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Dr. Arnold Foudin,
Deputy Director Evaluation,
USDA, APHIS, BSS,
4700 River Road, Unit 147,
Riverdale, MD 20737-1237

May 20, 1997

Re: Petition for Determination of Nonregulated Status for Radicchio rosso lines with Male Sterility (SEED LINK™).

Dear Dr. Foudin,

Bejo Zaden BV with headquaters in The Netherlands is submitting a Petition for Determination of Nonregulated Status to the Animal and Plant Health Inspection Service (APHIS) for Male Sterile plantlines of Radicchio rosso.

The petition request a determination from APHIS that the Male Sterile Radicchio rosso lines RM3-3, RM3-4 and RM3-6 and any progeny lines and hybrids obtained with those lines by conventional breeding methods, no longer be considered regulated articles under 7 CFR Part 340.

In the interest of an efficient selection procedure of the male sterile plants, selective marker genes coding for kanamycin-resistance and phosphinothricin-tolerance are linked to the gene of interest inducing the male sterility.
Fieldtesting of Male Sterile Radicchio rosso has been conducted since 1993 in several European countries and since 1995 in California.

Parent lines of Radicchio rosso expressing the barnase gene have shown complete male sterility, resulting in the production of 100% pure hybrid seeds after pollination with a conventional pollinator line.

The petition does not contain Confidential Business Information (CBI).

Please contact me at 31.226.396162 (phone), 31.226.393504 (fax) or 106231.2262@compuserve.com (E-mail) if you have any questions concerning this petition.

Yours sincerely,

Albertus J.M. Schrijver,
Manager Research Centre,
Bejo Zaden BV,
P.O. Box 50,
1749 ZH Warmenhuizen,
The Netherlands.

5/28/97

CONTENTS

Annex 15 Safety assessment of the bacterium-derived recombinant phosphinothricin acetyltransferase (PAT) protein. *H.P.J.M. Notenborn. RIKILT-DLO Wageningen, The Netherlands.*

Annex 16 Kanamycine resistentie in transgene planten. (Kanamycin resistance in transgenic plants). Conclusions

PETITION FOR DETERMINATION OF NONREGULATED STATUS FOR:

Radicchio rosso *(Cichorium intybus* L.*)* with a new male sterility system (SEED LINK ™).

The undersigned submits this petition under 7 CFR 340.6 to request that the Director, BEEP, makes a determination that this article should not be regulated under 7 CFR part 340.

Submitted by: Albertus J.M. Schrijver,
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This date:

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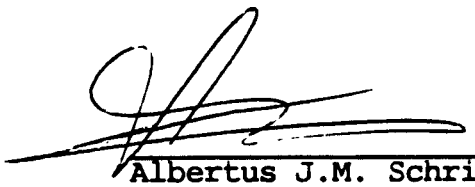
CERTIFICATION

The undersigned certifies, that to the best knowledge and belief of the undersigned, this petition includes all information and views on which to base a determination, and that it includes relevant data and information known to the petitioner which are unfavorable to the petition.



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1. NOTIFIER

Name of notifier: Bejo Zaden BV, which is a seedcompany with activities in breeding, production and marketing of vegetable seeds.
Contactperson: Albertus J.M. Schrijver.
Department: Research & Development.
Address of the notifier: PO Box 50, Trambaan 1, 1749 ZH Warmenhuizen, The Netherlands.
Telephone: 31.226.396162
Fax: 31.226.393504 and 31.226.394877
E-mail: 106231.2262@compuserve.com
Name of the product: Radicchio rosso with a new male sterility system (SEED LINK™).

2. INTRODUCTION

Up to 10 years ago breeding activities focused on Radicchio rosso (red hearted chicory) were very low. Growers in European production areas (mainly in Italy) produced their own seed for those crops. These so-called grower-selections generally demonstrate:

a. insufficient seed quality
b. low productivity
c. insufficient uniformity

About 10 years ago BEJO Zaden BV decided to improve these crops by means of an intensive breeding program emphasizing the development of hybrid varieties.
Essentially, the advantages of hybrid crops are :

a. pure seed quality with high germination rate
b. high productivity
c. uniformity of the crop

Till now the breeding of hybrid Radicchio rosso crops is exploited by self-incompatibility of the parental lines. However, the alleles regulating this self-incompatibility, often appear to be unstable, resulting in an unacceptable rate of self pollination in the parental lines during seed production. The resulting seed lots, contaminated with seed from the parental lines, are not marketable.

Thanks to a new male sterility system, existing of ***barnase*** linked to the selectable marker genes ***bar*** and ***neo***, parental lines can be developed which are incapable of producing pollen and performing pollinations. For the production of the hybrid seed a conventional male fertile parental line is used for cross-pollination of the transgenic male sterile parental line. Seed is harvested on the male sterile line only and accordingly a 100 % pure hybrid seed lot will be obtained. The male fertile line and its seed will be destroyed.

Radicchio rosso is the first vegetable crop in which this system for male sterility has been implemented. For growers this event opens possibilities for the cultivation of Radicchio rosso crops of a remarkably high quality.

Radicchio rosso (red hearted chicory) is a member of the *Cichorium intybus L* family. Together with green hearted chicory, endive and belgian endive (witloof) radicchio rosso belongs to a minor group of chicory-vegetables. The total acreage in Europe is 40,000 acres, of which 35,000 in Italy, and in USA 2,400 acres, mainly in California.

To date several releases in the field of the new transgenic plantlines and hybrid varieties have been effected:

COUNTRY	YEAR	PERMIT
The Netherlands	1993	B/NL/92/13
	1994	B/NL/93/06 B/NL/93/16
	1995	B/NL/93/16 B/NL/94/23
	1996	B/NL/93/16A B/NL/94/23A
Italy	1993	B/IT/93/07
	1994	B/IT/94/02 B/IT/94/04
	1995	B/IT/95/37 B/IT/95/43
	1996	B/IT/96/39
United Kingdom	1994	B/UK/94/R12/1
	1995	B/UK/95/R12/2
	1996	B/UK/95/R12/2
France	1994	B/F/94/03/09
	1995	B/F/95/03/01
	1996	B/F/96/04/01
Belgium	1994	B/B/94/W4
	1995	B/B/95/V5B
	1996	B/B/96/V5C
USA/California	1995	95-205-01
	1996	95-205-01

3. INFORMATION RELATING TO THE RECIPIENT OR PARENTAL ORGANISM FROM WHICH THE GMO IS DERIVED.

3.1 Scientific name and other names:

Family name: Compositae
Genus: *Cichorium*
Species: *Cichorium intybus*
Subspecies: Radicchio rosso (red hearted chicory).
Cultivar: Breeding line R3 (Ces473-1).

3.2 Phenotypic and genetic traits:

Chicory (*Cichorium intybus* L.) belongs to the family of the Compositae together with other species like *Lactuca sativa* (lettuce) and *Helianthus annuus* (sunflower). *Cichorium intybus* L. var. silvestre BISCHOFF is the wild-type form which is the origin of all cultivated varieties. The genus has a diploid number of 18 chromosomes.

The identification of *Cichorium* ssp. is effected on phenotypical characteristics. The plants have a typical composite morphology with ligulate, mostly blue corollas and herbaceous bracts in two rows. The flowers are grouped to form blue coloured flowerheads. They include 5 stamens and 2 styles.
Radicchio rosso (red hearted chicory) is phenotypically easy to identify due to the typical formation of the head and the red turning to green-red color of the leaves with striking thick, white veins.

In general only phenotypic aspects are utilized to distinguish the source organism from its nearest relatives. Herewith particularly those characteristics are observed that appear on the registration list of the 'General Netherlands Inspection Service for Flower and Vegetable Seeds' (NAKG), according to the UPOV guide-lines. Each individual (hybrid) variety is hereby described by means of the following characteristics, and differs in at least one of them from other (hybrid) varieties.

Characteristics :

- Formation of anthocyanin in the leaf
- Colour of the leaf, shape, etc.
- Heading
- Earliness / Harvest time
- Bolting resistance
- Flower colour

Annex 2 provides a description of characteristics for the distinction of varieties of Radicchio rosso, examined by the "General Netherlands Inspection Service for Flower and Vegetable Seeds". (NAKG), in respect of registration.
Annex 3 presents an example of an existant variety

(Scarlati) of BEJO Zaden BV, wich obtained plant variety protection rights on 29 march 1993.

Radicchio rosso plants can exchange genetic information with other lines and species of the same genus (*Cichorium*) due to cross pollination by insects.
Fecundation occurs essentially by insect cross pollination. Self pollination is rather exceptional, but occurs, depending on genotype. Asexual reproduction does not occur. Radicchio rosso has been selected for bolting tolerance. Therefore lower temperature is needed to induce flowering.

3.3 Geographical distribution, survival and dessimination:

The parental organism is indigenous to european countries, except the very north. To our knowledge it is also indigenous in the USA. Its natural habitats are waysides and land laying fallow.
Structures enhancing survival: pollen, seeds and roots. Essentially pollen represents a "genetic survival/dispersal source", merely of importance if pollination and seed formation follow.
Generally seeds survive properly under different circumstances. In optimal conditions (e.g. in conditioned seed storage rooms at -20^0C and low air humidity) germination can be guaranteed over a 5 year period. Under natural circumstances however, the probability of encountering those optimal conditions is very small and consequently the chances for seeds to conserve over a longer period are minimal. The natural conditions rather will encourage germination or be as such that seeds loose their germination capacity.
Generally, roots will not survive longer than one year, due to frost sensitivity. Yet, this depends strongly on the climatical conditions of the area where the vegetable is grown. In the South-European countries and the southern states of the USA more roots will survive as a result of higher temperatures during winter. Roots usually do not endure temperatures below -5^0C, through which survival in the Netherlands generally comes to less than one year.
In those areas where the product is grown, after seeds or vegetables have been harvested, the soil will be cultivated by ploughing and/or milling. Through this treatments roots, eventually arising from the source organism, are unlikely to survive. Possible seedlings/volunteers are treated as weeds. They will be treated by a herbicide application or a mechanical cultivation.

Ways of dissemination: Seeds and roots.
Factors affecting dissemination:
Biotic: For their maintenance, the cultivated types of Radicchio rosso completely rely on horticultural and agricultural practices. Thus, man is the most important biotic factor

in respect of survival of the organism.

Abiotic: Dispersal of seeds is essentially restricted to the immediate surroundings of the mother-plant since seeds stay in the torus after flowering. Only when the plant is completely decomposed, seeds will be released.

4. THE PRODUCT

4.1 Description of the product:

Productname: Radicchio rosso with a new male sterility system (SEED LINK™).

The product is developed by Bejo Zaden BV in The Netherlands.
The development of the gene constructs and the transformation of the Radicchio rosso line, improved by Bejo Zaden, were conducted in the laboratories of Plant Genetic Systems, Belgium.
Hereafter the transformed plants have been transferred to the greenhouse and laboratory facilities of Bejo Zaden in Warmenhuizen, The Netherlands.
Experimental hybrids and seeds from the parental lines are obtained in the Bejo Zaden-greenhouses. The commercial hybrid seed production is effected abroad, primarily in Italy, and in the future possibly also in other countries in- and outside Europe.

The product is a commercially grown vegetable crop, delivered in the form of seeds by Bejo Zaden to the seed industry, plantraisers and growers. The product, Radicchio rosso, is grown by farmers and growers for the production of vegetable chicory crops. After harvest the product will be put up for sale by the growers and subsequently distributed to supermarkets and vegetable-traders. The crop will be consumed by humans, mainly as a component of fresh mixed salads.
Radicchio rosso (red hearted chicory) belongs to the *Cichorium* family to which also endive, Belgian endive ("witloof") and Pan di Zucchero (green hearted chicory) belong. Industrial chicory, cultivated for its inulin production, does not belong to the group of vegetable chicories.

Definition of the product:
The product consists of the Radicchio rosso (red hearted chicory) lines RM3-3, RM3-4, and RM3-6 and all the hybrids obtained with those lines by conventional breeding methods.

The hybridization technique utilized for the production of the hybrids is based on the genetic male sterility of one of the parental lines. The technique results in the production of 100% pure hybrid seeds of Radicchio rosso.
In the interest of an efficient selection procedure of the male sterile parents, selective marker genes coding for kanamycin-resistance and phosphinothricin-tolerance are linked to the gene of interest inducing the male sterility.
In the product the new traits (male sterility, kanamycin-resistance and phosphinothricin-tolerance) are expected to be present in 50% of the plants. The others do not contain

the genes for these traits. This result is inherent to the rules of Mendelian inheritance. See also **annex 1**.
This means that growers cannot use glufosinate-ammonium for treatment of weeds. If they do, they will loose 50% of the crop.

4.2 Conditions of use and handling of the seeds:

The procedures for the usage, storage and transportation of the transgenic Radicchio rosso seeds do not differ from those which are in use for the conventional Radicchio rosso production.

Usage: About 75% of the commercial Radicchio rosso seeds are pelleted. These seeds are delivered to the professional plantraisers, who in their turn supply the professional growers with plant material. The remaining 25% is distributed as 'naked' seeds to the growers.

Storage: Storage of the seeds takes place in conditioned storage rooms at a constant temperature of 15°C and a relative humidity of 35%. During storage the seeds are packed in woven polypropylene bags. The seeds are delivered to the customer in two different packages. Pelleted seeds are packed in tins while naked seeds are packed in bags of aluminum laminate. This is composed of polyethylene on the inside, smooth kraft on the outside, and a thin aluminum layer in between. This air- and gas-tight package has been developed particularly for the storage and the transport of seeds.

Transportation: For the transport of the seeds from the production fields to the company building of Bejo Zaden BV and subsequently to the customers the same packages as described above are used.

For seeds, put on the market by the seed industry, EC- and US-regulations pertaining to the labeling of seed lots are in force. The most efficient approach would be to maintain this method of labeling. The labels contain information such as:

- Crop
- Species
- Variety
- Seed lot number
- Seedtreatment (Yes/No), applied product (active ingredient)
- Purity

- Germination
- Limit viability date
- Control mark NAKG (General Netherlands Inspection Service of Vegetable and Flower Seeds).

An additional statement will inform the user that possible volunteers can be destroyed by treatment with herbicides other than those containing glufosinate ammonium as active ingredient, because 50% of the plants are tolerant to glufosinate ammonium.

4.3 Annual estimated production:

In Europe, approximately 40,000 acres of Radicchio rosso are grown, of which about 35,000 acres in Italy. In the USA Radicchio rosso is a very minor crop with a estimated annual production of approximately 2,400 acres, mainly in California. If varieties obtained by the new hybridization system should enter the market, no influence on the acreage under culture is expected. In prospect of a market share of 50% in 10 years we expect the seed production acreage to raise from a few acres in the beginning to nearly 30 acres within 10 years, mainly in Italy and France. However, this is just a rough estimation inasmuch as a correct determination of the seed yield per acre is not predictable.

4.4 Current status of the product:

In accordance with the decision of the Commission of the European Communities, of 20 May 1996, taken in accordance with the procedures of Directive 90/220/EEC, Bejo Zaden BV has been given consent for placing on the market the product as described in this document.
According to this consent, for the moment of time, the product is excluded for the use as food or feed. The official text belonging to the above-mentioned decision of the Commission of the European Communities has been published on 13 July 1996.
The Ministry of Health of The Netherlands has already decided that the product is save for human consumption.
According to the european regulations the product will be evaluated in accordance with the new EU Novel Food Regulations which will be in force by june 1997.

5. THE GENETIC MODIFICATION

5.1 General information on the genetic modification

The genetic modification is based on the insertion of DNA sequences into the genome of the parental organismen by *Agrobacterium tumefaciens* disarmed Ti-plasmid mediated transformation.
The aim of the genetic modification is the introduction of a hybridization system in Radicchio rosso as a tool for the production of F1 hybrid varieties, including

1. *a pollination control system* based on a nuclear male sterility trait;
2. *an effective phenotypic selection technique* based on tolerance to phosphinothricin, the active ingredient in the commercial herbicide BASTA®;
3. *a functional in vitro selection method* based on kanamycin resistance.

5.2 Vectors

The vector used to transfer the new DNA-fragments is the plasmid pTTM8RE, carrying the genes for male sterility (*barnase*), kanamycin resistance (*neo*) and phosphinothricin tolerance (*bar*) between the T-DNA terminal repeats. The plasmid is an intermediate cloning vector which upon mobilization from *Escherichia coli* to *Agrobacterium tumefaciens* is integrated in the resident disarmed Ti-plasmid pGV2260.
The genes of interest are comprised between the T-DNA border sequences of the Ti-plasmid and the DNA fragment transferred to the plant genome is restricted to this part. This limitation has been confirmed by appropriate molecular analyses.
For the transformations a 'disarmed' *Agrobacterium tumefaciens* has been used. Two properties of the T-DNA on the Ti-plasmid of *Agrobacterium* make the latter ideal vectors for the introduction of new genes in plants :

1. *Agrobacterium* exhibits a very broad host range through which these organisms are capable of transforming plants cells of nearly all dicotyledons and some monocotyledons.
2. The introduced T-DNA is inherited according the rules of regular Mendelian inheritance.

The host range of the vector is limited to *E. Coli* en *Agrobacterium* species.
Besides the genes of interest to be transferred to the plant genome the plasmid contains genes conferring resistance to the antibiotics streptomycin-spectinomycin and ampiciline which are suitable for the identification of the bacterial strains containing the plasmid. These genes are only expressed in the bacterial genetic background and are not transferred to the plant genome.

For plant transformation the vector system as described by Deblaere et al. (1985) has been used.

The plasmid pTTM8RE carries three chimeric genes between the two T-DNA border repeats. These chimeric genes are denoted as PNos-*neo*-3'ocs, PSsuAra-tp-*bar*-3'g7 and PTA29-*barnase*-3'nos.

The table below gives an overview of the origin of the different introduced sequences.

Sequence	Origin	Reference
neo	*Escherichia coli*	Beck et al. (1982)
bar	*Streptomyces hygroscopicus*	Thompson et al. (1988)
barnase	*Bacillus amyloliquefaciens*	Hartley R. (1988-1989)
PNos	*Agrobacterium tumefaciens*	Depicker et al. (1982)
PSsuAra-tp	*Arabidopsis thaliana*	Krebbers et al. (1988)
PTA29	*Nicotiana tabacum*	Mariani et al. (1990)
3'ocs	*Agrobacterium tumefaciens*	Gielen et al. (1982)
3'g7	*Agrobacterium tumefaciens*	Velten et al. (1985); Dhaese et al. (1983)
3'nos	*Agrobacterium tumefaciens*	Depicker et al. (1982)

During the construction of the vector some characteristics of the vector changed: The *Agrobacterium tumefaciens* strain, used as transformation vector, is so called 'disarmed'. All plant pathogenic functions (the plant-tumorigenic functions of wild type Ti-plasmids) have been removed in the vectors used for research and development applications.
This adaptation of the Ti-plasmid was required in order to be able to introduce specific genes into the plant genome, other then the tumor-inducing genes.
The acceptor Ti-plasmid from which the T-region has been deleted is non-oncogenic. This plasmid still carries the genes required for transfer of T-DNA of the plant genome and is used as an acceptor plasmid for an intermediate vector, carrying the genes of interest between the 25 bp border sequences of the octopine T-region. The respective plasmid components are the non-oncogenic Ti-plasmid pGV2260 and the intermediate vector pGV825. Plasmid pGV2260 was derived from the octopine Ti-plasmid pTiB6S3 from which the T-region was

deleted and submitted by sequences from the plasmid pBR322 (Deblare et al., 1985).

Annex 4 provides a complete and detailed description of the design and properties of the vector.
Annex 5 provides a complete description of the results of the molucular analysis of the product.

5.3 The insert:

The male sterile lines contain the following chimeric gene constructs :

pSsuAra-tp-*bar*-3'g7 :
This sequence contains the promoter PSsuAra, regulating the expression of the *bar* coding sequence encoding phosphinothricin acetyl transferase, a transit peptide sequence for translocation to the chloroplasts and the 3' untranslated end of the T-DNA gene 7.

pTA29-*barnase*-3'nos :
The promoter pTA29 regulates the expression of the *barnase* coding sequence which upon expression in the tapetum confers male sterility. This sequence contains also the 3' untranslated end of the nopaline-synthase-gene.

pNos-*neo*-3'ocs :
This sequence contains the nopaline-synthase-promoter, regulating the expression of the *neo*-gene, conferring neomycin-phosphotransferase II (NPT II), resulting in resistance to kanamycin in the plant tissues.

A detailed description of the nucleotide sequences comprised between the two border repeats of the pTTM8RE plasmid and a plasmid map are shown in **annex 4 part 2 and 3.**

The table below gives an overview of the composition of all parts of the insert.

Overview of the composition of the inserts included in plasmid pTTM8RE.
The reference number of the donor-organisms refers to the numbers used in chapter 6.

		Donor organism	Ref. Donor org.	Function
1	Right Border//T-DNA rest gen 5	*Agrobacterium tumefaciens*	1	
2	3'gen 7	*Agrobacterium tumefaciens*	1	Terminater
3	*bar* gen	*Streptomyces hygroscopicus*	2	Herbicide tolerance
4	SSU promoter + transitpeptide	*Arabidopsis thaliana*	3	Promoter
5	3'nopaline synthase (nos)	*Agrobacterium tumefaciens*	1	Terminater
6	*barnase* gen	*Bacillus amiloliquefaciens*	4	Male sterility
7	TA29	*Nicotiana tabacum*	5	Promoter
8	Nopaline synthase (Nos)	*Agrobacterium tumefaciens*	1	Promoter
9	*Neo* gen	*Escherichia coli*	6	Kanamycin resistance
10	3' octopine synthase (ocs)	*Agrobacterium tumefaciens*	1	Terminater
11	T-DNA rest - ocs//Left Border	*Agrobacterium tumefaciens*		

The sequence of the T-DNA is fully known. We refer to the full description of the T-DNA in **annex 4**, indicating only a limited amount of linker sequences remaining in the insert. These sequences do not code for additional functions and are not expressed in the plant.
Agrobacterium-mediated plant transformation exploits the property of the bacterium to transfer its T-DNA, located on the Ti-plasmid, into the plant genome. Data are available showing that gene constructs comprised between the terminal border repeats of the T-DNA are integrated into nuclear chromosomal DNA (Rogers et al., 1988; Zambryski et al.,1989).
Once integrated, T-DNA behaves as common plant DNA and is inherited in a Mendelian way. Data demonstrating regular Mendelian inheritance of the transformed plants are comprised in **annex 6**.

Function of each constituent part of the insert in the GMO:

- **the *barnase* gene** encoding a protein (Ribonuclease (RNase)) which, when produced in the tapetum cell layer of the anther, disturbs the metabolism and functioning of this cell layer, resulting in male sterility of the plant;
- a tapetum-specific plant promoter capable of directing expression of the *barnase* gene selectively in the tapetum cell layer of the anthers early during anther development;
- the 3'nos untranslated region is present downstream the *barnase* coding region, providing polyadenylation signals;

- **a marker gene, *bar***, coding for a second protein (phosphinothricin acetyl transferase) which, when present in the tissue of the plant, renders the plant easily identifyable from other plants which do not contain this protein;
- a second plant promoter (PSsuAra) capable of directing expression of the *bar* gene;
- a transit peptide both necessary and sufficient for transport to the chloroplasts;
- the 3'untranslated region of TL-DNA gene 7 is present downstream of the *bar* coding region, providing polyadenylation signals;

- **a marker gene, *neo***, coding for a third protein (neomycin phosphotransferase II) which, when present in the tissue of the plant, renders the plant tissue easily identifiable from other plant tissues which do not contain this protein;
- a third promoter (PNos) capable of directing expression of the *neo* gene;
- the 3'ocs untranslated region is present downstream of

the *neo* coding region, providing polyadenylation signals.

A detailed description of the components and their specific functions is elaborated in **annex 4.**

The insert is limited to the required function. The sequence of the T-DNA is fully known. The insert does not contain parts whose product or function are not known. A report (assessing the results of extended molecular analyses) on the expression and protein activity of the new genes is presented in **annex 5 chapter 2.4., 2.5 and 2.6.**

We refer to the full description of the T-DNA indicating only a limited amount of linker sequences remaining in the insert. These sequences do not code for additional functions and are not expressed in the plant.

The results of a profound study on the occurence of "cryptic gene expression" have shown that beyond the genes of interest no other transcripts from the DNA fragment introduced in the plant genome are produced.

These transcripts might be produced due to the fact that putative transcription initiation signals are recognized in the inserted fragment or due to transcriptional initiation from flanking genome sequences.

The genes of interest are comprised between the T-DNA border sequences of the Ti-plasmid and the DNA fragment transferred to the plant genome is restricted to this part. This limitation has been confirmed by appropriate molecular analyses.

Primarily, PCR primers were used to verify that the T-DNA integration was limited by the left border repeat. These analyses demonstrated, in all cases, that the amplified fragments correspond to what was expected and consequently confirm a correct recognition of the left T-DNA border.

Secondly, a more extended Southern blot analysis of the progeny of one of the lines has been carried out to verify if the DNA, transferred into the plant genome, corresponds to the T-DNA region of the plasmid DNA. The experimental approach was based on digesting the DNAs with different restriction enzymes, probing with different fragments of the transferred DNA and comparing plasmid and genomic DNA fragments. The analysis displays for the tested transformant the expected hybridization patterns and, as a consequence, shows that the introduced DNA corresponds to the DNA configuration as designed in the plasmid vector.

6. INFORMATION ON THE DONORORGANISMS FROM WHICH THE INSERT IS DERIVED:

6.1 DONORORGANISM #1:

Agrobacterium tumefaciens, Ti plasmide: 3'ocs, 3'nos, 3'gene 7.
The donororganism is a bacterium.
Complete name:
Genus: *Agrobacterium.*
Species: *Agrobacterium tumefaciens.*
Strain: Ach5 (3'ocs), T37 (3'nos), Ach5 (3'g7), B6S3 (T-DNA remainder sequences).
Common name: crown gall disease.

Pathogenic characteristics of the donor organism:
Agrobacterium tumefaciens induces crown gall desease in several dicotyledonous plants. However, this ability could be abolished by deletion of oncogenes responsible for this pathogenicity.
The plant pathogenicity of *Agrobacterium tumefaciens* depends on the presence of a large extrachromosmal DNA element, the Ti-plasmid, part of which (the T-DNA) is transferred into the plant genome (Zaenen et al., 1974 ; Chilton et al., 1977 ; Schell et al., 1979). In the plant, the transferred DNA (T-DNA) directs the overproduction of/or hypersensitivity to plant growth hormones (Bergey's, 1986 ; Winans, 1992), as well as the production of novel compounds called opines, which provide a source of nutrients to the colonizing bacteria (Guyon et al., 1980 ; Tempé and Goldman, 1982).

