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NOTICE OF ENTITLEMENT

We, **INSTITUT NATIONAL DE LA RECHERCHE AGRONOMIQUE**, of 145 rue de l'Université, 75341 Paris Cedex 07, France, being the applicant in respect of Application No. 86631/91, state the following:-

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are the first application(s) made in a Convention country in respect of the invention

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Dated : 18 July 1994

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DNA SEQUENCE IMPARTING CYTOPLASMIC MALE STERILITY, MITOCHONDRIAL GENOME, NUCLEAR GENOME, MITOCHONDRIA AND PLANT CONTAINING SAID SEQUENCE AND PROCESS FOR THE PREPARATION OF HYBRIDS

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Ogura sterility DNA sequence that, when present in the cytoplasme of a plant, imparts cytoplasmic male sterility to said plant. The invention also concerns a mitochondrial genome, a nuclear genome, a mitochondria and a cytoplasme containing said sequence. It further relates to a plant belonging to the genus Brassica containing said genome, a process for preparing hybrid plants using such a plant, and a hybrid so obtained.

CLAIM

1. Ogura sterility DNA sequence, characterized in that:

a) it is carried by a DNA sequence bounded by the nucleotides numbered 928 and 2273 in Figure 1, or

b) it possesses an at least 50% homology with said sequence mentioned in a),

and confers, when it is present in the mitochondrial genome of a plant, a cytoplasmic male sterility on said plant.

.../2

3. Recombinant plant nuclear or mitochondrial genome, characterized in that it contains an Ogura sterility DNA sequence which consists

a) of a sequence bounded by the nucleotides numbered 928 and 1569 in Figure 1, or

b) of a sequence possessing an at least 50% homology with said sequence mentioned in a),

and confers, when it is present in the cytoplasm of a plant, a cytoplasmic male sterility on said plant, with the exclusion of the whole of the Ogura mitochondrial genome.

11. Mitochondrion, characterized in that it contains a genome according to Claim 10.

13. Plant belonging to the genus Brassica, characterized in that it contains Brassica chloroplasts which are compatible with the nuclear genome of said plant and mitochondria according to Claim 11.

26. Method for preparing hybrid plants, characterised in that a plant possessing the male-sterile cytoplasmic character according to one of Claims 13 to 25 is crossed with a plant of the same species possessing, where appropriate, a gene restoring fertility, Rf1.

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(54) Titre: SEQUENCE D'ADN CONFERANT UNE STERILITE MALE CYTOPLASMIQUE, GENOME MITOCHONDRIAL, GENOME NUCLEAIRE, MITOCHONDRIE ET PLANTE CONTENANT CETTE SEQUENCE, ET PROCEDE DE PREPARATION D'HYBRIDES

(57) Abstract

Ogura sterility DNA sequence that, when present in the cytoplasme of a plant, imparts cytoplasmic male sterility to said plant. The invention also concerns a mitochondrial genome, a nuclear genome, a mitochondria and a cytoplasme containing said sequence. It further relates to a plant belonging to the genus Brassica containing said genome, a process for preparing hybrid plants using such a plant, and a hybrid so obtained.

(57) Abrégé

La présente invention concerne une séquence d'ADN stérilité Ogura qui confère lorsqu'elle est présente dans le cytoplasme d'une plante, une stérilité mâle cytoplasmique à ladite plante. Elle concerne également une génôme mitochondrial, un génôme nucléaire, une mitochondrie et un cytoplasme contenant cette séquence. Elle concerne aussi une plante appartenant au genre Brassica contenant ce génôme et un procédé de préparation de plantes hybrides utilisant une telle plante, ainsi qu'un hybride ainsi obtenu.

+ Voir au verso

The present invention relates to a biological material possessing a male sterility which is useful for the development of hybrid varieties of species of agronomic importance.

It relates especially to a plant belonging to the Cruciferae family, in which the cytoplasm of the cells contains organelles possessing nucleotide sequences conferring a male sterility and good agronomic characteristics.

The development of hybrid varieties can be facilitated or made possible by the use of a cytoplasmic male sterility system. The hybrids are obtained by cross-fertilisation between two parent populations, one performing the role of male, the other of female. One of the stumbling blocks encountered when it is desired to obtain hybrid varieties of uniform quality by sexual crossing on self-fertile species is the capacity of the plant to self-pollinate. Male sterility systems enable female plants to be obtained which are incapable of self-fertilisation and from which, after pollination, the seeds, which are all hybrids, can be harvested directly without resorting to laborious techniques such as castration of the flowers.

The genetic determinants of male sterility include those which are carried by the cytoplasm. At each sexually produced generation, they are transmitted exclusively by the mother. 100% of male-sterile offspring are thereby produced at each generation with a cytoplasmic male sterility (CMS) system. These genetic determinants are carried by the genome of the mitochondria.

A suitable cytoplasmic male sterility system in Cruciferae is defined by the following characteristics:

1) The male sterility must be total, that is to say there must be no pollen production regardless of the culture conditions and regardless of the line which it is desired to use as a female parent. Were this not the case, the seeds harvested from these female plants would be derived in part from self-

fertilisation and would hence not be of the F1 hybrid type.

2) The production of these seeds must be carried out taking advantage of the natural pollen vectors, that is to say, in the case of these species, Hymenoptera, Diptera and wind. The pollen must be transported from the pollinating plants to the male-sterile (female) plants. In fact, only insects can effect this transport at a distance in Cruciferae.

The female plants must consequently be sufficiently attractive to the insects which come and collect the nectar in them. The morphology of the flowers must force the insect to perform this task via the top of the flower so that its thorax, principally, comes into contact with the stigma. In practice, this amounts to a situation where the base of the petals has to form a kind of tube around the base of pistil.

3) The morphology of the female organs (pistil) must be identical to that of a fertile plant, especially one single pistil per flower and rectilinear in shape. In effect, male sterilities often result also in a feminisation of the anthers, which are transformed into pseudopistils, and even in transformation of the nectaries into complete flowers. Sometimes the pistil or pistils thereby produced are also deformed. All these abberations do not permit good seed production, and this may be regarded as equivalent to some degree of female sterility.

4) For the production of F1 hybrid varieties in the species from which the seeds are harvested, such as colza or mustards, it is essential that the male parent of the hybrid completely negates the effect of the male-sterile cytoplasm, so that the hybrid plants are readily pollinated.

In Cruciferae, the first case of male-sterile cytoplasm or CMS was described by Ogura (1968) in the radish, Raphanus sativus. Bannerot (1974, 1977) transferred the Ogura cytoplasm in Brassicae, thereby obtaining plants which possess a cytoplasmic male sterility. These same plants did not possess satisfactory agronomic characteristics (chlorosis on lowering temperatures, poor female fertility), resulting in a poor yield making them unsuitable for commercial use.

In Cruciferae, to avoid this chlorosis, the nuclear and chloroplast genomes of one and the same genus should be combined in the same cell. Thus, Brassica plants possessing one of the chloroplast genomes of Brassicae no longer exhibit chlorosis. If they possess the whole of the Ogura mitochondrial genome, they exhibit total cytoplasmic male sterility but, however, the flowers will have an aberrant morphology making it impossible for them to be pollinated by the natural vectors.

In addition, for species in which the interest lies in the seeds, it is appropriate to restore the male fertility of hybrid varieties which are marketed by means of nuclear genes known as restorer genes.

It is difficult to restore the male fertility of a plant possessing the whole of the Ogura mitochondrial genome, since it is necessary to bring about the simultaneous participation of several restorer genes.

We set out to obtain a suitable male sterility system by removing the genes responsible for the undesirable characters of the Ogura cytoplasm while retaining a male sterility which is effective and easy to restore.

Thus, the present invention relates to a DNA sequence, which we shall refer to as Ogura sterility DNA sequence, characterised in that:

a) it is carried by a DNA sequence bounded by nucleotides 928 and 2273 in Figure 1, or

b) it possesses an at least 50% homology with the said sequence mentioned in a),

and confers, when it is present in the mitochondrial genome of a plant, a cytoplasmic male sterility on the

said plant.

In particular, the subject of the present invention is an Ogura sterility DNA sequence, characterised in that:

c) it is carried by the sequence bounded by nucleotides 928 and 1569 in Figure 1, or

d) it possesses an at least 50% homology with the said sequence mentioned in c),

and in that it is transcribed to RNA in the mitochondria of male-sterile plants.

In what follows, reference will be made to the following figures:

FIGURE 1: Nucleotide sequence of the Ogura radish mitochondrial DNA fragment carrying the CMS character.

FIGURE 2: Restriction map of the mitochondrial DNA fragment described in Fig. 1.

FIGURE 3: Electrophoresis of mitochondrial DNA after digestion with BglI (3a) and NruI (3b). The bands revealed correspond to a hybridisation with a Cox1 probe (Hiesel et al. 1987).

FIGURE 4: Electrophoresis of mitochondrial DNA after SalI digestion. The bands revealed correspond to a hybridisation with a probe bounded by nucleotides Nos. 389 and 1199 of the sequence described in Fig. 1.

FIGURE 5: Fruits produced by cabbage plants carrying different cytoplasmic genomes.

FIGURE 6: Electrophoresis of mitochondrial RNA. The bands revealed correspond to a hybridisation with a probe, an EcoRI-BamHI fragment, including a portion of the sequence referred to as ORF B.

The Ogura sterility DNA sequence is defined in relation to the sequence bounded by the numbers 1 and 2428 in Figure 1. It is carried by a transcribed sequence whose 3′ and 5′ ends are joined by a broken line in Figure 2, and which is observed only in male-sterile plants. ORF B corresponds to an open reading frame; this designation was given on the basis of the observed homology with a sequence described by Brennicke. In Figure 2, the sequence corresponding to one of the two

formylmethionine transfer RNA genes is shown shaded.

The DNA sequence bounded by the nucleotides numbered 928 and 2273 in Figure 1 corresponds to a transcript which can be visualised by molecular hybridisation (1.4) as seen in Figure 6. In this Figure 6, each well corresponds to a fertile plant (F) or male-sterile plant (S). Only the male-sterile plants synthesise a transcript of approximately 1,400 bases. This transcript begins at position 928 (± 10 bases) of the sequence in Figure 1 and ends at position 2273 (± 5) (transcription initiation and termination can take place at various positions in plant mitochondria).

Preferably, the present invention relates to a cytoplasm containing a DNA sequence possessing an at least 50% homology with the sequence bounded by nucleotides 928 and 2273 in Figure 1 conferring the CMS character, or a cytoplasm containing a DNA sequence possessing an at least 50% homology with the sequence bounded by nucleotides 928 and 1569 in Figure 1, and transcribed to RNA, conferring the CMS character and characterised in that it:

- contains chloroplasts of the same species as the nuclear genome or of another species but which are compatible with this nuclear genome,
- does not contain all or part of one or other (or both) fragment(s) of the Ogura mitochondrial genome, defined below:
  - carrying one of the two formylmethionine transfer RNA genes used for translation initiation,
  - carrying the Coxl gene coding for the subunit No. 1 of cytochrome oxidase.

The absence of these fragments, referred to as "undesirable sequences", is necessary in order to obtain mitochondrial genomes corresponding to a male sterility of good quality, corresponding to the 4 characteristics defined above.

According to another of its aspects, the invention relates to a recombinant plant nuclear or mitochondrial genome, characterised in that it contains an Ogura

sterility DNA sequence:

a) which is carried by a DNA sequence bounded by nucleotides 928 and 2273 of the sequence shown in Figure 1, or

b) which possesses an at least 50% homology with the said sequence mentioned in a),

and confers, when it is present in the cytoplasm of a plant, a cytoplasmic male sterility on the said plant.

In particular, one of the subjects of the present invention is a recombinant plant nuclear or mitochondrial genome, characterised in that it contains an Ogura sterility DNA sequence,

c) which is carried by a sequence bounded by the nucleotides numbered 928 and 1569 in Figure 1, or

d) which possesses an at least 50% homology with the said sequence mentioned in c),

and confers, when it is present in the cytoplasm of a plant and is transcribed to RNA, a cytoplasmic male sterility on the said plant.

A nuclear or mitochondrial genome according to the invention can be characterised in that the said recombinant mitochondrial genome is devoid of all or part of the Ogura genome fragments:

- carrying one of the two formylmethionine transfer RNA genes used for translation initiation,
- carrying the Cox1 gene coding for the subunit No. 1 of cytochrome oxidase,

or in which the said fragments are inactive.

More specifically, a recombinant mitochondrial genome according to the invention may be characterised:

1) in that it is devoid of all or part of an approximately 10.7-kb fragment after BglI digestion or of an approximately 11-kb fragment after NruI digestion, which fragments carry the Cox1 gene.

This is demonstrated, in particular, in Figure 3, by molecular hybridisation with a probe carrying the Cox1 sequence.

2) in that it is devoid of all or part of a 5.1-kb fragment after SalI digestion or of an approximately

15-kb fragment after NruI digestion or of an approximately 18.5-kb fragment after BglI digestion, which fragments carry one of the two formylmethionine transfer RNA genes.

This demonstrated, in particular, in Figure 4, by molecular hybridisation with a probe bounded by nucleotides Nos. 389 and 1199 of the sequence described in Figure 1.

In Figures 3 and 4, the genotypes designated by figures correspond to plants possessing a suitable cytoplasmic male sterility system.

GENOTYPES	CHLOROPLASTS	MITOCHONDRIA
B. n	B. napus	B. napus
27	B. napus	B. napus/Ogura
OGU.	R. sativus (OGU)	R. sativus (OGU)
9, 17, 21, 24, 27c	B. oleracea	B. oleracea/Ogura
B. o	B. oleracea	B. oleracea

Moreover, the existence of a CMS character of good quality necessitates the presence of a DNA sequence which can be identified by DNA/DNA hybridisations on digestions. Thus, the present invention relates to a DNA sequence which it has been possible to define and which is characterised in that it contains a sequence which gives a 2.5-kb fragment after NcoI digestion, gives a 6.8-kb fragment after NruI digestion and gives a 4.4-kb fragment after SalI digestion.

