant cell, and a coding region, particularly a heterologous coding region.

The term "gene" means any DNA fragment comprising a DNA region (the "transcribed DNA region") that is transcribed into a RNA molecule (e.g., a mRNA) in a cell operably linked to suitable regulatory regions, e.g., a promoter region which can direct expression in mitochondria. A gene capable of being expressed in mitochondria may thus comprise several operably linked DNA fragments such as a mitochondrial promoter region, a coding region, and a 3' region comprising a transcription termination site. A mitochondrial plant gene endogenous to a particular plant species (endogenous mitochondrial plant gene) is a gene which is naturally found in the mitochondria of that plant species or which can be introduced in that plant species by conventional breeding. A chimeric mitochondrial gene is any mitochondrial gene which is not normally found in a plant species or, alternatively, any gene in which the promoter region is not associated in nature with part or all of the transcribed DNA region or with at least one other regulatory region of the gene.

The term "coding region" refers to a DNA region which when operably linked to appropriate regulatory regions, particularly to a promoter region, is transcribed as part of an RNA which is biologically active i.e., which is either capable of interaction with another nucleic acid or which is capable of

being translated into a polypeptide or protein. A coding region is said to encode an RNA when the end product of the expression of the gene is biologically active RNA, such as e.g. inhibitory RNA like an antisense RNA or a ribozyme or a replicative intermediate. A coding region is said to encode a protein when the end product of the expression of the gene is a protein or polypeptide.

Promoters and promoter regions capable of being expressed in mitochondria of plant cells are known in the art (reviewed e.g. in Binder et al. 1996). As used herein "a promoter region capable of directing expression in mitochondria of plant cells" refers to regulatory DNA region which can initiate and support transcription of an operably linked DNA sequence in an *in vitro* system faithfully reflecting initiation of transcription in mitochondria, as described by Binder et al. (1996) and references therein.

Preferred examples of suitable mitochondrial promoter regions include the promoter regions of mitochondrial genes such as but not limited to the promoter regions of *cox2* genes as described by Newton et al. (1995) and Lupold et al. (1999), the promoter region upstream of the *rpl5* gene in pea (Hoffmann et al. 1999). Particularly preferred is a mitochondrial promoter region fragment comprising the nucleotide sequence of SEQ ID NO 2 from the nucleotide at position 370 to the nucleotide at position 378, such as the about 0.6 kb promoter region upstream of the *atp9-1* encoding ORF as described by Albaum et al. (1995), which comprises the nucleotide sequence of SEQ ID NO 2 from nucleotide 1 to 648 (see also Examples). Alternatively, mitochondrial promoter regions may be derived from other sources, particularly from chloroplast genes (Nakazono et al. 1996; Kanno et al. 1997).

The coding region of the chimeric mitochondrial gene may consist of one or more cistrons. As used herein "cistron" defines a nucleotide region coding for a single polypeptide, which can be a naturally occurring peptide or protein or a mutant peptide or protein including a chimeric protein or a fusion protein. As defined, cistron can refer to either a DNA

sequence or an RNA sequence encoding a single polypeptide, as will be understood from the context of the description by the person skilled in the art.

A "multicistronic" or "polycistronic" RNA, as used
5 herein, is an RNA molecule comprising at least two cistrons. It is expected however that the invention can be equally applied to RNAs containing more than two cistrons. For practical reasons, the number of cistrons in a multicistronic RNA should maximally be ten, preferably maximally five,
10 particularly three.

Preferred coding regions are those coding regions which when expressed result in an inhibitory RNA which reduces or abolishes the expression of mitochondrially encoded genes. Other preferred coding regions are those coding region which
15 when expressed result in cytoplasmic male sterility. Yet another type of preferred coding regions are those coding regions which when expressed result in a phenotype which allows mitochondria wherein the mitochondrial chimeric gene is expressed (and plant cells containing such mitochondria) to be
20 distinguished from mitochondria (and plant cells containing only such mitochondria) wherein such a mitochondrial marker gene is not expressed. Chimeric genes comprising the latter type of coding regions are referred to as "mitochondrial marker gene". A particularly preferred coding region resulting in a
25 marker polypeptide, is a GFP (green fluorescent protein) encoding DNA, quite particularly the sGFP encoding DNA as described by Chiu et al. (1996). A particularly preferred chimeric marker gene is the sGFP coding region under control of the atp9-1 promoter region.

30 The presence of the foreign DNA in the mitochondria of plant cells and plants can always be traced by any method allowing recognition of specific DNA sequences, such as e.g. Southern hybridization using foreign DNA specific probes, or PCR amplification based methods using foreign DNA specific
35 primers. Particularly preferred primers for embodiments of the invention involving foreign DNA with an mp1 derived origin of replication, are the mp1 specific primers of SEQ ID NOS 3

and 4.

The availability of the direct manipulation of mitochondrial genomes by the methods and means of the invention will allow high expression of a particular polypeptide or protein by introduction of a mitochondrial chimeric gene encoding such a polypeptide or protein of interest. Indeed, plants contain from 100 to 50000 copies of the organelle genomes per cell, and gene expression in mitochondria is mainly regulated by copy number.

The invention is also directed to a method for high level expression of a polypeptide or a protein in a cell of a plant, comprising the steps of introducing a foreign DNA into the mitochondria of plant cells, and optionally regenerating the transgenic plant cell, wherein the foreign DNA comprises a chimeric gene capable of expression in mitochondria encoding the polypeptide or protein of interest. Preferably, the foreign DNA also comprises an origin of replication for mitochondria.

Transcription and translation machinery of mitochondria are prokaryotic in nature. Moreover, transcription of several mitochondrial genes results in polycistronic RNA molecules allowing simple coordinated expression of different cistrons by introduction of one foreign DNA into the mitochondria. These methods may find particular applications in resistance management strategies (aiming at preventing the development of pathogens resistant to the defense proteins expressed by plants) envisioning the coordinated expression of several defense proteins. Likewise, the expression of proteins consisting of several peptides assembled in a specific quaternary structure (such as heterodimeric transcription factors, multimeric enzymes or enzymatic complexes) or the expression of different enzymes of a metabolic pathway for *de novo* production of metabolites in plants will often rely on coordinated expression of the genes encoding the different subunits.

The invention is thus, also directed to a method for coordinated expression of polypeptides or proteins in plant

cells or plants, comprising the steps of introducing a foreign DNA into the mitochondria of plant cells, preferably according to the methods of the invention, and optionally regenerating the transgenic plant cell, wherein the foreign DNA comprises a
5 chimeric gene capable of expression in mitochondria comprising two or more cistrons encoding the polypeptides or proteins of interest. Preferably, the foreign DNA also comprises an origin of replication for mitochondria.

It is clear that the methods of the invention can be
10 used to express different kinds of cistrons from a multicistronic nucleic acid. Preferred applications are the production of pest resistance proteins and/or pathogen resistance proteins from multicistronic RNA in plant cells. Particularly preferred pest resistance proteins are insect
15 resistance proteins such as the insecticidal crystal proteins (ICP) from *Bacillus thuringiensis* (Bt), particularly a Bt ICP having insecticidal activity to at least one insect species. Especially preferred is a truncated Bt ICP, comprising the minimal toxic fragment. Particularly preferred Bt ICP are
20 CRY1Ab5, CRY9C, CRY1Ba, CRY3C, CRY3A, CRY1Da and CRY1Ea. As used herein, CRY1Ab5 represents the CRYIAb described by Höfte et al. (1986); CRY9C represents the CRYIH described by Lambert et al. (1996); CRY1Ba represents the CRYIB described by Brizzard and Whitely (1988); CRY3C represents the CRYIIID
25 described by Lambert et al. (1992); CRY3A represents the CRYIIIA described by Höfte et al. (1987); CRY1Da and CRY1Ea represent the *bt4* and *bt18* encoded ICPs, respectively, described in WO 90/02801, according to the classification proposed by Crickmore et al., Abstract presented at the 28th
30 annual meeting of the Society for Invertebrate Pathology, 16-21 July 1995. Other preferred insecticidal resistance proteins are the vegetative insecticidal proteins from *B. thuringiensis*, insecticidal toxins from *Photorhabdus* or *Xenorhabdus* spp., insecticidal α -amylase and protease inhibitors (Hilder et al.,
35 1987; Huesing et al. 1991), spider venom toxin or scorpion venom toxin.

In most crop plants, mitochondria are maternally

inherited and are not transmitted via pollen. Transfer of foreign DNA comprising chimeric genes encoding a polypeptide or protein of interest into mitochondria, therefore represents a method for genetic containment of chimeric genes. The maternal inheritance may also represent an advantage in maintaining lines expressing the chimeric gene of interest.