Potential for natural exchange of genetic material between the donor and recipient organism:
Agrobacterium is able of transferring DNA to higher organisms. Members of the genus invade the crown, roots and stems of a great variety of dicotyledonous, and some monocotyledonous plants. *Cichorium intybus* is a dicotyledonous plant and thus susceptible for infection by *Agrobacterium*.

6.2 DONORORGANISM #2:

Streptomyces hygroscopicus: bar.
The donororganism is a bacterium.
Complete name:
Genus: *Streptomyces*
Species: *Streptomyces hygroscopicus*
Strain: ATCC21705
Common name: not known

Pathogenic characteristics of the donor organism: Not applicable.

Potential for natural exchange of genetic material between the donor(s) and recipient organism: No natural exchange of genetic material.

6.3 **DONORORGANISM #3:**
Arabidopsis thaliana: PSsuAra plus transit peptide.
The donororganism is a plant.
Complete name:
Family name (for plants): Cruciferae
Genus: *Arabidopsis*
Species: *Arabidopsis thaliana*
Cultivar: Colombia
Common name: Thale cress

Pathogenic characteristics of the donor organism: No pathogenic characteristics.

Potential for natural exchange of genetic material between the donor(s) and recipient organism: No natural exchange of genetic material.

6.4 **DONORORGANISM #4:**
Bacillus amiloliquefaciens: barnase.
The donororganism is a bacterium.
Complete name:
Genus: *Bacillus*
Species: *Bacillus amiloliquefaciens*
Common name: not known

Pathogenic characteristics of the donor organism: No pathogenic characteristics.

Potential for natural exchange of genetic material between the donor(s) and recipient organism: No natural exchange of genetic material.

6.5 **DONORORGANISM #5:**
Nicotiana tabacum: PTA29.
The donororganism is a plant.
Complete name:
Family name (for plants): Solanaceae
Genus: *Nicotiana*
Species: *Nicotiana tabacum*
Cultivar: Samsun NN
Common name: Tobacco

Pathogenic characteristics of the donor organism: No pathogenic characteristics.

Potential for natural exchange of genetic material between the donor(s) and recipient organism: No natural exchange of genetic material.

6.6 **DONORORGANISM #6:**
Escherichia coli: neo.
The donororganism is a bacterium.
Complete name:
Genus: *Escherichia*
Species: *Escherichia coli*
Strain: CSR603

Pathogenic characteristics of the donor organism: Strains of *E. coli* can cause human gut- and stomach infection. The donororganism can be pathogenic occasionally and is easy to control.
The neo-gene sequence, coding for the neomycin phosphotransferase II enzyme and leading to resistance to some aminoglycoside antibiotics, is not involved to the pathogenic properties of the bacterium.

Potential for natural exchange of genetic material between the donor(s) and recipient organism: No natural exchange of genetic material.

7. CHARACTERIZATION OF THE GENETICALLY MODIFIED PRODUCT

7.1 History:

In 1991 and 1992 some transgenic male sterile vegetable chicory lines have been tested at the experimental farm of Plant Genetic Systems, Belgium. Besides the confirmation of the presence of the male sterility trait by RNAse activity in the anthers (coded by the barnase gene), agronomical aspects associated to seed production have been evaluated. The new male sterile lines on wich the new product is build have been tested in the 1992-1996 seasons in the BEJO greenhouses in the Netherlands. The male sterile phenotype has been confirmed and no irregularities in morfology (color and shape of the leaves) or inflorescence (color and shape of the flowers) have been discovered. Additionally the transgenic male sterile lines and the hybrids created with those lines have been evaluated in several fieldtrials from 1993 till 1996 in The Netherlands, Belgium, France, Italy, United Kingdom and California (see page 8 also). Flies and bees were used throughout cross-pollination experiments with trangenic plants. No deviation in behaviour has been observed - in respect of the pollination activity on non-transgenic control lines. These observations are always done during the conventional breeding program too. The results on insect behaviour are presented in **annex 7**.
Experimental hybrid seed production has successfully been accomplished in Italy in 1994, 1995 and 1996. All selected plants arising from the sterile parents showed the male sterile characteristic. The seed production was comparable with the non-transgenic control lines and the seed quality was good. No significant deviations in insect visits on the sterile lines in comparison with the non-transgenic pollinator lines could be detected. The germination and vigour of the seed were comparable with the non-transgenic controls.
Furthermore fieldtrials have been carried out in Italy, France, Belgium, United Kingdom, The Netherlands and California for the purpose of an evaluation of the specific aptitude of the hybrids for cultivation in the particular countries.
As a result some hybrids have been presented for registration to the "General Netherlands Inspection Service for Flower and Vegetable Seeds (NAKG)" in Roelofarendsveen. For this purpose the NAKG effected fieldtrials in 1994, 1995 and 1996. **Annex 7** provides a schematic overview of the development of the product up to the season 1996. Now the project has reached the stage in which, technically spoken, a commercial introduction of the product is possible.

7.2 New characteristics of the product and their activity:

The new traits, male sterility, kanamycin resistance and phosphinothricin tolerance, segregate in the plants of the GMO-product. One part, 50% of the plants, contains these traits; the other part, also 50%, does not. One characteristic, kanamycin resistance, will not be used anymore. The other characteristics, male sterility and phosphinothricin tolerance will only be used during the breeding and seed production phase.

Kanamycin resistance:
A variety of chimeric selectable marker genes that express bacterial coding sequences under the control of plant promoters have been developed (Walden et al., 1990). By far the most widely used selectable marker has been the neomycin phosphotransferase II enzym (NPTII), isolated from the transposon *Tn5* (Beck et al., 1982; Hayford et al., 1988). The *neo* gene under the control of a plant promoter has successfully allowed the selection of a large number of stable transformants, especially in dicotyledonous systems.
Introduction of the *neo* gene into the plant chromosome can permit plant cells to withstand kanamycin concentrations of up to a few mg/ml, depending on the expression level of the *neo* gene in plant cells (Hall et al., 1988; De Block et al., 1989). On the contrary, the addition of kanamycin, G418 or neomycin to non-transformed plant tissue in culture media typically results in bleaching and death of the cells (Hall et al., 1988).
The *neo*-gene coding for neomycin-phosphotransferase II (NPTII) is included in the construct as a selectable marker. This allows selection of cells carrying the inserted DNA segment in the *in vitro* phase.
The NPTII enzyme confers resistance to several components of a group or subclass of antibiotics, named the aminoglycosidic amino-cyclitols (Davies, 1980; Philips, 1982; Sande et al. 1985; Reynolds, 1989; Kors, 1990).
The aminoglycosidic amino-cyclitols are antibiotic compounds that are produced synthetically or by microorganisms (Davies, 1980). They are primarily used as therapeutic drugs in human and veterinary medicine to reduce the number of pathogenic bacteria in serious infections to a level that can be managed by natural humoral and cellular defenses of the body (Osweiler et al. 1985, Pollock, 1988, Reynolds, 1989).
Although resistance to the aminoglycosides can occur by several mechanisms, the most important cause of resistance to these drugs is the inactivation by enzymatic modification. The NPTII enzyme, encoded on transposable element *Tn*5 (*Escherichia coli*), is a phosphotransferase that catalyzes the phosphorylation of the 3' hydroxylgroep of the aminohexose of the target antibiotics (Davies et al., 1978).

More recently, a few compounds of the aminoglycosidic amino-cyclytols have been applied for *in vitro* use in plant tissue culture (Caplan et al., 1983). This is especially the case for the aminoglycosides which can be inactivated and metabolized by the NPTII enzyme. The aminoglycoside antibiotics kanamycin, neomycin, ribostamycin, butinosin, paramomycin and to a lesser extent gentamicin A and B were reported to be the substrates for the enzyme (Davies et al. 1977; Davies, 1980; Davies, 1986). Only kanamycin, neomycin and G418 (structurally related to gentamicin) have frequently been used for selection in plant tissue culture (Höfte et al., 1988, Potrykus et al., 1985; Herrera-Estrella et al., 1983; Colbère et al, 1981).

Phosphinothricin tolerance:
The fact that the bar gene forms an integral part of the hybrid system is fundamental to breeding and high quality seed production of Radicchio rosso. Since the *barnase* gene is physically linked to the *bar* gene, these genes will segregate as a single locus. Consequently, the male sterile line can be maintained by pollination crossing with wild type plants followed by the application of the herbicide (Mariani et al., 1990). The design of the insertion confines the use of this herbicide application strictly to breeding and selection purposes.
The introduced gene codes for a phoshinothricin acetyl transferase (PAT) which detoxifies phosphinothricin (glufosinate-ammonium) in 50% of all plants.
The relatively new class of herbicides with glufosinate-ammonium as the active ingredient acts by the inhibition of a specific amino acid biosynthesis pathway in plants (Wild et al., 1984; De Block et al., 1987; Wild et al., 1987). PPT inhibits glutamine synthetase. That causes rapid accumulation of ammonia which leads to death of the plant cell. PAT acetylates the free NH_2 group of PPT and thereby prevents autotoxicity in the producing organism. Transgenic plants expressing PAT are tolerant to field application of the commercial formulations of PPT (Basta, Finale).
The herbicides were discovered as antibiotics produced by *Streptomyces* species (Bayer et al., 1972; Leason et al., 1982; Sadaaki Mase, 1984; Murakami al., 1986). They are highly effective against plants, but are safe to humans and animals and are rapidly biodegraded in the environment (Hoechst info brochure, 1992).

Male sterility:
In flowering plants, the male gamete formation is a highly regulated developmental process that occurs in the anther. One of the tissues of the anther, the tapetum, plays a vital nutritive role during and after microsporogenesis. Defects in the tapetal function are considered as the primary causes of male sterility (Kaul, 1988). Preventing

viable pollen formation by disrupting the normal anther development therefore per definition results in male sterile plants.
The expression of the *barnase* gene, regulated by the tapetum specific TA29 promoter, starts at the vacuolated microspore stage of the pollen formation, which results in the degradation of RNA in the tapetum. This RNA degradation is almost immediately followed by a complete loss of RNA in all the developing microspores, after which they die. After the lysis of the microspores in the sterile anthers, the *barnase* gene is expressed in the vascular tissue of the anther filament.
This expression results in a deposition of wound callose in the phloem, so that nutrients no longer can reach the anther tissues, causing a precocious wilting of the anther. The degeneration of the endothecium connectivum and vascular tissue of the male sterile anther can then be observed.
The *barnase*-gene codes for a ribonuclease. Ribonucleases are capable of cleaving RNA into shorter oligonucleotides or degrade it completely into its constituent ribonucleotide subunits by catalyzing the hydrolysis of phosphodiester bonds in RNA chains.
The promoter PTA29 limits the activity of the *barnase*-gene in place (tapetum cells of the pollen sac) as well as in time (only when flowering, during anther development).
Barnase is a small single-chain protein originating from the bacterium *Bacillus amyloliquefaciens*. Barnase is the common name of the extracellular ribonuclease enzyme secreted by *Bacillus amyloliquefaciens*.
Because of its relatively small and simple structure, it has been the subject of intensive study for several years (Smeaton et al., 1967; Mauguen et al., 1982). The major interest in this protein, among others, has been a model for protein folding (Hartley et al., 1986). This gene, under the control of a specific plant promoter that exclusively expresses it in the tapetal cell layer during anther development, has been used to develop the described hybrid system (Mariani et al., 1990).

7.3 Expression of the new characteristics:

Expression of the *neo*-gene:
The *neo*-gene is under control of the nopaline synthase promoter (pNos). Literature data illustrates that this promoter reveals a weak constitutive expression of the gene. Constitutive promoters are useful in those cases where expression of a trait throughout the whole plant is desirable (Harpster et al., 1988 ; Kuhlmeier et al., 1987).
The PNos promoter has been used extensively for the expression of genes in plants, such as the *neo* gene (Herrera-Estrella et al., 1983; Fraley et al., 1983; Sanders et al., 1987; Ebert et al., 1987; De Block et al.,

1989; An et al., 1990). The activity of the PNos promoter has been observed in leaves, stems, cotyledons, various reproductive organs and calli. However, its activity seemed to be organ specific and developmentally regulated in different plant organs of tobacco (An et al. 1988; Mitra et al., 1989; An et al. 1990).

Expression of the *bar*-gene:
The gene is under control of the promoter originating from one of ribulose-1,5-biphosphate carboxylase small subunit genes, isolated from the total genomic DNA or *Arabidopsis thaliana*. The expression pattern of the small subunit genes is predominantly restricted to green tissues.
The expression of the PSsuAra promoter fused to the bar gene has been fully examined in plant tissues. Both the RNA and protein levels revealed that this promoter is capable of directing higher levels of expression than the PNos promoter. The activity of the PSsuAra promoter is most abundant in green tissues (leaves, stems and sepals), though it is also active to a lesser but still significant extent in petals, anthers and stigmas. In roots a wide range of expression levels has been observed (De Almeida et al., 1989).
The activity of the PSsuAra promoter is most abundant in green tissues (leaves, stems and sepals), though it is also active to a lesser but still significant extent in petals, anthers and stigmas. In roots a wide range of expression levels has been observed (De Almeida et al., 1989).
A transit peptide (tp) from the *Arabidopsis thaliana* Rubisco small subunit gene 1A is fused in frame with the bar coding sequence. It has been demonstrated that gene products encoded by chimeric gene constructs can be targeted to chloroplasts when the coding sequence of the gene of interest is fused to a transit peptide sequence from a plant gene (Vandenbroeck et al., 1985).

Expression of the *barnase*-gene:
The use of the promoter from the TA29 gene allows one to restrict the expression of the *barnase*-gene in place (tapetum cells of the pollen sac) as well as in time (only when flowering, during anther development). It does not seem to be expressed detectably at any other time of the sporophytic life cycle. At the detection limit of RNA gel blots, no TA29 mRNA was found to be present in heterologous vegetative and floral organ systems (Koltunow et al., 1990 ; Goldberg et al., 1993).

7.4 Rate and level of expression:

In order to study the rate and level of expression, transcripts were quantified and enzymatic activity was analized in different organs and different stages of plant development. Complete data are provided in annex 5,

chapter 2.4, 2.5 and 2.6.
In seedlings (leaves) transcription levels of the bar gene varied between 12.5 and 75 pg/ug total RNA. Protein levels varied between 5 ug PAT/mg protein with exeptions of 0.8 and 14.7 ug PAT/mg protein.
In the heads the contents of mRNA is about 5 times lower. PAT activity in the heads of Radicchio rosso is substantial lower than in the green leaves of seedlings. The PAT content in the heads vary from 0.2 to 1.6 ug PAT/mg protein (see table 2.6.4), which means that PAT-activity in mature heads is strongly reduced. Although the bar gene is under the control of the PSsu-promotor, which normally shows only activity in green tissues, low activity of m-RNA was detected in some roots.
Neo mRNA levels vary between 0.1 and 0.2 pg/ug RNA in seedlings, which can be translated in protein contents between 0.02 and 0.47 ug NPTII/mg eiwit. In heads transcription level is about 0.4 pg/ug RNA, which means 0.12 to 0.29 ug NPTII/mg protein.
In some root samples 1 pg/ug RNA was detected. In other samples no mRNA was detected. No enzymatic assays have been performed on root samples.
Barnase messengers were not detected. This is according to what could be expected, because the barnase gene is under the control of a tapetum-specific promotor.

7.5 Genetic stability of the insert in the product:

Stable incorporation of the genes into the plant genome has been confirmed by the demonstration of standard Mendelian genetics for the inheritance of these traits in subsequent progenies. The Radicchio rosso lines at issue have been maintained for the male sterility trait and were used in a backcrossing program and for hybrid production, throughout five generations now. Since the number of inserted copies has been determined for each line, it is possible to predict and check the segregation patterns. In each generation the anticipated segregation frequences have been confirmed. Experimental data - resulting from a trial evaluating phosphinothricin tolerance of seedlings - are available in annex 6. Maintaining of, and crossings with RM3-3 and RM3-4 showed a 1:1 tolerance / susceptibility ratio at every turn. Through the maintaining of line RM3-6 a peculiar segregation ratio was found in one single case. It should be mentionned that the original transformant RM3-6 contained two copies of the gene construct. After crossing and maintaining it appeared that just one copy was present in the next generations. Accordingly the RM3-6 line used for the ultimate seed production contains one single copy of the insert.

Segregation was checked both at the phenotypic level as well as at the genetic level. For each line the integration pattern of the T-DNA in the plantgenome was

investigated. These analyses were carried out on the primary transformant (T_0) as well as on the seed propagated progeny in the same genetic background (T_x) and in a new genetic background (BC_x). From each generation different individual plants were analyzed. In the lines RM3-3 and RM3-4 only one T-DNA copy was detected. Identical integration patterns were observed in individual plants that were tested. This means that the gene construct is integrated stable in the plantgenome.
Southern blot analysis of the primarily transformant RM3-6 has proved that two copies (M1 and M2) of the T-DNA are integrated in the plantgenome. These copies are not linked to each other and segregate independently. Only one of these copies contain a functional bar gene and has been used for further development in a conventional breeding program. The southern blot analysis of the T_x and BC_x generation of RM3-6, as well as the segregation data, provided in annex 6, show that this T-DNA copy has a stable integration in the plantgenome.
The results of the molecular analyses considering the stability of the integration of the new DNA is given in annex 5, chapter 2.1.

8. INTERACTIONS OF THE GMO-PRODUCT WITH THE ENVIRONMENT

8.1 Survival, multiplication and dissemination of the GMO-product in the environment:

From greenhouse trials in the 1992 till 1996 season and field trials from 1993 till 1996 in the Netherlands, Belgium, France, Italy, United Kingdom and California it appeared that the concerned Radicchio rosso lines and hybrids differ from the source organism only by the presence of the male sterility trait, the tolerance to phosphinothricin and the resistance to kanamycin. These new traits have no influence on the survival of the GMO. It is possible that cross-pollinations occur with other, non modified vegetable chicory plants grown in the proximity or with wild relatives which possibly emerge in the surroundings of the cultivation areas. However, no dispersal can be expected by the male sterile plants - of which the uncapability of producing pollen already has been explained. Accordingly, transfer of the genetic material of the GMO towards wild relatives is unlikely to happen, the opposite is possible. However, the probability for such outcrossings to occur is considered rather small, since for hybrid seed production those sites are selected where emergence of wild relatives is as little as possible, which is indispensable due to the high demands upon the purity of the seed. Throughout the practical cultivation of Radicchio rosso, the crop will be in the vegetative stage and not attain the flowering stage, unless inappropriate flowering occurs due to climatical circumstances. **Annex 8** gives a description of the pathway of the product after release into the environment.

8.2 Environmenal impact of the GMO-product:

As already mentioned, the GMO is characterized by its male sterility trait, linked to resistance to kanamycin and tolerance to phosphinothricin. As a consequence these plants can only be used as female parents in cross pollinations, since they cannot produce pollen themselves. For the production of hybrid seed on the other hand, the very goal is to attain a 100 % flowering rate. All the plants originating form the GMO line are male sterile after glufosinate ammonium treatment and will be pollinated by a non transgenic male fertile line. For the professional cultivation as well as for seed production outcrossing with wild relatives only can take place by migration of pollen from these wild relatives in the concerned field. Seed production takes place on carefully selected locations which have to be liberated from wild populations, in order to yield pure hybrid seed, free of so-called bastards. In Europe hybrid seed production involving the GMO will be limited to a maximum of a few tens of hectares per year, namely in Italy and France.

In the common cultivation as well as through hybrid seed production the remaining plant parts will be destroyed and ploughed in. Since Radicchio rosso plants and roots are sensitive to frost and susceptible to soil microorganisms, possible remaining plant parts will die off. In conclusion to what is described above, no excessive plant population growth is to be expected.
As already mentioned the concerned GMOs have attentively been compared with the original non transgenic lines in diverse greenhouse and field trials in 1992 till 1996.
A major advantage hereby was that the original line already existed a few years and already had been used in other conventional hybrid varieties. Therefore the company plantbreeders were capable of making a quick conclusion that the GMOs do not differ from the source organism, except for the new introduced characteristics.
Our general conclusion is that the GMO - throughout its release into the environment - will behave normally and as predicted, as it has been the case for the non transgenic source organism.

9. HEALTH CONSIDERATIONS

9.1 Toxic or allergenic effect of the product:

Experience of this GMO has been built up since 1992. Several employees have been involved in the sowing, planting, maintenance of the crop and harvest of the seed in the greenhouses in the Netherlands (1992 till 1996) and in the field in the Netherlands, Belgium, France, the United Kingdom and Italy (1993 till 1996), and California since end of 1995. The employees have been in contact with the crop for the purpose of normal cultural operations. Hereby no peculiarities have been observed.
Throughout pollination activities in the greenhouse in the Netherlands and in the field in Italy special attention was paid to the flying behaviour of the flies and the bees. This is also one of the normal observations during the classical breeding process. In hybrid seed production of cross-pollinating crops, insects are not supposed to show a preference towards one of the parental lines. If the flowers of the sterile plants are visited less or not at all by insects, this could be detrimental for the seed yield. In all cases no alteration in the behaviour of the insects or in their preferences towards flower visits could be observed. See also 7.1 and annex 7.
In conclusion we can state that neither through normal cultural operations with seed and plants executed by employees in the greenhouse (in the Netherlands) as well as in the field (in several EU member states and California, under different climatic conditions), nor in insect behaviour, any aberrant events could be observed associated with the use of the GMO.
We do not have any indication that the NPTII, PAT and barnase proteins give rise to any allergenic effect: A computerscreening was carried out on the homology of the three polypeptides (NPTII, PAT and barnase) coded by the transgene DNA of the genetic modified Radicchio rosso. The homology of the PAT, NPTII and barnase polypeptides with other polypeptides in HIVAA7, PIR42 and Swiss-Prot30 databases was very low and spreaded over the polypetide-chains.
In order to get information on the influence of the transgenic traits of Radicchio rosso on the simple chemical components in the mature stage of the crop, heads of the transgenic lines, RM3-3, RM3-4 and RM3-6, and heads of the non-transgenic contol line, R3, were harvested and freeze-dried. Sixteen parameters were analyzed by a toxicological laboratory (with GLP status). The results are presented in annex 9. Despite some small differences, the results show no important changes in these 16 parameters in the GMO-lines.

Gene products from the introduced genes

With the consumption of transgenic Radicchio rosso we have to take into account that the crop contains the *neo*-gene for kanamycin resistance and the *bar*-gene for phosphinothricin tolerance.
Besides, the crop contains the gene products neomycine phosphotransferase II (NPTII) and phosphinothricin-N-acetyltransferase (PAT).
For NPTII we refer to the conclusions (chapter VI) of the "kanamycin report", published by the Ministry of Housing, Spatial planning and the Environment in The Netherlands (SVS/GGO/1), which are presented in annex 16.
We conclude from the information that is available that kanamycin-resistant transgenic plants are safe for human consumption.

The *bar*-gene

The *bar*-gene consists, like all other genes, of normal DNA-structures, which are present in organisms. With the consumption of food, including vegetables, a large amount of DNA passes the digestive tract daily. This indicates that DNA in itself is not intrinsically toxic to human beings. Most DNA is efficiently degraded and no functional genes are assumed to remain present (Berkowitz, 1990).
In this respect, *bar*-DNA will not differ from any other DNA and normally be degraded, not posing any (additional) threats after consumption.

Phosphinothricine-N-acetyltransferase (PAT)

Biochemical studies of the PAT enzyme show a high substrate specificity and a strong affinity to phosphinothricin (Thompson et al., 1987).
No enzyme activity was detected with other acetyltransferase substrates, such as pyruvate, choline or serine (confidential data Ciba Seeds, 1994).
It can be concluded that the presence of PAT will not result in the establishment of pools of unfamiliar secondary metabolites from activity in other pathways. Also, the crop does not contain the specific substrate for PAT.
The optimum pH for the enzyme is 7.5 and rapid thermo inactivation is observed at temperatures exceeding 35°C (Botterman et al., 1991; Walter et al., 1992). The gastric fluid has a pH of 2 to 4, at which PAT loses all enzymatic activity within one minute of exposure. In addition, the required co-factor (acetyl-CoA) is not stable in such acidic conditions (Privalle, 1994).

Toxic or allergenic effects of the PAT-protein

The PAT-protein did not show homology with know toxic

peptides.
In addition it is rapidly degraded in the gastro-intestinal tract. In simulated gastric fluid experiments, the enzyme was degraded rapidly and irreversibly. The enzyme could not be detected upon immediate sampling of the reaction mix using standard simulated gastric fluid (Privalle, 1994). A 1000-fold dilution of the standard amount of pepsin was required to establish a halflife of the PAT protein of 1-2 minutes (Privalle, 1994).
It can be concluded, that the PAT-protein is readily digested and, therefore, allergenic effects are not-expected.
The fast *in vitro* digestibility (Privalle, 1994) indicates the absence of potential allergenicity of the protein or its degradation products, as allergenic food proteins are typically resistant to proteolytic degradation (Taylor et al., 1992).
The PAT-protein concentration in the heads of the transgenic Radicchio rosso plants is very low and varies between 0.02 and 0.16% of the total soluble protein (see annex 5, chapter 2.6). The protein has no glycosylation sites.
We have already described in this chapter that employees, who worked with the transgenic seeds and plants and members of taste-panel (see 9.3 and annex 10) did not show any allergenic effects in the past few years.
Additionally we provide a report recently published by the RIKILT-DLO Institute in Wageningen, The Netherlands in annex 15. This report describes a study on "*Safety assessment of the bacterium-derived recombinant phosphinothricin acetyltransferase (PAT) protein*".

9.2 Pathogenicity:

Neither the source organism nor the GMO are known to express pathogenic characteristics. The vector does not contain sequences which show pathogenicity. The pathogenicity of *Agrobacterium tumefaciens* is not encoded on the DNA of the donor organism (nos promoter and nos terminater) which is present in the insertion. Between the T-DNA borders no sequences for pathogenic characteristics are present.