This sequence can also be identified by hybridisations on the total RNA of male-sterile plants. A transcript of approximately 1,400 base pairs is demonstrated. It is absent from plants returning to fertility.

The definition of "undesirable" nucleotide sequences and of nucleotide sequences "essential" to an Ogura sterility according to the invention enables a plant material carrying chloroplasts which are compatible with the nuclear genome and carrying mitochondria of good quality to be selected, by DNA hybridisation techniques well known to those skilled in the art, without waiting to have an adult plant and the appearance of flowers and

fruits. A highly efficacious tool for selecting plants possessing a male-sterile cytoplasm having good agronomic characteristics is hence at the user's disposal.

According to another aspect, the invention relates to a mitochondrion, characterised in that it contains a nucleotide sequence corresponding to a DNA exhibiting an at least 50% homology with the sequence bounded by the bases numbered 928 and 2273 in Figure 1 and coding for Ogura cytoplasmic male sterility; or alternatively the mitochondrion contains a DNA sequence carried by the sequence bounded by nucleotides 928 and 1569 in Figure 1 or possessing a 50% homology with this sequence, and which is transcribed to RNA in the mitochondria of male-sterile plants. This DNA can possess, in addition, the characteristics defined above, in particular the absence of undesirable sequences.

The present invention also relates to a Cruciferae cytoplasm, characterised in that it contains an "Ogura sterility" DNA sequence; this cytoplasm contains, in addition, chloroplasts of the same species or of another species but which are compatible with the nuclear genome.

The Ogura sterility sequence is characterised in that:

a) it is carried by the 2428-base pair DNA sequence shown in Figure 1,

b) it is bounded by nucleotides 928 and 2273 in Figure 1 and corresponds to a transcript indicated by the broken line in Figure 2 and visualised by molecular hybridisation (1.4) in Figure 6,

c) it possesses an at least 50% homology with the said sequence mentioned in b), and confers, when it is present in the mitochondrial genome of a plant, a cytoplasmic male sterility on the said plant,

or

d) it is carried by the sequence bounded by nucleotides 928 and 1569 in Figure 1 and is transcribed to RNA in the mitochondria of sterile plants, or

e) it possesses an at least 50% homology with the

sequence described in d) and is transcribed to RNA in the mitochondria of sterile plants.

The present invention also relates to a plant of the Cruciferae family, characterised in that it contains chloroplasts and a nucleus of the same species or which are compatible, and mitochondria carrying a genome conferring the CMS character as defined above.

More specifically the present invention also relates to a plant belonging to the genus Brassica, characterised in that it contains chloroplasts and a nucleus of Brassica, and mitochondria carrying a genome conferring the CMS character as has been defined above.

This mitochondrial genome must also carry a number of genes of the Brassica species in question. This is achieved by recombination between the Ogura genome and the Brassica genome.

In particular, the present invention relates to a plant belonging to the species Brassica napus, characterised in that it contains a Brassica nucleus and in that the cytoplasm contains Brassica chloroplasts and male-sterile mitochondria carrying a DNA according to the present invention as has been defined above; these mitochondria can also carry the majority of the mitochondrial genes of Brassica napus (18S, Atp9, Atp6, CoxII, ndh1, cob). Brassica napus corresponds to colza or canola and swede.

The present invention relates to a plant of the species Brassica oleracea, characterised in that it contains a Brassica nucleus and in that the cytoplasm contains Brassica chloroplasts and mitochondria containing a DNA sequence coding for the CMS character as has been defined.

Brassica oleracea covers the various types of cabbage: headed cabbage, Brussels sprouts, kohl-rabi, broccoli, fodder kale and cauliflower.

The present invention also relates to a plant of the species Brassica campestris, characterised in that it contains a Brassica nucleus and in that the cytoplasm contains Brassica chloroplasts which are compatible with

the nuclear genome and mitochondria containing a DNA sequence coding for the CMS character as has been defined.

Brassica campestris corresponds to rape, turnip and Chinese, Peking and Japanese cabbage.

Similarly, the present invention relates to a plant chosen from the group comprising B. juncea, B. nigra, B. hirta and B. carinata, characterised in that it contains a Brassica nucleus and in that the cytoplasm contains Brassica chloroplasts which are compatible with the nuclear genome and mitochondria containing a DNA sequence coding for the CMS character as has been defined.

According to another of its aspects, the subject of the present invention is a plant belonging to the genus Brassica and whose nuclear genome contains an Ogura sterility sequence as defined above as well as elements effecting its expression and the transport of the translation product into the mitochondrion. This plant can, in particular, belong to one of the following species: B. napus, B. oleracea, B. campestris, B. nigra, B. juncea, B. hirta and B. carinata.

The presence of the "Ogura sterility sequence" is necessary and sufficient for inducing a total absence of pollen in the absence of restorer genes. The pollination of these plants is effected normally as a result of a good production of nectar.

The morphology of the female organs is normal, and the fruits (siliques) formed contain a normal number of seeds. Figure 5 shows the morphology observed in a normal control plant (z), a plant having aberrant morphology possessing the whole Ogura genome (z(6)) and Brassica oleracea chloroplasts, a cabbage plant carrying Brassica napus chloroplasts and male-sterile mitochondria having Brassica napus genes (z(A)), and plants carrying Brassica oleracea chloroplasts and recombinant mitochondria no longer containing the undesirable sequences (z(9) and z(17)). The plants have the following characteristics:

GENOTYPE	CHLOROPLASTS	MITOCHONDRIA
z	B. oleracea	B. oleracea
z(A)	B. napus	B. napus/Ogura
z(6)	B. oleracea	Ogura
z(9)	B. oleracea	B. oleracea/Ogura
z(17)	B. oleracea	B. oleracea/Ogura

The genotypes z(A) and z(6) do not possess a suitable cytoplasmic male sterility system.

Such plants may be obtained, for example, by the protoplast fusion technique or by all other techniques which effect good recombination between the mitochondrial genome of the species in question and the Ogura mitochondrial genome. In such plants, fertility is restored by a single restorer gene, referred to as Rf1, originating from radish, which is not the case with plants which carry the whole of the unsuitable mitochondrial genome.

Such plants may also be obtained by natural or artificial sexual reproduction.

Plants possessing a mitochondrial genome according to the invention may also be obtained by gene transfer in the mitochondrion.

In all cases, these plants possess a suitable CMS system, namely:

- a total male sterility,
- a morphology permitting good pollination and good seed production as illustrated in Table 1 and Table 2.

Thus, the present invention also relates to a method for preparing hybrid plants, characterised in that a plant possessing a suitable CMS character, containing the Ogura sterility sequence in its mitochondrial or nuclear genome, is crossed with a normal plant in the case of an edible or fodder crop, or with a plant providing a gene restoring fertility, Rf1, in the case where seeds are to be harvested. It also relates to a hybrid plant obtained by this method.

Generally speaking, the best agronomic characteristics are obtained with male-sterile plants

possessing chloroplasts of the same species as the nucleus and mitochondria possessing a suitable male sterility system.

Table 1 shows the productivity of a cabbage line with different cytoplasms (the genotypes Z9 or Z17 are suitable). Table 2 shows the productivity of colza line with different cytoplasms (the genotypes Fu 27, Fu 58 and Fu 85 are suitable).

TABLE 1: PRODUCTIVITY OF THE z LINE (SAUERKRAUT CABBAGE) WITH DIFFERENT CYTOPLASMS

Cytoplasm		Genotypes	Seed harvest
Chloroplasts	Mitochondria		Grams/plant
B. oleracea (fertile control)	B. oleracea	(z)	53.1
B. napus	Ogura	(zC)	0
B. napus	Ogura/napus	(zA)	22.7
B. oleracea	Ogura	(z6)	9.3
Ogura	Ogura	(z0)	20.1
B. oleracea	Ogura/oleracea	(z9 or z17)	91.8

TABLE 2: PRODUCTIVITY OF THE DARMOR LINE (WINTER COLZA) WITH DIFFERENT CYTOPLASMS

* Yield
• Male-fertile and male-sterile (Fu) DARMOR winter colza

	Yield (% DARMOR)	Chloroplasts	Mitochondria
DARMOR	100 (35 qx)	B. napus	B. napus
Fu 27	118	B. napus	B. napus/Ogura
Fu 58	120	B. napus	B. napus/Ogura
Fu 77	96	B. campestris	Ogura
Fu 85	114	B. napus	B. napus/Ogura
Fu 118	103	B. napus	B. napus/Ogura
BIENVENUE	108		
JET NEUF	89		

TABLE 2 (continued)

Components of the yield (Clermont-Ferrand)

	NSq	W1Sq	NSdPSq	NSd	W1Sd	TDM	HI	YLD	HT
DARMOR	7183	81.9	9.9	70028	4.31	1077	0.256	31.6	120
BIENVENU	7334	80.7	11.2	81841	3.88	1064	0.269	30.7	109
JET NEUF	7977	87.7	8.6	67291	4.98	1176	0.262	33.3	115
Fu 27 DARMOR	9292	82.8	11.6	106188	4.09	1337	0.293	42.2	122
Fu 58 DARMOR	8617	76.5	11.7	99947	3.65	1228	0.270	36.0	131
Fu 118 DARMOR*	8389	84.6	11.0	92428	4.11	1243	0.276	38.1	132

NSq: Number of siliques/m^2 - W1Sq: Weight of one silique (mg) - NSdPSq: Number of seeds/silique
NSd: Number of seeds/m^2 - W1Sd: Weight of one seed (mg) - TDM: Total dry matter (g/m^2)
HI: Harvest index (%) - YLD: Yield (qx/ha) - HT: Height (cm)

* These plants often possess deformed fruits (siliques).

The subject of the present invention is also a probe containing a sequence of at least 10 boxes, and preferably 15 boxes, of a sequence bounded by the nucleotides numbered 928 and 1569 in Figure 1; the said probe can be labelled, for example by means of a radioactive base, or by any other means such as, for example, by fluorescence. This probe may be used for the demonstration of male sterility, and may be used, in particular, in the selection of clones.

Some characteristics and advantages of the present invention will be more clearly demonstrated in the examples which follow.

EXAMPLE 1: DEMONSTRATION OF THE DNA SEQUENCE RESPONSIBLE FOR OGURA CYTOPLASMIC MALE STERILITY

1. Plant.

"Cybrid" denotes forms obtained by the fusion of isolated protoplasts followed by regeneration of the whole plant. This method of production enables cytoplasmic information originating from both parents to be mixed in the cell. Cybrid No. 13 was obtained from among 820 plants regenerated by protoplast fusions between an Ogura-cms, triazine-resistant B. napus cybrid (progeny of cybrid 77 described in Pelletier et al., 1983 and Chétrit et al., 1985) and the triazine-sensitive and fertile variety of Brutor origin. A triazine resistance test (Ducruet and Gasquez, 1978) carried out on a leaf sample of each regenerant enabled the type of chloroplast (triazine-resistant chloroplasts originating from the parent 77 or triazine-sensitive chloroplasts originating from the Brutor line) to be determined. The plants were cultivated and the flowering stage was observed. Plants exhibiting non-parental combinations (either sensitive/male-sterile or resistant/male-fertile) were selected as cybrids. Cybrid No. 13 was of the sensitive/male-sterile type. Cybrid 1 was of the resistant/male-fertile type.

2. Nucleic acid isolation.

The total DNA was isolated from leaves originating from 4-week-old plants according to the method described by Dellaporta (1983). The mitochondrial DNA was

extracted from leaves of 8-week-old plants as has been described by Vedel and Mathieu, 1982, with the following variants:

the mitochondria were not purified on a sucrose gradient before lysis, and lysis was carried out in 4% sarcosyl with 0.5 mg/ml proteinase K (Boehringer Mannheim GmbH) in 50 mM Tris pH 8, 20 mM EDTA. After precipitation, the mitochondrial DNA was purified by centrifugation on an ethidium bromide/caesium chloride gradient (method 1 - Vedel and Mathieu, 1982) in polyallomer centrifuge tubes.

The total RNA was isolated from leaves or floral buds according to Logemann et al., 1987.

The mitochondrial RNA was extracted from 8-week-old cauliflowers according to the technique of Stern and Newton, 1986.

3. Restriction analyses of the mitochondrial DNA and agarose gel electrophoresis.

These were carried out as described in Pelletier et al., 1983. The total or mitochondrial RNA was loaded onto electrophoresis gels containing formaldehyde, as has been described by Sambrook et al., 1989.

4. Hybridisation.

Transfer of DNA or RNA onto nylon filters (Hybond-N, Amersham) was carried out by capillary absorption with 6 x SSC or 10 x SSPE, respectively, according to the manufacturer's instructions. Prehybridisation and hybridisation were performed according to Amersham, using probes labelled by the multiprimer DNA labelling system (Amersham) after purification on Sephadex G-50 columns (Sambrook et al., 1989).

5. Cloning of the mitochondrial DNA.

Two genomic libraries of male-sterile (13-7) and revertant (13-6) cybrid lines were constructed in a phage lambda EMBL3 vector cultured on the restrictive E. coli strain Nm539 (Frischauf et al., 1983). Approximately 2.5×10^4 clones per μg of mitochondrial DNA were obtained.

The mitochondrial DNA libraries were assayed and

plated out in order to isolate the plaques, which were transferred onto nylon filters as described in Sambrook et al., 1989. The hybridisation probe used to screen the two libraries of mitochondrial DNA was prepared as follows: the mitochondrial DNA fragment specific for the cms was eluted using the Gene clean™ procedure (BIO 101 INC.) from a mitochondrial DNA digestion product loaded onto a preparative agarose gel. The eluted DNA was then labelled as has been described.