Foreign DNA comprising an origin of replication for mitochondria will normally replicate in the mitochondria in an independent way with regard to other DNA molecules making up the mitochondrial genome. However, occasionally, the foreign DNA may integrate partly or completely into other replicating DNA molecules from the mitochondrial genome by homologous or illegitimate recombination. It is expected that such recombinational integration may be enhanced by inclusion of sequences homologous to parts of the mitochondrial genome into the foreign DNA.

The obtained plant comprising a foreign DNA in mitochondria of its cells, can be further used in a conventional breeding scheme to produce more transformed plants with the same characteristics or to introduce the foreign DNA of interest of the invention in other varieties of the same or related plant species, or in hybrid plants. Seeds or propagules obtained from the transformed plants contain mitochondria comprising the foreign DNA of the invention.

Foreign DNA of the invention comprised within the mitochondria of plant cells, particularly chimeric mitochondrial plasmids, can also be transferred between plant cells by fusing donor protoplast comprising mitochondria with such foreign DNA, with recipient protoplasts, to obtain recipient protoplasts with mitochondria comprising the foreign DNA of the invention.

In another embodiment, the invention is also related to plants and plant cells as they may be obtained by the methods of the invention. Thus the invention is related to plants and plant cells comprising transgenic mitochondria, particularly to plants and plant cells with mitochondria comprising chimeric mitochondrial plasmid. Different

embodiments for foreign DNA and chimeric mitochondrial plasmids are as described elsewhere.

It is also clear that the methods and means of the invention are suited for the introduction of exogenous DNA in mitochondria of all plant cells of all plants, whether they are
 5 monocotyledonous or dicotyledonous plants, particularly crop plants such as but not limited to corn, rice, wheat, barley, sugarcane, cotton, oilseed rape, soybean, vegetables (including chicory, brassica vegetables, lettuce, tomato), tobacco,
 10 potato, sugarbeet but also plants used in horticulture, floriculture or forestry.

The following non-limiting Examples describe the construction of chimeric mitochondrial plasmids and the use of such DNA molecules for transforming mitochondria of plants.
 15 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.
 20 (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,
 25 UK.

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

- SEQ ID NO 1: sequence of the mitochondrial plasmid of *Chenopodium album*.
- 30 SEQ ID NO 2: sequence of the atp9 locus from *Brassica napus* with Tokumasu cytoplasm from *Rhaphanus sativus*.
- SEQ ID NO 3: mp1 specific primer
- SEQ ID NO 4: mp1 specific primer
- SEQ ID NO 5: oligonucleotide for the introduction of a NotI
 35 restriction site around the start codon of atp9
- SEQ ID NO 6: oligonucleotide for the introduction of a NotI

restriction site around the stop codon of atp9.

EXAMPLES

Example 1. Experimental procedures

1.1. Construction of chimeric mitochondrial plasmids used in the Examples.

1.1.1. pMP2

Construction of the chimeric mitochondrial plasmid pMP2 has been described in detail by Backert et al. (1996). Briefly, the mitochondrial plasmid mp1 from *Chenopodium album* (SEQ ID NO 1) was opened by BamHI restriction and ligated to the BamHI restricted vector pGEM3zf(+) (commercially available from Promega, Madison, Wisc.).

1.1.2. MP-atp9-GFP

The chimeric plasmid MP-atp9-GFP comprises the following operably linked elements (in this order):

- an origin of replication of mp1 (SEQ ID NO 1, in permutation);
- a DNA fragment from the atp-9 locus from *Brassica napus* with Tokumasu cytoplasm comprising a mitochondrial promoter region and the atp9-1 ORF, having the nucleotide sequence from SEQ ID NO 2 from the nucleotide at position 1 to the nucleotide at position 943 which has been modified by site specific mutation to include a NotI restriction site 66 bp downstream of the stop codon of atp9-1;
- a coding region containing the sGFP from *Aequorea victoria* (Chiu et al. 1996);
- a DNA fragment from the atp-9 locus from *Brassica napus* with Tokumasu cytoplasm comprising a transcription terminator functional in mitochondria, having the nucleotide sequence SEQ ID NO 2, from the nucleotide at position 944 to the nucleotide at position 1880

all cloned in pGEM3zf (Promega).

The plasmid was constructed in the following way using standard cloning techniques:

The about 1800 bp EcoRI/BamHI fragment (described by Albaum
 5 *et al.*, 1995) comprising the *atp9* gene locus was isolated from
Brassica napus with Tokumasu cytoplasm from *Raphanus sativus*,
 cloned into pBlueSkriptSK (Promega) and sequenced (SEQ ID NO 2)
 to yield a plasmid designated pSK-*atp9*. Using an
 10 oligonucleotide with the sequence of SEQ ID NO 6, a NotI
 restriction site was introduced 66 bp downstream of the
 stopcodon of *atp9-1* (pSK-*atp9mN*). The NotI restriction site
 from the polylinker of the vector was eliminated by SacI/SpeI
 restriction, rendering the ends blunt, and religation.
 Into the remaining NotI site, the coding region for sGFP,
 15 located on a NotI cassette was introduced, and the about
 2600 bp BamHI fragment was isolated from the resulting plasmid,
 blunt-ended, and ligated to blunt-ended XbaI restricted pMP2,
 to yield MP-*atp9*-GFP.

In MP-*atp9*-GFP, the GFP coding region is under the
 20 same transcriptional control as the *atp9* cistron.

1.1.3. MP-GFP

The chimeric plasmid MP-GFP comprises the following operably linked elements (in this order):

- an origin of replication of *mp1* (SEQ ID NO 1, in
 25 permutation);
- a DNA fragment from the *atp-9* locus from *Brassica napus*
 with Tokumasu cytoplasm comprising a mitochondrial
 promoter region, having the nucleotide sequence from
 30 SEQ ID NO 2 from the nucleotide at position 1 to the
 nucleotide at position 641 which has been modified by site
 specific mutation to include a NotI restriction site 8 bp
 upstream of the start codon of *atp9-1*;
- a coding region containing the sGFP from *Aequorea victoria*
 (Chiu *et al.* 1996);
- 35 • a DNA fragment from the *atp-9* locus from *Brassica napus*
 with Tokumasu cytoplasm comprising a transcription

terminator functional in mitochondria, having the nucleotide sequence SEQ ID NO 2 (modified by site specific mutation to include a NotI restriction site 66 bp downstream of the stop codon of atp9-1) from the nucleotide at position 944 to the nucleotide at position 1880

all cloned in pBlueskriptSK (Promega).

The plasmid was constructed in the following way using standard cloning techniques:

- 10 Using an oligonucleotide with the sequence of SEQ ID NO 5, a NotI restriction site was introduced 8 bp upstream of the startcodon of atp9-1 in pSK-atp9mN (see 1.1.2). The about 300 bp NotI fragment encoding the atp9-1 ORF was removed, and exchanged for the coding region encoding sGFP, located on a
- 15 NotI cassette. Into the resulting plasmid, the about 1.3 kb BamHI fragment from pMP2 (see 1.1.1) comprising the mp1 sequence was introduced as a blunt-ended fragment into the blunt-ended EcoRI site, yielding MP-GFP, wherein the GFP coding region is now under direct control of the atp9 mitochondrial
- 20 promoter region.

1.2. Protoplast transformation method

1.2.1. Isolation of mesophyll protoplasts

- Five young leaves were transferred into 15 ml enzyme solution in a Petri dish. Two millimetre stripes were cut into
- 25 the leaves with a scalpel. The leaves were incubated 15 to 17 h in the dark at room temperature. After 15 h incubation the enzymatic digestion of the leaves was estimated macroscopically. The Petri dishes were further incubated on a rotary shaker (40 rpm) for about 1 to 2 hours. The protoplast
 - 30 suspension was sieved through a 100 μ m mesh sieve to eliminate undigested material. Petri dish and sieve were rinsed with an additional 30 ml W5 solution. The protoplast solution was transferred into 15 ml centrifuge tubes and centrifuged for 5 minutes at 600 rpm/min (53g). The protoplast pellet was
 - 35 resuspended in circa 500 μ l W5 solution. The combined

resuspended pellets of both tubes (about 1 ml) were carefully layered onto 8 ml of a 20% sucrose solution (CPW20S) and centrifuged for about 10 min at 800 rpm. Intact protoplasts can then be collected as one band located on top of the sucrose cushion. The protoplast band was removed with a 1 ml plastic pipette and transferred to a fresh tube. To remove the sucrose, the protoplasts were diluted in W5 solution and centrifuged for 5 min at 600 rpm. The protoplasts were diluted in 10 ml of W5 medium and the number of protoplasts was calculated in a Fuchs-Rosenthal chamber. Protoplasts were again pelleted (5 min centrifugation at 600 rpm) and resuspended in a sufficient amount of F3 medium to obtain a final concentration of about 10^6 protoplasts per centrifuge tube. After centrifugation (5 min, 600 rpm) the protoplasts were directly used for transformation. If required, the osmolarity of the media was adjusted to 450 mOsm/kg H₂O.