9.3 Human health effects of the product:

Regarding possible effects of the GMO on human beings and the environment, the fact that none of the new introduced genes are known to be detrimental, should be taken in consideration. Taking into account the lack of potential health risks for human beings and animals, we also refer here to the "kanamycin-report" (report number SVS/GGO/1) published by order of the Ministry of housing, Spatial Planning and the Environment in The Netherlands, of which the conclusions are presented in annex 16. The farmer or

grower cannot use a herbicide on the basis of glufosinate ammonium, to control the weeds, because of the susceptibility of 50% of the crop to this ingredient. The product used for human consumption never has any residue of herbicides. In a special laboratory study it has been proved that the product is free from the *Agrobacterium tumefaciens* strain that had been used for transformation (**Annex 5, chapter 2.7**).
The crop, which will be grown by growers and farmers, has 50% plants that are tolerant to phosphinothricin. This tolerance is based on phosphinothricin acetyl transferas (PAT) activity, caused by the *bar* gene. This gene has been isolated from the bacterium *Streptomyces hygroscopicus*.
The genus *Streptomyces*, formerly *Actinomyces*, is, also because of its wide distribution in nature, one of the best documented genera, belonging to the family Streptomycetae. The genus was described first by Waksman et al. (Kutzner, 1981; Bradburry, 1986). The information on this organism has been described in several reviews, monographs and symposia (see Kutzner, 1981). The natural habitat of most members of the Streptomycetae is the soil, in which they find the optimal conditions for growth and reproduction. The microorganisms can be isolated from fresh water very easily, although *Streptomyces* spp. that are found here probably originate from soil and exist as a living, but inactive arthrospore (Kutzner, 1981). Some *Streptomyces* have been isolated from animal or human sources but are not pathogenic. Only a few *Streptomyces* are known as being a plantpathogen (Bradburry, 1986; Kutzner, 1981). Some *Streptomyces* produce very active antibiotics, which eliminate prokaryote cellfunctions or enzymes. These *Streptomyces* cannot survive without a self-defence mechanism. This mechanism is based on an enzyme that inhibits the antibiotic-effect, to avoid autotoxicity (De Block et al, 1987).
Streptomyces hygroscopicus (ATCC 27438) was first described by Waksman and Curtis in 1916. The spore-surface of this microorganism is undulated, the spore-mass is grey and becomes blackened in the mature stage. Diffusion- and melanine pigments are not produced (Bergey's, 1989). There is no growth under 10°C or in presence of fenol (0.1% w/v).
As far as is known phosphinothricin acetyl transferase (PAT) does not occur normally in plants while different types of other acetyl transferases are common in plants.
At the end of 1994, to a protocol agreed with the Institute of Food Research in Norwich, United Kingdom, and a British seedscompany Elsoms Seeds Ltd. in Spalding, United Kingdom, a taste test with GMO-Radicchio rosso and conventional varieties was carried out. The protocol of this experiment and the results are described in **annex 10**.
In our opinion the conclusion can be stated that the texture, taste and overall palatability of the genetic modified Radicchio rosso does not differ from the

conventional crop. Volunteers did not note any stomach or other discomfort during the days after the taste test. We foresee no influence on public health arising from the consumption of the genetic modified Radicchio rosso.

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ANNEXES

Annex I PLANT GENETIC SYSTEMS NEW HYBRIDIZATION SYSTEM BASED ON A DOMINANT NUCLEAR MALE STERILITY (NMS) GENE LINKED TO A CONVENIENT MARKER.

Development of the male sterile female parent (line A) :

Transformation of line A with the new DNA sequences including the *barnase* gene (MS) and the herbicide resistance marker gene *bar* (H), giving rise to the female parent phenotype :

$A^{msh/msh}$

Transformation ↓

$A^{MSH/msh}$

Maintenance of the male sterile female parent (line A) :

Maintaining of $A^{MSH/msh}$ with $A^{msh/msh}$ gives rise to :

$A^{MSH/msh}$

Crossing x

$A^{msh/msh}$

↓

50 % $A^{MSH/msh}$ and 50 % $A^{msh/msh}$

of which the latter phenotype can be eliminated by spraying glufosinate ammonium on the plantlets.

Production of the new hybrid :

Crossing of the female parental line $A^{MSH/msh}$ with male parental line $B^{msh/msh}$ gives rise to:

$A^{MSH/msh}$

Crossing x Seed Harvest on the female parent

$B^{msh/msh}$

↓

50 % $AB^{MSH/msh}$ and 50 % $AB^{msh/msh}$

Annex 2

DESCRIPTION OF CHARACTERISTICS

used by the "General Netherlands Inspection Service for Vegetable and Flower seeds (NAKG)" for the distinction of Radicchio rosso (red hearted chicory) varieties for registration purposes.

Description of characteristics of leafchicory varieties for registration purposes.

1. Group or type of chicory.

2. Description of the way how to use and cultivate the variety.

3. Characteristics of the variety:

   a. Anthocyan intensity in juvenile stage
   b. Leaf: color
      anthocyan intensity
      lumpy intensity
      shape

   c. Head: head production, yes or no
      shape

   d. Earlyness of the variety; time between sowing and harvesting date.

   e. Bolting tolerance

   f. Color of the flowers

4. Conformities and differences with other varieties of the same crop.

5. Extra comments.

COMMISSIE TOELATING
GROENTERASSEN
BESCHRIJVINGSBLAD

BLADCICHOREI
(Cichorium intybus L.
(partim))

Ras:

1. Groep of type
roodlof/groenlof/snijtype/stengeltype

2. De voor het ras geschikte teeltwijze(n)

3. Eigenschappen van het ras

Anthocyaan in de kiemplant:	afwezig/aanwezig
Blad: kleur:	licht/middel/donker/geel/groen/rood
anthocyaan:	diffuus/in vlekken/diffuus en in vlekken
bobbeling:	afwezig of zeer zwak/zwak/middel/sterk
vorm:	smal elliptisch/elliptisch/breed elliptisch/rond
Krop: vorming:	afwezig/aanwezig
vorm:	elliptisch/eivormig/rond/plattrond
Oogstrijpheid:	zeer vroeg/vroeg/middel/laat/zeer laat vroeger dan/gelijk met/later dan
Schiettijdstip:	zeer vroeg/vroeg/middel/laat/zeer laat vroeger dan/gelijk met/later dan
Bloemkleur:	wit/blauw/roze

4. Overeenkomst met andere rassen
Het ras vertoont overeenkomst met
maar verschilt hiervan door

5. Verdere opmerkingen

Aldus zo volledig mogelijk en naar beste weten opgemaakt.

Te :

Op :

Handtekening

SEE FOR TRANSLATION PAGE 49

Annex 3 **BREEDERS RIGHT DESCRIPTION OF THE RADICCHIO ROSSO VARIETY "SCARLATI".**

Breeders right description of the variety 'Scarlatl'.

Summary:

The description is based on phenotypical characteristics of the variety.

Institution: Centre for Plant Breeding and Reproduction Research (CPRO-DLO) in Wageningen.

Investigation period: 1991 and 1992.

Characteristics:

Plant:	Size
	Intensity of head formation
Leaf:	Arrangement of leaves
	Lenght
	Maximum width
	Shape
	Colour
	Colour of the main vein
	Anthocyan
	Distribution of anthocyan in the leaves
	Intensity of anthocyan
	Glossyness of leaves
	Profile outer leaves
	Lumpy intensity
	Description of leaf margin
Head:	Shape
	height
	Colour
	Earlyness of head formation

Conformities and differences with other varieties.

RAAD VOOR HET KWEKERSRECHT

BEWIJS VAN INSCHRIJVING

voor één ras van het gewas	roodlof
benaming	Scarlati 1383
volgnummer	12791

THIS "BREEDERS RIGHT" DESCRIPTION IS TRANSLATED AND SUMMARIZED ON PAGE 52

RAAD VOOR HET KWEKERSRECHT

De Raad voor het Kwekersrecht verklaart,

dat op aanvrage nummer RDL 4

ingediend op 1/02/91

het hierna beschreven ras van het gewas roodlof

onder de benaming Scarlati

onder volgnummer 12791

per datum 29/03/93

in het Nederlands Rassenregister is ingeschreven met aantekening van verlening van kwekersrecht ten name van:

Bejo Zaden B.V., Postbus 50, 1749 ZH Warmenhuizen

Wageningen, 30/03/93

DE SECRETARIS VAN DE
RAAD VOOR HET KWEKERSRECHT,

mr. P.H.M van Beukering.

Rasbeschrijving

1.	Aanvraagnummer	: RDL 4
2.	Aanvraagnummer verzoekende instantie	: -
3.	Voorlopige aanduiding	: Bejo 1383
4.	Aanvrager	: Bejo Zaden B.V., Warmenhuizen

5.	Botanische soortnaam	: Cichorium intybus L.
6.	Nederlandse gewasnaam	: Roodlof
7.	Rasnaam	: SCARLATI
8.	Nummer en datum van UPOV richtlijn	: -
9.	Nummer en/of datum van nationale richtlijn	: -
10.	Onderzoekende instantie	: CPRO-DLO, Wageningen
11.	Onderzoek station(s) en plaats(en)	: CPRO-DLO, Wageningen
12.	Periode van onderzoek	: 1991 en 1992
13.	Plaats en datum van rapportering	: Wageningen, 17 november 1992

14. Groep: (indien kenmerken genoemd onder 15 gebruikt voor groepering, zijn ze met een G aangeduid).

No.	Kenmerken	Klasse	Code	Opmerkingen
9.	Blad: anthocyaan	aanwezig	9	
18.	Krop: vorm	rond	2	

Aanvraagnummer rapporterende instantie: RDL 4

15. Kenmerken

No.	Kenmerken		Klasse	Code	Opmerkingen
1.	Plant:	omvang	midden	5	tot groot (6)
2.	Plant:	mate van kropvorming	sterk	7	
3.	Blad:	houding	halfopgericht tot horizontaal	6	
4.	Blad:	lengte	kort	3	
5.	Blad:	maximum breedte	midden tot breed	6	
6.	Blad:	vorm	rond	4	
7.	Blad:	kleur	donkergroen	4	
8.	Blad:	kleur middennerf	groenachtig	2	
9.G	Blad:	anthocyaan	aanwezig	9	
10.	Blad:	verdeling anthocyaan	over het blad verspreid	2	
11.	Blad:	anthocyaan vorm	diffuus	1	
12.	Blad:	glans	midden	5	
13.	Blad:	profiel buitenblad	vlak	5	
14.	Blad:	bobbeling	zwak	3	
15.	Bladrand:	golving	zwak	3	
16.	Bladrand:	insnijdingen	sterk	7	
17.	Bladrand:	diepte insnijdingen	zeer ondiep tot ondiep	2	
18.G	Krop:	vorm	rond	2	
19.	Krop:	lengte	kort	3	
20.	Krop:	kleur buiten-blad	rood	5	

Aanvraagnummer rapporterende instantie: RDL 4

16. Overeenkomstige rassen en verschillen met deze rassen
RDL 4 vertoont overeenkomst met Elios; maar verschilt daarvan door de iets grotere plant en de meer ingesneden bladrand.

17. Extra informatie

a) Extra gegevens: -

b) Opmerkingen : -

ANNEX 4: THE AGROBACTERIUM VECTOR SYSTEM

Part 1: *Agrobacterium* mediated transformation system

A. Disarmed *Agrobacterium tumefaciens* vectors for use in plant transformation

1. Introduction

Two properties of the T-DNAs of Ti-plasmids make them virtually ideal vectors for introducing foreign genes into plants : firstly, the host range of *Agrobacterium* is very broad which implies that these microorganisms are capable of transforming cells of virtually all dicotyledonous plants and some monocotyledonous plants; secondly, the integrated T-DNA is inherited in a Mendelian way.

Wild-type Ti-plasmids have nevertheless made their practical use as experimental gene vectors rather troublesome. One of the limitations of using the natural occurring Ti-plasmid for gene transfer in plants was its large size. The size of these big plasmids (more than 200 kilobases) was such that it was very difficult or rather impossible to find adequate and unique restriction sites within the T-region for the cloning of foreign DNA sequences. As a solution to solve this problem, intermediate vectors have been developed (Leemans et al., 1981; Leemans et al., 1982; Matzke et al., 1981; Garfinkel et al., 1981).

Another limitation that first hindered the use of the wild Ti-plasmid as a DNA vector, was due to the fact that the transformed plants received the T-DNA genes coding for the formation of tumorous plant cells, resulting in abnormal growth and differentiation properties. This tumor phenotype can be maintained in tissue culture as it results from the expression of genes on the T-DNA that alters the normal balance of the growth substances in the transformed plant cells. To use the *Agrobacterium* mediated gene transfer for obtaining transgenic plants from totipotent plant cells, it was essential that the transformed cells were normal rather than tumorous (Schell et al., 1983; Goodman et al., 1987).

The ability of the Ti-plasmid to cause crown gall disease could be abolished by deletion of oncogenes within the T-DNA without the loss of T-DNA transfer and integration capacity (Leemans et al., 1982). An *Agrobacterium* strain that does not cause crown gall diseases anymore, is designated as 'disarmed'. The construction of a disarmed Ti-plasmid requires a detailed knowledge of the plasmid (Schell et al., 1979; Leemans et al., 1983; Deblaere et al., 1985; Klee et al., 1987; Goodman et al., 1987).

Important from a practical point of view was the discovery that *Agrobacterium* can still transfer the T-DNA when the *vir*

region and the T-DNA are located *in trans* (Figure 1). An *Agrobacterium* cell harboring a Ti-plasmid containing the *vir* region and another plasmid containing the T-DNA, still can transform plant cells by incorporating the physically separated T-DNA into the plant genome (de Framond et al., 1983; Hoekema et al., 1983).

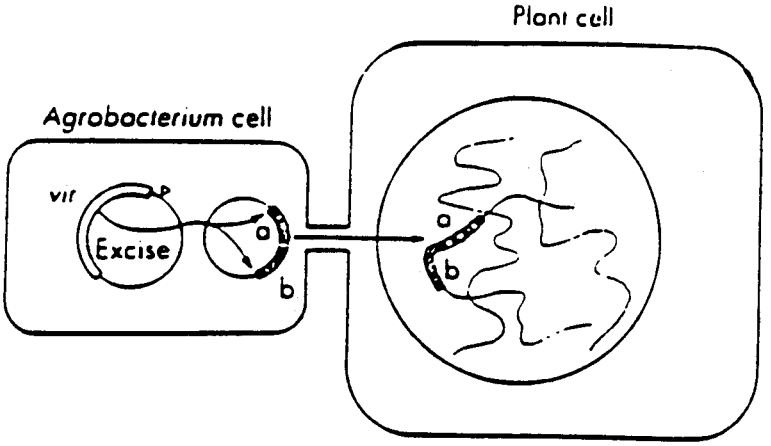


Figure 1. ***Agrobacterium*** **mediated gene transfer** ***in trans*** **(binary vector system)**

2. Vectors for *Agrobacterium* mediated plant transformation

A variety of plant transformation vectors which exploit the process of tumor formation, but have the sequence responsible for the establishment of the tumor phenotype deleted, have been developed. Each of these vectors capitalize on the experimental observations that the DNA transferred to the plant cell is defined by the 25bp T-DNA border repeats and that the transfer and integration into the plant genome does not require the presence of any of the genes encoded by the T-DNA. Plant transformation vectors based on *Agrobacterium* can generally be divided into two categories : those that cointegrate into a resident Ti-plasmid and those that replicate autonomously (Deblaere et al., 1987; Klee et al., 1987; Rogers et al., 1988; Walden et al., 1990).

Cointegrative vectors are derivatives of the Ti-plasmid from which the majority of the T-DNA between the border repeats has been replaced by a defined sequence of DNA (Zambryski et al., 1983; Fraley et al., 1985; Walden et al., 1990). To insert the defined sequence of DNA into a cointegrative vector, the DNA to be transferred is cloned into an intermediate vector. This intermediate vector contains selectable markers and a sequence homologous with the disarmed Ti-plasmid. Because *Escherichia coli* is generally used as a host cell in recombinant DNA techniques, construction of the intermediate vectors is

carried out in *E. coli*. Using specific helper plasmids the intermediate vector is subsequently transferred into *Agrobacterium* (Ditta et al, 1980).

Following introduction into the recipient *Agrobacterium* strain, the *E. coli* plasmids are lost because they are unable to replicate in this host. Although the intermediate vector does not contain an appropriate origin of replication , the plasmid can be maintained in *Agrobacterium* upon cointegration through homologous recombination with the acceptor Ti-plasmid. Both acceptor Ti-plasmid and the intermediate vector are designed in such a way that they share a substantial region of homology. Therefore, selection for an antibiotic resistance marker carried by the intermediate vector will enable the isolation of bacteria in which homologous recombination has resulted in the foreign DNA being integrated between the T-DNA border of the disarmed Ti-plasmid. Transconjugants harboring such a cointegrate Ti-plasmid are then further used in plant transformation experiments (Zambryski et al., 1983; Klee et al, 1987; Walden et al, 1990).

Upon cointegration as described above, the resulting T-DNA is rather large and contains moreover a duplication of part of or the complete plasmid part of the intermediate vector (see Zambryski et al, 1983). To overcome these shortcomings, an alternative Ti-cointegration system was developed (Deblaere et al, 1985).

This set up also consists of two plasmids. A first plasmid constitutes a disarmed Ti-plasmid in which the complete T-DNA, including the border sequences is deleted and replaced by a region homologous to the intermediate vector (e.g. plasmid pBR322-derived sequences). A second plasmid, forming the intermediate vector on the basis of the *E. coli* plasmid pBR322, contains a disarmed T-DNA. In essence it only contains the T-DNA border sequences, between which unique cloning sites are available for the introduction of foreign DNA. After transfer to the appropriate *Agrobacterium* strain the intermediate vector will cointegrate into the acceptor Ti-plasmid, giving rise to a simple T-DNA configuration without duplications between the border sequences.

A further improvement consisted in the introduction of a binary vector system. This strategy was designed based on the observation that the T-DNA could still be transferred to the plant cell when physically separeted from its Ti-plasmid (de Framond et al, 1983). Therefore it was sufficient to provide the intermediate vector, carrying a disarmed T-DNA, with an origin of replication that functions in *Agrobacterium*. This approach ommits the need for cointegration in a specific acceptor Ti-plasmid. Several binary vector systems have been described (Bevan, 1984; Klee et al, 1985; Simoens et al, 1986; Deblaere et al, 1987).

B. Construction of the vectors used to transform Radicchio rosso (red hearted chicory).

1. Introduction

The general process for manipulating genes to be transferred into the genome of plant cells is carried out in two phases. First, all the cloning and DNA modification steps are done in *Escherichia coli*, and then the plasmid containing the gene construct of interest is transferred by conjugation to *Agrobacterium tumefaciens*. The resulting *Agrobacterium* strain is finally used to transform plant cells (Herrera-Estrella et al., 1988).

A disarmed plasmid was constructed to transfer the DNA sequences of interest to Radicchio rosso. The plasmid, named pTTM8RE, was constructed to engineer male sterility. The plasmid is a cointegrate type Ti vector. In part 2 an overview of the origin of the sequences is given. It has to be kept in mind that the applied transformation method guarantees that these donor organisms were not involved in the modification process.
Via *Agrobacterium tumefaciens* mediated transformation, the *barnase* gene under the control of a plant promoter, and two marker DNA sequences under the control of two other different promoters were introduced into *Radicchio rosso*. The marker genes are also located between the border sequences so that they are transferred together into the nuclear genome of the plant cell. This resulted in male sterile Radicchio rosso plants.

2. Components of the *Agrobacterium* vector system

For plant transformation, the vector system as described by Deblaere et al. (1985) has been used. This vector system consists of two plasmid components : a non-oncogenic Ti-plasmid and an intermediate vector.

The acceptor Ti-plasmid from which the T-region has been deleted is non-oncogenic. This plasmid still carries the *vir* genes required for transfer of T-DNA to the plant genome and is used as an acceptor plasmid for an intermediate vector, carrying the genes of interest between the 25bp border sequences of the octopine T-region. The respective plasmid components are the non-oncogenic Ti-plasmid pGV2260 and the intermediate vector pGV825. Plasmid pGV2260 was derived from the octopine Ti-plasmid pTiB6S3 from which the T-region was deleted and substituted by sequences from the plasmid pBR322 (Deblaere et al., 1985).

The *Agrobacterium tumefaciens* host strain is a rifampicin (Rif) resistant derivative of C58, cured for pTiC58 ($C58C1Rif^R$) (Van Larebeke et al., 1974). The plasmid pGV825 is

derived from pBR322 and is composed of the origin of replication of pBR322 and genes conferring resistance to ampicillin (Ap) and streptomycin/spectinomycin (Sm/Sp) for selection in bacteria (Deblaere et al., 1985). The T-region of pGV825 consists of the DNA regions surrounding the left and right border sequences of the TL-DNA from pTiB6S3 (Deblaere et al., 1985).
Between the T-DNA border repeats, there are still residual sequences left from the TL-DNA: 265 bp at the right border and 689 bp at the left border. The region at the right border is part of the octopine synthase gene which is localized immediately at the right border sequence of the TL-DNA (De Greve et al., 1982). This sequence comprises the octopine synthase upstream activator sequence, but does not contain the TATA box (Leisner et al., 1988; Leisner et al., 1989; Bouchez et al., 1989).

The sequence at the left border is part of gene 5 localized immediately at the left border repeat of the TL-DNA (Gielen et al., 1984). A major part of the gene 5 promoter including the putative regulatory boxes such as CCAAT and TATA box are still present. Expression pattern analysis of chimeric gene constructs with this promoter are described by Koncz et al. (1986). Genes under the control of this promoter were expressed in callus tissues and in stems of transformed plants and at barely detectable levels in fully developed leaves. Promoter analysis of gene 5 in plants revealed that its expression was inducible by auxin and confined to the vascular phloem cells. Cis-regulatory elements required for auxin regulation and phloem specific expression of gene 5 were mapped to a 90 bp promoter region (Körber et al., 1991).

Vectors derived from pGV825 contain multilinker cloning sites allowing the insertion of chimeric genes between the T-DNA border repeats (Deblaere et al., 1987). These intermediate plasmid vectors carrying chimeric genes of interest, can be introduced into the acceptor Ti-plasmid pGV2260 by a single homologous recombination, using the Sm/Sp resistance gene as selectable marker for cointegration. The mobilization is based on a triparental mating between an *Escherichia coli* strain carrying the intermediate vector derived from pGV825, the *Agrobacterium* strain C58C1Rif^R (pGV2260) and an *E. coli* strain carrying a mobilization helper plasmid (Van Haute et al., 1983; Deblaere et al., 1987). The resulting *Agrobacterium* strain contains a pGV2260::pGV825 cointegrate plasmid. The structure of the resulting T-region is confirmed by Southern blot hybridization (Deblaere et al., 1985).

3. Description of the plasmid pTTM8RE

The plasmids pTTM8RE comprises three chimeric genes between the T-DNA border repeats. The chimeric genes consist of a promoter sequence for initiation of transcription, the coding sequence of the gene of interest and a fragment containing a

3' untranslated region from a T-DNA gene providing the signals for transcription - termination and polyadenylation. A schematical representation of the intermediate vector pTTM8RE is given in annex 4, part 3. The nucleotide sequence of the DNA fragment comprised between the T-DNA border repeats is completely known. The plasmid carries chimeric genes conferring resistance to the antibiotic kanamycin and to the herbicide glufosinate-ammonium, respectively. These chimeric genes are denoted as PNos-*neo*-3'ocs and PSsuAra-tp-*bar*-3'g7. Additionally, pTTM8RE carries a chimeric *barnase* gene construct denoted as PTA29-*barnase*-3'nos. The following description gives an overview of the design of the different gene constructs in order to allow to identify all nucleotide sequences present in the transferred T-DNAs of the plasmid vectors pTTM8RE.

The chimeric *bar* gene consists of the promoter from the atS1A ribulose-1,5-biphosphate carboxylase small subunit gene (*ssu*) from *Arabidopsis thaliana*, the *bar* coding sequence and the 3' untranslated fragment of the TL-DNA gene 7. The PSsuAra promoter fragment comprises the 1.7 kb fragment upstream of the atS1A ATG codon and the transit peptide encoding sequence (Krebbers et al., 1988). The *bar* gene contains the complete coding sequence of the *bar* gene as described by Thompson et al. (1987). The 3'g7 fragment is derived from the TL-DNA gene 7 (Velten et al., 1985; Dhaese et al., 1983). The construction of the chimeric PSsuAra-tp-*bar*-3'g7 gene has been described by De Almeida et al. (1989) and can be summarized as follows. The plasmid pATS3 (Krebbers et al., 1988) contains a 1.7 kb EcoRI-SphI fragment which includes the promoter region and the transit peptide encoding sequence of the atS1A *ssu* gene. The plasmid pGSFR2 is derived from pGSFR1 (De Block et al., 1987) and carries the *bar* coding sequence in which an NcoI site has been created at the initiation codon. Accordingly, the second codon of the *bar* gene - AGC (Ser) - has been modified to a GAC (Asp) codon (Botterman et al., 1991).

The tp-*bar* fusion was obtained by ligating the filled-in NcoI ends to the SphI ends treated with Klenow DNA polymerase. This yielded a fusion of the *bar* coding sequence at the transit peptide encoding sequence with the same Cys-Met transit peptide cleavage site as present in the wild-type atS1A *ssu* gene. The PSsuAra-tp-*bar* gene was cloned in the polylinker region of pLK56-2 (Botterman et al., 1987) and could be retrieved as a BamHI fragment. In order to have the chimeric gene completed with a 3' untranslated end providing signals for transcription termination and polyadenylation, the BamHI PSsuAra-tp-*bar* fragment was cloned in the BamHI site localized immediately in front of a 3' untranslated end of the TL-DNA gene 7. This yielded the chimeric PSsuAra-tp-*bar*-3'g7 gene construct.