The extraction of lambda DNA, subcloning of the 2.5 NcoI fragment into the NcoI site of pTrc99A (Amann et al., 1988) and extractions of plasmid DNA were performed according to the protocols of Sambrook et al., 1989. The recombinant plasmids were introduced into an E. coli strain NM522 (Gough and Murray, 1983).

6. Genetic study of cybrid 13 and its progeny.

In the first generation of progeny obtained by pollination of cybrid 13 with Brutor, composed of 13 plants, 5 are totally male-sterile (including plants 13-2 and 13-7), one is male-fertile (No. 13-6) and 7 are almost completely sterile with a few male-fertile flowers.

The fertile plant 13-6 was self-pollinated and crossed with Brutor. In both cases, only fertile plants (43 and 42, respectively) are obtained.

In the crosses between the male-sterile plant No. 13-7 and Brutor, 24 progeny are completely sterile and 6 possess a few fertile flowers, a similar result to that observed with the cybrid itself. The plant 13-2 was crossed with the restorer line RF, which is heterozygotic for the specific restorer genes for Ogura male sterility (Chétrit et al., 1985). The progeny of this cross is composed of 53 male-sterile plants, 37 male-fertile plants and 9 plants which are almost completely sterile although they possess a few fertile flowers. These results suggest that the male-sterile plants of the cybrid family 13 contain the Ogura-cms determinant, like the other cybrids studied beforehand with a simpler restoration profile (Chétrit et al., 1985).

At this stage of the study, two possibilities may be envisaged: either cybrid 13 contains a mixture of "male-fertile" and "male-sterile" mitochondrial genomes, and it is possible to select further for both phenotypes, or cybrid 13 contains a recombinant mitochondrial genome of unstable structure which reverts to a more stable "fertile" configuration, and it will be impossible to maintain a homogeneous male-sterile phenotype among subsequent generations.

Male-sterile plants obtained from the progeny of the male-sterile plant No. 13-7 were developed both by taking cuttings and by sexual crossing with Brutor. After a variable number of generations (1 to 5) by both methods, all the families give fertile plants. In contrast, the completely fertile plants thereby obtained never give rise again to sterile plants.

In the light of these results, it may be considered that the second explanation proposed above, that is to say that cybrid 13 carries an unstable mitochondrial genome which loses the Ogura-cms determinant during the process leading to a "fertile" configuration, without the possibility of a return to a sterile phenotype, is the correct one.

7. Comparison between the mitochondrial DNAs of male-sterile and fertile revertant offspring. Isolation of a fragment specific to the male-sterile plants.

The mitochondrial DNA was extracted from the leaves of male-sterile 13-7 progeny and of fertile revertants (13-6 or 13-7 progeny), and digested with several restriction enzymes in order to compare their restriction profiles. The mitochondrial genomes of the two types are very similar, since no difference can be observed between the restriction profile of the male-sterile mitochondria and fertile revertants using various enzymes. However, a 6.8-kb restriction fragment was detected in the restriction profile of the mitochondrial DNA of the male-sterile plants digested with NruI, and was never observed in the corresponding profiles of the fertile revertants.

The fragment (referred to as N6.8) was eluted from an agarose gel, labelled and used as a probe on NruI mitochondrial DNA restriction profiles: a large signal at 6.8 kb was observed in all the male-sterile progeny of cybrid 13, whereas no fragment of this size hybridised with the probe in the genomes of mitochondria of fertile revertants. In addition, the probe N6.8 hybridises with a 6.8-kb fragment in the Ogura mitochondrial DNA digested with NruI, but not in B. napus cv Brutor, showing the Ogura origin of this fragment.

A lambda library containing mitochondrial DNA extracts originating from male-sterile plants (13-7) was tested with the eluted labelled fragment, and out of 8 clones hybridising, 2 recombinant phages were isolated, containing the whole N6.8 fragment and adjacent sequences. A detailed restriction map of this region was obtained. Hybridisation of the restriction profiles of the mitochondrial DNA originating from fertile and sterile progeny of cybrid 13 with N6.8 as a probe enabled the region specific for the male-sterile genotype to be limited to a 2.5-kb NcoI fragment.

The 2.5-kb NcoI fragment was labelled and used as a probe with respect to mitochondrial DNA originating from 13-7 and 13-6 progeny digested with NcoI. Apart from the signal at 2.5 kb which is specific for the male-sterile profile, several NcoI fragments hybridise in both the fertile revertant and male-sterile profiles; these fragments are at 2.2, 10 and 14 kb. A 2.7-kb NcoI fragment hybridises strongly in the mitochondrial genome of the fertile progeny and not in that of the sterile progeny. Analysis of this hybridisation profile leads to the conclusion that the 2.5-kb NcoI fragment, although specific for the male-sterile mitochondrial DNA, contains sequences which are repeated elsewhere in the mitochondrial genome (on 2.2-, 10- and 14-kb fragments after NcoI), and these repeated sequences are also present in the mitochondrial DNA of fertile revertants apart from the specific 2.7-kb fragment.

The total RNA is extracted from leaves or buds of

progeny of cybrids 13, or of male-sterile or fertile cybrids (originating from other fusion experiments) and of Brutor line. Northern blotting was carried out and the blots were hybridised with a probe corresponding to the insert of the lambda clone containing N6.8 described in Example 3. A major 1.4-kb transcript was detected in all the male-sterile cybrids, including cybrid 13-7, whereas no transcript of this size was observed in the Brutor line, or in the two fertile cybrids (different from 13). Moreover, fertile plants possess a 1.1-kb transcript which hybridises with the probe, which is absent or present at a very low level in all the male-sterile cybrids tested. Several transcripts common to all the samples hybridise weakly with the probe on account of the large size of the labelled insert. It was confirmed that the mitochondrial transcripts can be detected in samples of total RNA by hybridisation of the same Northern blot with a DNA fragment containing the atpa gene sequence.

The same specific 1.4 transcript was found in the Ogura mitochondrial RNA extracted from cauliflowers, using the 2.5 NcoI fragment as a probe. The exact limits of this transcript were determined using subclones of the 2.5 NcoI fragment as a probe.

8. Study of cybrid 1 and its progeny.

Cybrid 1 was male-fertile. In its progeny, the plant 1.12 was fertile and the plant 1.18 sterile. The plant 1.12 gave in its progeny sterile plants (S_3) and fertile plants (RF_3). The plant 1.18 gave sterile plants (S_2) and a fertile branch (RF_2). The plants S_2 and S_3 are restored by the same nuclear gene that restores pollen fertility as the sterile cybrid 13.

On hybridisation with the labelled 2.5-kb NcoI fragment, the mitochondrial DNA of the plants S_2 and S_3 does not give a signal at 2.5 kb on NcoI digestion, or a signal at 6.8 kb on NruI digestion.

Similarly, hybridisation with the total RNA (Northern blotting) with a probe corresponding to the ORFB sequence does not give a signal at 1.4 kb as occurs with the sterile cybrid 13. In contrast, a probe corres-

corresponding to the sequence bounded by nucleotides 928 and 1569 in Figure 1 gives a signal in Northern blotting at approximately 1.3 kb. This signal is absent from the RNA of the plants RF_1, RF_2, RF_3 or Brutor. Similarly, it is possible to use this sequence (928-1569) as a probe in dot-blotting of total RNA, and in this case, only the male-sterile plants, and all of them, give a signal.

These results indicate that the S_2 and S_3 plants, although male-sterile, have not retained the nucleotide sequence described in Figure 1 in its original conformation, and demonstrate that, in this sequence, the portion bounded by nucleotides 928 and 1569 is that which carries the "Ogura sterility" specific determinant, which makes the plants male-sterile when this sequence is transcribed.

This sequence has no significant homology with the sequences present in the data banks.

EXAMPLE 2: DEMONSTRATION OF UNDESIRABLE SEQUENCES IN THE OGURA MITOCHONDRIAL GENOME

A collection of cybrids was obtained in the species B. napus by protoplast fusion between a colza carrying Ogura cytoplasm and a normal colza. The former is male-sterile and chlorophyll-deficient at low temperature, while the latter is normally green and fertile. The cybrids were sorted from among the regenerated plants and those which were male-sterile and normally green were adopted.

In the same manner, a collection of cybrids was obtained in the species B. oleracea by protoplast fusion between a cabbage carrying Ogura cytoplasm and a normal cabbage. The cybrids which were male-sterile and normally green were adopted from among the regenerated plants.

These cybrids were crossed with different varieties, of colza in the first case and cabbage in the second. The crosses were repeated at each generation with the same varieties so as to obtain a defined genotype close to that of the recurring variety.

These different varieties, thereby converted with the cytoplasms of different cybrids, were subjected to

agronomic tests to measure seed production, which depends on several factors: a sufficient production of nectar to effect pollination by insects, and a normal floral morphology in order that this pollination is effective and the fruits develop normally.

The collection of cybrids could thus be divided into two batches:

- a batch of cybrids possessing a male sterility suited to commercial seed production,
- a batch of cybrids not possessing all the characteristics favourable to a good commercial seed production.

The colza cybrids Nos. 27, 58 and 85 and the cabbage cybrids Nos. 9, 17, 21, 24 and 27c, for example, belong to the first batch.

The colza cybrids Nos. 23s, 77 and 118 and the cabbage cybrids Nos. 1, 6 and 14, for example, belong to the second batch.

The total DNA of these cybrids was subjected to enzymatic digestions with SalI, NcoI, NruI, BglI, PstI and KpnI. The Southern blots obtained were hybridised with various mitochondrial probes Atpa, Cob, Cox1, Atp6, 26S and 18S and two fragments of Ogura genome, of 2.5 kb derived from an NcoI digestion and of 19.4 kb derived from an NruI digestion.

The two batches of cybrids differ in that:

a) Nos. 23s, 77 and 11s in colza and 1, 16 and 11 in cabbage <u>do possess</u> the region of the Ogura genome which surrounds the Cox1 gene, recognisable by 10.7-kb BglI or 11-kb NruI fragments, and the region of the Ogura genome which surrounds one of the formylmethionine transfer RNA genes, recognisable by 5.1-kb SalI or 15-kb NruI fragments.

b) Nos. 27, 58 and 85 in colza and 9, 17, 21, 24 and 27c in cabbage do not possess the corresponding regions, which have been replaced, as a result of recombinations between the genomes of the two parents which have been fused, by analogous regions of the mitochondrial genome of colza in Nos. 27, 58

and 85 and of cabbage in Nos. 9, 17, 21, 24 and 27c.

It is deduced from this that the two regions in question of the Ogura genome are undesirable if it is desired to have a male sterility system suited to commercial seed production.

EXAMPLE 3

This example illustrates the value of knowing the "Ogura male sterility" sequences and the undesirable sequences for performing an immediate sorting of the cybrids obtained without having to wait several years for back-crossing and agronomic tests.

Protoplasts of a Brassica plant carrying the Ogura cytoplasm are fused with protoplasts of the Brassica species in question. The colonies derived from fusion are cultured in vitro and set up to regenerate on a medium which promotes bud formation (see Pelletier et al., 1983).

From one gram of fresh material, either a callus or a fragment of the regenerated plantlet, it is possible by the techniques described above to isolate the total DNA. After SalI digestion, Southern type hybridisation with the probe bounded by nucleotides Nos. 389 and 1199 (see Figure 1) should give a signal only for a size of 4.4 kb (should not give a signal at 5.1 kb). Similarly, after NruI digestion and hybridisation with a probe carrying the Cox1 gene, a signal should be obtained for a size different from 11 kb.

These hybridisations enable it to be predicted that the plant which has been obtained will indeed be male-sterile and suited to commercial seed production.

EXAMPLE 4

This example is a variant of Example 3, based on the idea of carrying out sexual crossing between the two parents under special conditions or with particular genotypes instead of carrying out protoplast fusions, with the result that, in contrast to the known processes of fertilisation in plants, there is mixing of the cytoplasms of the oosphere and of the pollen tube or the male gamete. If such methods were described, an early

sorting could be performed in the same manner on young plants derived from these artificial fertilisations, using the same probes and the same criteria as in Example 3.

EXAMPLE 5

This example illustrates the value of knowing the Ogura sterility sequence in a type of genetic manipulation which has already been described in yeast (Johnston et al., 1988).

Starting with a normal Brassica plant, meristems or alternatively cells in vitro are bombarded with microparticles coated with DNA carrying the Ogura sterility sequence. The plants obtained in the progeny of the treated meristems or the regenerated plantlets will be cytoplasmic male-sterile if the DNA has been able to enter the mitochondria and become integrated in the genome of these organelles. This procedure will avoid the problems that are created by the undesirable sequences when Ogura radish chloroplasts or the sequences so defined of the Ogura mitochondrial genome are involved.

EXAMPLE 6

This example illustrates the value of knowing the "Ogura sterility" sequence in the construction by genetic engineering of a nuclear, instead of a cytoplasmic, male sterility conforming to Mendelian inheritance.

Starting with the mitochondrial DNA sequence bounded by nucleotides 928 and 1569, a chimeric gene may be constructed which will be transcribed, after genetic transformation of Brassica cells or cells of another genus, in the nucleus of the cells of the transformed plants obtained. If the chimeric gene contains a presequence which enables its protein translation product to be imported into the mitochondrion, these transformants will be male-sterile and this character will behave as a dominant Mendelian character.

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CLAIMS

1. Ogura sterility DNA sequence, characterized in that:

a) it is carried by a DNA sequence bounded by the nucleotides numbered 928 and 2273 in Figure 1, or

b) it possesses an at least 50% homology with said sequence mentioned in a),

and confers, when it is present in the mitochondrial genome of a plant, a cytoplasmic male sterility on said plant.