1.2.2. Transformation of mesophyll protoplasts.

The osmolarity of the media was adapted to the source material. In the middle of a 10 cm Petri dish (5 cm dish, if protoplasts will be cultured in liquid Kao8p medium) a droplet of 150 μ l MaMg medium was mixed with 50 μ l DNA of interest (50 μ g/l) (pMP2; MP-atp9-GFP or MP-GFP). Six droplets of each 50 μ l of MaMg PEG solution were placed around this central droplet. To the central droplet, a 100 μ l of the protoplast suspension was added. The protoplasts were carefully resuspended in the MaMg solution. Next, the protoplast DNA solution was mixed, droplet by droplet with the PEG solution. Protoplasts were incubated for 20 min in the dark. Then, over a period of 15 to 20 minutes, the protoplasts were carefully diluted with 6 x 1 ml of Kao8p medium. In case the protoplasts were directly embedded in agarose medium, they were diluted over a 15 to 20 min. period with 6 x 2 ml W5 solution. The protoplast suspension was centrifuged 5 min. at 600 rpm and the pellet was diluted in 10 ml warm (32°C) of Kao8p agarose medium. The protoplast agarose mixture was divided into 100 to 150 μ l droplets in 15 cm Petri dishes (around 50 droplets per

dish). After solidification of the droplets 15 ml of liquid Kao8p medium were added to each Petri dish.

1.2.3 Media for Protoplast Isolation and Transformation

All media were filtered sterilized except W5 which was autoclaved. The osmolarities of all solutions were measured in an osmometer.

CPW

	CaCl ₂ ·2H ₂ O	1480 mg/l
	KH ₂ PO ₄	27.2 mg/l
10	KNO ₃	101.1 mg/l
	MgSO ₄ ·7H ₂ O	24.6 mg/l
	MES	250 mg/l
	Ad 1000 ml H ₂ O dest. /pH 5.6 - 5-8	

CPWM

15 The osmolarity was adjusted with mannitol (to 100 ml CPW the given amount of mannitol was added:

	400 mOsm	6.8 g	
	450 mOsm	7.5 g	tobacco mesophyll protoplasts
	500 mOsm	8.4 g	rapeseed mesophyll protoplasts
20	550 mOsm	9.2 g	
	570 mOsm	9.7 g	rapeseed hypocotyl protoplasts

CPW20S

20 g sucrose ad 100 ml CPW (w/v)

W5

25	CaCl ₂ ·2H ₂ O	18.4 g	
	NaCl	9.0 g (600 mOsm) or	4.5 g (500 mOsm)
	KCl	0.8 g	
	MES	0.5 g	
	ad 1000 ml H ₂ O		
30	pH 5.6 - 5.8		

F3 medium

	NaCl	6.4 g (600 mOsm) or 3.0 g (500 mOsm)
	KCl	0.373 g

25

NaH₂PO₄•2H₂O 0.113 g

CaCl₂•2H₂O 22.05 g

MES 0.250 g

ad 1000 ml H₂O

5 pH 5.6 - 5.8

MaMg medium

Mannitol 5.64 g

MES 0.195 g

MgCl 2.033 g

10 ad 100 ml H₂O

pH 5.6

MaMgPEG

4 g PEG 1500 ad 10 ml MaMg

1 ml aliquots were stored at -20°C

15 pH 7.0

Enzyme Solution.

1% Cellulase, 0.3% Macerozym in CPWM

300 mg cellulase R10 and 90 mg macrozyme R10 were added in a

50 ml Falcon tube and filled ad 30 ml with CPWM. The solution

20 was shaken for 1 h and filtered sterilized.

Kao8p (protoplast medium)

3.7 g Kao8p salt mixture (Sigma KO878)

1 mg Kao8p vitamin solution (Sigma G1019)

250 mg casamino acids

25 organic acids:

20 mg Na-pyruvate

40 mg malic acid

40 mg fumaric acid

40 mg citric acid

30 sugars:

250 mg xylose

250 mg ribose

250 tug fructose
250 mg mannose
250 mg rhamnose
250 mg cellobiose
5 250 tug sucrose
250 mg sorbitol
250 mg mannitol
500 mg myo-inositol

68.4 g glucose
10 1 mg 2,4-D
0.1 mg NAA
0.1 BAP

pH 5.6 - 5.8

**EXAMPLE 2. INTRODUCTION OF PMP2 INTO MITOCHONDRIA OF TOBACCO
15 PLANT CELLS AND PLANTS.**

Tobacco protoplasts were transformed with pMP2, using the protoplast procedure as described in Example 1 (modified after Krans *et al.*, 1982). As controls, protoplasts were transformed with the control construct pRT101 (Töpfer *et al.*,
20 1987) carrying the GFP gene under the control of the CaMV 35S promoter.

Protoplasts were further cultivated and analyzed at different times for the presence of either the pRT101 DNA or the pMP2 DNA by PCR using specific primers.

25 Fourteen days after transformation, no GFP specific PCR product could be found. Surprisingly, in total DNA of cells cultured for 12, 18, 23 and 26 days after transformation a mp1 (and thus also a pMP2) specific PCR fragment was amplified using as primers oligonucleotides with the sequence
30 of respectively SEQ ID NOS 3 and 4. Also in shoots, regenerated from pMP2 treated protoplasts, the specific band could be observed (Fig 1 upper gel). Shoots were further regenerated to mature plants.

EXAMPLE 3. SOUTHERN BLOT ANALYSIS OF PLANTS REGENERATED FROM PMP2 TREATED PROTOPLASTS.

Southern blot analysis of total DNA of regenerated shoots confirmed the stable transformation with the
5 mitochondrial plasmid pMP2. The DNA was digested with XbaI, an enzyme with a single restriction site in the 4.5 kb pMP2 shuttle plasmid. Two plants (designated 1a and 1b) showed a mp1 specific signal at 4.5 kb after hybridization with a mp1 specific probe (Fig 2). This result indicated that the shuttle
10 vector was not integrated into other DNA, such as nuclear DNA, but was maintained as a separate, autonomous replicon.

The two positive plants were propagated *in vitro* to obtain sufficient amounts of leaves allowing chloroplast and mitochondria isolation.

15 Eight weeks after the transfer of the plants to soil, nuclear, mitochondrial and chloroplast DNA was separately isolated from at least 50 g young leaf material from 5 to 8 plants cloned from the positive plants.

Southern blot analysis of the XbaI digested nuclear,
20 chloroplast and mitochondrial DNA clearly showed a fragment of about 4.5 kb only in the mitochondrial DNA fraction. (Fig 3).

EXAMPLE 4. PROGENY ANALYSIS.

Progeny of the plants of the "1a " plant line, were analyzed for the presence of the chimeric mitochondrial plasmid
25 pMP2, by Southern blot analysis of the mitochondrial DNA fraction (mt-DNA).

In one sample of mt-DNA out of 5, a strong mp1-specific signal of about 8.0 kb was detected (Fig 4), indicating that the pMP2 is integrated into the mitochondrial
30 genome.

To test the cytoplasmic inheritance of the transgenic mitochondria, pMP2 containing plants were pollinated by pollen from an untransformed plant (wt), and vice versa. In Southern blots of mitochondrial DNA isolated from the different

progenies, the mp1 specific band was detected exclusively in progeny plants derived from PMP2 containing plants pollinated with wild type pollen (Fig 5). Maternal inheritance of the mp1 plasmid is an additional proof for mitochondrial transformation.

EXAMPLE 5. INTRODUCTION OF A CHIMERIC MITOCHONDRIAL PLASMID INTO MITOCHONDRIA OF TOBACCO CELLS AND EXPRESSION OF A CHIMERIC MITOCHONDRIAL GENE ENCODING GFP.

In several experiments, tobacco protoplasts were transformed using the methods described in Example 1 with the chimeric mitochondrial plasmid MP-atp9-GFP and MP-GFP, comprising a GFP coding region under control of a mitochondrial promoter region.

To test mitochondria-specific transcription of the GFP gene, RNA from two months old calli derived from the treated protoplasts were analyzed by RT-PCR. In all RT-PCR approaches, DNase treated RNA was used.

GFP specific transcripts were detected by RT-PCR in RNA from calli, regenerated from protoplasts treated with either MP-GFP or MP-atp9-GFP, indicating that transcription of the GFP gene occurred in mitochondria, whether the gene was directly under control of the mitochondrial atp9 promoter (as in MP-GFP) or was transcribed in a polycistronic manner (see Fig 6).

The presence of the GFP translation product in mitochondria is verified.