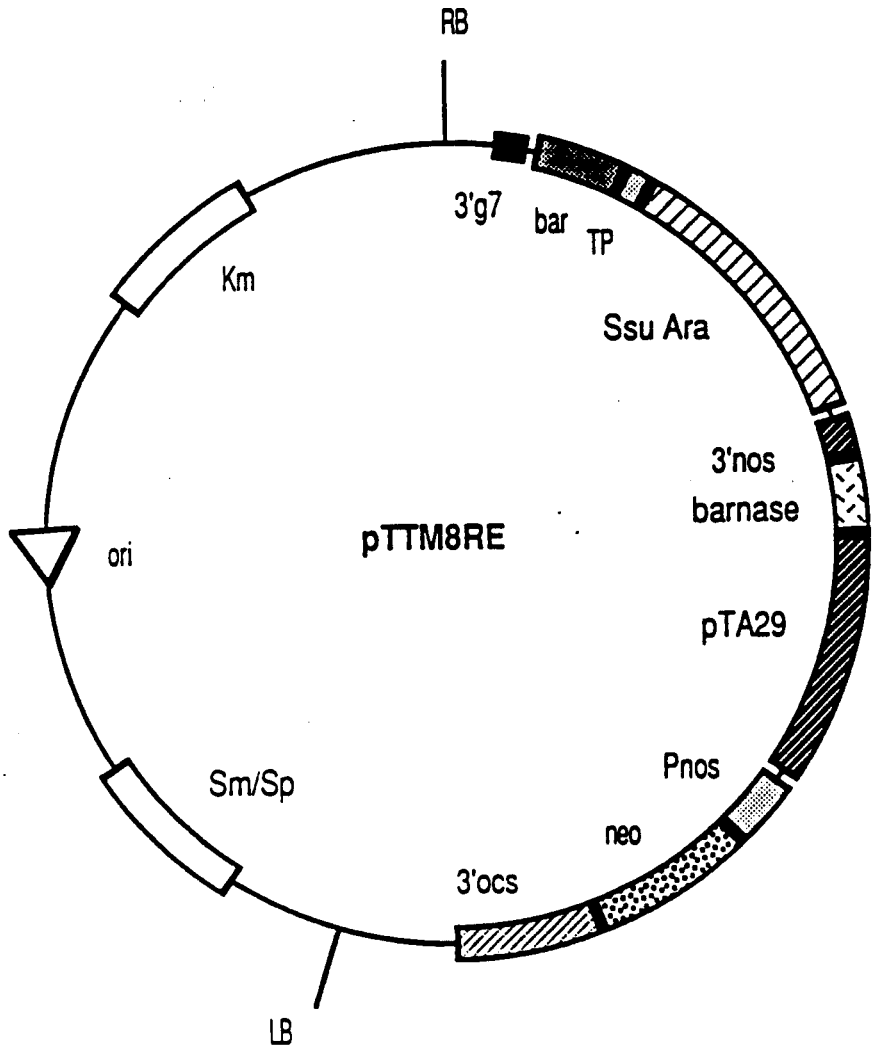
The chimeric *neo* gene consists of the promoter from the T-DNA nopaline synthase gene (PNos), the coding region of the *neo*

gene encoding neomycin phosphotransferase II from *Tn*5 and the 3' untranslated end from the octopine synthase gene (3'ocs). The nucleotide sequence of the PNos promoter is described by Depicker et al. (1982). The *Tn*5 sequence comprises the *neo* coding sequences and a part of the *Tn*5 sequence downstream from the *neo* coding region. This sequence corresponds with the sequence as described by Beck et al. (1982). The ATG initiation codon of the *neo* coding region has been substituted for a linker sequence as described by Reiss et al. (1984). The 3' end from the octopine synthase gene corresponds to the 706 bp PvuII fragment from the octopine synthase gene which has been cloned into sequences originating from *Tn*5 downstream from the *neo* coding region. The nucleotide sequence of the PvuII fragment can be found in Gielen et al. (1984). The construction of the chimeric PNos-*neo*-3'ocs gene has been described by Hain et al. (1985).
The plasmid pTTM8RE also contains a chimeric *barnase* construct. The latter consists of a promoter fragment from the tobacco anther-specific gene TA 29 (PTA29), the coding region of the *barnase* gene (*barnase*) and the 3' untranslated end of the nopaline synthase gene (3'nos). The cloning and characterization of the TA29 genomic clone from tobacco has been described by Koltunow et al. (1990) and Seurinck et al. (1990) and is also in detail described within the European Patent Application 89401194.9. A 2.5 kb ClaI-AccI fragment carrying the promoter and part of the TA29 coding region was cloned in the polylinker AccI site of pMAC 5-8 (Stanssens et al., 1989). The sequence surrounding the ATG initiation codon - AAAATGGTA - was modified to ACCATGGTA by substituting two A residues for C residues by site directed mutagenesis according to Stanssens et al. (1989). The resulting plasmid was named pMB3 and the 1507 bp ClaI-NcoI fragment was denoted as the PTA29 promoter fragment. Subsequently, the PTA29 coding region in pMB3 was deleted and substituted for a 900 bp Asp718-HindIII fragment. The latter comprises the *barnase* coding region as described in Hartley (1988), with an Asp718 site engineered at the start of the coding sequence of the mature *barnase*. The fragment was cloned between NcoI and HindIII sites of PMB3. Before cloning, Asp718 and NcoI sites were rendered blunt end by treatment with Klenow DNA polymerase. This yielded the plasmid pTM8 with a PTA29-*barnase* gene fusion carrying the *barnase* coding region fused at the ATG initiation codon of the TA29 gene. Subsequently, the EcoRI-XbaI fragment from pTM8 was isolated and ligated to a XbaI-EcoRI fragment from PNos2. The latter plasmid carries a restriction fragment with the 3'untranslated end of nopaline synthase gene (Depicker et al., 1982) cloned in the polylinker of pUC18 (Yanish-Perron, 1985). Both fragments simultaneously cloned within an EcoRI site of a plasmid vector allows to retrieve the PTA29-*barnase*-3'nos fragment as an EcoRI fragment.

Part 2 Nucleotide sequence of the DNA comprised between the T-DNA border repeats of PTTM8RE and origin of the different sequences

Nucleotide sequence		Origin of the sequence
# 1-25	:	Right border repeat from the TL-DNA from pTiB6S3.
# 26-283	:	Residual sequences from the TL-DNA at the right border repeat. The junction is at the HpaI restriction site in the TL-DNA
# 284-290	:	Synthetic polylinker derived sequences
# 502-291	:	A 212 EcoRV-ClaI fragment containing plant polyadenylation signals and obtained from the 3'untranslated end from the TL-DNA gene 7 (3'g7) of pTiB6S3 (Velten and Schell., 1985; Dhaese et al., 1983).
# 503-523	:	Synthetic polylinker derived sequences
# 1075-524	:	The coding sequence of the bialaphos resistance gene (*bar*) of *Streptomyces hygroscopicus* (Thompson et al., 1987).
# 2966-1076	:	The promoter from the atS1A ribulose-1,5-biphosphate carboxylase small subunit gene from *Arabidopsis thaliana* (PSsuAra). The promoter fragment comprises the 1.7 kb fragment upstream from the atS1A ATG codon (Krebbers et al., 1988) and the transit peptide (tp) sequence (1240-1076) for targeting to the chloroplast.
# 2967-2995	:	Synthetic polylinker derived sequences.
# 3256-2996	:	A 260 bp TaqI fragment from the 3' untranslated end of the nopaline synthase gene (3'nos) from the T-DNA of pTiT37 and containing plant polyadenylation signals (Depicker et al., 1982).
# 3257-3272	:	Synthetic polylinker derived sequences.
# 3273-3367	:	Sequences from the 3'untranslated end of the *barnase* gene from *Bacillus amyloliquefaciens*
# 3704-3368	:	Region encoding mature *barnase* from *Bacillus amyloliquefaciens*.
# 5214-3705	:	The promoter region of the anther-specific gene TA29 from *Nicotiana tabacum*. The PTA29 promoter comprises the 1.5 kb of the sequence upstream from the ATGinitiation codon (Seurinck et al., 1990).
# 5215-5250	:	Synthetic polylinker derived sequences
# 5251-5655	:	The promoter from the nopaline synthase gene from the T-DNA of pTiT37 of *Agrobacterium tumefaciens* (PNos); the nucleotide sequence of the PNos promoter is described by Depicker et al.(1982).
# 5656-6633	:	The coding sequence from the *neo* gene encoding neomycin phosphotransferase II. This sequence corresponds with the sequence from *Tn*5 of *Escherichia coli* as described by Beck et al. (1982). The ATGinitiation codon of the *neo* coding region has been substituted for a linker sequence as described by Reiss et al. (1984). There are 171 bp of the *Tn*5 sequence downstream from the *neo* coding region present (Beck et al., 1982).
# 6634-7509	:	The 3' untranslated end from the octopine synthase gene (3'ocs). This corresponds with a 706 bp PvuII fragment from the octopine synthase gene (Gelen et al., 1984). The PvuII fragment has been cloned in the SmaI site of the *Tn*5 sequence. There are an additional 169 bp of the *Tn*5 sequence present downstream from the 3'ocs fragment.
# 7510-7513	:	Synthetic linker derived sequences
# 7514-8202	:	Residual sequences from the TL-DNA at the left border repeat. The junction is at the former BglII site in the TL-DNA.
# 8203-8227	:	Left border repeat from the TL-DNA from pTiB6S3.

Part 3: Map of the plasmid pTTM8RE



Annex 5 MOLECULAR ANALYSES

Part 1 DESCRIPTION OF MOLECULAR TECHNIQUES USED TO INVESTIGATE GENE STRUCTURE, GENE EXPRESSION AND FUNCTION.

1.1 ANALYSIS OF DNAs BY SOUTHERN BLOT HYBRIDIZATIONS

1.2 ANALYSIS OF RNAs BY NORTHERN BLOT HYBRIDIZATIONS

1.3 AMPLIFICATION OF DNAs BY THE POLYMERASE CHAIN REACTION (PCR)

Part 2 CHARACTERIZATION OF THE MALE STERILE RADICCHIO ROSSO LINES IN THE LABORATORY

2.1 STABILITY OF INTEGRATION OF THE TRANSFORMING DNA OVER A NUMBER OF GENERATIONS VIA SOUTHERN BLOT ANALYSIS

2.2 VERIFICATION THAT THE TRANSFORMING DNA CORRESPONDS TO PLASMID DNA

2.3 CHARACTERIZATION OF THE T-DNA INTEGRATION AT THE T-DNA TERMINAL REPEATS BY PCR

2.4 EVALUATION OF THE EXPRESSION OF THE INTRODUCED GENES AND ANALYSIS OF THE POSSIBLE OCCURENCE OF CRYPTIC GENE EXPRESSSION

2.5 EVALUATION OF THE EXPRESSION AND INHERITANCE OF THE *NEO* GENE BY A NPTII ENZYMATIC ASSAY

2.6 QUANTIFICATION OF PHOSPHINOTRICIN-ACETYL-TRANSFERASE IN PLANT EXTRACTS BY A SPECTROPHOTOMETRIC PAT ASSAY

2.7 STUDY ON THE OCCURENCE IN PLANT MATERIAL OF THE *AGROBACTERIUM TUMEFACIENS* STRAIN APPLIED IN TRANSFORMATION EXPERIMENTS

2.8 VERIFICATION THAT THE TRANSFORMING DNA LACKS GENE SEQUENCES CONFERRING RESISTANCE TO STREPTOMYCIN/SPECTINOMYCIN (Sm/Sp).

Part 1 DESCRIPTION OF MOLECULAR TECHNIQUES USED TO INVESTIGATE GENE STRUCTURE, GENE EXPRESSION AND FUCNTION.

1.1 ANALYSIS OF DNAs BY SOUTHERN BLOT HYBRIDIZATIONS

1.1.1 Introduction

Total genomic DNA is isolated from plant tissue. A fraction of the isolated DNA is digested with appropriate restriction enzymes and the digested DNA fragments are separated by electrophoresis in agarose. After a depurination step, the fragments are denatured and transferred to nylon filters. The DNA fragments attached to the membranes are hybridized with P^{32}-labeled purified DNA fragments. Subsequently, the membranes are washed and autoradiography was carried out to visualize the hybridizing bands. Based on the mobility of the respective fragments, results are interpreted.

1.1.2 Preparation of total genomic DNA (according to Dellaporta et al. (1983). Plant Molecular Biology Reporter, 1, 3, 19-21)

- Weigh 0.5 g of leaf tissue, freeze in liquid nitrogen, grind with morter and pestle, and transfer the powder into a 30 ml Oak Ridge tube.
- Add 15 ml extraction buffer (100 mM Tris.HCl pH 8, 50 mM EDTA, 500 mM NaCl, 10mM β mercaptoethanol).
- Mix well.
- Add 1 ml 20% SDS, vortex and incubate at 65°C for 10 min.
- Add 5 ml 5 M potassium acetate, vortex and leave at 0°C for 20 min.
- Spin tubes at 25 000 x g (gravitation constant) for 20 min (13 000 rpm in Sorvall SA 600 rotor).
  Pour supernatant trough Miracloth filter (Calbiochem) into a clean 30 ml tube, containing 10 ml isopropanol.
  Mix and incubate at -20°C for 30 min .
- Pellet the DNA at 20 000 x g for 15 min.
  Gently pour off the supernatant and dry pellets by inverting the tubes on paper towels for 10 min.
- Redissolve DNA pellets with 700 µl of TE20 buffer (50 mM Tris.HCl pH 8, 20 mM EDTA), and transfer to an Eppendorf tube.
- Add 2.5 µl RNase (10 mg/ml) and incubate for 10 min at 37°C.
- Spin tubes for 10 min in an Eppendorf centrifuge to remove insoluble debris.

- Transfer the supernatant to a new eppendorf tube and add 75 µl 3 M sodium acetate and 500µl isopropanol. Mix well and pellet the DNA for 30 seconds in an eppendorf centrifuge.
- Wash pellets with 80% ethanol, dry and redissolve DNA in 100 µl TE buffer (10 mM Tris.HCl pH 8, 1 mM EDTA).
- Determine the concentration of the DNA by measuring the UV absorbance at 260 nm.
- Use the required genomic DNA amount to set up the digestion.

1.1.3 Restriction digests of total genomic DNA

- Mix together in an eppendorf tube :
  10 µg of genomic DNA
  4 µl 10 x RE buffer (*)
  20 units of restriction enzyme
  H_2O to 40 µl
- Incubate digest overnight in an oven at recommended temperature.

* composition of 10 x RE buffer
- 100 mM Tris.HCl pH 8
- 50 mM $MgCl_2$
- 60 mM β mercaptoethanol
- 1 mM EDTA
- 1 mg/ml BSA

RE 50 - 0.5 M NaCl
RE 100 - 1 M NaCl
RE 150 - 1.5 M NaCl

1.1.4 Separation of the restriction fragments on agarose gels

- Prepare 1% agarose gel in TAE buffer (40 mM Tris, 5 mM sodium acetate, 1 mM EDTA, pH 7.8 with acetic acid), containing 0.3 µg/ml Ethidium Bromide.
- Pour the gel into the gelsupport and let solidify.
- Add 5 µl of loading dye to the digested DNA samples and load the gel. Include a marker (lambda DNA digested with restriction endonuclease PstI on the gel
- Run the gel at an electric currency of 20 mA overnight .
- Cover the gel with Saran-wrap after the samples have migrated about 1 cm into the gel.

1.1.5 Blotting of the restriction fragments on nylon membranes

- After electrophoresis is completed, cut the gel from the support and photograph the gel. Place a transparent ruler

alongside the gel so that the migration distance of the fragments can be read directly from the photograph.
- Blot the separated DNA fragments on Nylon membrane by capillary transfer or by vacuum transfer.
  * Depurination of the gel : put the gel in 0.25 M HCl until the dyes have changed colour.
  * Alkali transfer : transfer is done in 0.4 M NaOH (for capillary blotting) or 1 M NaOH (for vacuum blotting)
  * Membrane : Hybond - N+
  * Duration of transfer : minimum 3 hours to overnight for capillary transfer; 1 hour for vacuum transfer.
  * Rince the membrane briefly in 2 x SSC buffer (20 x SSC = 3M NaCl + 0.3M Sodium Citrate), wrap in Saran-wrap and store at 4°C.

1.1.6 Purification of fragments for probe preparation

- Digest +/- 20 µg of the plasmid DNA with the appropriate restriction enzyme as to generate the desired double stranded DNA fragment.
- Separate the DNA fragments on a 1% Low Melting Agarose gel, prepared in TAE buffer and containing 0.3 µg/ml Ethidium bromide.
- After electrophoresis is completed, cut the desired fragment from the gel with a scalpel. Put the gel slice in an Eppendorf tube.
- Add an equal volume of TE buffer (10 mM Tris.HCl pH 8 , 1mM EDTA) to the gel slice.
- Melt the gel slice in a 65°C waterbath for 10 min.
- Preheat an equal volume of phenol (equilibrated with TE buffer) 30 sec. at 65°C.
- Add the phenol to the melted gel slice and put the mixture on an Eppendorf shaker for 15 min.
- Centrifuge for 10 min in an Eppendorf centrifuge to separate the two phases.
- Transfer the water phase to a new Eppendorf tube and extract for a second time with an equal volume of phenol.
- Precipitate the DNA from the water phase with 0.1 volume of 5 M Sodium perchlorate and 1 volume of isopropanol.
- Pellet the precipitated DNA by spinning for 15 min in an Eppendorf centrifuge.
- Dry pellets and redissolve in 50 µl of TE.
- Measure the concentration of the DNA solution and dilute with H_2O, to a concentration of 25 ng/µl.

1.1.7 Labeling the DNA fragment with radioisotope (according to Feinberg and Vogelstein (1983) Analyt. Biochem., 132, 6-13 and Feinberg and Vogelstein (1984). Analyt. Biochem., 137, 266)

- Mix 25 ng DNA fragment + H_2O (total volume = 12 µl) in an Eppendorf.
- Denature the DNA fragment for 5 min in a boiling waterbath and cool quick in ice-water.
- Add to the tube :
  18 µl LS buffer (*)
  1 µl 5 mg/ml BSA (DNAse free)
  3 µl 1 mM dATP, dGTP, dTTP
  4 µl αP^{32} dCTP (specific activity = 3000 mCi/mmole)
  2 µl Klenow DNA Polymerase (5U/µl)

  40 µl

- Leave at room temperature for 5 hrs.
- Remove the unincorporated nucleotides by purifying the labeled DNA fragment over a BIORAD biospin-30 column.

* Composition of the LS buffer:
Mix together:
- 25 µl 1M HEPES pH 6.6
- 25 µl TM buffer (250 mM Tris.HCl pH 8, 25 mM $MgCl_2$, 50 mM β- Mercaptoethanol)
- 7 µl OL (45 O.D. units Hexamers /ml TE - PL Biochemicals)

1.1.8 Hybridization of the membrane with the labeled probe

- Make up a hybridization solution :
  6 x SSC (20 x SSC = 3 M NaCl + 0.3 M Sodium citrate)
  5 x Denhardt's solution (100 x Denhardt's = 2% BSA + 2% Ficoll + 2% PVP)
  0.5% (w/v) SDS
  20 µg/ml denatured sonicated Herring Sperm DNA
- Prehybridize the membrane at 65°C for minimum 1 hour.
- Denature the labeled probe by heating for 5 min. at 95°C.
- Replace the hybridization solution and add the denatured probe
  (do not exceed a probe concentration of 20 ng/ml)
- Hybridize at 65°C overnight
- Upon hybridization, wash the filters as follows:
  * 15 min in 6 x SSC
  * 30 min in 2 x SSC / 0.1% SDS
  * 30 min in 0.1 x SSC / 0.1% SDS
- Remove excess of washing solution from the membrane (probed membranes may not dry out after hybridization) and wrap in Saran-wrap.

1.1.9 Autoradiography

- Put the membrane, wrapped in Saran-wrap into the X-ray cassette , between two Kodak intensifying screens.
- Expose a Kodak X-ray film to the membrane for an appropiate time period at -70°C.
- Develop film in a X-ray film processor.

1.2 ANALYSIS OF RNAs BY NORTHERN BLOT HYBRIDIZATIONS

The following procedure has been used to demonstrate the expression of the introduced transgenes in the male sterile progenies. The same procedure was used to analyze the possible occurrence of cryptic gene expression.

1.2.1 Extraction and purification of total RNA

Total RNA is isolated according to the improved single-step RNA isolation method developed by Chomczynski and Sacchi (1987) Anal. Biochem. 162, p.156. The commercial available TRIzol™ Reagent (patent pending) was purchased from Life Technologies.

1. Homogenization

- Weigh 1 gram of leaves or heads. Use 2 grams for the RNA isolation from roots.
- Grind the tissue to a fine powder in liquid nitrogen.
- Transfer the powder to a 50ml Falcon tube containing 15ml TRIzol™ reagent.
- Homogenize sample by vortexing.
- Incubate the homogenized samples for 30 to 60 minutes at room temperature to permit the complete dissociation of nucleoprotein complexes.

2. Phase separation

- Add 3ml chloroform-isoamylalcohol (24:1) to the Falcon tube and shake vigorously by hand for about 15 seconds.
- Transfer the solution to a 30ml Corex tube and incubate for 2 to 3 minutes at room temperature.
- Centrifugate the sample in the HB4 Sorvall Rotor at 12000g (± 8500 rpm) for 15 minutes at 4°C.
- The RNA remains exclusively in the aqueous phase.

3. RNA precipitation

- Transfer the aqueous phase to a new 30ml Corex tube and precipitate the RNA by mixing with 7.5ml isopropanol.
- Incubate samples at room temperature for 10 minutes.
- Centrifugate the samples in the HB4 Sorvall Rotor at 12000g (± 8500 rpm) for 10 minutes at 4°C.
- The RNA precipitate forms a gel-like pellet at the bottom of the tube.

4. RNA wash

- Remove the supernatant and wash with 15ml 75% ethanol.
- Mix by vortexing and centrifugate in the HB4 Sorvall Rotor at 7500g (± 7000 rpm) for 10 minutes at 4°C.

5. Redissolving of the RNA

- Briefly air-dry the RNA pellet.
- Dissolve the pellet in DEPC treated H_2O.
- Transfer the solution to an Eppendorf tube.

For quantitating the amount of RNA spectrophotometric readings are taken at wavelengths of 260 nm and 280 nm. The reading at 260 nm allows the calculation of the concentration of nucleic acids in the sample. An OD of 1 corresponds to 40µg/ml RNA. The ratio OD 260/OD280 provides an estimate of the purity of the nucleic acid.

At this stage the RNA can be concentrated by ethanol precipitation.

- Add 1/10 volume 2M NaOAc pH 4.8 and add 2 volumes ethanol.
- Mix well and keep at least 1 hour at -20°C.
- Pellet the precipitate by centrifugating the tube in a microfuge at maximum speed for about 10 minutes.
- Wash the pellet with 75% ethanol, centrifuge and invert the tube to dry the pellet.
- Finally dissolve the pellet in DEPC-treated H_2O.
- Quantitate the RNA concentration by spectrophotometric reading.

1.2.2 In vitro synthesis of control RNA transcripts

For the synthesis of control RNA transcripts (used as positive hybridization controls and for the quantification of the hybridization signals), all four ribonucleoside triphosphates are used. During "cold" transcription reactions, substrate levels are not limiting and the synthesis continues longer, producing higher amounts of RNA.

Templates

A. Plasmids for preparing RNA probes

pVE113: barnase-barstar in pGem1 vector (see Figure 1.)
- HindIII digested pVE113 DNA transcribed with T7 DNA polymerase gives sense barstar/barnase RNA transcripts.
- EcoRI digested pVE113 DNA transcribed with SP6 DNA polymerase gives anti-sense barstar/barnase RNA transcripts.

pFM146: neo in pGem1 vector (see Figure 2.)
- BstBI digested pFM146 DNA transcribed with T7 DNA polymerase gives sense neo RNA transcripts.
- BamHI digested pFM146 DNA transcribed with SP6 DNA polymerase gives anti-sense neo RNA transcripts.

pGemBar: bar in pGem2 vector (see Figure 3.)
- EcoRI digested pGemBar DNA transcribed with T7 DNA polymerase gives sense bar RNA transcripts.
- HindIII digested pGemBar DNA transcribed with SP6 DNA polymerase gives anti-sense RNA transcripts.

B. PCR amplification of DNA templates for in vitro RNA synthesis

For the analysis of occurrence of cryptic gene expression we amplified specific T-DNA fragments, by means of PCR, to serve as templates for in vitro RNA synthesis.
For every template, two primers are designed: an upstream primer which comprises the T7 promotor (including the 6 nucleotides GGGAGA that are present at the 5' end of transcripts) adjacent to specific T-DNA sequences and a downstream primer which comprises the SP6 promotor (including the 6 nucleotides GAATAC that are present at the 5' end of transcripts) adjacent to specific T-DNA sequences (Figure 4.)
The sequence of the different synthesized primers can be found in Table 1. Amplified fragment lengths and the region of the T-DNA they cover, can be found in Table 2.
PCR is carried out by using the thermostable Vent DNA polymerase (New England Biolabs, Inc.). This polymerase contains a 3'--->5' proofreading exonuclease activity, resulting in much higher fidelity of base incorporation compared to Taq DNA polymerase.

100ng of EcoRV or EcoRI linearized pTTM8RE DNA and 30pmoles of upstream and downstream primer were mixed in a 50µl PCR reaction containing 10mM KCl, 10mM $(NH4)_2SO_4$, 20mM Tris-HCl (pH8.8 at 25°C), 2mM $MgSO_4$, 0.1% Triton-X-100, 200µM of each deoxyribonucleoside triphosphate and 1 unit of Vent DNA polymerase. DNA amplification occurred during 23 cycles.

Thermocycling profile:

4 min. at 95°C
Followed by: 1 min. at 95°C
1 min. at 57°C
2 min. at 75°C
For 5 cycles

Followed by: 30 sec. at 92°C
30 sec. at 60°C
1 min. at 75°C
For 18 cycles
Followed by: 10 min. at 75°C

Some primer pairs required optimization of Mg^{2+} levels and primer extension times (For primer pairs MDB170-MDB171 and MDB172-MDB173, 4mM $MgSO_4$ and 2 minutes primer extension time at 75°C were found to be optimal).
The synthesized fragments were checked on agarose gels. After phenol-chloroform extractions the fragments were precipitated , washed and subsequently dissolved in water. The concentration of the DNAs was spectrophotometrically measured.

In vitro synthesis

- Mix the following components in the given order in a microfuge tube at room temperature:

DEPC-treated water	23µl	
Template DNA (500ng/ml)	8µl	
10x Transcription buffer		5µl
0.5M DTT	1µl	
RNase Inhibitor (25 units/µl)		2µl
NTP mix (2.5mM each)	10µl	
DNA-dependent RNA polymerase	1µl	

(10x Transcription buffer: 400 mM Tris-HCl pH7.5 at 37°C; 60 mM $MgCl_2$; 20 mM spermidine and 50 mM NaCl)

- Incubate at 37°C for 120 minutes.
- Add 1µl 10x Transcription buffer, 8µl NTP mix and 1µl polymerase. Incubate of another 120 minutes at 37°C.
- The template DNA is removed by treatment with DNase I for 10 minutes at 37°C.
- The synthesized RNA transcripts are extracted with phenol-chloroform and precipitated with ethanol. After washing with 70% ethanol, the transcripts are resuspended in DEPC-treated water. The concentration is spectrophotometrically measured.
- 1µg of the synthesized RNA transcripts are checked on a 1.5% agarose-formaldehyde gel.