2. DNA sequence according to Claim 1, characterized in that it is carried by the sequence bounded by nucleotides 928 and 1569 in Figure 1, or in that it possesses an at least 50% homology with said sequence, and in that it is transcribed to RNA in the mitochondria of male-sterile plants.

3. Recombinant plant nuclear or mitochondrial genome, characterized in that it contains an Ogura sterility DNA sequence which consists

a) of a sequence bounded by the nucleotides numbered 928 and 1569 in Figure 1, or

b) of a sequence possessing an at least 50% homology with said sequence mentioned in a),

and confers, when it is present in the cytoplasm of a plant, a cytoplasmic male sterility on said plant, with the exclusion of the whole of the Ogura mitochondrial genome.

4. Recombinant plant nuclear or mitochondrial genome according to Claim 3, characterized in that it contains an Ogura sterility DNA sequence which is carried by a sequence bounded by the nucleotides numbered 928 and 1569 in Figure 1 or which possesses an at least 50% homology with said sequence.

5. Nuclear or mitochondrial genome according to one of Claims 3 and 4, characterized in that said recombinant mitochondrial genome does not contain all or part of one and/or other of the two fragments of the Ogura genome:

- carrying one of the two formylmethionine transfer RNA genes used for translation initiation,
- carrying the coxl gene coding for the subunit No. 1 of cytochrome oxidase,

or in which said fragments are inactive.

6. Nuclear or mitochondrial genome according to one of Claims 3 to 5, characterized in that it is devoid of part of the sequence which is carried by a 10.7-kb fragment after BglI digestion and an 11-kb fragment after NruI digestion, these fragments being revealed by hybridization with a coxl probe.

7. Nuclear or mitochondrial genome according to one of Claims 3 to 6, characterized in that it is devoid of part of the sequence which gives a 15-kb fragment after NruI digestion, gives a 5.1-kb fragment after SalI digestion and gives an 18.5-kb fragment after BglI digestion, and which is revealed by hybridization with a probe corresponding to the sequence bounded by bases 389 and 1199 of the sequence shown in Figure 1.

8. Nuclear or mitochondrial genome according to one of Claims 3 to 7, characterized in that it contains a sequence which gives a 2.5-kb fragment after NcoI digestion, gives a 6.8-kb fragment after NruI digestion and gives a 4.4-kb fragment after SalI digestion.

9. Nuclear genome, characterized in that it contains, apart from the DNA sequence according to one of Claims 1 to 8, a presequence enabling the translation product of said sequence to be imported into the mitochondria.

10. Genome according to one of Claims 3 to 8, characterized in that it is a mitochondrial genome.

11. Mitochondrion, characterized in that it contains a genome according to Claim 10.

12. Cytoplasm, characterized in that it contains a mitochondrial genome according to Claim 10, and in that it contains chloroplasts of the same species as the nuclear genome or of another species but which are

compatible with this nuclear genome.

13. Plant belonging to the genus Brassica, characterized in that it contains Brassica chloroplasts which are compatible with the nuclear genome of said plant and mitochondria according to Claim 11.

14. Plant belonging to the genus Brassica, characterized in that its nuclear genome comprises a genome according to Claim 9 as well as elements effecting its expression and the transport of the translation product into the mitochondrion.

15. Plant according to Claim 13, characterized in that it belongs to the species Brassica napus.

16. Plant according to Claim 13, characterized in that it belongs to the species Brassica oleracea.

17. Plant according to Claim 13, characterized in that it belongs to the species Brassica campestris.

18. Plant according to Claim 13, characterized in that it belongs to the species Brassica juncea.

19. Plant according to Claim 13, characterized in that it belongs to the species Brassica nigra.

20. Plant according to Claim 13, characterized in that it belongs to the species Brassica hirta.

21. Plant according to Claim 13, characterized in that it belongs to the species Brassica carinata.

22. Plant according to Claim 14, characterized in that it belongs to one of the following species: B. napus, B. oleracea, B. campestris, B. nigra, B. juncea, B. hirta and B. carinata.

23. Plant according to one of Claims 13 to 22, characterized in that it is obtained by protoplast fusion.

24. Plant according to one of Claims 13 to 22, characterized in that it is obtained by sexual reproduction.

25. Plant according to one of Claims 13 to 21, characterized in that it is obtained by gene transfer in the mitochondrion.

26. Method for preparing hybrid plants, characterised in that a plant possessing the male-sterile cytoplasmic character according to one of Claims 13 to 25 is crossed with a plant of the same species possessing, where appropriate, a gene restoring fertility, Rf1.

27. Hybrid plant obtained by the method according to Claim 26.

28. Nucleic acid probe, characterised in that it contains a sequence of at least 10 bases of the sequence bounded by the nucleotides numbered 928 and 1569 shown in Figure 1, and responsible for the male-sterile cytoplasmic character, labelled by a radioactive or non-radioactive means.

29. Ogura sterility DNA sequence according to Claim 1 or 2 substantially as hereinbefore described.

30. Recombinant genome according to any one of Claims 3 to 10 substantially as hereinbefore described.

31. Mitochondrion according to Claim 11 substantially as hereinbefore described.

32. Cytoplasm according to Claim 12 substantially as hereinbefore described.

33. A plant according to any one of Claims 13 to 25 or 27 substantially as hereinbefore described.

34. A method according to Claim 26 substantially as hereinbefore described.

35. A nucleic acid probe according to Claim 28 substantially as hereinbefore described.

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CARTER SMITH & BEADLE

Patent Attorneys for the Applicant:

INSTITUT NATIONAL DE LA REDIERCHE AGRONOMIQUE

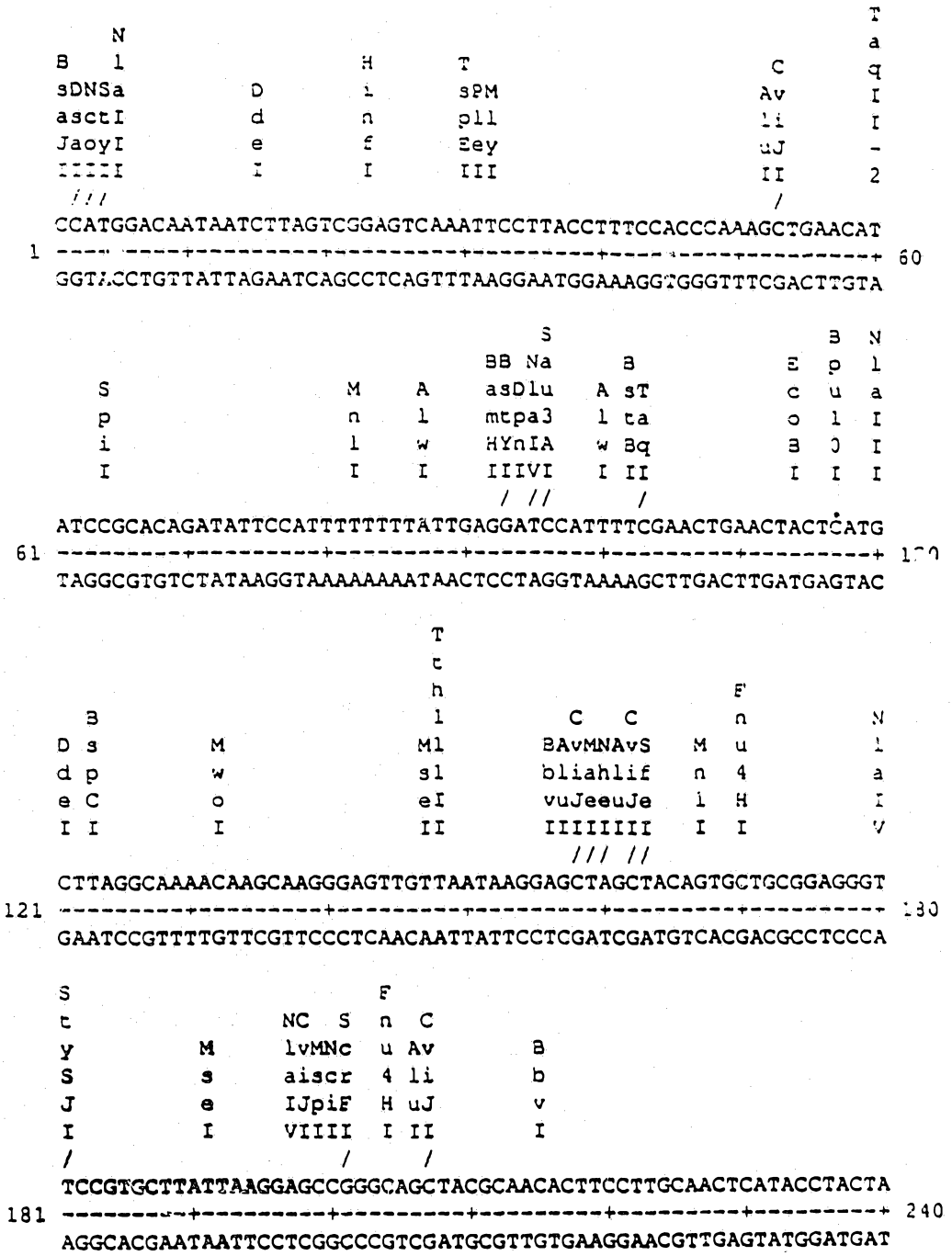
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Fig. 1

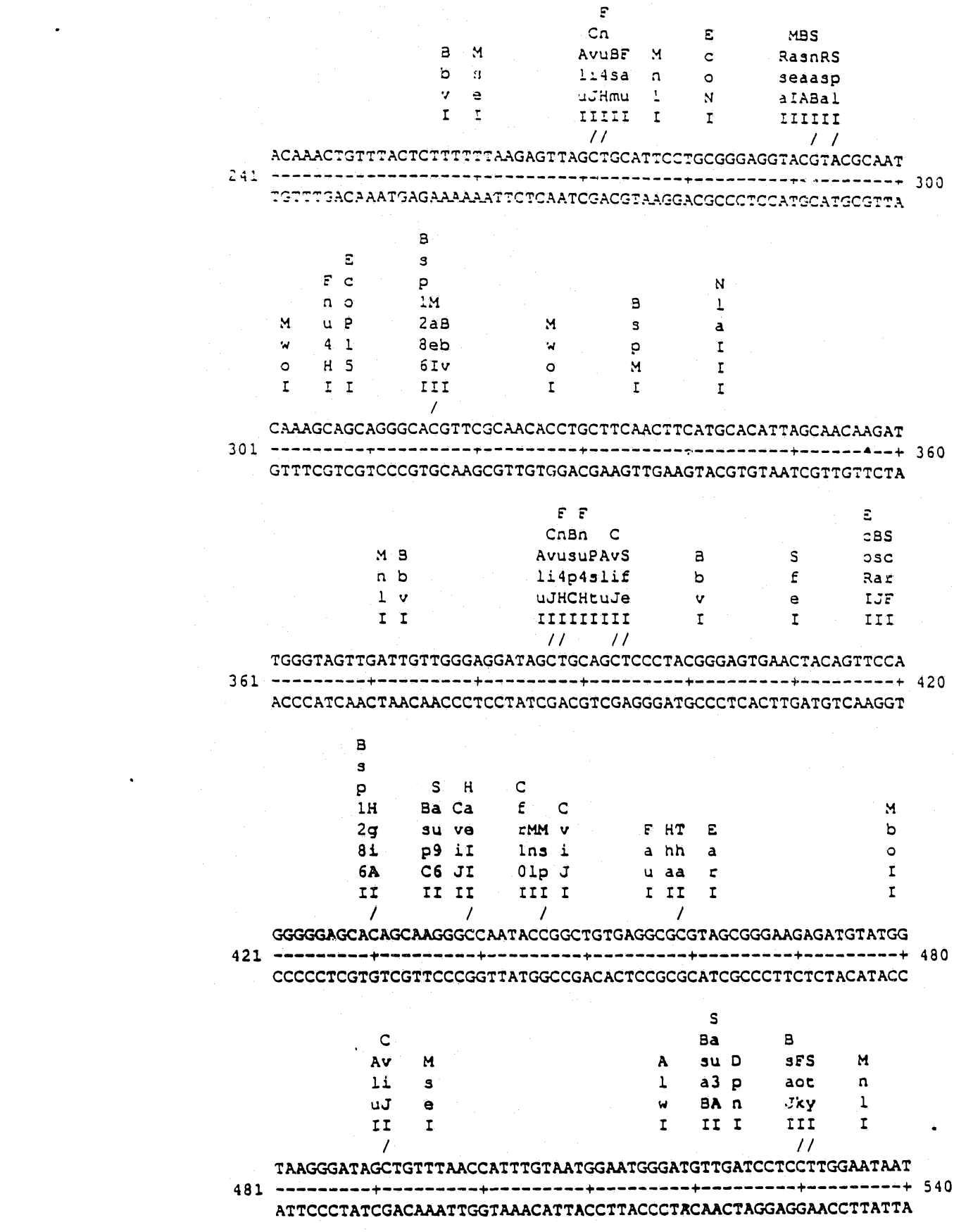
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Fig. 1