Shoots are regenerated and analyzed for the presence of MF-atp9-GFP or MP-GFP by PCR, using PCR with mp1-specific primers or GFP-specific primers.

Positive shoots, containing the chimeric mitochondrial plasmid are regenerated into mature plants, which are pollinated by wt pollen.

Some progeny plants contain the chimeric mitochondrial plasmids in their mitochondria, and transcription and translation of the GFP product is detected.

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SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: GARCHING INNOVATION GMBH

(ii) TITLE OF INVENTION: MEANS AND METHODS FOR TRANSFORMATION OF PLANT
MITOCHONDRIA

10 (iii) NUMBER OF SEQUENCES: 6

(iv) CORRESPONDENCE ADDRESS:

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(E) COUNTRY: CANADA

(F) ZIP: K1P 5Y6

(v) COMPUTER READABLE FORM:

(A) MEDIUM TYPE: Floppy disk

20 (B) COMPUTER: IBM PC compatible

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(D) SOFTWARE: ASCII (text)

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(B) FILING DATE: 11-JUN-1999

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(A) APPLICATION NUMBER:

(B) FILING DATE:

30 (viii) ATTORNEY/AGENT INFORMATION:

(A) NAME: SMART & BIGGAR

(B) REGISTRATION NUMBER:

(C) REFERENCE/DOCKET NUMBER: 75749-5

(ix) TELECOMMUNICATION INFORMATION:

31

(A) TELEPHONE: (613)-232-2486

(B) TELEFAX: (613)-232-8440

(2) INFORMATION FOR SEQ ID NO.: 1:

(i) SEQUENCE CHARACTERISTICS

(A) LENGTH: 1309

(B) TYPE: nucleic acid

(C) STRANDEDNESS:

(D) TOPOLOGY:

10 (ii) MOLECULE TYPE: DNA

(vi) ORIGINAL SOURCE:

(A) ORGANISM: Artificial Sequence

(ix) FEATURE

(C) OTHER INFORMATION: Description of Artificial Sequence: sequence of
the mp1 mitochondrial plasmid from
Chenopodium

(ix) FEATURE

(A) NAME/KEY: misc_feature

(B) LOCATION: (776)..(781)

20 (C) OTHER INFORMATION: BamH1 site used for insertion of mp1 in PGEM3zf
to produce pMP2

(ix) FEATURE

(A) NAME/KEY: misc_feature

(B) LOCATION: (1126)..(1145)

(C) OTHER INFORMATION: sequence corresponding to MP-2A (mp1 specific
primer)

(ix) FEATURE

(A) NAME/KEY: misc_feature

(B) LOCATION: (715)..(734)

30 (C) OTHER INFORMATION: sequence corresponding to the complement of MP-2b
(mp1 specific primer)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO.: 1:

ACGCGTACAA GTTCTTATGT TCGGGTTTAG CAAAATGAAG TGAACCGGAC TAAACGTGTT 60
ACCGAGATAA AAAGGGGAAA GGACG TTCAG TTTTAAATTT AAACAGGGCT GATGCTGTGA 120
AGAATCGTAT ACCATGGGCA TGGTTGCCAT GGGCTCTCAA CTGGCTGAGT CAGTTGTTTG 180
TGGAAGTCCC ACTACATAGA GGAAGGCTAA CCCACTGCAG CTGACGGCGG ATTGAGGCTT 240
AAACTATGAT TAAAATTAGG CGCTTGGGTG GGGTCTGGGG GGTCCCTTCG AAACGTAAGT 300
GTTTCGGAGGT ACCAGGGAAG TACTCCAGCC TCGAGAAAGC GCCTTTGCTC GAGCGATATG 360
CAGAAATACG ACTCTATCCA TTCATATGAG TCACTCAAAA CCCTTATTCC CCCGGGGTCT 420
ATTATAAACG TGAGCGCTCC GGAACCTGAA ATGCCTATCG ACAACACGGA GACTTGTATA 480
10 TGCCATCTAA AACGAGCGAC GCGACGGGGG TAACAAAATA TAGCAGCACG GCGGTCCCTT 540
ATTTTCCTTA AAAAACAGGA AAAAAGGGAC AAATCGGCAA GAAAACCGCA AGAACGGCCC 600
TGAGAAAACC GAATTCGCCC CAACCTCGAA TTCGGATTTT CGAAGGACGC GACCCCCACC 660
CACGGGGGGA GCACAACCGA GTAGCGAAGC TACGACCTTG TGAGAAACTT GTTTCGAACG 720
ACGGCTGCTA TACCCTTAGA GCAGAGCGAA GTGGAGTTTG GCCCTCGCCC CCGGAGGATC 780
CAGATAAAGA ATACTACGCT CGTACATTCA CTGTTTTTTGA AGCTTTAAAT TCAAGGAATT 840
CAAAGAACT GTTTAACAAA ACCTTGAATT TAAAGAAACC TTCCTTATCG AAGGAGGCAG 900
GAAAGGAACG TTTCCCCCA AAACCCCTC GGGGGGATGT TTACAAGGAA TCATTAAGTT 960
GATTTAGAAA ATCAAGTAAG ATTAGGATTT TGTAATTA CTAAGAGACC ATAGAAACGA 1020
AGCGTACTTC CCTCGAGGAT AACACTCCTG ACTATCTAAA CGGAAGTTTT AGTGGCAGGA 1080
20 AACATGTAAG CCGCCCAAAA GGCGGTGCCT CGGGTTGGTG GAGGACAGGC TAGGCTGTGG 1140
AATCGCCAGG GCCTCTAGTA AAATAGTGGC CGAAACTTAA CCGTTTCGTC AGTTCGACCG 1200
GTATCATGCA TGTACGTGGA GGAAGAAGGC TATGGTTGCG TGAGGAAATT CAGAAATAGC 1260
CATTATAGCT AAGGAACTTT TCCGAAGCTC GGCTACTTCG CGAAGGAGC 1309

(2) INFORMATION FOR SEQ ID NO.: 2:

(i) SEQUENCE CHARACTERISTICS

(A) LENGTH: 1880

(B) TYPE: nucleic acid

30 (C) STRANDEDNESS:

(D) TOPOLOGY:

(ii) MOLECULE TYPE: DNA

(vi) ORIGINAL SOURCE:

(A) ORGANISM: Artificial Sequence

(ix) FEATURE

(C) OTHER INFORMATION: Description of Artificial Sequence: sequence of
the mitochondrial atp9 gene from brassica napus
with Tokumasu cytoplasm from Raphanus sativus

(ix) FEATURE

(A) NAME/KEY: misc_feature

10 (B) LOCATION: (371)..(379)

(C) OTHER INFORMATION: mitochondrial promoter core sequence

(ix) FEATURE

(A) NAME/KEY: misc_feature

(B) LOCATION: (6500)..(652)

(C) OTHER INFORMATION: atp9-1 ORF

(ix) FEATURE

(A) NAME/KEY: misc_feature

(B) LOCATION: (872)..(874)

(C) OTHER INFORMATION: stop codon atp9-1 ORF

20 (xi) SEQUENCE DESCRIPTION: SEQ ID NO.: 2:

GAATTCCAAA AATAGGATAC TCTTTAACCC AAAGAATAGG AAAAGCTTGA AAGACTTTAA 60
TCAAAACTCA TCGGGATTCA AAGATCCCTT CCTTATTGAT ACAATTAGAC CCGCCGCGGT 120
ATGCTCAGCT ACGCCATTGA TCCTTGAGTA CTTGTGCTAA GTACATGCTC TCTCTCTTTC 180
TTTACTTATG CTTTACGCTT ACAGGACTAT GCTCTTCATT TCTCGAGGAA CGCCTTTGAA 240
TTATAGCGGA AAAGTCCCGC ACTTCATTAC GAAAGACTGG GCCTTCTTAA TCCTCCAATC 300
GTGCAGTAGC TCTCGTATAT AAGAGAAGGG CAGCATTTAG GAGTAATCGA TCTCACAAAC 360
TATCAATTTT ATAAGAGAAG ACGAAGACGG ATCAAATTGA ATAATCGAAG AGATATGGGA 420
CCCTAGCTAC GAGTCAGTCC CTCTAACGTC GAATGATCTA CTTGCTTGTA CTTCTCTTTG 480
TCGAGATTCA GTTGGTCTTC AGTCTACCAC TCCGTGGGTA CAAGATCGAA AAGAATGCAT 540
30 TCCAAGTGAG ATGTCCAAGA TTAAAGGAAC GAGGGTAAGA ATCGACGAGG AATCAATAAG 600
ATAGAAGATC AGTGAATGAC AAAGCGTGAG GAGAATTATC AACCCGAGAT GTTAGAAGGT 660