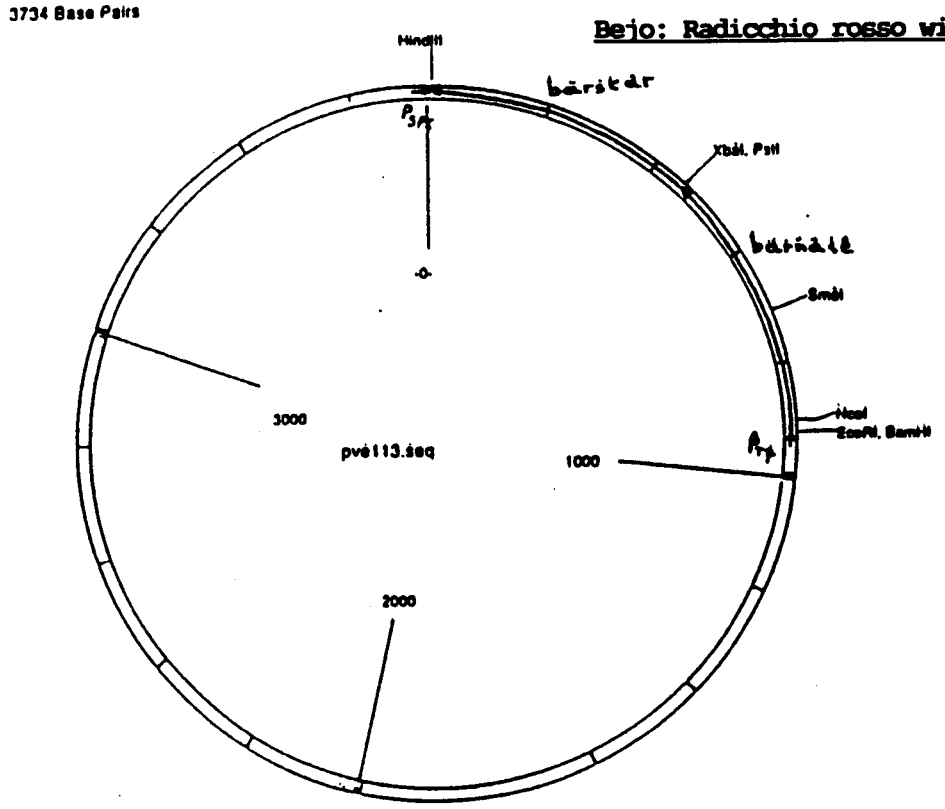


Figure 1. pVE113 : barnase-barstar in pGem1 vector

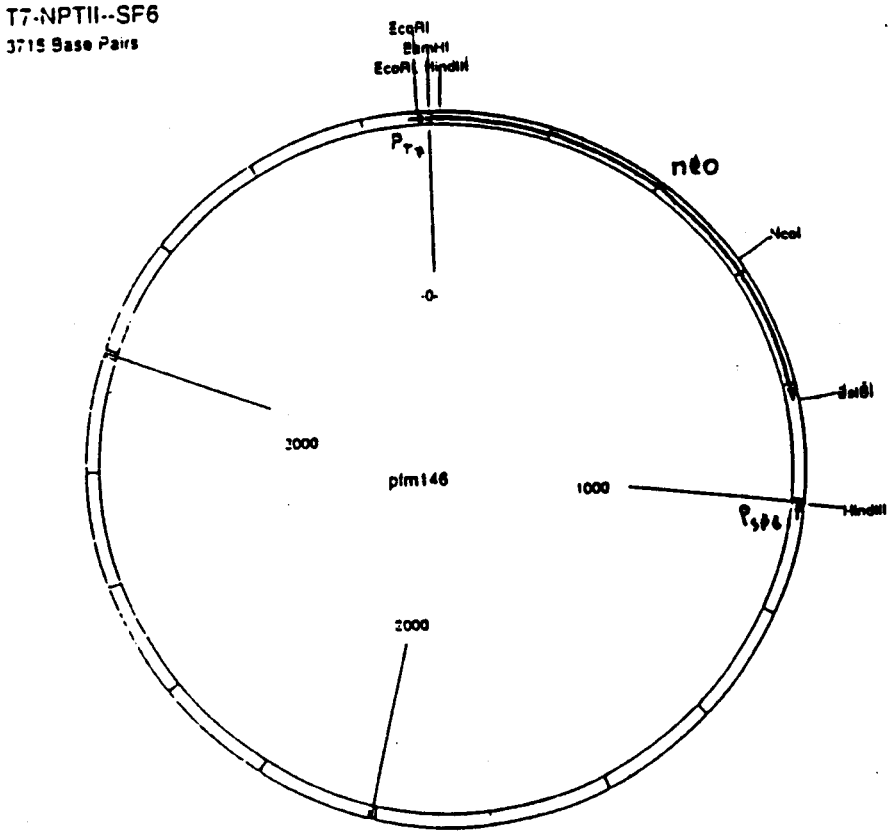


Figure 2. pFM146 : neo in pGem1 vector

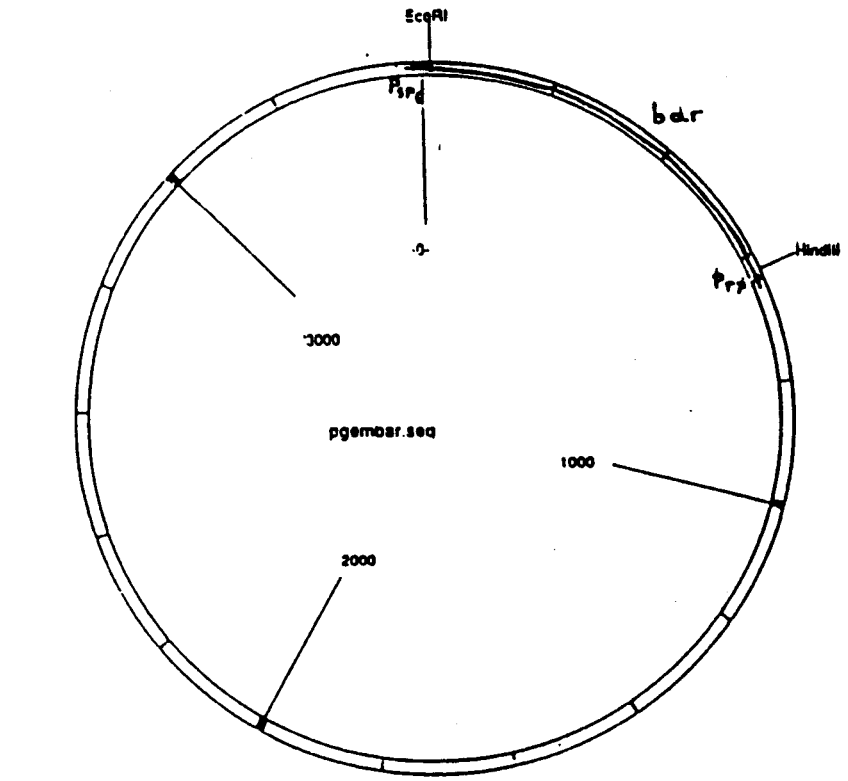


Figure 3. pGemBar : bar in pGem2 vector

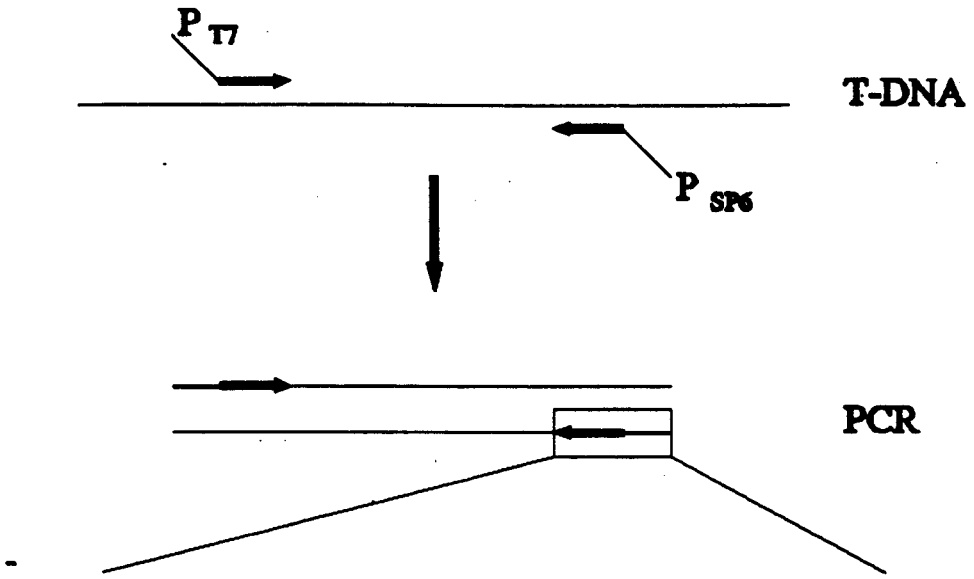


Figure 4.
Outline for the generation of specific T-DNA fragments for use in the in vitro transcription of RNA probes (The oligonucleotide sequence shown is MDB160)

1.2.3 Fractionation of RNA

The RNA is separated according to size by electrophoresis through a denaturing agarose gel containing formaldehyde.

The gels are prepared by melting agarose (1.5% final concentration) in water, cooling it to 60°C, adding 10x formaldehyde gel-running buffer (0.2M MOPS, 0.05M NaOAc pH7.0 and 0.01M EDTA) and formaldehyde to give a final concentration of 1x and 2.2 M respectively. Cast the gels in a chemical hood and allow the gel to set for at least 30 min. at room temperature.

Samples are prepared by mixing the following in a sterile microfuge tube:

- RNA (5µg)	3.5µl
- 10x formaldehyde gel-running buffer	2µl
- formaldehyde	3.5µl
- formamide	10µl
- Ethidium bromide (1mg/ml)	1µl

Note: The control RNA dilutions are complemented with 5µg control leaf RNA.

Incubate the samples for 15 minutes at 55°C and then chill them on ice. Add 2µl of sterile DEPC-treated dye (50% glycerol, 0.5% bromophenol blue and 0.5% xylene cyanol FF). Run the gel submerged in 1x formaldehyde gel-running buffer at ±5V/cm.
To avoid unnecessary inhalation of volatile formaldehyde vapors, documentation of the fractionation of the RNA is done after the transfer and fixation of the RNA to the nylon membrane.

1.2.4 Transfer of denaturated RNA to nylon membranes

The RNAs are transferred immediately after electrophoresis from the agarose gel to nylon membranes (Hybond-N, Amersham International) by capillary elution.

- Fill a glass dish with blotting buffer (20xSSPE = 3.6M NaCl, 0.2M Sodium phosphate, 0.02M EDTA pH 7.7). Make a platform and cover it with a Whatman 3MM filter paper wick, saturated with buffer.
- Place the gel on the wick and avoid trapping air bubbles beneath it. A sheet of Hybond-N membrane, cut to the exact size of the gel, is placed on top of the gel. Avoid trapping bubbles beneath the membrane.
- Place a sheet of Whatman 3MM cut to size and wetted with blotting buffer, on top of the Hybond-N membrane.
- Surround the gel with SaranWrap foil to prevent the blotting buffer being absorbed directly into the paper towels above.

- Place a stack of absorbent paper towels on top of the 3MM paper.
- Place a glass plate on top of the paper towels and a 0.5 - 1Kg weight on top. Allow the transfer to proceed for 12 to 20 hours.
- After blotting carefully dismantle the setup. Before removing from the gel, mark the membrane with a pencil to allow later identification of the tracks.
- The samples are fixed to the membrane by baking in an oven at 80°C for 2 hours.

Documentation of the fractionation of the RNA is done at this stage. The image is acquired, processed and copied to thermal paper using the Foto/Analyst™ Visionary imaging system from FOTODYNE (CCD camera: charge-coupled device).

1.2.5 In vitro synthesis of RNA probes

Single-stranded RNA probes of high specific activity are prepared by using either plasmid vectors containing polycloning sites downstream from powerful promoters derived from the *Salmonella typhimurium* bacteriophage SP6 or from the E. coli bacteriophage T7 or by either using PCR generated templates with 5' extensions containing the sequences from the before mentioned promoters.

<u>In vitro labeling</u>

-Mix the following components in the order given in a microfuge tube at room temperature:

DEPC-treated water	4µl
Template DNA (250ng/ml)	2µl
10x Transcription buffer	2µl
NTP mix (- UTP), 2.5 mM each	3µl
1mM UTP	1µl
0.2M DTT	1µl
RNase inhibitor (25units/µl)	1µl
[α-^{32}P]UTP (20 mCi/ml)	5µl
Bacteriophage DNA-dependent RNA polymerase (7 - 12 units/µl)	1µl

(10x Transcription Buffer: 400mM Tris-HCl pH7.5 at 37°C, 60mM $MgCl_2$, 20mM spermidine and 50mM NaCl).

- Mix the reagents by gentle tapping.
- Incubate the reaction for 1 hour at 40°C (SP6 RNA polymerase) or 37°C (T7 RNA polymerase).
- Add 1µl RNase inhibitor and 1µl of RNase-free pancreatic DNaseI (20 units/µl). Mix and incubate for 15 min. at 37°C.
- Analyze 0.5µl on a 6% denaturing acrylamide gel.
- The rapid removal of unincorporated nucleotides from the labeling reaction mixture is done by using Bio-Spin 30 chromatography columns (Bio-Gel P-30 polyacrylamide gel, Bio-Rad).

1.2.6 Hybridization and autoradiography

- The filters are prehybridized for 1 - 2 hours in a hybridization oven using 10ml prehybridization buffer (for 3 filters of 14cmx19cm) at 65°C.
  Prehybridization buffer: 50% formamide, 5x SSPE, 5x Denhardt's, 0.1% SDS and 100µg/ml carrier DNA at 65°C.
  (20xSSPE: 3.6M NaCl, 0.2M Sodium phosphate, 0.02M EDTA pH7.7)
  (100xDenhardt's solution: 2%(w/v) BSA, 2%(w/v) ficoll and 2%(w/v) Polyvinylpyrrolidone)
- Remove the prehybridization buffer
- Add fresh prehybridization buffer supplemented with the denaturated radiolabeled probe to the hybridization tube and continue the incubation over night.
- Wash the filters for 5 min. in 5xSSPE, followed by 2 - 3 washes of 20 - 30 minutes each in 2xSSPE, 0.1%SDS and 1 wash of 10 - 20 minutes in 0.1xSSPE, 0.1%SDS.
- Establish an autoradiograph by exposing the filter for 3 up to 96 hours to X-Ray film at -70°C with an intensifying screen. The shorter exposures are performed for accurate quantification and for reproduction of the results. The longer exposures are performed to assure the absence of any signals in control samples or in the analysis of occurrence of cryptic gene expression.
- Reproduction of the results in this document is done by using the iphoto deluxe software (U-lead Systems, Taipei, Taiwan, ROC) and the Harvard Graphics Software.
- After the exposure, the membranes are stripped to remove the probes. For this purpose a 0.5% SDS solution is boiled. Membranes are submerged in this solution and allowed to cool to room temperature.
- To check that the probe was removed completely, an autoradiograph for the normal exposure time was established.
- Subsequently, the filters can be prehybridized and hybridized with a new probe.

Table 1:
Oligonucleotide sequences for PCR amplification of DNA templates suited for *in vitro* RNA synthesis. (5' end: the 6 nucleotides that are present at the 5' end of transcripts).

Oligo	Promotor	5'end	T-DNA	Pos. in pTTM8.RE
MDB159	T7 5' TAA.TAC.GAC.TCA.CTA.TA	GGGA.GA	C.CTT.CGC.TGC.CCA.TAT.TAT.TGG 3'	7532 → 7553
MDB160	SP6 5' ATT.TAGGTGACA.CTA.TA	GAAT.AC	GTAA.ATGGCT.TCA.TGT.CCG 3'	8208 → 8190
MDB168	T7 5' TAA.TAC.GAC.TCA.CTA.TA	GGGA.GA	C.AGT.CAGCAT.CAT.CAC.ACC 3'	24 → 42
MDB169	SP6 5' ATT.TAGGTGACA.CTA.TA	GAAT.AC	A.CCC.TTGAGGAAA.CTGGTA.GC 3'	223 → 203
MDB170	T7 5' TAA.TAC.GAC.TCA.CTA.TA	GGGA.GA	GAGA.TTC.AAGTGGACT.AGG 3'	1356 → 1374
MDB171	SP6 5' ATT.TAGGTGACA.CTA.TA	GAAT.AC	GCAGATC.AAT.CGC.AAC.ATA.TAT.GG 3'	2802 → 2779
MDB172	T7 5' TAA.TAC.GAC.TCA.CTA.TA	GGGA.GA	C.TGT.TAC.ACT.TGC.ACC.ACA.AGG 3'	3755 → 3776
MDB173	SP6 5' ATT.TAGGTGACA.CTA.TA	GAAT.AC	GACT.CTA.ATT.GGA.TAC.CGA.GG 3'	5624 → 5604

Table 2:
Primer-pairs for the analysis of occurrence of cryptic gene expression.

Primer-pair	Amplified fragment	Position in pTTM8RE	Features
MDB159 - MDB160	723bp	7532 - 8208	3' ocs + T-DNA leftover seq.
MDB168 - MDB169	246bp	24 - 223	T-DNA leftover seq. + 3' g7
MDB170 - MDB171	1493bp	1356 - 2802	PSsuARA
MDB172 - MDB173	1915 bp	3755 - 5624	PTA29 + PNos

1.3 AMPLIFICATION OF DNAS BY THE POLYMERASE CHAIN REACTION (PCR)

1.3.1 Preparation of Plant Genomic DNA

The rapid extraction of small amounts of plant genomic DNA suitable for PCR analysis is done according to the method described by Edwards et al. (K. Edwards et al., Nucleic Acids Research, 19 (6), page 1349, 1991).

- Collect samples for PCR analysis (usually leaf tissue) by using the lid of a Eppendorf tube to pinch out a disc of material into the tube.
- Macerate the tissue with a plastic pestle at room temperature, without buffer for 5 to 15 sec.
- Add 400 µl extraction buffer (EB: 200 mM Tris HCl pH 7.5, 250 mM NaCl, 25mM EDTA, 0.5% SDS). The mixture can be left at room temperature until all samples have been extracted (> 1 hour).
- Centrifuge the extracts for 1 minute at max. speed and transfer 300 µl of the supernatant to a fresh Eppendorf tube.
- Mix with 300 µl isopropanol and leave at room temperature for 2 minutes.
- Centrifuge at max. speed for 5 minutes.
- Dry pellet and dissolve in 100 µl water.
- Centrifuge for 2 minutes and transfer supernatant to a new Eppendorf tube.
- Use 5 µl of this sample in a 50 µl PCR reaction.

1.3.2 Polymerase chain reaction

5µl of the isolated DNA is used in a 50µl PCR reaction containing 10 mM Tris-HCl (pH8.3); 50 mM KCl; 1.5 mM $MgCl_2$; 200µM of each dNTP; 0.001% (w/v) gelatin; 1 unit *Taq* DNA polymerase (Boehringer Mannheim); 10 pmole of barnase gene specific oligonucleotide primers; and/or 10 pmole of barstar gene specific oligonucleotide primers; 5 pmole each of acetolactate synthase specific oligonucleotide primers CVZ9 and CVZ11 (acting as a positive endogenous control) yielding a 319bp product. Alternatively 5 pmole each of *Brassica napus* 12S seed storage protein gene specific primers CVZ7 and CVZ8, yielding a 394bp product, can be used as a positive endogenous control.
A master mix of reagents (water, buffer, dNTP's, primers and enzyme) for all samples is prepared first and then aliquoted to the individual samples. The reaction mixtures are overlayed with 50µl mineral oil and thermocycling is started.

Thermocycling profile:

4 min. at 95°C
Followed by: 1 min. at 95°C
1 min. at 57°C
2 min. at 72°C
For 5 cycles
Followed by: 30 sec. at 92°C
30 sec. at 57°C
1 min. at 72°C
For 22 cycles
Followed by: 10 min. at 72°C

15µl of each PCR sample is separated on a 1.5% agarose gel. The BRL 123bp ladder or the Pharmacia 100bp ladder is used as a MW marker. Results are documented by Polaroid photography.

1.3.3 Left Border internal primers

MDB74	5'	AGT.TGA.CAG.ACT.GCC.TAG.C	3'
MDB75	5'	CTA.CAT.TCA.CGT.CCA.AAT.GGG	3'

1.3.4 External primer

MDB76	5'	GCT.CAG.TTC.TGC.GTA.GAA.ACC	3'

Part 2 CHARACTERIZATION OF THE MALE STERILE RADICCHIO ROSSO LINES IN THE LABORATORY

Plant Material

Radicchio rosso male sterile lines RM3-3, RM3-4 and RM3-6.
R = Radicchio rosso.
M = Transformed with the construct for male sterility.
3-3 = Transformant number 3 from inbred line number 3.
3-4 = Transformant number 4 from inbred line number 3.
3-6 = Transformant number 6 from inbred line number 3.

Detailed description of plant material used in molecular analyses.

RM3-3 :

- T122 = original transformant RM3-3 (To), juvenile leaf tissue from the greenhouse.
- T13 = RM3-3 x R3 (Tx), juvenile leaf tissue from the greenhouse.
- T326 = RM3-3 x R3 (Tx), mature heads from field crop.
- T33 = RM3-3 x CRP609-3 (BCx), juvenile leaf tissue from the greenhouse.

RM3-4 :

- T123 = original transformant RM3-4 (To), juvenile leaf tissue from the greenhouse..
- T14 = RM3-4 x R3 (Tx), juvenile leaf tissue from the greenhouse.
- T327 = RM3-4 x R3 (Tx), mature heads from field crop.
- T30 = RM3-4 x Mar1-1 x Mar 1-1 (BCx), juvenile leaf tissue from the greenhouse.

RM3-6 :

- T124 = original transformant RM3-6 (To), juvenile leaf tissue from the greenhouse.
- T17 = RM3-6 x R3 (Tx), juvenile leaf tissue from the greenhouse.
- T328 = RM3-6 x R3 (Tx), mature heads from field crop.
- T309 = RM3-6 x Mar1-1 (BCx), mature heads from field crop.

Control :

- T201 = R3, juvenile leaf tissue from the greenhouse.
- T325 = R3, mature heads from field crop.

2.1 STABILITY OF INTEGRATION OF THE TRANSFORMING DNA OVER A NUMBER OF GENERATIONS VIA SOUTHERN BLOT ANALYSIS

Responsible : C. Opsomer, Researcher

2.1.1. Goals of the experiment

Analyzing the stability of integration and the inheritance of the introduced chimeric barnase gene construct in the progenies of 3 lines of Radicchio Rosso (red hearted chicory), respectively RM3-3, RM3-4 and RM3-6. The experimental approach is based on a molecular analysis using the Southern blot procedure.

2.1.2. Plant material

To demonstrate the stability of the integrated gene construct, a molecular analysis has been performed on the plants from subsequent progenies. For every line, 3 generations have been tested; T_0 (copy of original transgenic plant), T_X (maintained progeny), BC_X (backcross in other line), for detailed information, see annex 7. The analysis has been performed on two individual plants (A and B) from each progeny. Non-transgenic lines have been used as a negative control.

Table : Plant material

Plant material	Notation
Radicchio Rosso transformed with pTIM8RE	
RM3-3 progeny :	
T_0 (RM3-3)	T122 (leaves)
T_X (RM3-3)	T13 (leaves)
BC_X of RM3-3	T33 (leaves)
RM3-4 progeny :	
T_0 (RM3-4)	T123 (leaves)
T_X (RM3-4)	T14 (leaves)
BC_X of RM3-4	T30 (leaves)
RM3-6 progeny :	
T_0 (RM3-6)	T124 (leaves)
T_X (RM3-6)	T328 (head)
BC_X of RM3-6	T309 (head)

2.1.3 Methods

Southern analysis was performed according to the protocol as described in annex 5, part 1.1. Total DNA of the respective plants was isolated from plant tissue according to Dellaporta et al. (1983) and has been digested with EcoRV. This enzyme has a single cleavage site in the transferred T-DNA fragment and is localized in the TA29 promoter fragment.
In Figure 1 a schematic representation of the DNA fragment comprised between the T-DNA border repeats of pTTM8RE is given. The localization of the EcoRV site within the transferred DNA, the TA29 promoter fragment used as probe and the expected crosshybridizing bands are indicated.

2.1.4 Results and conclusion

Results

Upon hybridization with a TA29 promoter fragment, two hybridizing bands overlapping with the genomic DNA towards the right and left integration sites of the transferred DNAs were expected, when only one copy of the T-DNA has been integrated. This means for the line RM3-3 ,fragments of >14 and 4.2 Kb. Two bands of respectively 8 and 4.7 Kb are expected for the line RM3-4. The line RM3-6 contains more than one copy of the T-DNA, with fragments of respectively >14Kb, 10Kb and a doublet of 3.8Kb. An identical hybridization pattern should be found, analyzing the different generations of each individual line.

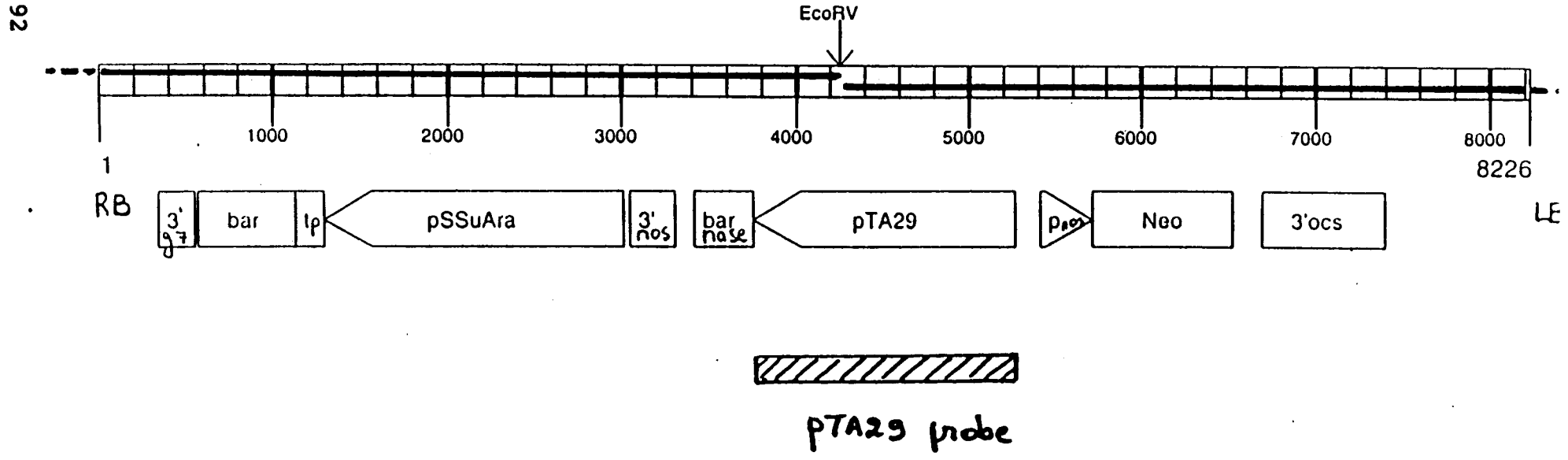
The autoradiograms from the Southern blot analysis done on the 3 lines and on two individual plants (A and B) from different generations (T_0, T_X, and BC_X), carrying the male sterility gene, with the hybridization pattern using the TA29 probe is shown in Figure 2 and 3.