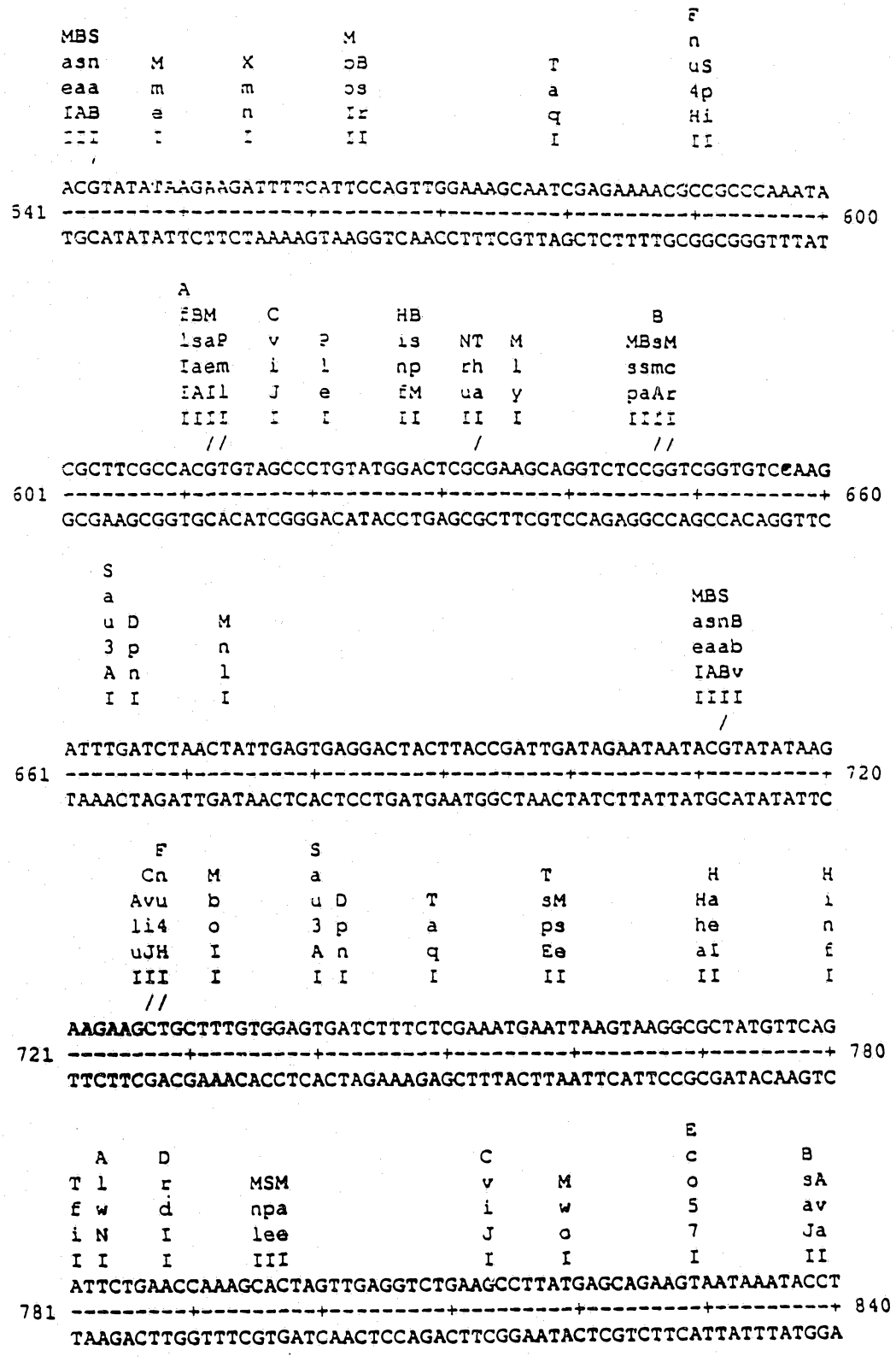
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FIG. 1

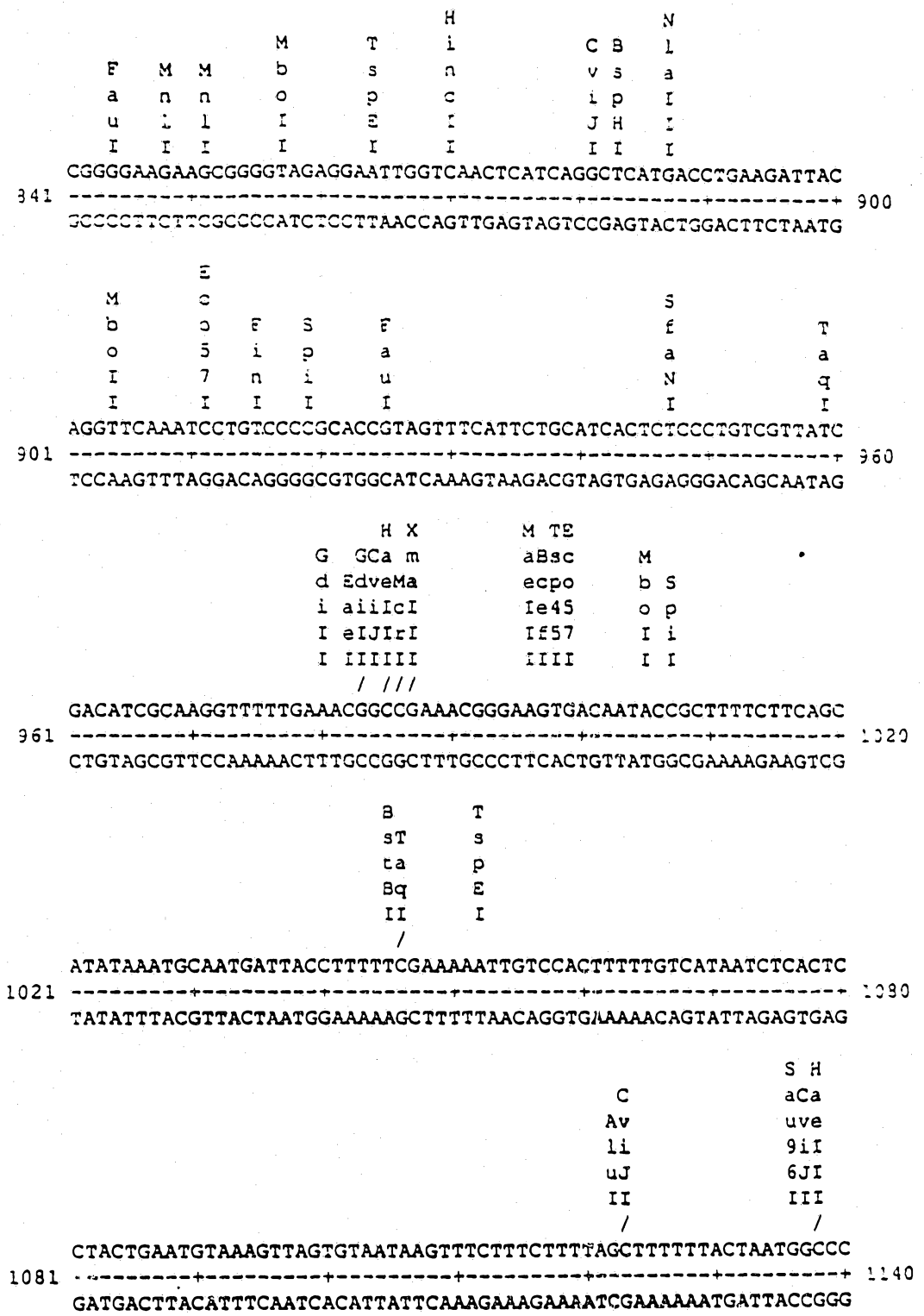
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FIG. 1

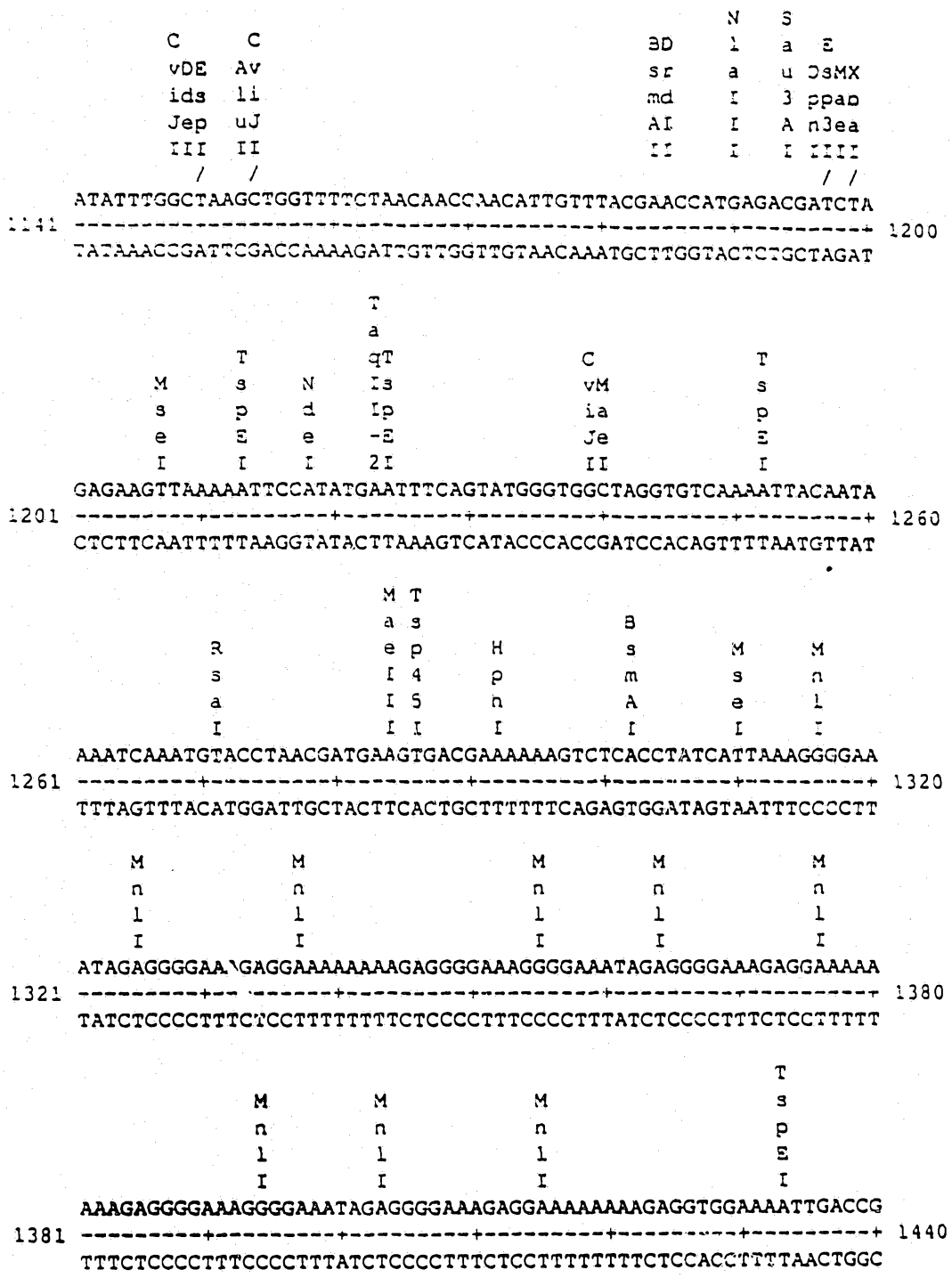
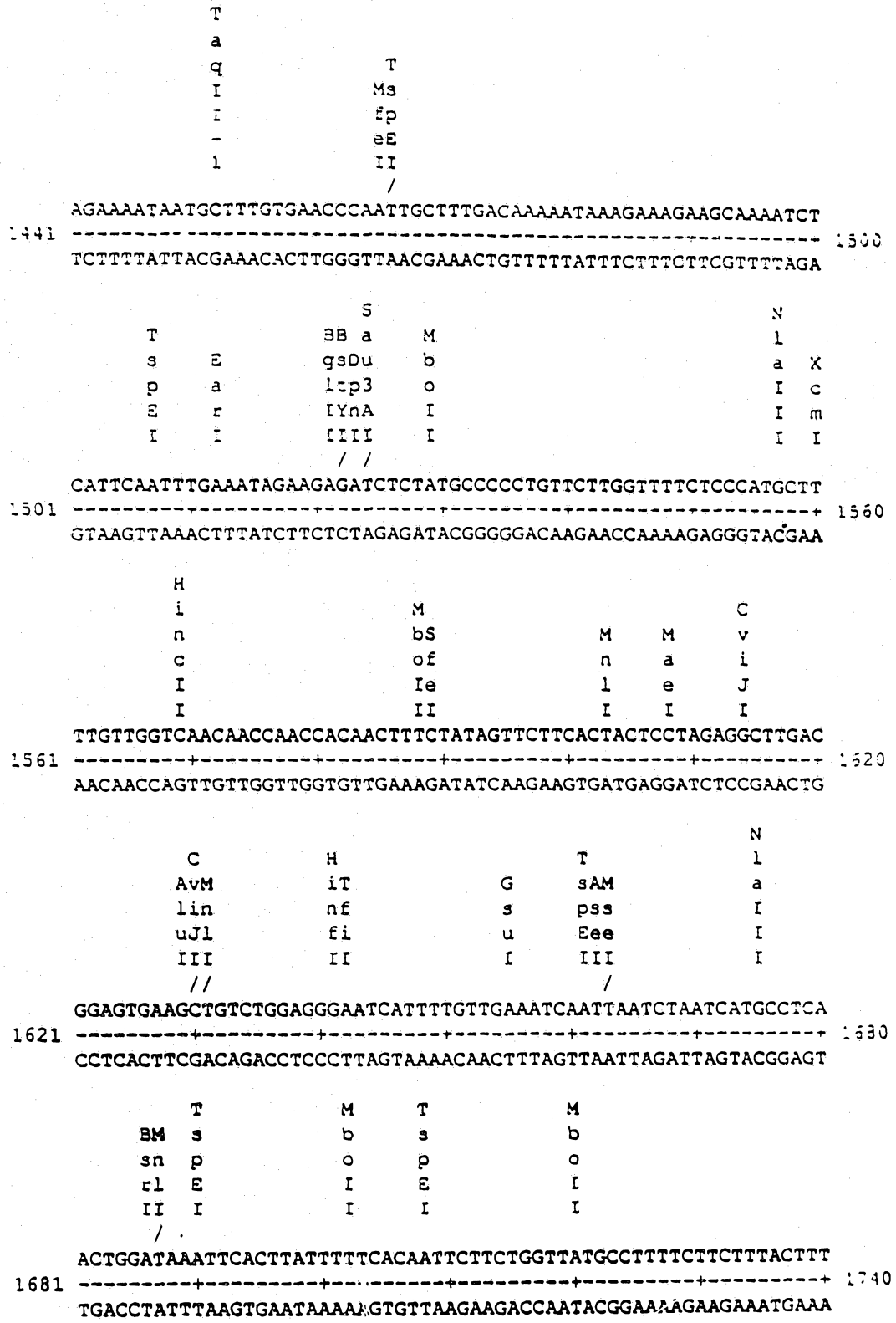