GCAAAATCAA TAGGTGCCGG AGCTGCTACA ATTGCTTCAG CGGGAGCTGC TATCGGTATT 720
GGAAACGTCT TCAGTTCTTT GATCCATTCT GTGGCGCGAA ATCCATCATT GGCTAAACAA 780
TCATTTGGTT ATGCCATTTT GGGCTTTGCT CTAACCGAAG CTATTGCATT GTTTGCCCCA 840
ATGATGGCCT TTTTGATCTT ATTCGTATTC TGATCGAAGA AAGAAGGTTT CATTCACTCT 900
CATAAAGCAA GCACCTCTTT CACATAAGAA AGTGGAGGCA GGCTTGGATA CGATCTAAAA 960
TGATTCCACA TGAAAGAGGA CCGGGCAATC GCCCTCTTGA GTAATGGGAA GCGGGCTAGT 1020
CCCCGAAAAT GCCCGTTAAT CAAGCAAGTT GGGGAACAAA ATCTTCCTTG TTAGTTACTC 1080
ATTTCTTCGG TCGAGCGTTC TCCGGACGTC GAGAAATCTA TCACTCAATC GCTGGCCGCT 1140
CTGTCATTGT CTGATTTTAG GTTTCTGATC ACACTCGAAA TTATGTATCT ACTTATCGTA 1200
10 TTTTACCCC TGCTCGGTAG TTCCGTAGCA GGTTTTTTCG GACGTTTTCT AGGATCAGAA 1260
GGAAGCGCTA TTCTGACCAC TACGTGCGTT TCATTCTTCG CACTGGTGGG CTCCTATTT 1320
GGATTTACA TTTCTTCCTT TCGTTTGAAA GGACCACTGA GGGGGATTCT CAATCTCTTC 1380
TTGCTCTTTT TCATCGCCGT AGTAATATCT TTGATACGGA TCAAAGTCAT CCACCTACTG 1440
GGTGGTCAGG CTTTGCCCCT GTTGGAGCCC ATTATATGGG CTGCAGTAGG AGGGGGAGCA 1500
CTTCCTTCTA CGGGCCCTAA CGGGGCGGAG AGGGACGCCA TGTGGGAGGA GGATGACTTT 1560
GAACTTCGAG TTCTCGAGGA ATCATTCTCG GACTCCCCC CGGCAGGGGA GTCCCAGACG 1620
GCAGAGGGTG AACCCTCGGT CAATCAGCTT CCTCCCCAGG AAGCTGGGCC ATCTGTCCCC 1680
TATCAGGACC AGGGCCTACC TACTGATAGG AATGGTAACC CGATGGATCT TAATGCTCGG 1740
CCCTCCCTCT CGTCGTTGTA CAACGAAATA GAGAGTCCG ACTCTTTACG AGCGCGGAAT 1800
20 CTCCAATTGG GGGAGGATCT GGAGCGAATC CATGAAATGG AACGGAACCT CCAGAACGAG 1860
AGGGATCCTC TAGAGTCGAC 1880

(2) INFORMATION FOR SEQ ID NO.: 3:

(i) SEQUENCE CHARACTERISTICS

(A) LENGTH: 20

(B) TYPE: nucleic acid

(C) STRANDEDNESS:

(D) TOPOLOGY:

30 (ii) MOLECULE TYPE: DNA

(vi) ORIGINAL SOURCE:

35

(A) ORGANISM: Artificial Sequence

(ix) FEATURE

(C) OTHER INFORMATION: Description of Artificial Sequence: MP-2a:

oligonucleotide for mp1 specific PCR

(xi) SEQUENCE DESCRIPTION: SEQ ID NO.: 3:

CAGGCTAGGC TGTGGAATCG

20

(2) INFORMATION FOR SEQ ID NO.: 4:

10 (i) SEQUENCE CHARACTERISTICS

(A) LENGTH: 20

(B) TYPE: nucleic acid

(C) STRANDEDNESS:

(D) TOPOLOGY:

(ii) MOLECULE TYPE: DNA

(vi) ORIGINAL SOURCE:

(A) ORGANISM: Artificial Sequence

(ix) FEATURE

(C) OTHER INFORMATION: Description of Artificial Sequence: MP-2b

20 oligonucleotide for mp1 specific PCR

(xi) SEQUENCE DESCRIPTION: SEQ ID NO.: 4:

GGTATAGCAG CCGTCGTTTCG

20

(2) INFORMATION FOR SEQ ID NO.: 5:

(i) SEQUENCE CHARACTERISTICS

(A) LENGTH: 38

(B) TYPE: nucleic acid

(C) STRANDEDNESS:

30 (D) TOPOLOGY:

(ii) MOLECULE TYPE: DNA

36

(vi) ORIGINAL SOURCE:

(A) ORGANISM: Artificial Sequence

(ix) FEATURE

(C) OTHER INFORMATION: Description of Artificial Sequence:

oligonucleotide for site specific mutagenesis to
introduce a NotI site around the start codon of
atp9

(xi) SEQUENCE DESCRIPTION: SEQ ID NO.: 5:

GCGTGAGGAG AATTAGCGGC CGCGATGTTA GAAGGTGC

38

10

(2) INFORMATION FOR SEQ ID NO.: 6:

(i) SEQUENCE CHARACTERISTICS

(A) LENGTH: 30

(B) TYPE: nucleic acid

(C) STRANDEDNESS:

(D) TOPOLOGY:

(ii) MOLECULE TYPE: DNA

(vi) ORIGINAL SOURCE:

20 (A) ORGANISM: Artificial Sequence

(ix) FEATURE

(C) OTHER INFORMATION: Description of Artificial Sequence:

oligonucleotide for site specific mutagenesis t
introduce a NotI site around the stop codon of
atp9

(xi) SEQUENCE DESCRIPTION: SEQ ID NO.: 6:

GAAAGTGGAG GCGGCCGCGG ATACGATCTA

30

30

CLAIMS:

1. A method for producing a transgenic plant comprising a foreign DNA fragment in the mitochondria of its cells, said method comprising

- 5 (a) contacting an untransformed plant cell with said foreign DNA, under conditions allowing uptake of DNA into said plant cell to generate a transgenic plant cell; and
- 10 (b) regenerating a transgenic plant from said transgenic plant cell wherein said foreign DNA comprises an origin of replication for mitochondria.

2. The method of claim 1 wherein said untransformed plant cell is derived from a multicellular plant.

3. The method of claim 1, wherein said foreign DNA further
15 comprises a gene of interest, said gene of interest comprising
- (a) a promoter region which can direct expression in mitochondria of a plant cell
- (b) a coding region; and
- 20 (c) optionally, a transcription terminator region functional in mitochondria.

4. The method of claim 3, wherein said coding region comprises two or more cistrons.

5. The method of claim 3, wherein said promoter region is selected from the group consisting of the promoter region upstream
25 of the cox2 gene, the promoter region upstream of the rpl5 gene in pea, the about 0.6 kb promoter region upstream of the atp9-1 encoding ORF and the promoter region with the nucleotide sequence of SEQ ID NO 2 from the nucleotide at position 1 to the nucleotide at position 650.

6. The method of claim 3, wherein said coding region, when expressed, results in the production of a marker polypeptide or protein.

7. The method of claim 3, wherein said coding region
5 encodes inhibitory RNA.

8. The method of claim 3, wherein said coding DNA when expressed, results in cytoplasmic male sterility.

9. The method of claim 1, wherein said foreign DNA further comprises a mitochondrial marker gene.

10 10. The method of claim 1, wherein said plant cells are protoplasts and said contacting is performed by treating plant protoplasts with a polyalcohol in the presence of said foreign DNA.

11. The method of claim 1, wherein said contacting is
15 performed by electroporation of said plant cells in the presence of said foreign DNA.

12. The method of claim 11, wherein said plant cells are protoplasts.

13. The method of claim 1, wherein said contacting is
20 performed by bombardment of said plant cells with microprojectiles coated with said foreign DNA.

14. The method of claim 1, wherein said origin of replication is derived from a mitochondrial plasmid.

15. The method of claim 14, wherein said mitochondrial
25 plasmid is selected from the group consisting of the mitochondrial plasmid mp1 of *Chenopodium album*, mt-plasmid 1 from *Vicia faba*, mt-plasmid 2 from *Vicia faba* and mt-plasmid 3 from *Vicia faba*.

16. The method of claim 14, wherein said origin of replication comprises the nucleotide sequence of SEQ ID NO 1.

17. The method of claim 14, wherein said origin of replication comprises a nucleotide sequence selected from the
5 group of the nucleotide sequence of SEQ ID NO 1 from the nucleotide at position 628 to the nucleotide at position 781, the nucleotide sequence of SEQ ID NO 1 from the nucleotide at position 411 to the nucleotide at position 781 and the nucleotide sequence of SEQ ID NO 1 from the nucleotide at position 308 to the
10 nucleotide at position 781.