Conclusion

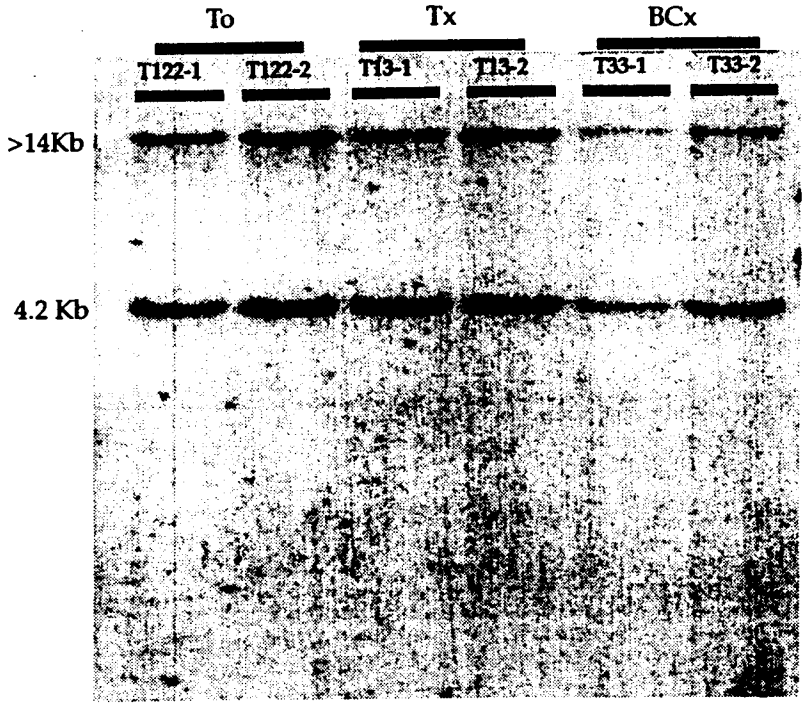
For the lines RM3-3 and RM3-4 this analysis clearly demonstrates that the transferred DNA is stably inherited in subsequent generations and in different genetic backgrounds. For the line RM3-6, wich has originally integrated 2 copies of the transferred DNA, this analysis demonstrates that in subsequent generations and in different genetic backgrounds, only one copy of the transferred DNA is stably inherited, The other copy (with overlapping hybridizing fragments of 2 x 3.8 Kb towards RB and LB) is lost after the first generation, as no functional *bar* gene was present.

Spliced PTTM8.SEQ 193:8419 into 8.SEQ

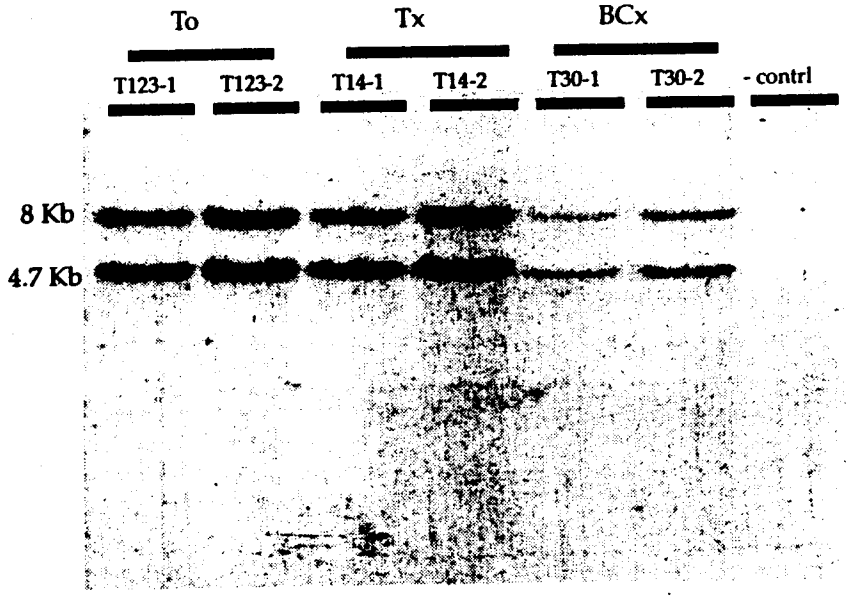
8.seq 8226 Base Pairs



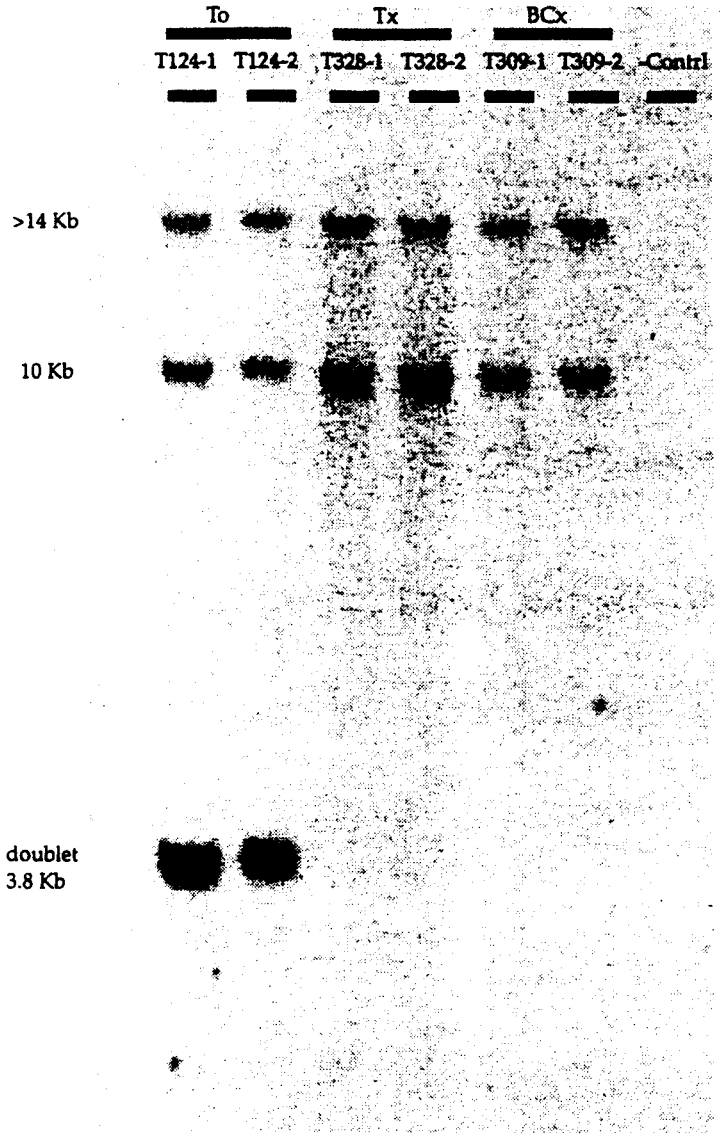
pTA29 probe



RM3-3
EcorV digest
TA29 probe



RM3-4
EcorV digest
TA29 probe



RM3-6:
EcorV digest
TA29 probe

2.2 VERIFICATION THAT THE TRANSFORMING DNA CORRESPONDS TO PLASMID DNA

Responsible : C. Opsomer, Researcher

2.2.1 Goals of the experiment

To demonstrate that the DNA transferred into the plant genome is identical to the T-DNA region of the plasmid vector, a more extended molecular analysis based on Southern blotting has been performed for the plantlines RM3-3, RM3-4 and RM3-6.

2.2.2 Plant material

Molecular analysis has been performed on the progeny of the lines carrying the male sterility construct, RM3-3, RM3-4 and RM3-6. Two individual plants of each line have been analysed and compared with total plasmid DNA. A Non-transgenic plant has been used as negative control.

Table 1 : Plant material

Plant material	Notation
RM3-3 progeny : T_x(RM3-3)	T326 (head)
RM3-4 progeny : T_x(RM3-4)	T327 (head)
RM3-6 progeny : T_x(RM3-6)	T328 (head)

2.2.3 Methods

Southern analysis was performed according to the protocol as described in annex 5, part 1.1. Genomic DNA has been prepared from leaf tissue of a plant carrying the male sterility construct and compared with total plasmid DNA. The experimental approach is based on digesting the respective DNAs with different restriction enzymes, probe with different fragments of the transferred DNA and make a comparison between plasmid and genomic DNA fragments. The comparative analysis is based on the determination of the sizes of the respective hybridizing bands. For this reason, restriction digests were chosen to have restriction fragments between 0.8 to 3.4 kb. Within this range, restriction fragments can be sized within a resolution of 0.1 kb. The use of different probes - comprising the bar gene, neo gene and ssuAra and TA29 promoter - allows to visualize different fragments of the transferred DNA. Digest and probe combinations were chosen in such a way that the whole transferred region was covered.

2.2.4 Results and conclusion

Results

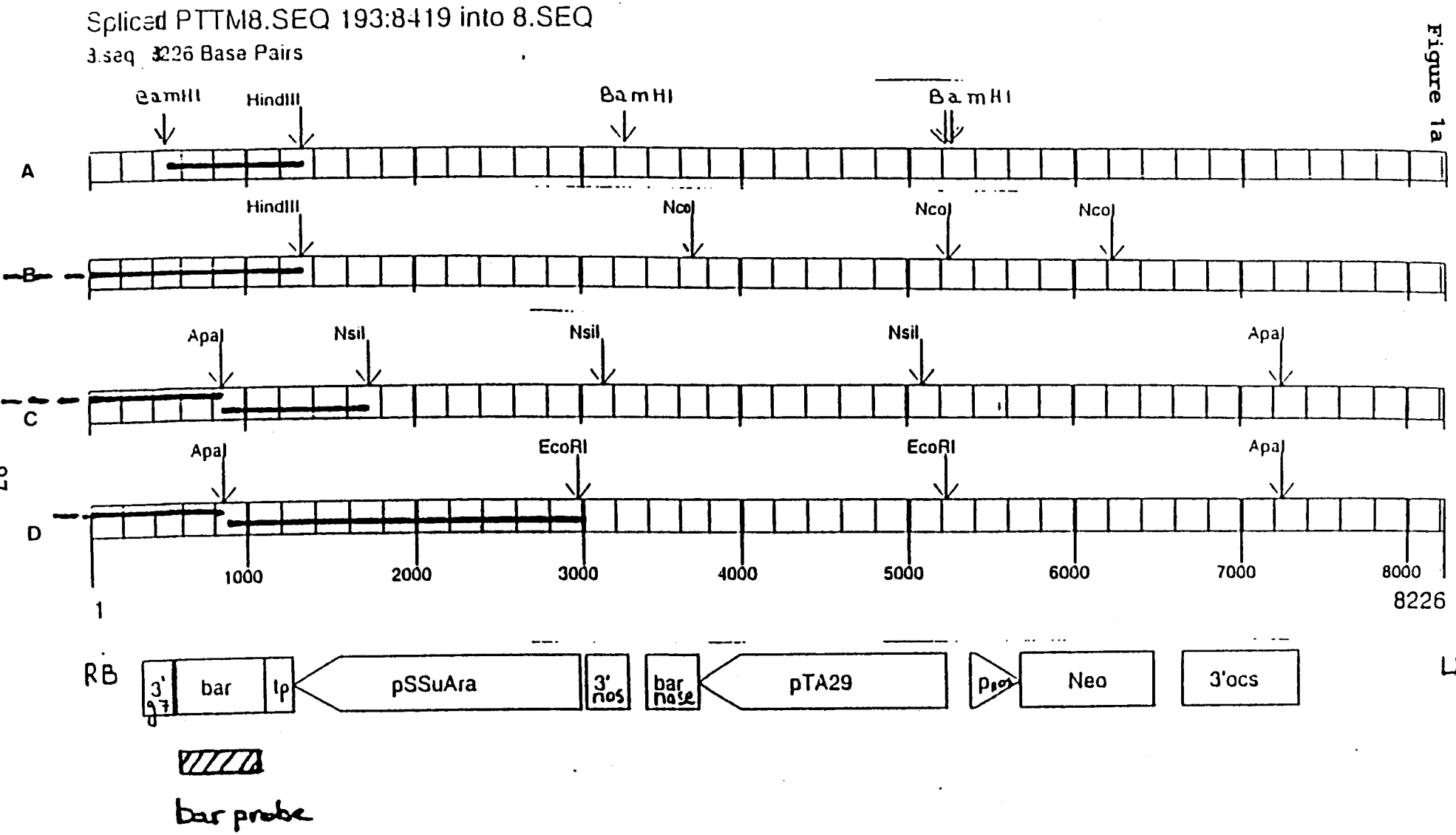
Table 2 gives an overview of the different digests performed on total genomic DNA from the male sterile line and the plasmid vector pTTM8RE. Four probes have been used in the hybridizations and the expected fragments based on the plasmid map are indicated. As an example, the schematic representation of the DNA fragment comprised between the right and left border repeat of pTTM8RE with the respective chimeric gene constructs, is outlined in Figure 1a.,1b.,1c. and 1d.. The respective restriction enzymes used in the consecutive hybrizations are indicated and the crosshybridizing bands with the respective probes are highlighted.
In Figures 2a,b,c,d, 3a,b,c,d and 4a,b,c,d some typical autoradiograms of the different probe-digest combinations are shown. Bands with size below 1000 bp are weak and in some cases hardly visible especially when homology is only partial. In the case of RM3-3 all autoradiograms are showing all bands as expected, with exception of the hybridization with the bar probe. In case of the NcoI-HindIII digest, 2 additonal fragments are shown to the expected right border fragment, which can only be explained by partial digest of the DNA, as all other hybridizations are showing the expected pattern. When using a EcoRI/ApaI enzyme combination, partial digest have been observed, on genomic DNA from the line RM3-6. Additional hybridizing bands with bar, SSuAra and TA29 probe can be explained, since the EcoRI site at position 2960 in the T-DNA insertion is not completely cut.

Conclusion

In conclusion, this analysis demonstrates that the transferred DNA in the plant genome corresponds to the DNA configuration as designed in the plasmid vector.

Table 2 : OVERVIEW OF THE DIFFERENT DIGESTS PERFORMED ON TOTAL GENOMIC DNA

digests	bar probe	PSsuAra probe	PTA29 probe	neo probe
NcoI+HindIII	RB fragment	2379 bp	1543 bp	981 bp
EcoRI+ApaI	2113 bp + RB fragment	2113 bp	2262 bp	2019 bp
NsiI+ApaI	877 bp + RB fragment	877 bp + 1402 bp	1962 bp	2153 bp
BamHI+HindIII	817 bp	1934 bp	1962 bp	LB fragment



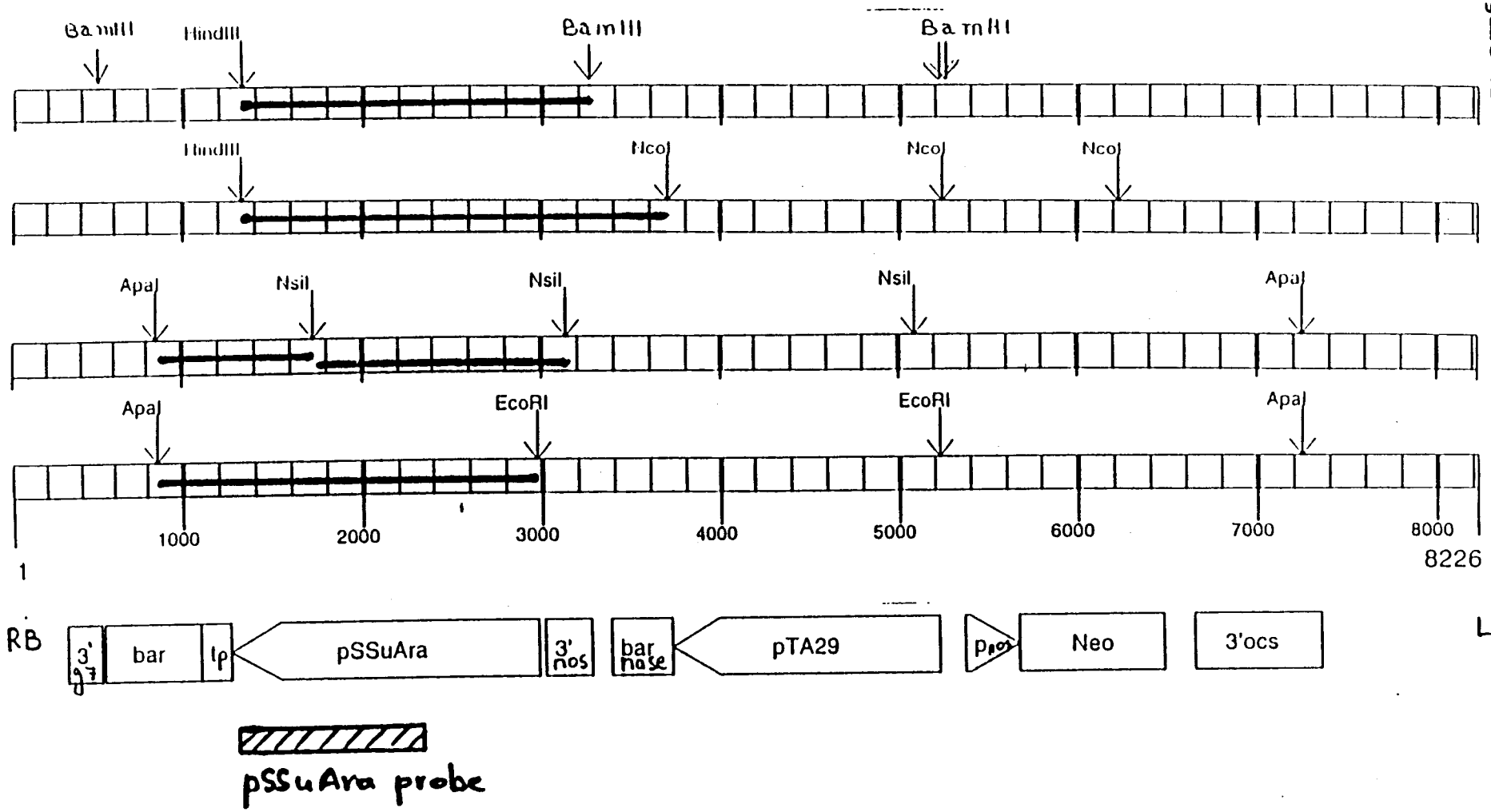
Schematic representation of the DNA fragment between the borders of plasmid pTTM8RE showing the localisation of the different chimeric genes.
The plant DNA was digested with 4 combinations of restriction enzymes (restriction sites shown on the scheme)

A. BamHI - HindIII
B. NcoI - HindIII
C. ApaI - NsiI
D. ApaI - EcoRI

The localisation of the bar probe and the expected hybridising fragments are shown.

Spliced PTTM8.SEQ 193:8419 into 8.SEQ

8.seq 8226 Base Pairs



pSSuAra probe

Schematic representation of the DNA fragment between the borders of plasmid pTTM8RE showing the localisation of the different chimeric genes.
The plant DNA was digested with 4 combinations of restriction enzymes (restriction sites shown on the scheme)

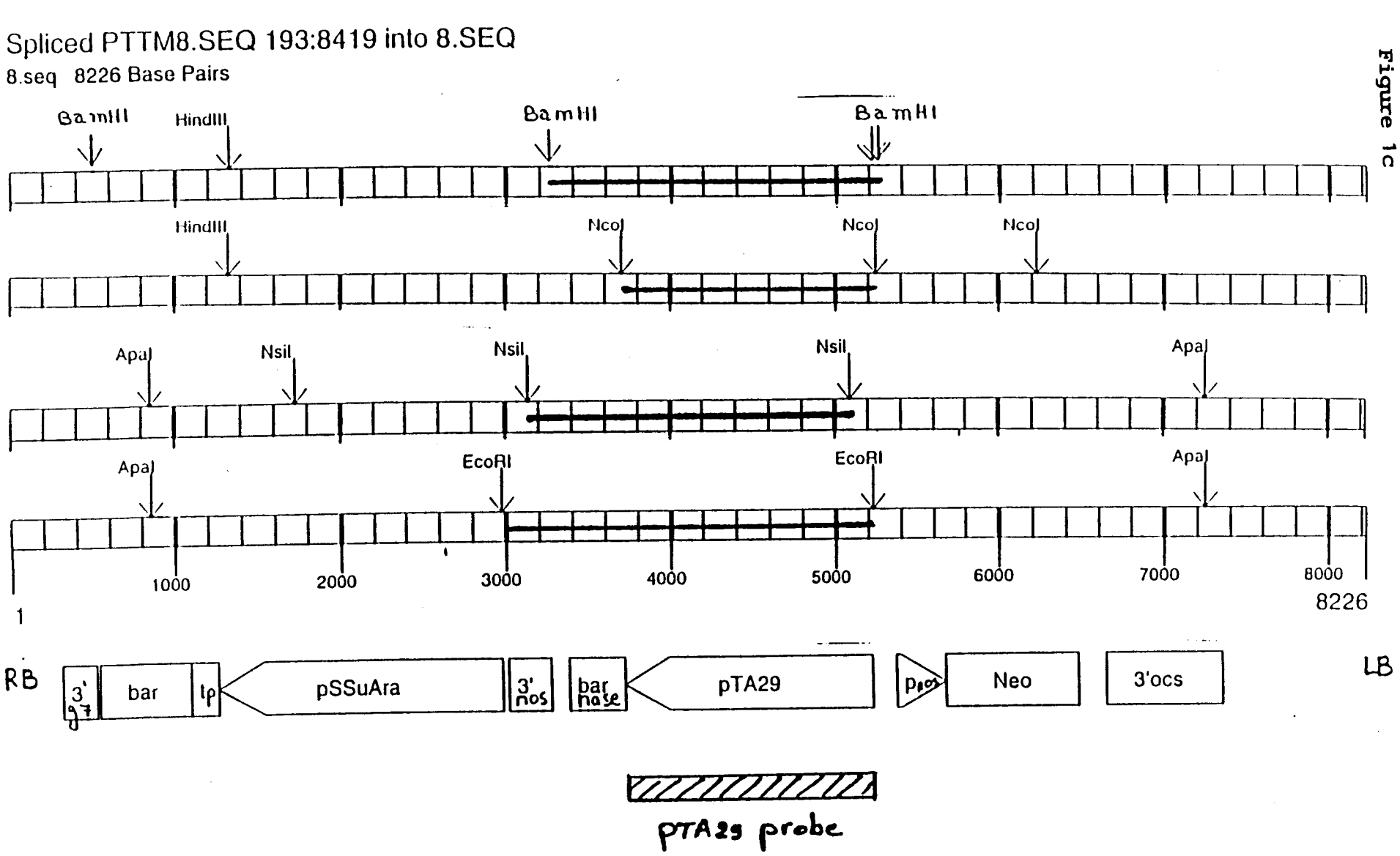
A. BamHI - HindIII
B. NcoI - HindIII
C. ApaI - NsiI
D. ApaI - EcoRI

The localisation of the PSSUAra probe and the expected hybridising fragments are shown

Figure 1b

Bejo: Radicchio rosso with male sterility

Figure 1c

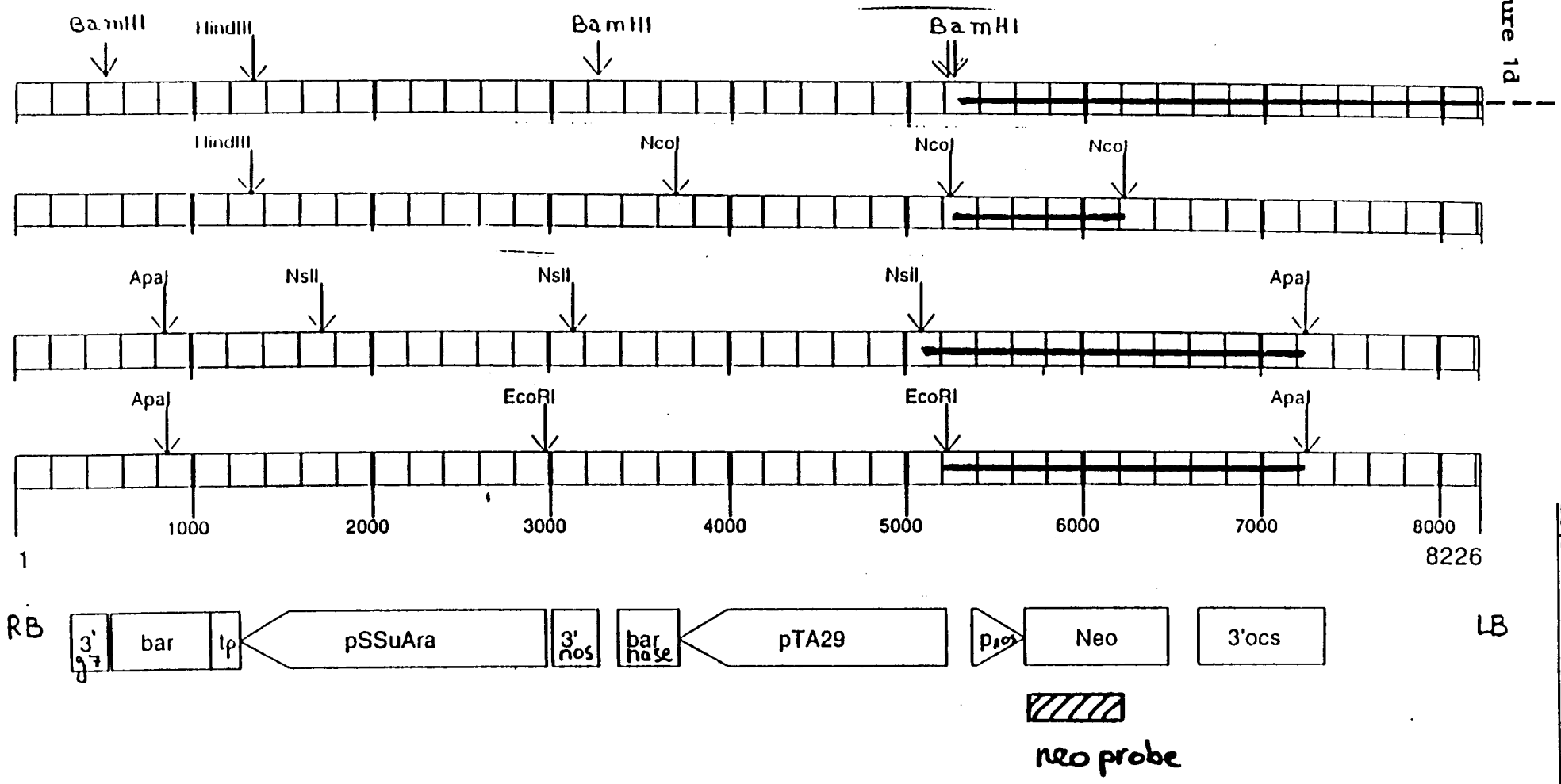


Schematic representation of the DNA fragment between the borders of plasmid pTTM8RE showing the localisation of the different chimeric genes.
The plant DNA was digested with 4 combinations of restriction enzymes (restriction sites shown on the scheme)