FIG. 1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GTAAGTTAAACTTTATCTTCTCTAGAGATACGGGGGACAAGAACCAAAAGAGGGTACGAA
TTGTTGGTCAACAACCAACCACAACTTTCTATAGTTCTTCACTACTCCTAGAGGCTTGAC
1561 ---------- 1620
AACAACCAGTTGTTGGTTGGTGTTGAAAGATATCAAGAAGTGATGAGGATCTCCGAACTG
GGAGTGAAGCTGTCTGGAGGGAATCATTTTGTTGAAATCAATTAATCTAATCATGCCTCA
1621 ---------- 1680
CCTCACTTCGACAGACCTCCCTTAGTAAAACAACTTTAGTTAATTAGATTAGTACGGAGT
ACTGGATAAATTCACTTATTTTTCACAATTCTTCTGGTTATGCCTTTTCTTCTTTACTTT
1681 ---------- 1740
TGACCTATTTAAGTGAATAAAAAGTGTTAAGAAGACCAATACGGAAAAGAAGAAATGAAA

Fig. 1

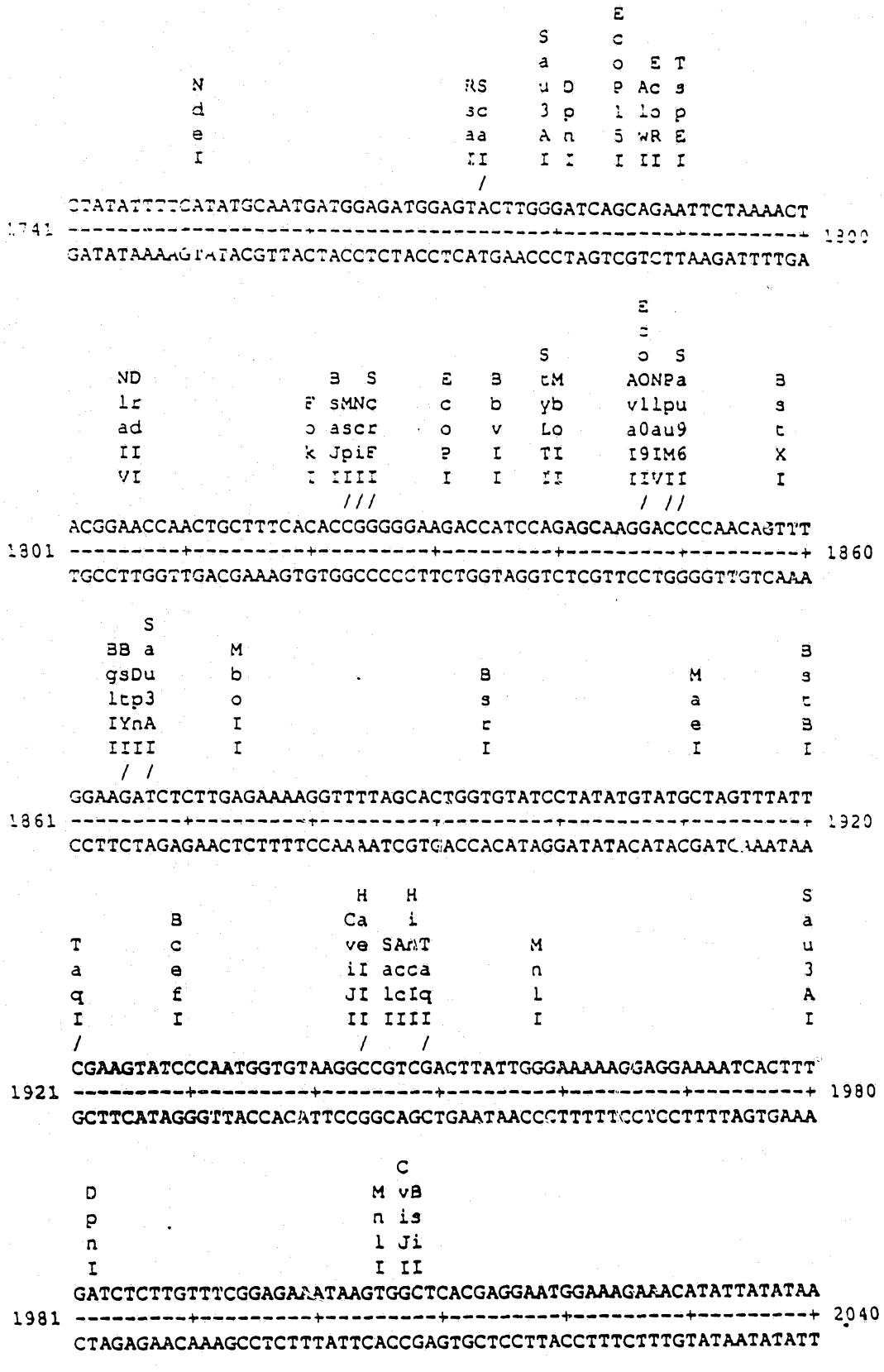

1741
GATATAAAAGTATACGTTACTACCTCTACCTCATGAACCCTAGTCGTCTTAAGATTTTGA
1800
ACGGAACCAACTGCTTTCACACCGGGGGAAGACCATCCAGAGCAAGGACCCCAACAGTTT
1801
TGCCTTGGTTGACGAAAGTGTGGCCCCCTTCTGGTAGGTCTCGTTCCTGGGGTTGTCAAA
1860
GGAAGATCTCTTGAGAAAAGGTTTTAGCACTGGTGTATCCTATATGTATGCTAGTTTATT
1861
CCTTCTAGAGAACTCTTTTCCAAAATCGTGACCACATAGGATATACATACGATCAAATAA
1920
CGAAGTATCCCAATGGTGTAAGGCCGTCGACTTATTGGGAAAAAGGAGGAAAATCACTTT
1921
GCTTCATAGGGTTACCACATTCCGGCAGCTGAATAACCCTTTTTCCTCCTTTTAGTGAAA
1980
GATCTCTTGTTTCGGAGAAATAAGTGGCTCACGAGGAATGGAAAGAAACATATTATATAA
1981
CTAGAGAACAAAGCCTCTTTATTCACCGAGTGCTCCTTACCTTTCTTTGTATAATATATT
2040

FIG. 1

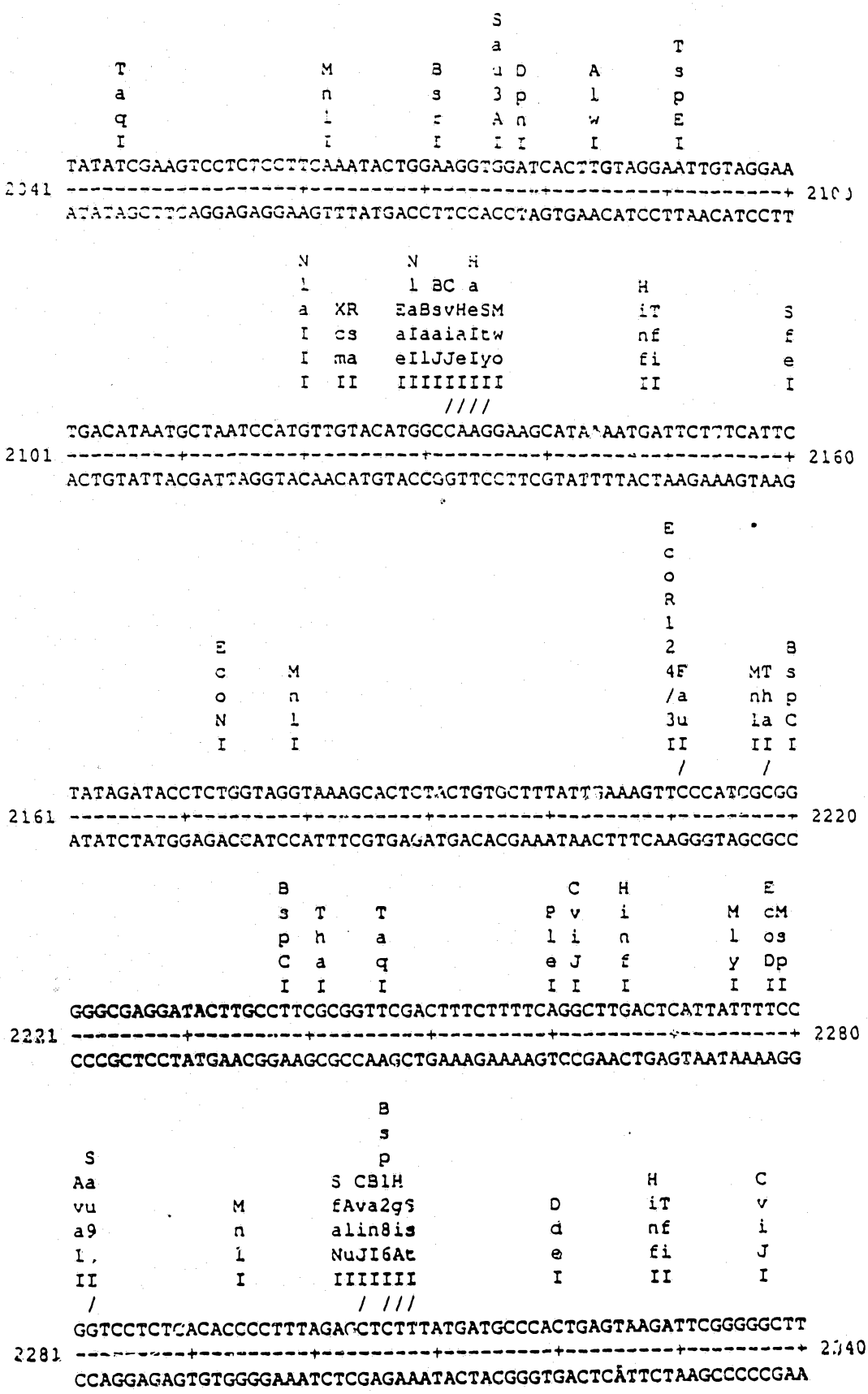
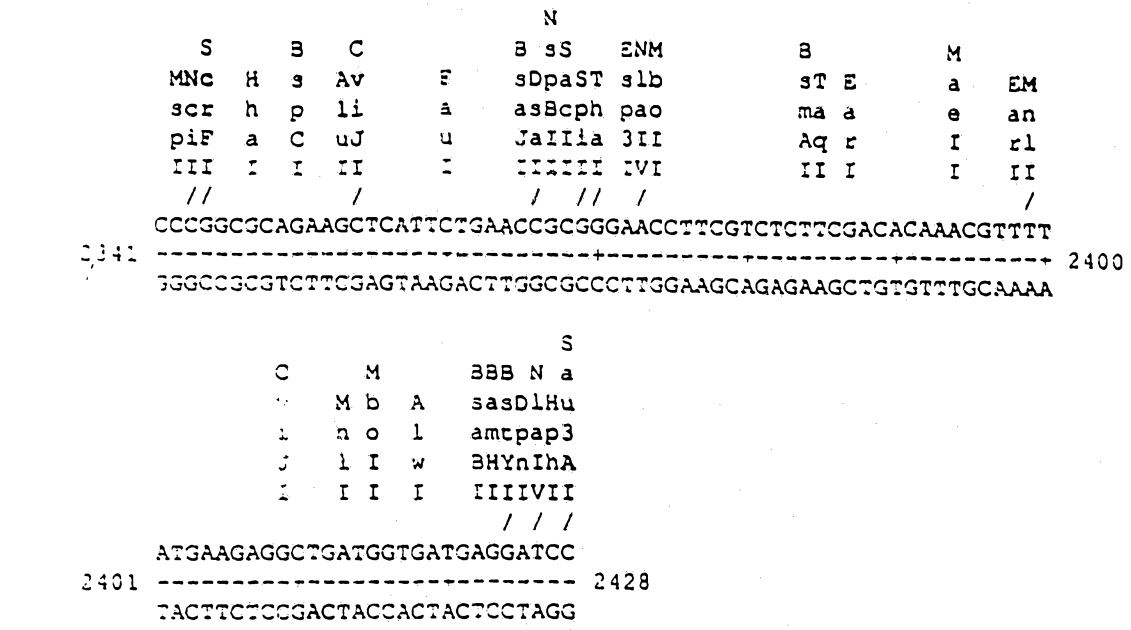

TATATCGAAGTCCTCTCCTTCAAATACTGGAAGGTGGATCACTTGTAGGAATTGTAGGAA
2041
ATATAGCTTCAGGAGAGGAAGTTTATGACCTTCCACCTAGTGAACATCCTTAACATCCTT
TGACATAATGCTAATCCATGTTGTACATGGCCAAGGAAGCATAAAATGATTCTTTCATTC
2101
2160
ACTGTATTACGATTAGGTACAACATGTACCGGTTCCTTCGTATTTTACTAAGAAAGTAAG
TATAGATACCTCTGGTAGGTAAAGCACTCTACTGTGCTTTATTGAAAGTTCCCATCGCGG
2161
2220
ATATCTATGGAGACCATCCATTTCGTGAGATGACACGAAATAACTTTCAAGGGTAGCGCC
GGGCGAGGATACTTGCCTTCGCGGTTCGACTTTCTTTTCAGGCTTGACTCATTATTTTCC
2221
2280
CCCGCTCCTATGAACGGAAGCGCCAAGCTGAAAGAAAAGTCCGAACTGAGTAATAAAAGG
GGTCCTCTCACACCCCTTTAGAGCTCTTTATGATGCCCACTGAGTAAGATTCGGGGGCTT
2281
2340
CCAGGAGAGTGTGGGGAAATCTCGAGAAATACTACGGGTGACTCATTCTAAGCCCCCGAA