18. The method of claim 1, wherein said foreign DNA is a chimeric mitochondrial plasmid.

19. The method of claim 1, further comprising the step of crossing the said transgenic plant to obtain progeny plants
15 comprising said foreign DNA in their mitochondria.

20. A plant cell comprising transgenic mitochondria.

21. The plant cell of claim 20, wherein said transgenic mitochondria comprise a chimeric mitochondrial plasmid.

22. The plant cell of claim 21, wherein said chimeric
20 mitochondrial plasmid further comprises a gene of interest, said gene of interest comprising

- (a) a promoter region which can direct expression in mitochondria of a plant cell; and
- (b) a coding region.

25 23. The plant cell of claim 22, wherein said coding region comprises two or more cistrons.

24. The plant cell of claim 22, wherein said promoter region is selected from the group consisting of the promoter region upstream of the cox2 gene, the promoter region upstream of the

rpl5 gene in pea, the about 0.6 kb promoter region upstream of the atp9-1 encoding ORF and the promoter region with the nucleotide sequence of SEQ ID NO 2 from the nucleotide at position 1 to the nucleotide at position 650.

5 25. The plant cell of claim 21, wherein said chimeric mitochondrial plasmid further comprises a mitochondrial marker gene.

26. The plant cell of claim 21, wherein said mitochondrial plasmid comprises an origin of replication comprising a nucleotide
10 sequence selected from the group of the nucleotide sequence of SEQ ID NO 1 from the nucleotide at position 628 to the nucleotide at position 781, the nucleotide sequence of SEQ ID NO 1 from the nucleotide at position 411 to the nucleotide at position 781, the nucleotide sequence of SEQ ID NO 1 from the nucleotide at position
15 308 to the nucleotide at position 781 and the nucleotide sequence of SEQ ID NO 1.

27. A plant comprising the plant cells of any one of claims 20 to 26.

28. Seed of the plant of claim 27, wherein the cells of said
20 seed comprise transgenic mitochondria.

29. An isolated DNA molecule, comprising an origin of replication for mitochondria and a gene of interest, said gene of interest comprising the following operably linked DNA fragments:

- 25 (a) a promoter region which can direct expression in mitochondria of a plant cell;
- (b) a coding region; and
- (c) optionally, a transcription terminator region functional in mitochondria.

30. The isolated DNA molecule of claim 29, wherein said
30 origin of replication is derived from a circular mitochondrial plasmid.

31. The isolated DNA molecule of claim 29, wherein said mitochondrial plasmid is mp1.

32. The isolated DNA molecule of claim 29, wherein said coding region comprises two or more cistrons.

5 33. The isolated DNA molecule of claim 29, wherein said promoter region is selected from the group consisting of the promoter region upstream of the cox2 gene, the promoter region upstream of the rpl5 gene in pea, the about 0.6 kb promoter region upstream of the atp9-1 encoding ORF and the promoter region with
10 the nucleotide sequence of SEQ ID NO 2 from the nucleotide at position 1 to the nucleotide at position 650.

34. The isolated DNA molecule of claim 29, wherein said chimeric mitochondrial plasmid further comprises a mitochondrial marker gene.

15 35. The isolated DNA molecule of claim 29, wherein said mitochondrial plasmid comprises an origin of replication comprising a nucleotide sequence selected from the group of the nucleotide sequence of SEQ ID NO 1 from the nucleotide at position 628 to the nucleotide at position 781, the nucleotide sequence of
20 SEQ ID NO 1 from the nucleotide at position 411 to the nucleotide at position 781, the nucleotide sequence of SEQ ID NO 1 from the nucleotide at position 308 to the nucleotide at position 781 and the nucleotide sequence of SEQ ID NO 1.

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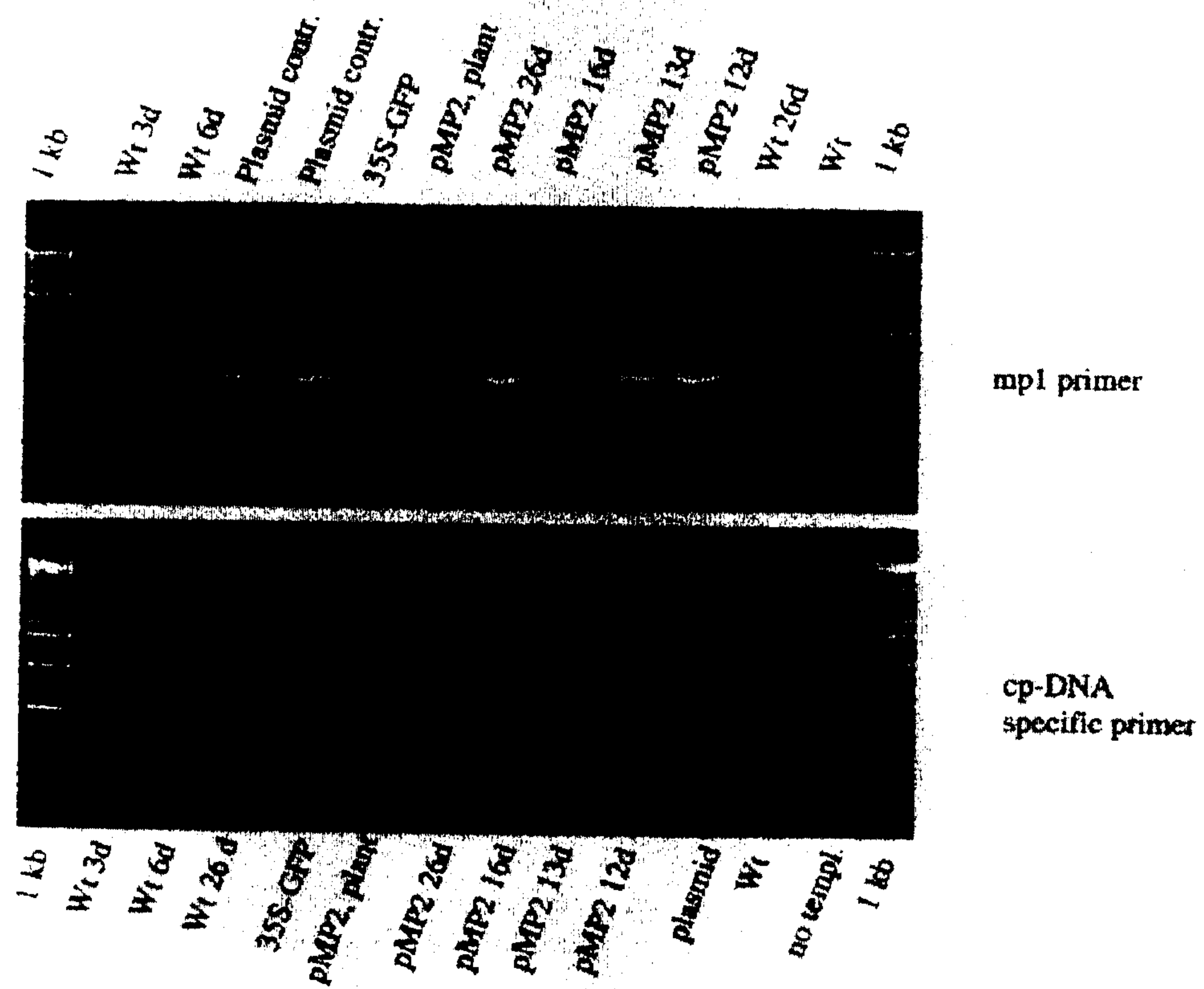