A. BamHI - HindIII
B. NcoI - HindIII
C. ApaI - NsiI
D. ApaI - EcoRI

The localisation of the PTA29 probe and the expected hybridising fragments are shown

Figure 1d



Schematic representation of the DNA fragment between the borders of plasmid pTTM8RE showing the localisation of the different chimeric genes.
The plant DNA was digested with 4 combinations of restriction enzymes (restriction sites shown on the scheme)

A. BamHI - HindIII
B. NcoI - HindIII
C. ApaI - NsiI
D. ApaI - EcoRI

The localisation of the neo probe and the expected hybridising fragments are shown

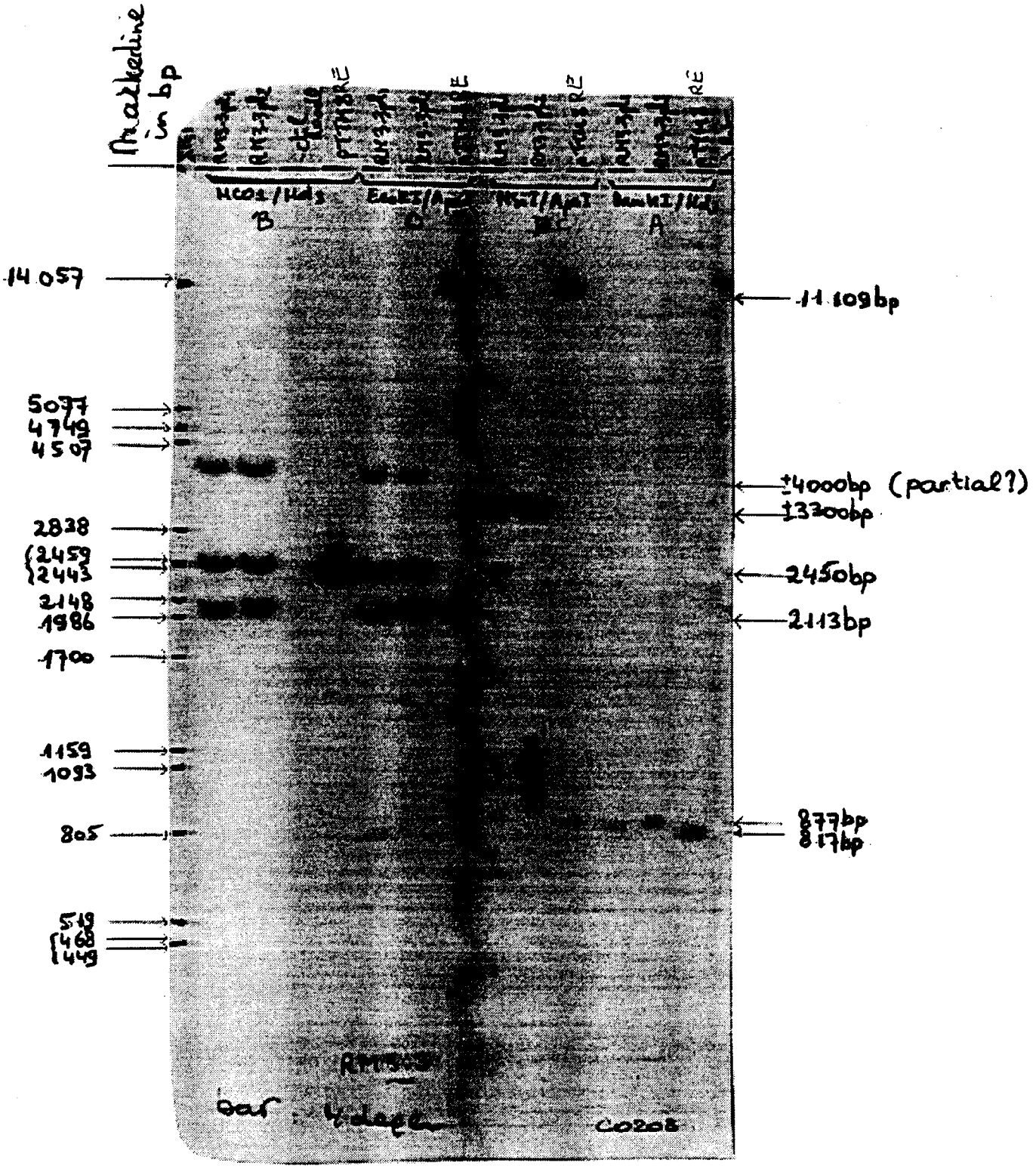


Fig. 2a

Southern blot hybridisation of RM3-3 using a bar probe. The DNA was digested with different restriction enzyme combinations as indicated in fig. 1a. For each digest DNA extracted from 2 individuals was used (pl 1/pl2). DNA extracted from a nontransgenic control plant is included (ctrl) as a negative control and plasmid DNA (pTTM8RE) is added as a positive control.

A. BamHI - HindIII
B. NcoI - HindIII
C. ApaI - NsiI
D. ApaI - EcoRI

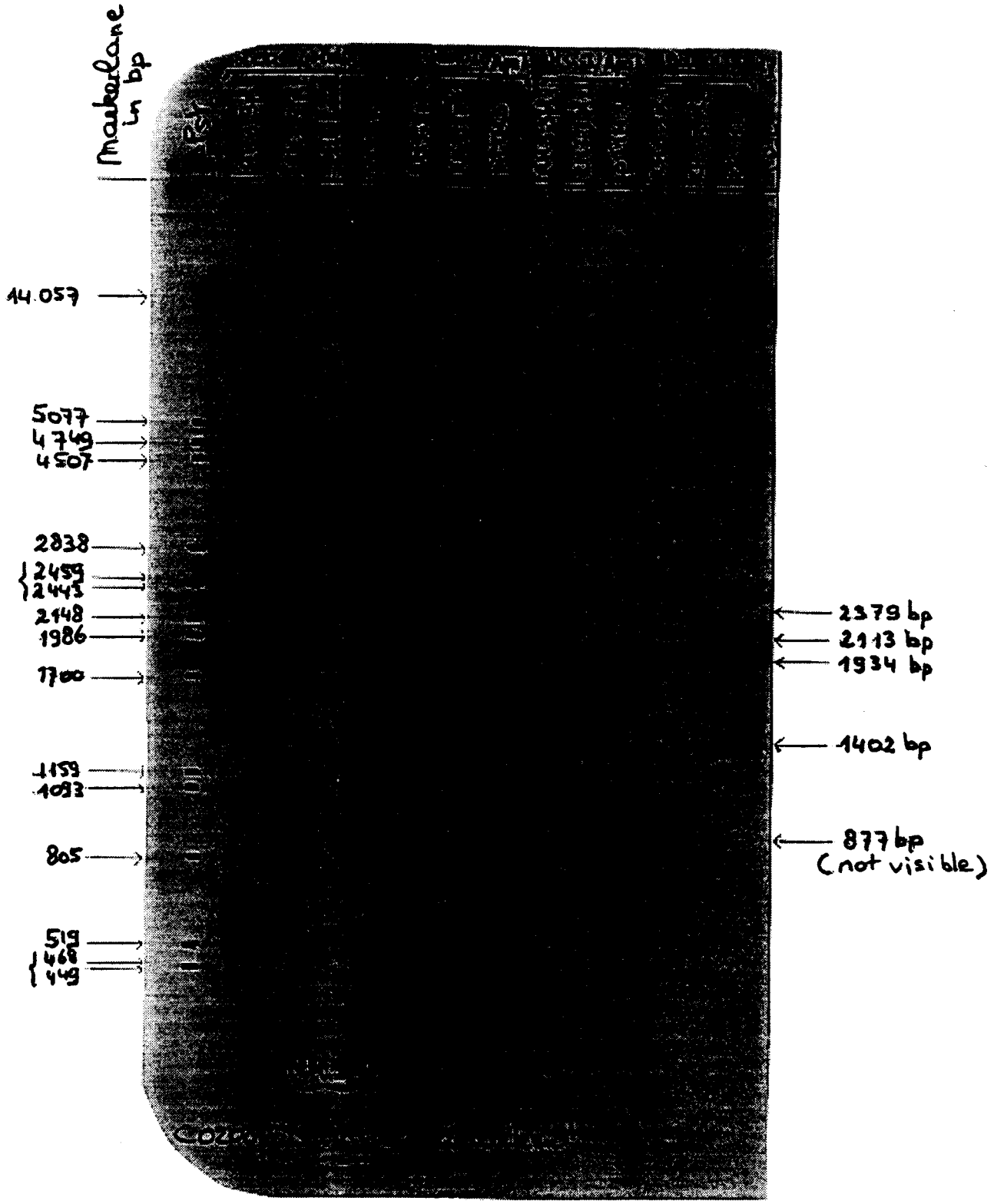


Fig. 2b

Southern blot hybridisation of RM3-3 using a PSSUAra probe. The DNA was digested with different restriction enzyme combinations as indicated in fig. 1b. For each digest DNA extracted from 2 individuals was used (pl 1/pl2). DNA extracted from a nontransgenic control plant is included (ctrl) as a negative control and plasmid DNA (pTTM8RE) is added as a positive control.

A. BamHI - HindIII
B. NcoI - HindIII
C. ApaI - NsiI
D. ApaI - EcoRI

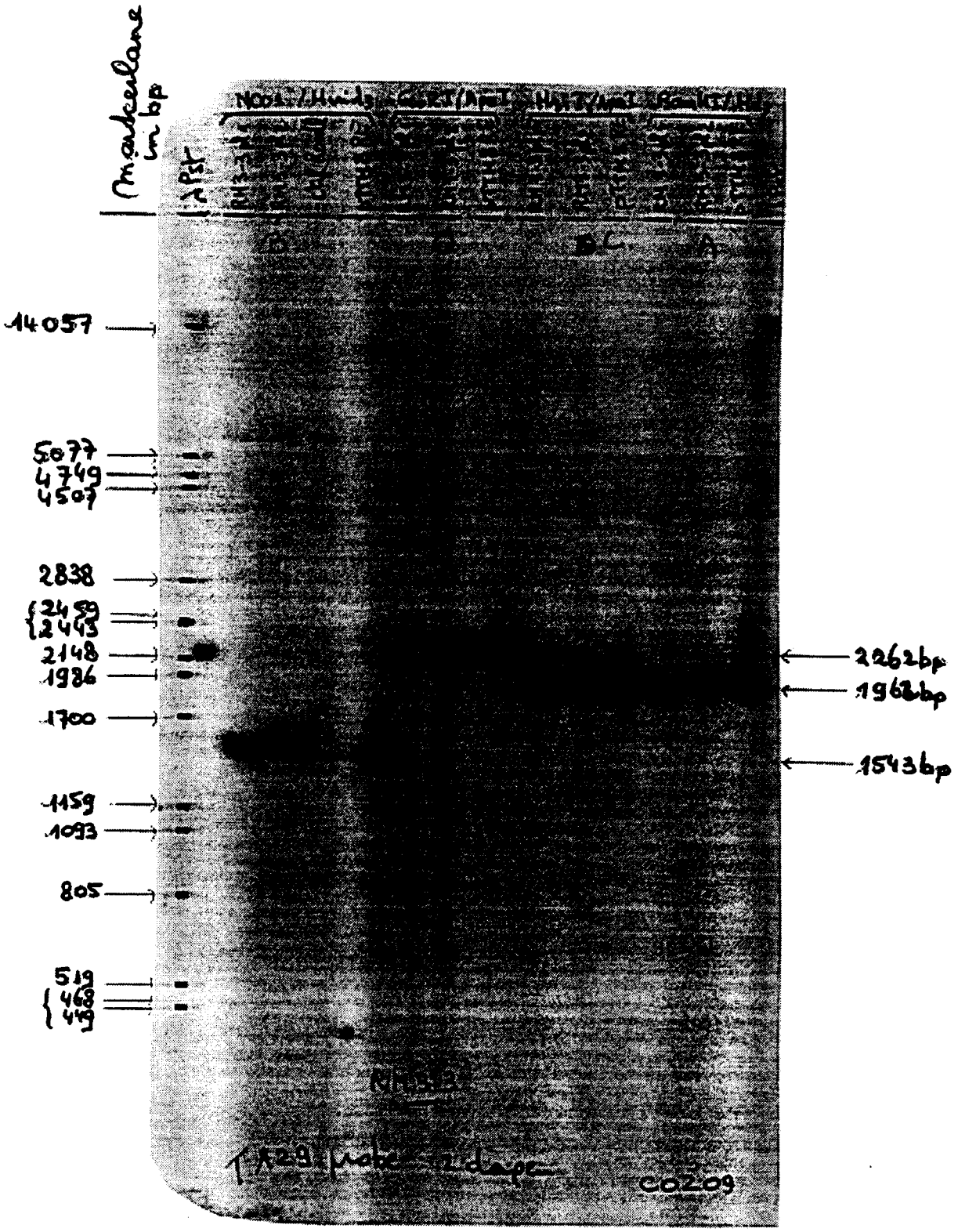


Fig. 2c

Southern blot hybridisation of RM3-3 using a PTA29 probe. The DNA was digested with different restriction enzyme combinations as indicated in fig. 1c. For each digest DNA extracted from 2 individuals was used (pl 1/pl2). DNA extracted from a nontransgenic control plant is included (ctrl) as a negative control and plasmid DNA (pTTM8RE) is added as a positive control.

A. BamHI - HindIII
B. NcoI - HindIII
C. ApaI - NsiI
D. ApaI - EcoRI

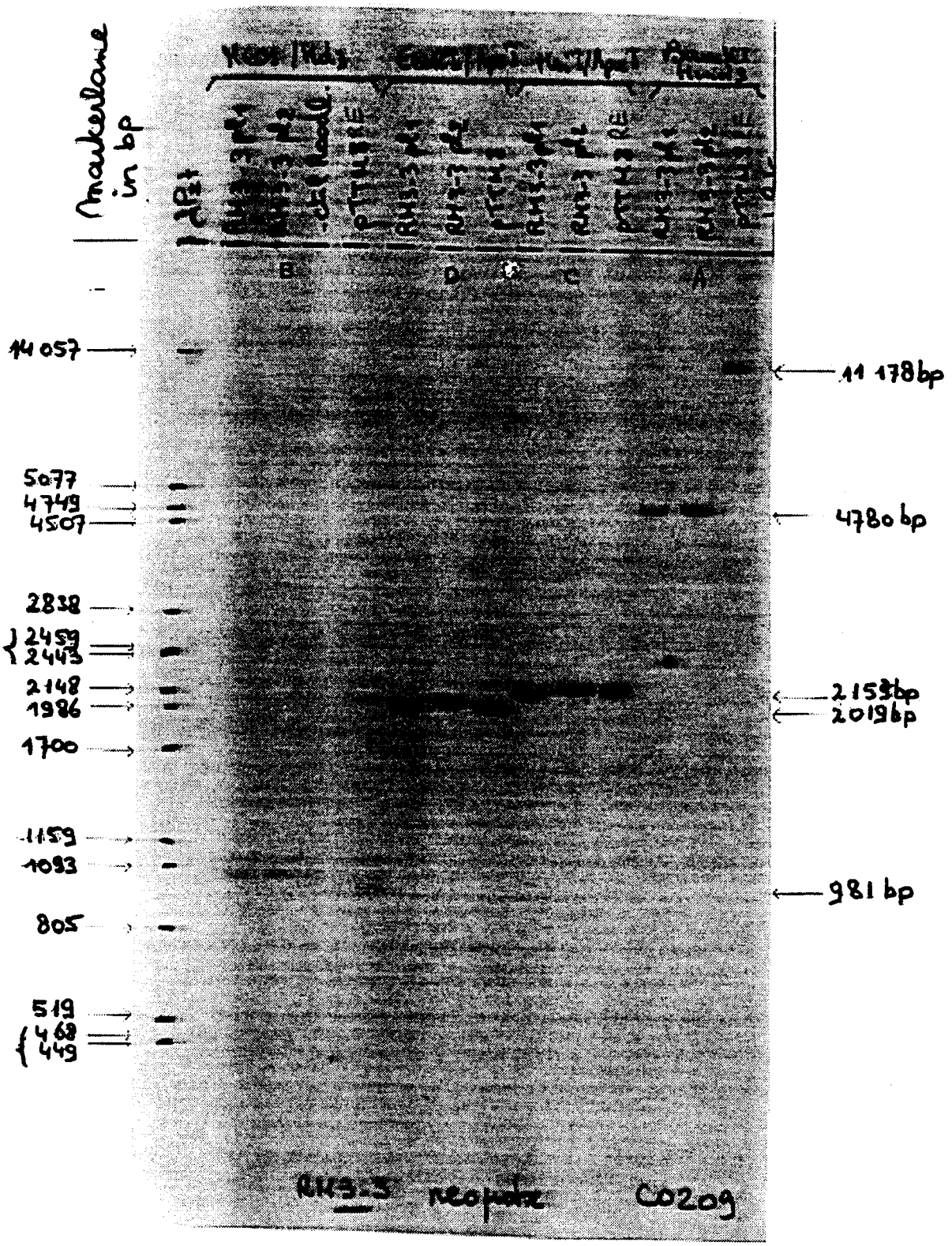


Fig. 2d

Southern blot hybridisation of RM3-3 using a neo probe. The DNA was digested with different restriction enzyme combinations as indicated in fig. 1d. For each digest DNA extracted from 2 individuals was used (pl 1/pl2). DNA extracted from a nontransgenic control plant is included (ctrl) as a negative control and plasmid DNA (pTTM8RE) is added as a positive control.

A. BamHI - HindIII
B. NcoI - HindIII
C. ApaI - NsiI
D. ApaI - EcoRI

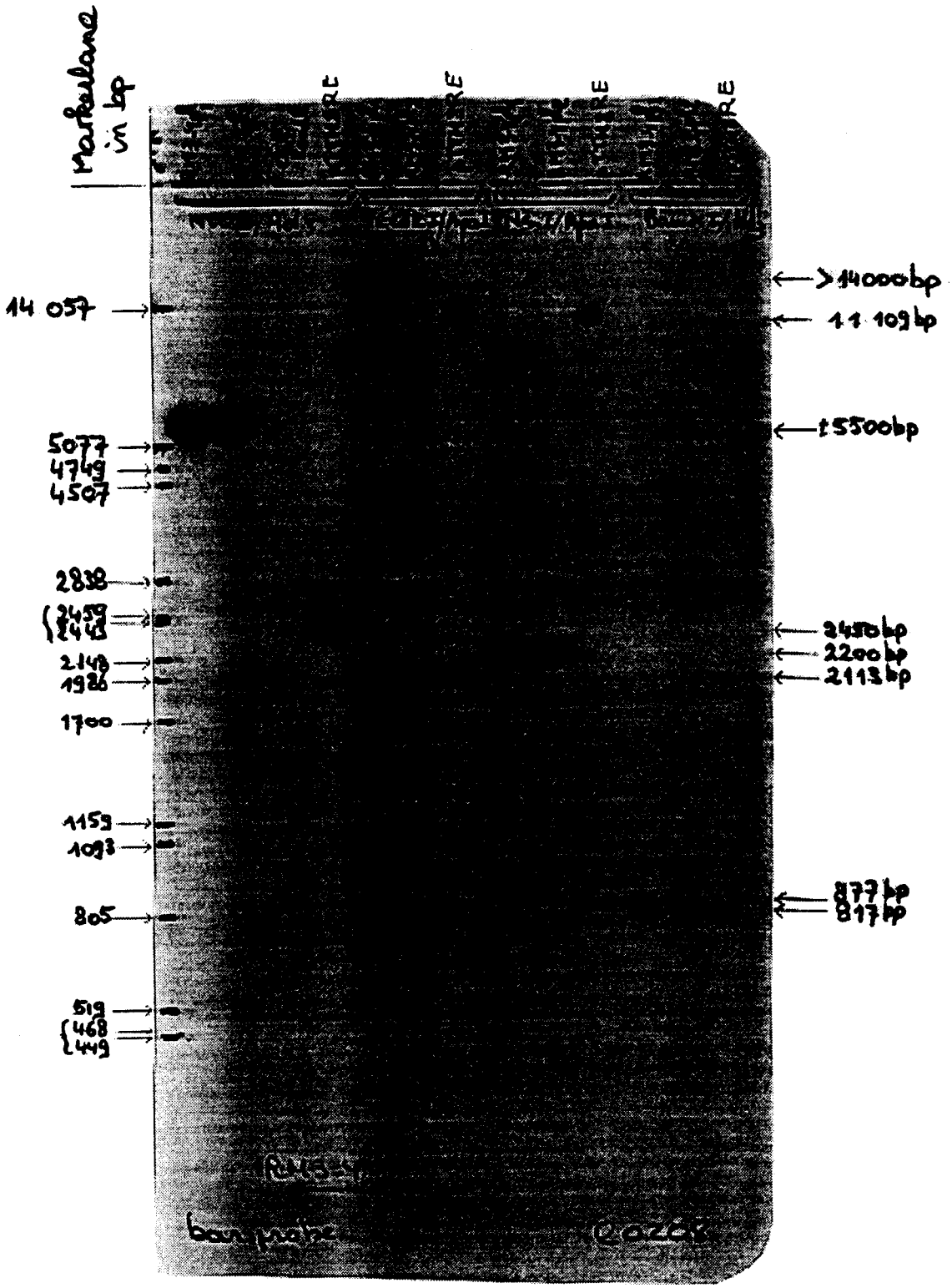


Fig. 3a

Southern blot hybridisation of RM3-4 using a bar probe. The DNA was digested with different restriction enzyme combinations as indicated in fig. 1a. For each digest DNA extracted from 2 individuals was used (pl 1/pl2). DNA extracted from a nontransgenic control plant is included (ctrl) as a negative control and plasmid DNA (pTTM8RE) is added as a positive control.

A. BamHI - HindIII
B. NcoI - HindIII
C. ApaI - NsiI
D. ApaI - EcoRI

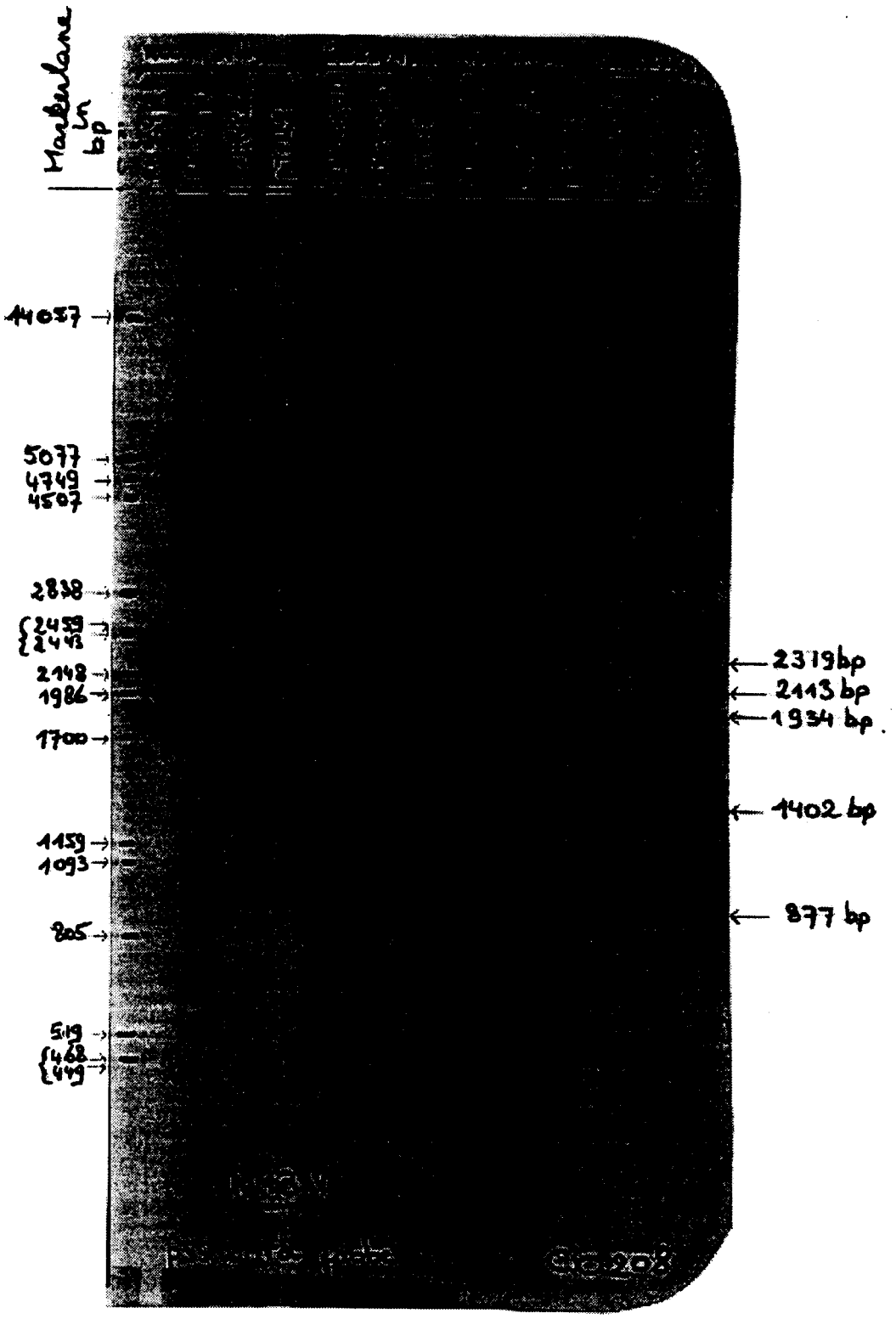


Fig. 3b

Southern blot hybridisation of RM3-4 using a PSSUAra probe. The DNA was digested with different restriction enzyme combinations as indicated in fig. 1b. For each digest DNA extracted from 2 individuals was used (pl 1/pl2). DNA extracted from a nontransgenic control plant is included (ctrl) as a negative control and plasmid DNA (pTTM8RE) is added as a positive control.