FIG. 1



Enzymes that do cut:

AccI	AflIII	AluI	AlwI	AlwNI	AseI	AvaI	AvaII
BalI	BamHI	BanII	BbvI	BbvII	BcefI	BglII	Bpu10I
BsaI	BsaAI	BsaBI	BsaJI	BsiI	BsmI	BsmAI	Bsp1286I
BspCI	BspHI	BspMI	BsrI	BstBI	BstXI	BstYI	Cfr10I
CviJI	DdeI	DpnI	DrdII	DsaI	EaeI	EarI	Eco57I
EcoBI	EcoDI	EcoNI	Eco0109I	EcoPI	Eco15I	EcoRI	EcoRII
EcoR124/3I	EspI	Esp3I	FauI	FinI	Fnu4HI	FokI	GdiII
GsuI	HaeI	HaeII	HaeIII	HgiAI	HhaI	HincII	HinfI
HphI	MaeI	MaeII	MaeIII	MboII	McrI	MfeI	MlyI
MmeI	MnlI	MseI	MspI	MwoI	NciI	NcoI	NdeI
NheI	NlaIII	NlaIV	NruI	NspBII	PleI	PmlI	PpuMI
PstI	RsaI	SacII	SalI	Sau96I	Sau3AI	ScaI	ScrFI
SfaNI	SfeI	SmaBI	SpeI	SpiI	SplI	SstI	StyI
StyLTI	StySJI	TaqI	TaqII-1	TaqII-2	TfiI	ThaI	Tsp45I
TspEI	Tth111II	XbaI	XcmI	XmaIII	XmnI		

Enzymes that do not cut:

AatII	AflII	AgeI	AhaII	ApaI	ApaLI	AvrII	BanI
BcgI	BclI	BglI	BspGI	BspMII	BssHII	BstEII	Bsu36I
CfrAI	ClaI	DraI	DraIII	DrdI	EciI	Eco47III	EcoAI
EcoDXXI	EcoEI	EcoKI	EcoR124I	EcoRV	FseI	FspI	HgaI
HgiEII	HindIII	HinfIII	HpaI	KpnI	MluI	NaeI	NarI
NotI	NsiI	NspI	PflMI	PshAI	PvuI	PvuII	RleAI
RsrII	SfiI	SgrAI	SmaI	SnaI	SphI	SspI	StuI
StySBI	StySPI	StySQI	Tth111I	Uba1105I	Uba1108I	XhoI	

Fig.1

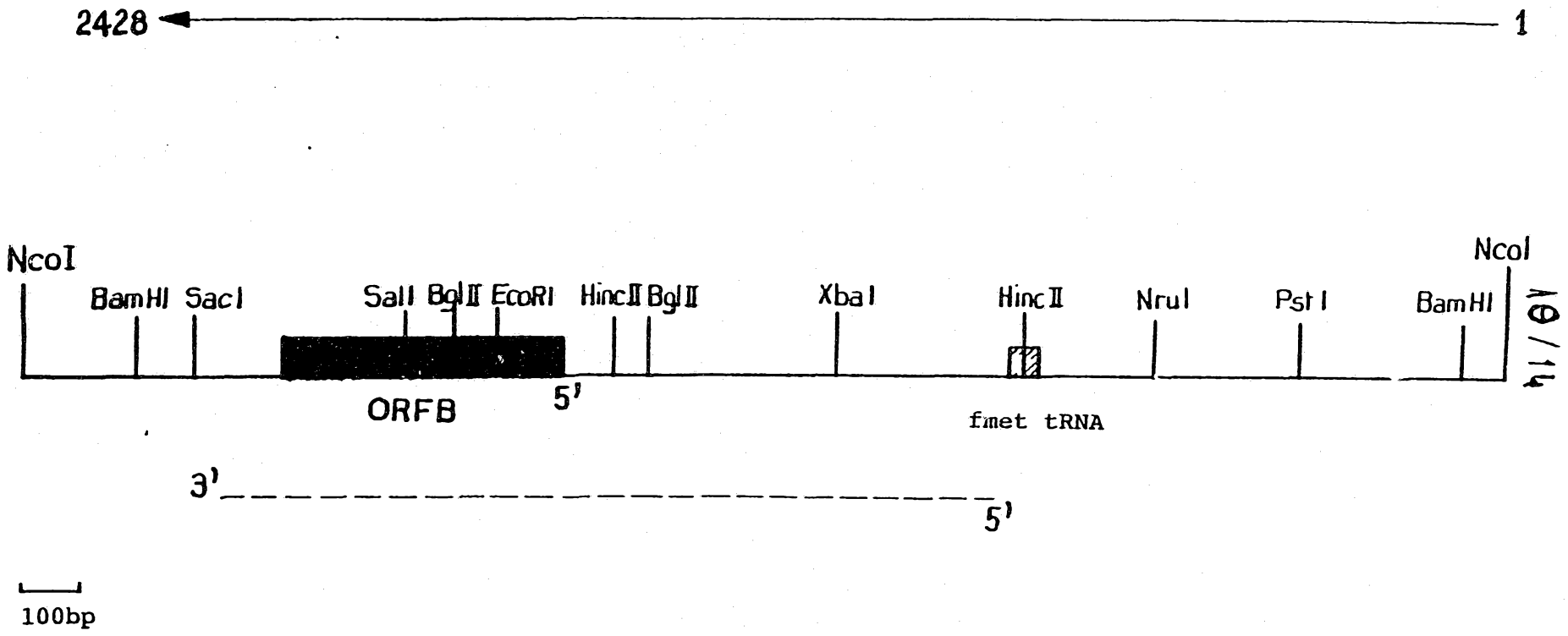

2428
1
NcoI
BamHI
SacI
SalI
BglII
EcoRI
HincII
BglII
XbaI
HincII
NruI
PstI
BamHI
NcoI
4.0 / 1.4
ORFB
5'
fmet tRNA
3'
5'
100bp