FIG. 1

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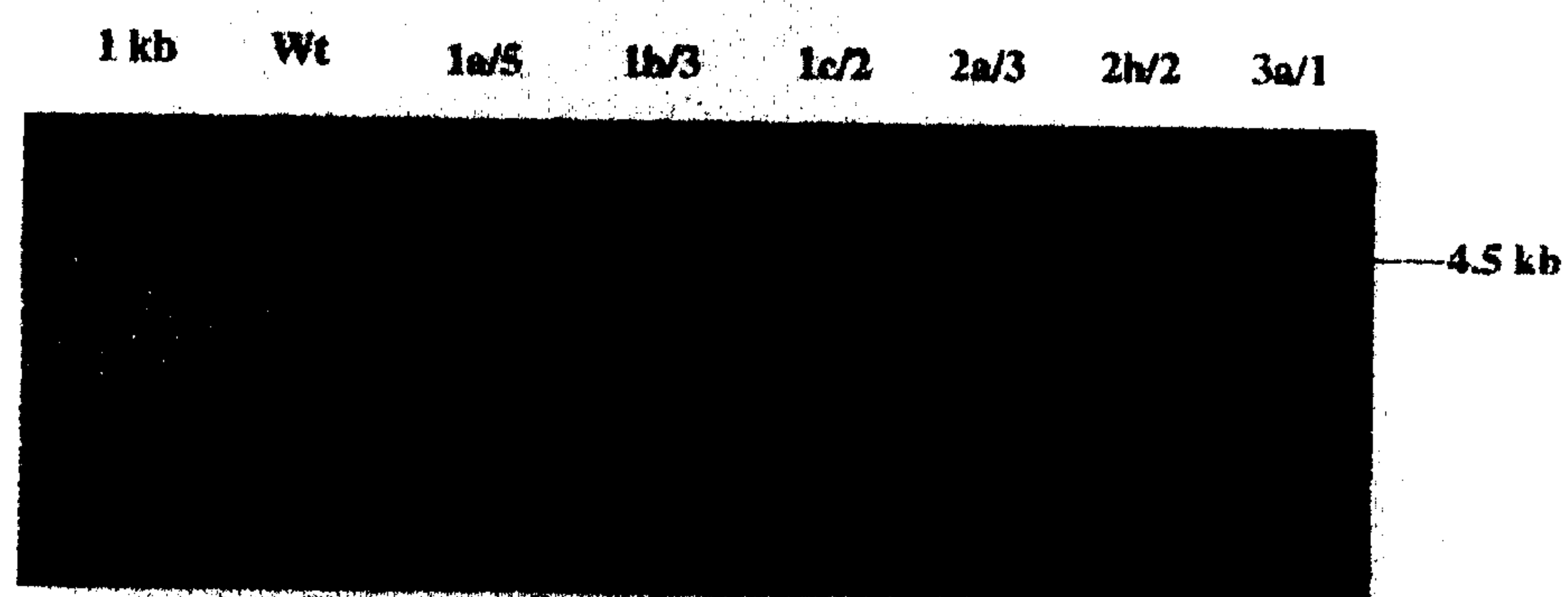


FIG. 2

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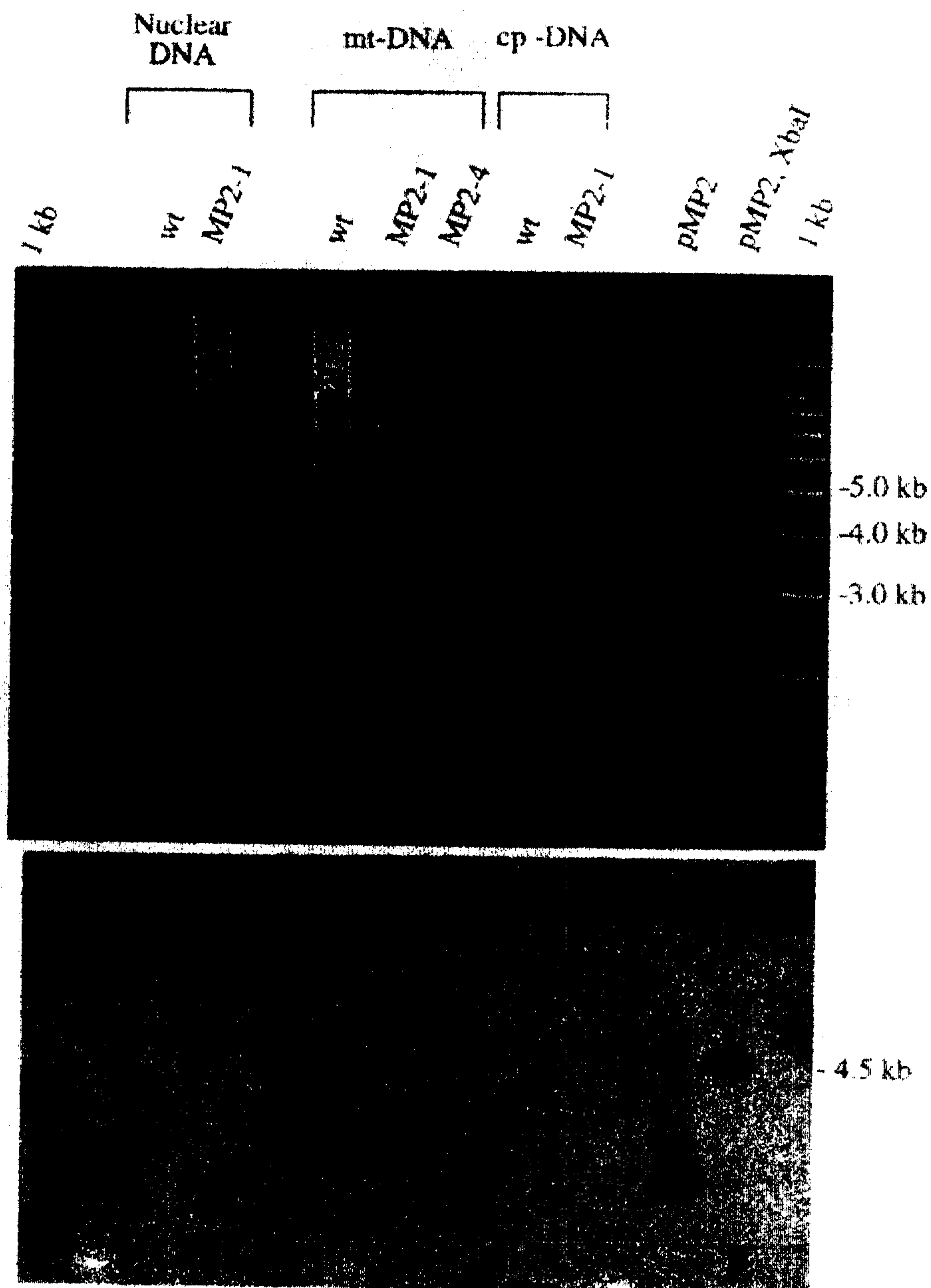
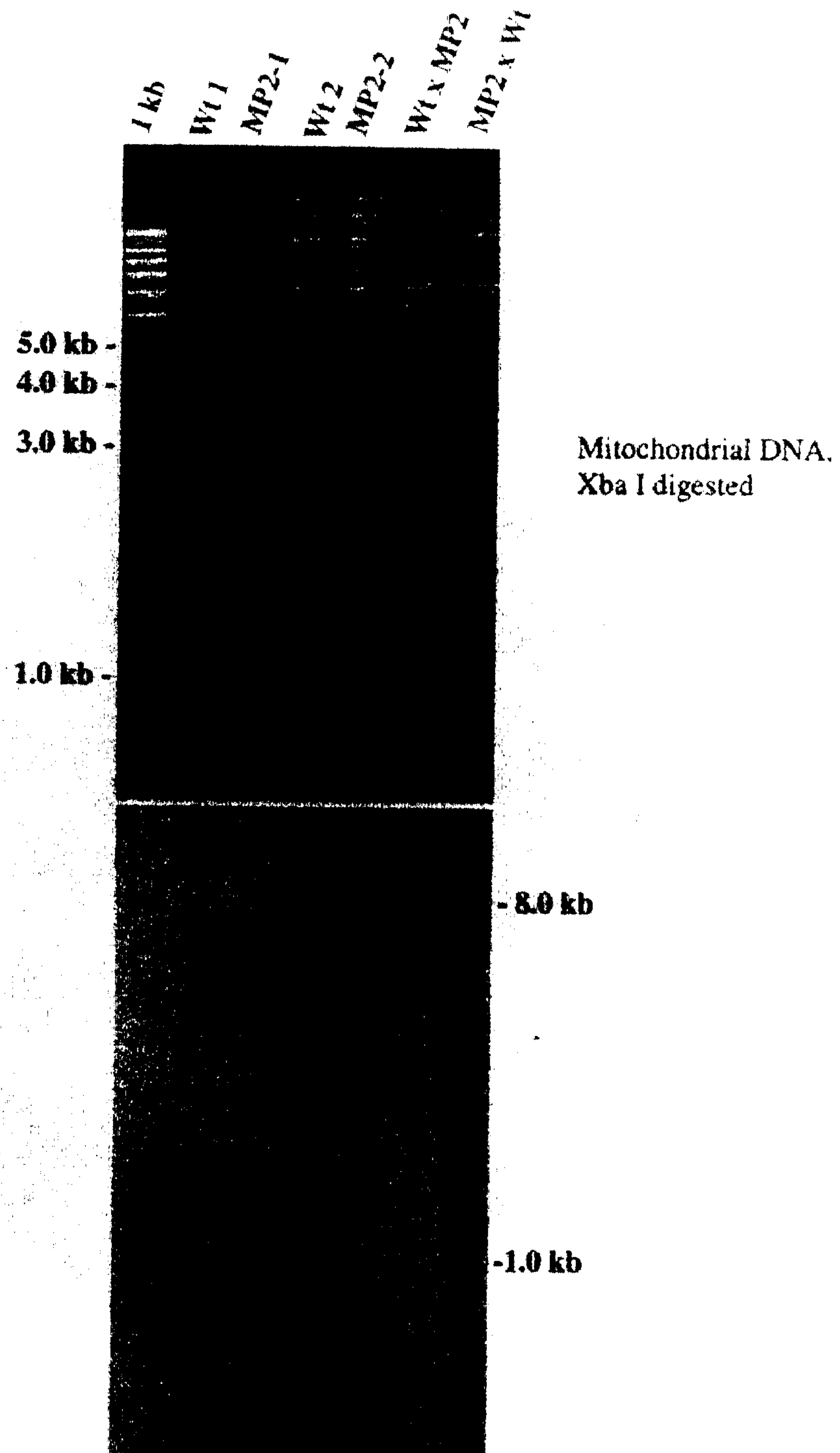