FIG. 2

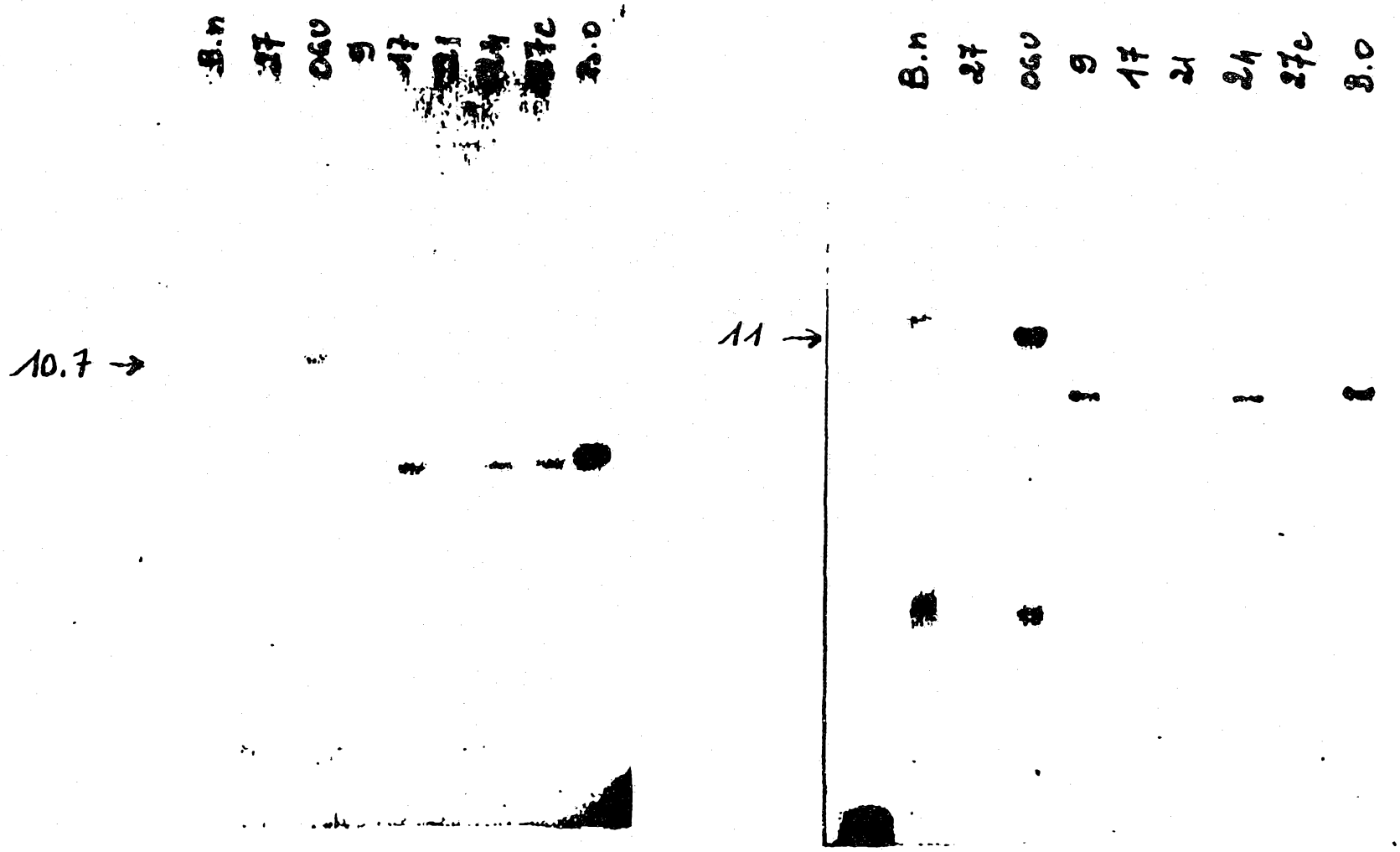


FIG. 3

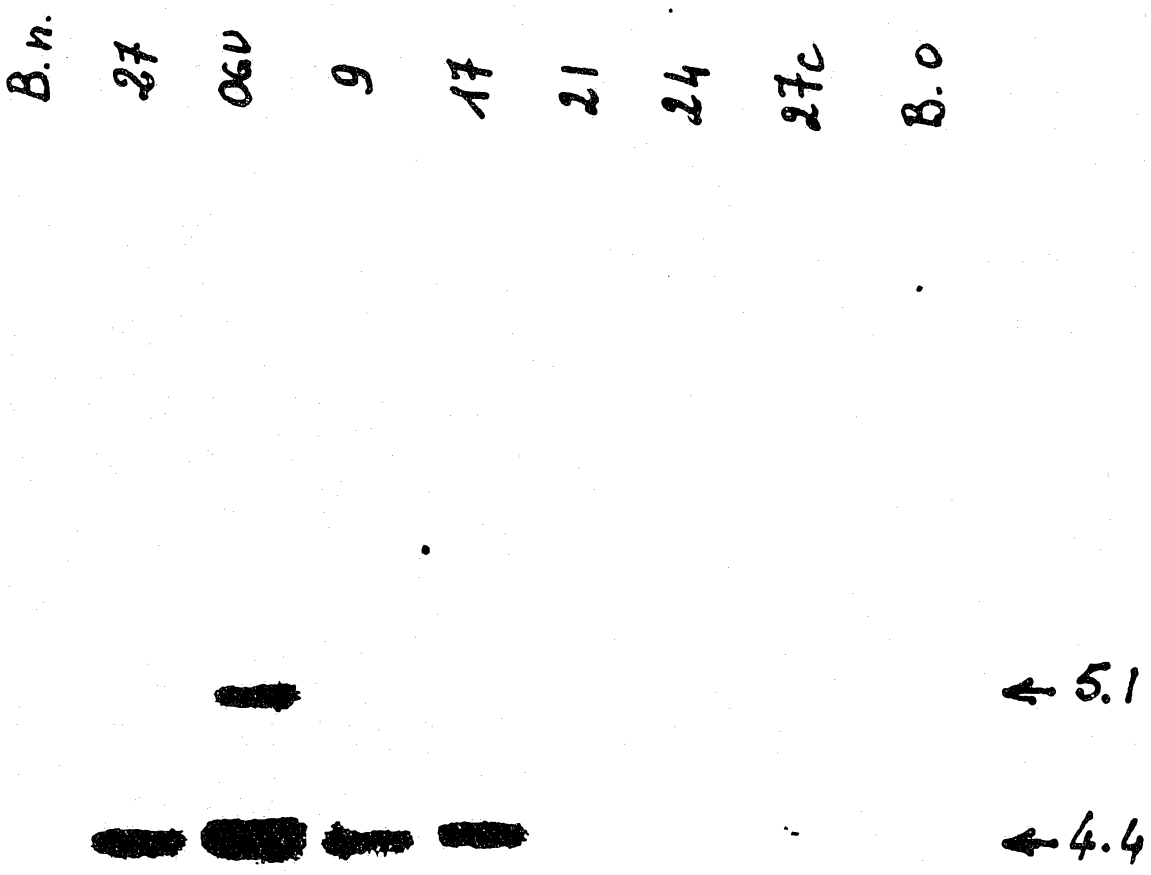


FIG.4

FIG.5

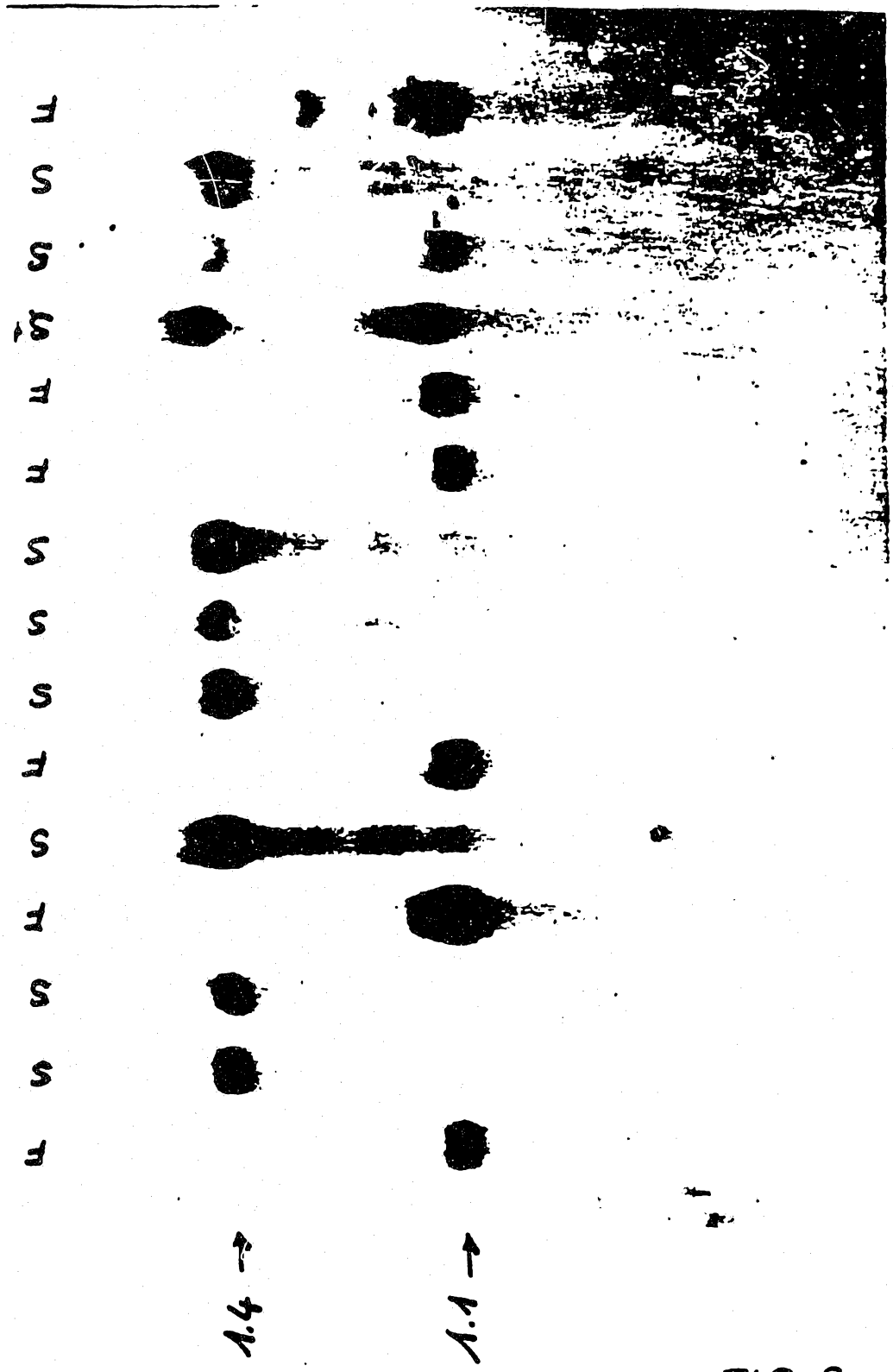


FIG. 6

INTERNATIONAL SEARCH REPORT

International Application No PCT/FR91/00741

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [6]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC[5] : C12N 15/11; A01H 5/00; C12N 15/05; C12Q 1/68

II. FIELDS SEARCHED

Minimum Documentation Searched [7]

Classification System	Classification Symbols
IPC[5]	C12N; A01H; C12Q

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT [9]

Category [10]	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No. [13]
X	MOLECULAR & GENERAL GENETICS volume 191, 1983, pages 244-250; PELLETIER, G., ET AL.,: "Intergeneric cytoplasmic hybridization in cruciferae by protoplast fusion" see page 247, left-hand column	1-8,10-13, 15,23-27
X	THEOR. APPL. GENET volume 69, 1985, pages 361-366; CHETRIT, P., ET AL.,: "Mitochondrial DNA polymorphism induced by protoplast fusion in Cruciferae" see page 364; tables 1,2	1-8,10-13, 15,23-27
X	PLANT PHYSIOL. BIOCHEM. volume 25, No. 3, 1987, pages 249-257; VEDEL, F., ET AL.,: "Mitochondrial DNA variation in cytoplasmic male sterile somatic hybrids of Brassica napus" see page 250, right-hand column	1-8,10-13, 15,23-27
X	JOURNAL OF BIOLOGICAL CHEMISTRY. volume 264 No. 20, 15 July 1989, BALTIMORE US pages 11706-11713; MAKAROFF, C. A., ET AL.,: "The atp6 coding region has been disrupted and a novel reading frame generated in the mitochondrial genome of cytoplasmic male- ./.	28

[10] Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
6 January 1992 (06.01.92)	16 January 1992 (16.01.92)
International Searching Authority	**Signature of Authorized Officer**
European Patent Office	

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
	sterile radish" see figure 2 sequences -169 to -58 and figure 7 sequences -169 to -58	
P,X	CURR. GENET. volume 19, No. 2, February 1991, pages 121-127; BONHOMME, S., ET AL.,: "A 2.5kb NcoI fragment of Ogura radish mitochondrial DNA is correlated with cytoplasmic male-sterility in Brassica cybrids" see the whole document	1-8,10-13, 15,23-28
A	J. CELL. BIOCHEM. SUPPL. volume 13D, 1989, page 299; CONNETT, M. B., ET AL.,: "Plant transformation as a test of the relarionship between cytoplasmic male sterility, respirator phenotype, and the PCF gene" see abstract M310	9,14
A	Biological abstracts v. 89, 1990, No. 14710 & THEOR. APPL. GENET. volume 78, No. 3, 1989, pages 445-455; JOURDAN, P. S., ET AL.,: "Synthesis of male sterile, triazine-resistant Brassica napus by somatic hybridization between cytoplasmic male sterile Brassica oleracae and atrazine-resistant Brassica campestris" see abstract	1-28
A	GB, A, 2211205 (ZAADUNIE) 28 June 1989 see the whole document	1-28
A	WO, A, 8701726 (ALLELIX) 26 March 1987 see the whole document	1-28

ANNEX TO THE INTERNATIONAL SEARCH REPORT ON INTERNATIONAL PATENT APPLICATION NO. FR 9100741 SA 51902

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report. The members are as contained in the European Patent Office EDP file on
The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information. 06/01/92

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
GB-A-2211205	28-06-89	DE-A- 3842473	29-06-89
		FR-A- 2628601	22-09-89
		NL-A- 8803089	17-07-89
WO-A-8701726	26-03-87	AU-A- 6407786	07-04-87
		EP-A- 0238596	30-09-87

RAPPORT DE RECHERCHE INTERNATIONALE

Demande Internationale No PCT/FR 91/00741

I. CLASSEMENT DE L'INVENTION (si plusieurs symboles de classification sont applicables, les indiquer tous) 7

Selon la classification internationale des brevets (CIB) ou à la fois selon la classification nationale et la CIB

CIB 5 C12N15/11; A01H5/00; C12N15/05; C12Q1/68

II. DOMAINES SUR LESQUELS LA RECHERCHE A PORTE

Documentation minimale consultée[8]

Système de classification	Symboles de classification
CIB 5	C12N ; A01H ; C12Q

Documentation consultée autre que la documentation minimale dans la mesure où de tels documents font partie des domaines sur lesquels la recherche a porté[9]

III. DOCUMENTS CONSIDERES COMME PERTINENTS[10]

Catégorie °	Identification des documents cités, avec indication, si nécessaire,[12] des passages pertinents[13]	No. des revendications visées[14]
X	MOLECULAR & GENERAL GENETICS vol. 191, 1983, pages 244 - 250; PELLETIER, G.,ET AL.,: 'Intergeneric cytoplasmic hybridization in cruciferae by protoplast fusion' voir page 247, colonne de gauche ---	1-8, 10-13, 15,23-27
X	THEOR. APPL. GENET vol. 69, 1985, pages 361 - 366; CHETRIT, P., ET AL.,: 'Mitochondrial DNA polymorphism induced by protoplast fusion in Cruciferae' voir page 364; tableaux 1,2 ---	1-8, 10-13, 15,23-27

-/--

° Catégories spéciales de documents cités:[11]

"A" document définissant l'état général de la technique, non considéré comme particulièrement pertinent

"E" document antérieur, mais publié à la date de dépôt international ou après cette date

"L" document pouvant jeter un doute sur une revendication de priorité ou cité pour déterminer la date de publication d'une autre citation ou pour une raison spéciale (telle qu'indiquée)

"O" document se référant à une divulgation orale, à un usage, à une exposition ou tous autres moyens

"P" document publié avant la date de dépôt international, mais postérieurement à la date de priorité revendiquée

"T" document ultérieur publié postérieurement à la date de dépôt international ou à la date de priorité et n'appartenenant pas à l'état de la technique pertinent, mais cité pour comprendre le principe ou la théorie constituant la base de l'invention

"X" document particulièrement pertinent; l'invention revendiquée ne peut être considérée comme nouvelle ou comme impliquant une activité inventive

"Y" document particulièrement pertinent; l'invention revendiquée ne peut être considérée comme impliquant une activité inventive lorsque le document est associé à un ou plusieurs autres documents de même nature, cette combinaison étant évidente pour une personne du métier.

"&" document qui fait partie de la même famille de brevets

IV. CERTIFICATION

Date à laquelle la recherche internationale a été effectivement achevée	Date d'expédition du présent rapport de recherche internationale
06 JANVIER 1992	16 JAN 1992
Administration chargée de la recherche internationale	**Signature du fonctionnaire autorisé**
OFFICE EUROPEEN DES BREVETS	MADDOX A.D.

3

III. DOCUMENTS CONSIDERES COMME PERTINENTS [14] (SUITE DES RENSEIGNEMENTS INDIQUES SUR LA DEUXIEME FEUILLE)

Catégorie °	Identification des documents cités, [16] avec indication, si nécessaire des passages pertinents [17]	No. des revendications visées [18]
X	PLANT PHYSIOL. BIOCHEM. vol. 25, no. 3, 1987, pages 249 - 257; VEDEL, F.,ET AL.,: 'Mitochondrial DNA variation in cytoplasmic male sterile somatic hybrids of Brassica napus' voir page 250, colonne de droite	1-8, 10-13, 15,23-27
X	JOURNAL OF BIOLOGICAL CHEMISTRY. vol. 264, no. 20, 15 Juillet 1989, BALTIMORE US pages 11706 - 11713; MAKAROFF, C. A.,ET AL.,: 'The atp6 coding region has been disrupted and a novel reading frame generated in the mitochondrial genome of cytoplasmic male-sterile radish' voir fig 2 séquence -169 à -58 et fig. 7 séquence -169 à -58	28
P,X	CURR. GENET. vol. 19, no. 2, Février 1991, pages 121 - 127; BONHOMME, S., ET AL.,: 'A 2.5kb NcoI fragment of Ogura radish mitochondrial DNA is correlated with cytoplasmic male-sterility in Brassica cybrids' voir le document en entier	1-8, 10-13, 15,23-28
A	J. CELL. BIOCHEM. SUPPL. vol. 13D, 1989, page 299; CONNETT, M. B.,ET AL.,: 'Plant transformation as a test of the relarionship between cytoplasmic male sterility, respirator phenotype, and the PCF gene' voir abrégé M310	9,14
A	Biological abstracts v. 89, 1990, no.14710 & THEOR. APPL. GENET. vol. 78, no. 3, 1989, pages 445 - 455; JOURDAN, P. S., ET AL.,: 'Synthesis of male sterile,triazine-resistant Brassica napus by somatic hybridization between cytoplasmic male sterile Brassica oleracae and atrazine-resistant Brassica campestris' voir abrégé	1-28
A	GB,A,2 211 205 (ZAADUNIE) 28 Juin 1989 voir le document en entier	1-28

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III. DOCUMENTS CONSIDERES COMME PERTINENTS [14] (SUITE DES RENSEIGNEMENTS INDIQUES SUR LA DEUXIEME FEUILLE)

Catégorie °	Identification des documents cités, [16] avec indication, si nécessaire des passages pertinents [17]	No. des revendications visées [18]
A	WO,A,8 701 726 (ALLELIX) 26 Mars 1987 voir le document en entier ---	1-28

ANNEXE AU RAPPORT DE RECHERCHE INTERNATIONALE RELATIF A LA DEMANDE INTERNATIONALE NO.

FR 9100741
SA 51902

La présente annexe indique les membres de la famille de brevets relatifs aux documents brevets cités dans le rapport de recherche internationale visé ci-dessus.
Lesdits membres sont contenus au fichier informatique de l'Office européen des brevets à la date du 06/01/92
Les renseignements fournis sont donnés à titre indicatif et n'engagent pas la responsabilité de l'Office européen des brevets.

Document brevet cité au rapport de recherche	Date de publication	Membre(s) de la famille de brevet(s)	Date de publication
GB-A-2211205	28-06-89	DE-A- 3842473	29-06-89
		FR-A- 2628601	22-09-89
		NL-A- 8803089	17-07-89
WO-A-8701726	26-03-87	AU-A- 6407786	07-04-87
		EP-A- 0238596	30-09-87

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