FIG. 3

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12. Southern blot analysis of mitochondrial DNA isolated from progenies of

FIG. 4

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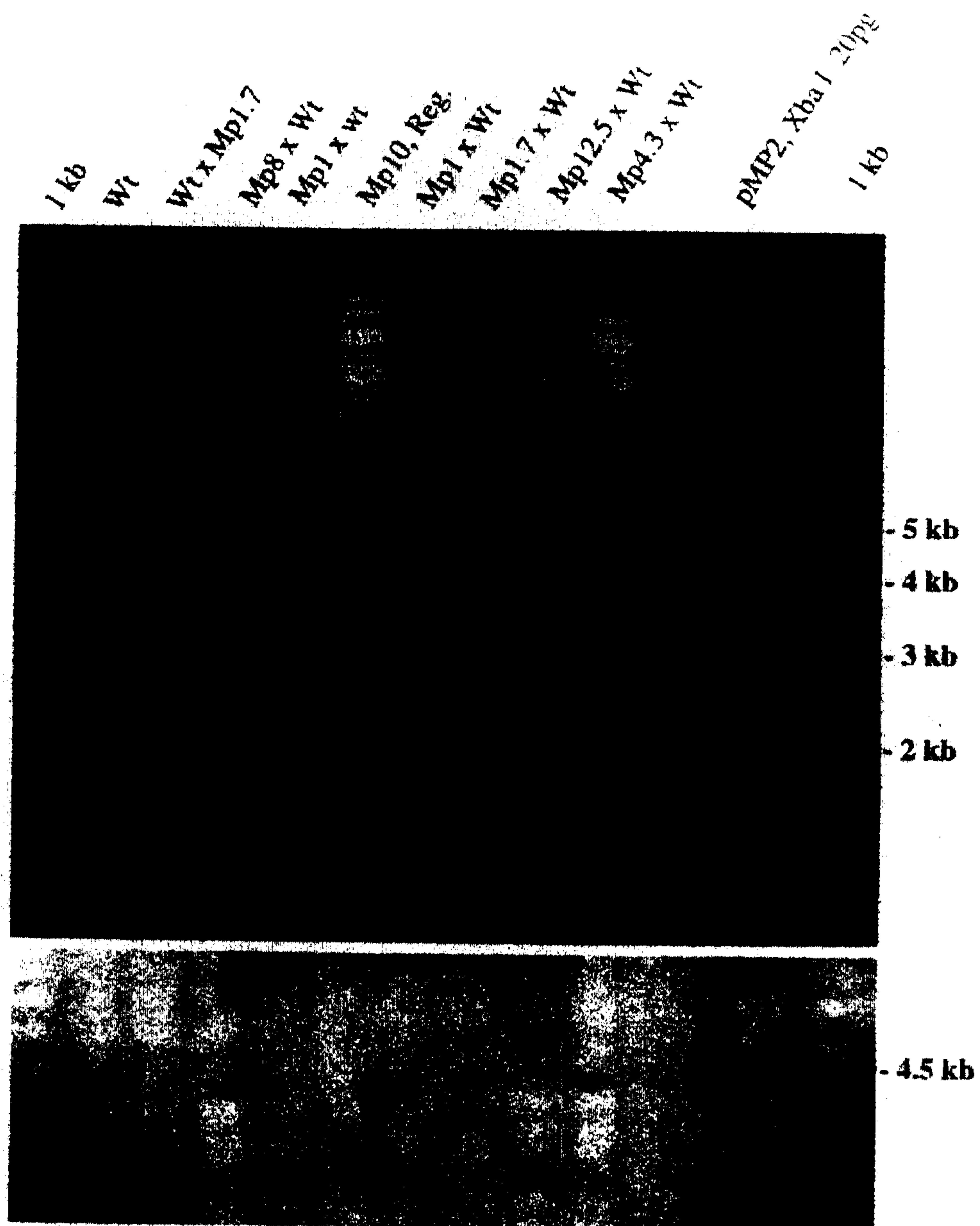


FIG. 5

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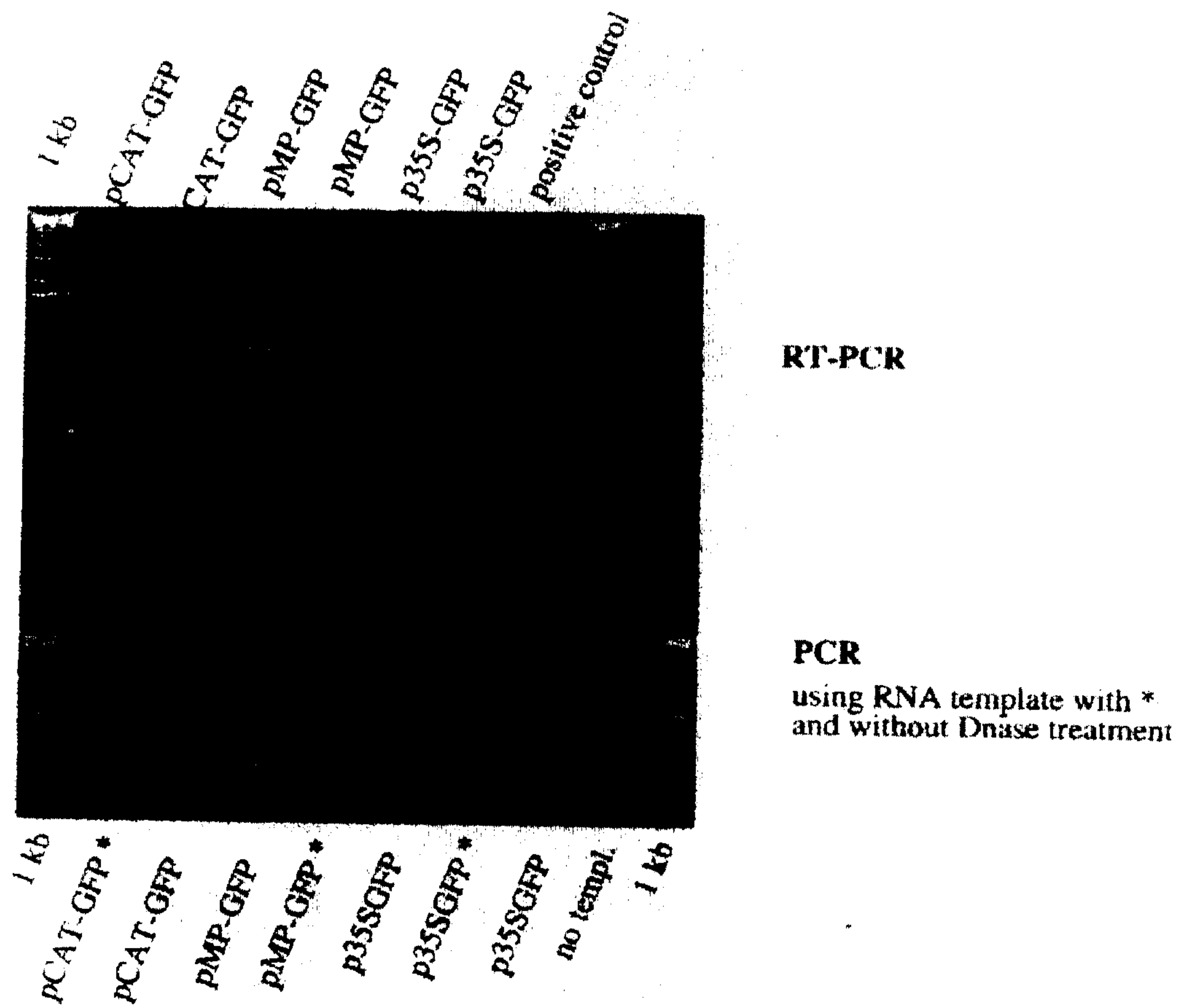


FIG. 6