A. BamHI - HindIII
B. NcoI - HindIII
C. ApaI - NsiI
D. ApaI - EcoRI

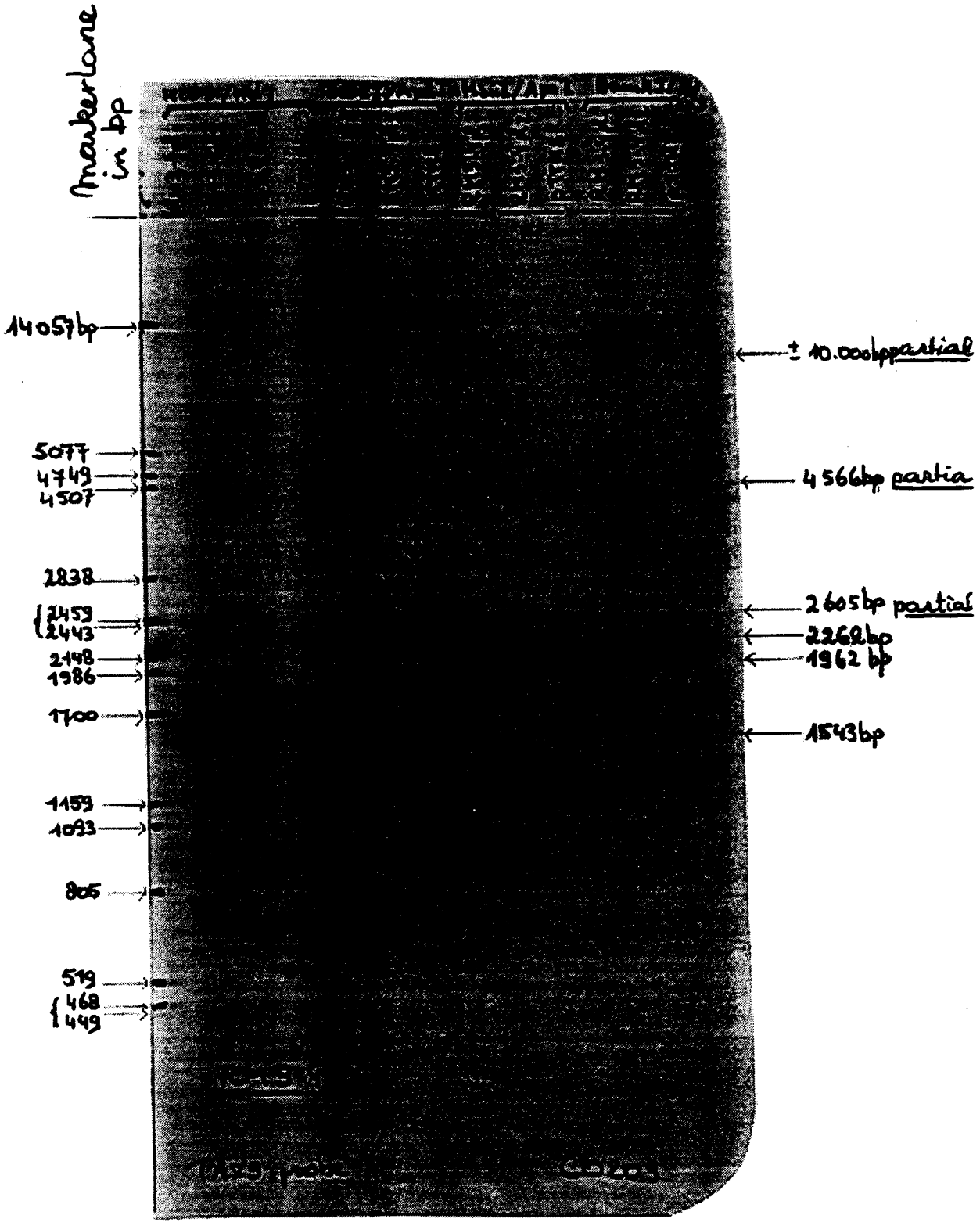


Fig. 3c

Southern blot hybridisation of RM3-4 using a PTA29 probe. The DNA was digested with different restriction enzyme combinations as indicated in fig. 1c. For each digest DNA extracted from 2 individuals was used (pl 1/pl2). DNA extracted from a nontransgenic control plant is included (ctrl) as a negative control and plasmid DNA (pTTM8RE) is added as a positive control.

A. BamHI - HindIII
B. NcoI - HindIII
C. ApaI - NsiI
D. ApaI - EcoRI

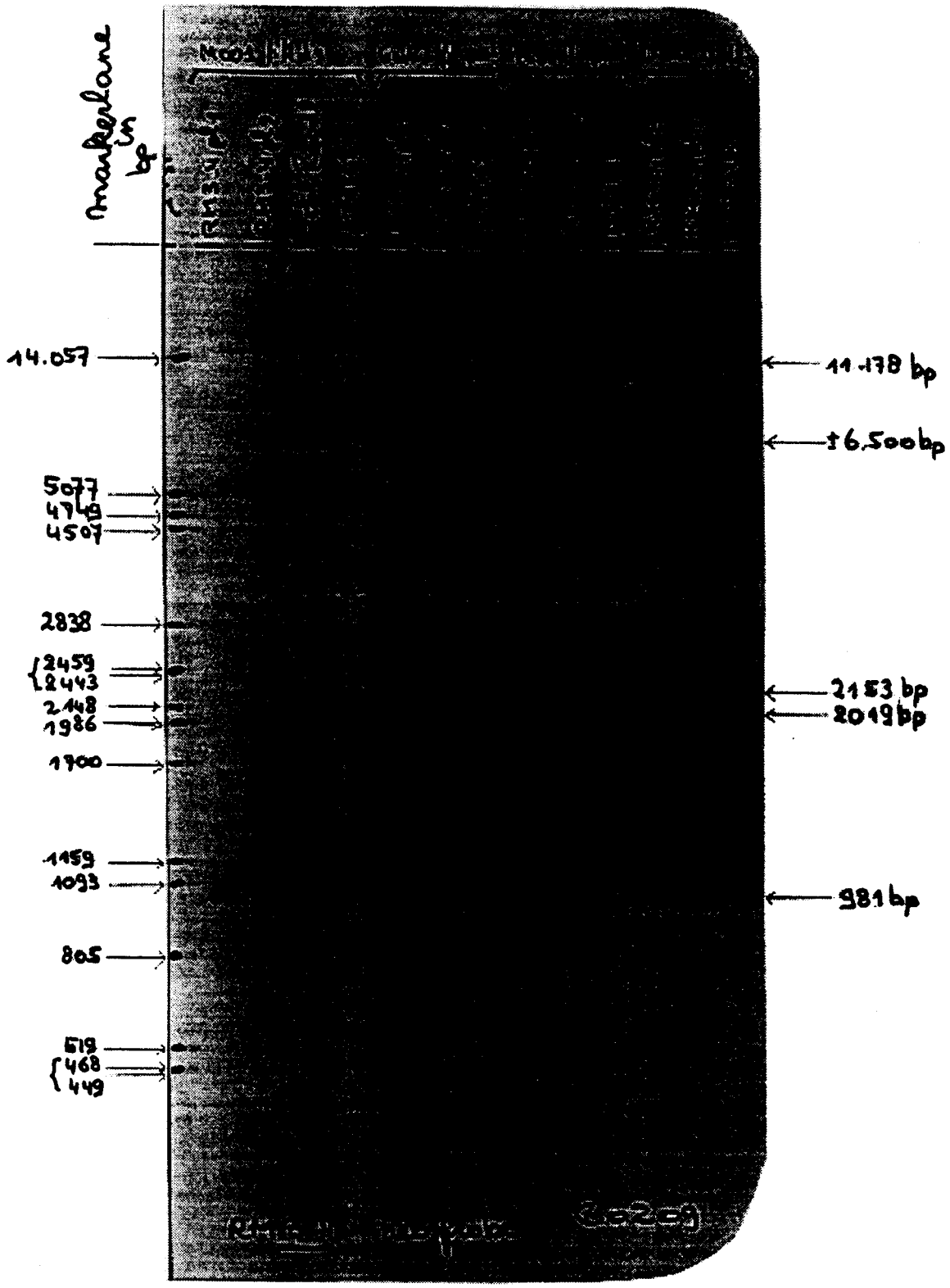


Fig. 3d

Southern blot hybridisation of RM3-4 using a neo probe. The DNA was digested with different restriction enzyme combinations as indicated in fig. 1d. For each digest DNA extracted from 2 individuals was used (pl 1/pl2). DNA extracted from a nontransgenic control plant is included (ctrl) as a negative control and plasmid DNA (pTTM8RE) is added as a positive control.

A. BamHI - HindIII
B. NcoI - HindIII
C. ApaI - NsiI
D. ApaI - EcoRI

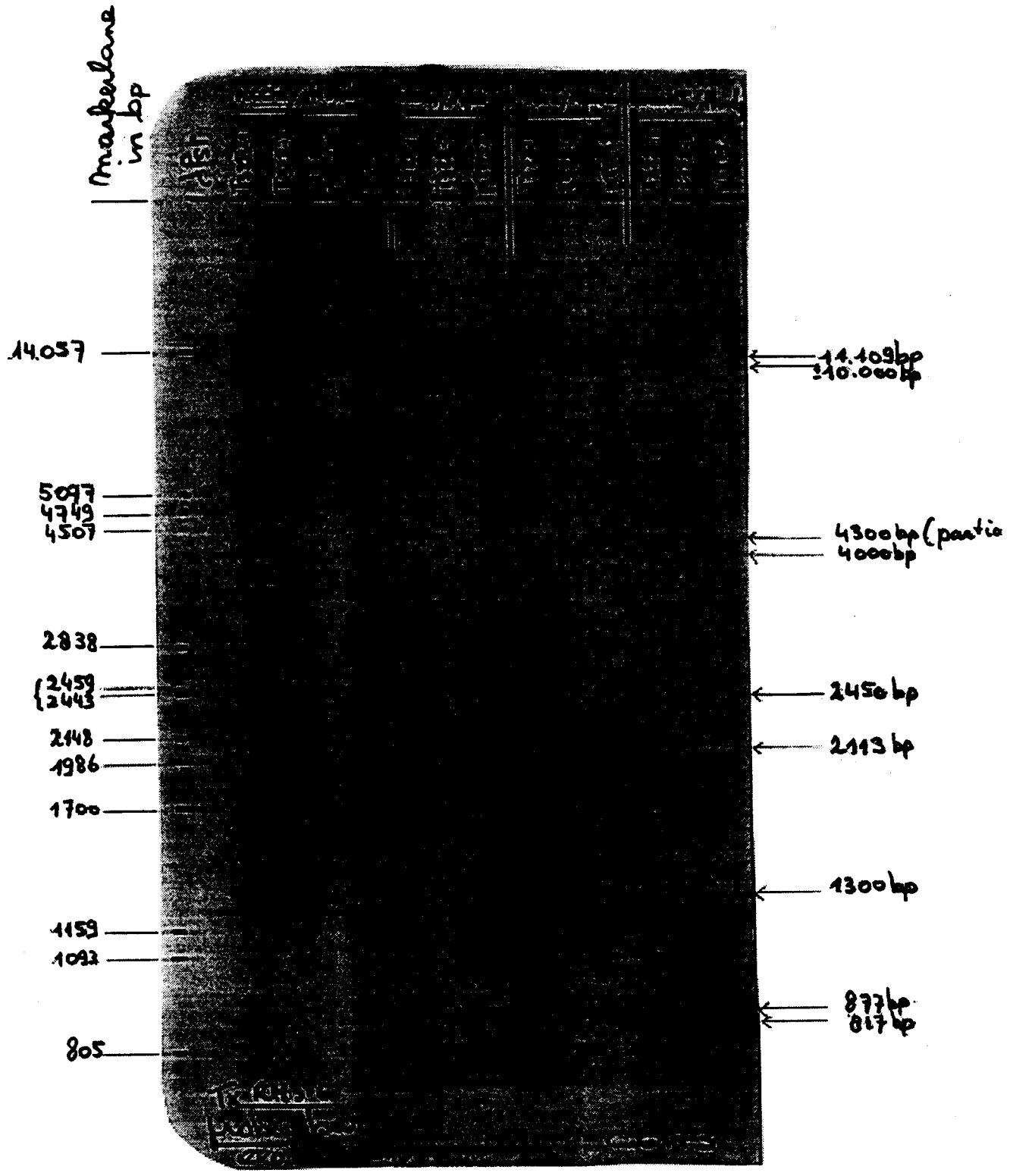


Fig. 4a

Southern blot hybridisation of RM3-6 using a bar probe. The DNA was digested with different restriction enzyme combinations as indicated in fig. 1a. For each digest DNA extracted from 2 individuals was used (pl 1/pl2). DNA extracted from a nontransgenic control plant is included (ctrl) as a negative control and plasmid DNA (pTTM8RE) is added as a positive control.

A. BamHI - HindIII
B. NcoI - HindIII
C. ApaI - NsiI
D. ApaI - EcoRI

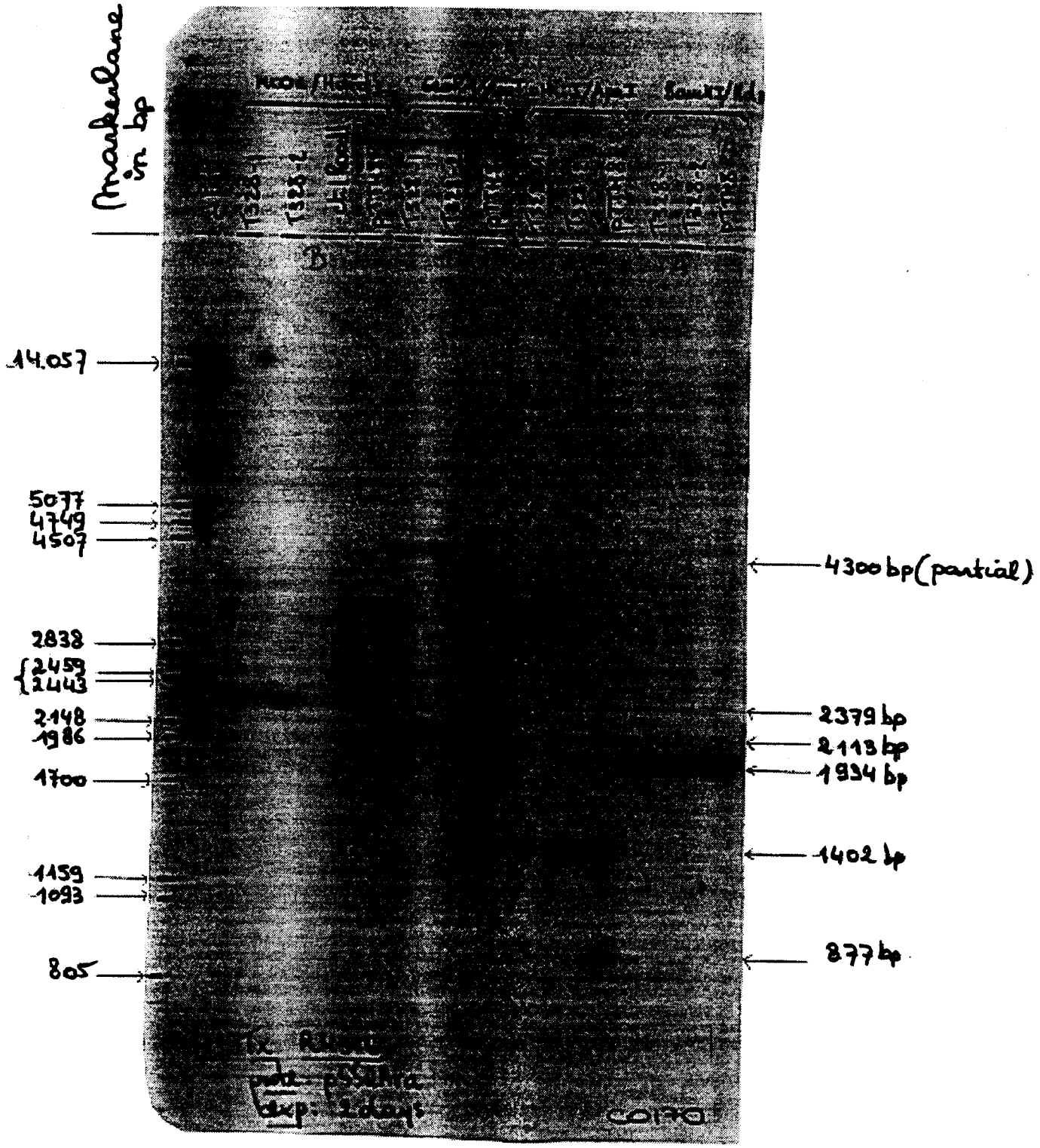


Fig. 4b

Southern blot hybridisation of RM3-6 using a PSSUAra probe. The DNA was digested with different restriction enzyme combinations as indicated in fig. 1b. For each digest DNA extracted from 2 individuals was used (pl 1/pl2). DNA extracted from a nontransgenic control plant is included (ctrl) as a negative control and plasmid DNA (pTTM8RE) is added as a positive control.

A. BamHI - HindIII
B. NcoI - HindIII
C. ApaI - NsiI
D. ApaI - EcoRI

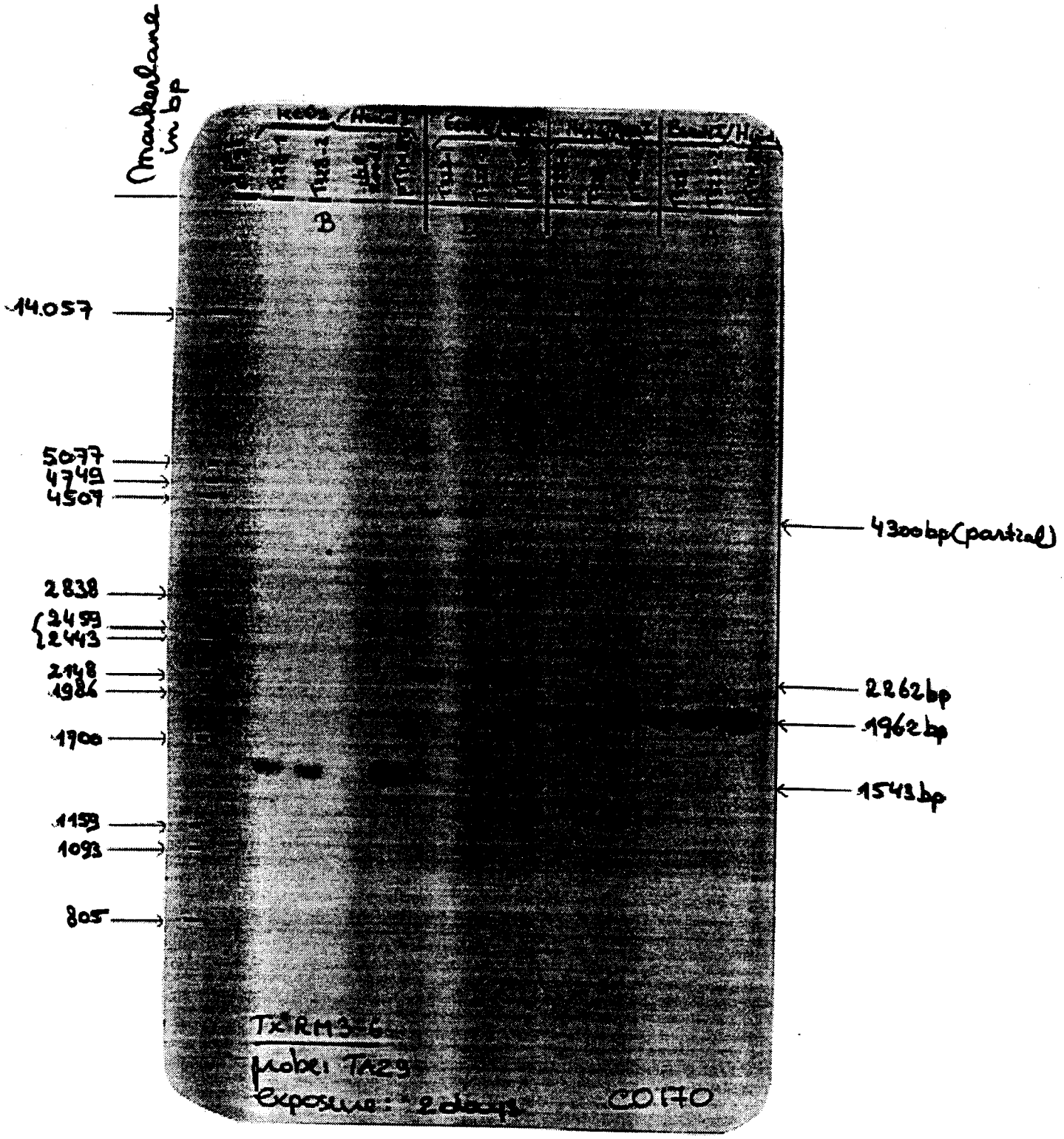


Fig. 4c

Southern blot hybridisation of RM3-6 using a PTA29 probe. The DNA was digested with different restriction enzyme combinations as indicated in fig. 1c. For each digest DNA extracted from 2 individuals was used (pl 1/pl2). DNA extracted from a nontransgenic control plant is included (ctrl) as a negative control and plasmid DNA (pTTM8RE) is added as a positive control.

A. BamHI - HindIII
B. NcoI - HindIII
C. ApaI - NsiI
D. ApaI - EcoRI

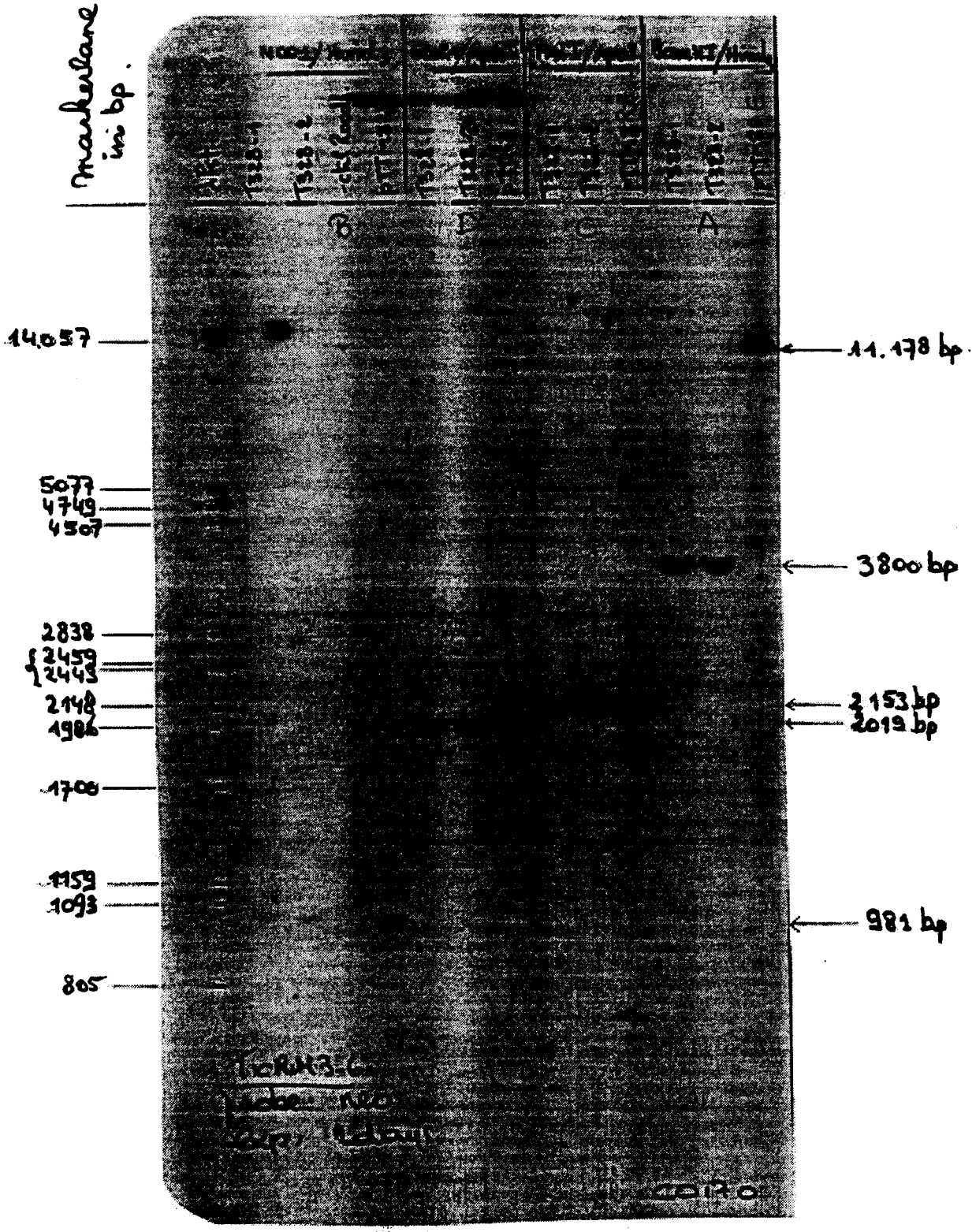


Fig. 4d

Southern blot hybridisation of RM3-6 using a neo probe. The DNA was digested with different restriction enzyme combinations as indicated in fig. 1d. For each digest DNA extracted from 2 individuals was used (pl 1/pl2). DNA extracted from a nontransgenic control plant is included (ctrl) as a negative control and plasmid DNA (pTTM8RE) is added as a positive control.

A. BamHI - HindIII
B. NcoI - HindIII
C. ApaI - NsiI
D. ApaI - EcoRI

2.3 CHARACTERIZATION OF THE T-DNA LEFT BORDER INTEGRATION AT THE T-DNA TERMINAL REPEATS BY PCR

Responsible : C. Opsomer, Researcher

2.3.1 Goals of the experiment

The aim of the experiment is to demonstrate that the T-DNA integration is limited by the left border repeat.

2.3.2 Plant material

Molecular analysis has been performed on radicchio rosso plants carrying the male sterility gene.

2.3.3 Methods

Three different primers were used for left border PCR analysis. Primers 74 & 75 are homologous with internal sequences of the T-DNA near the left border, primer 76 is homologous to plasmid sequences outside of the T-DNA, beyond the left border. Amplification using primers 74 & 75 will be positive as long as intact T-DNA copies are integrated, amplification using probes 74 & 76 will be negative except when imperfect integration has occurred and sequences beyond the left border are integrated.

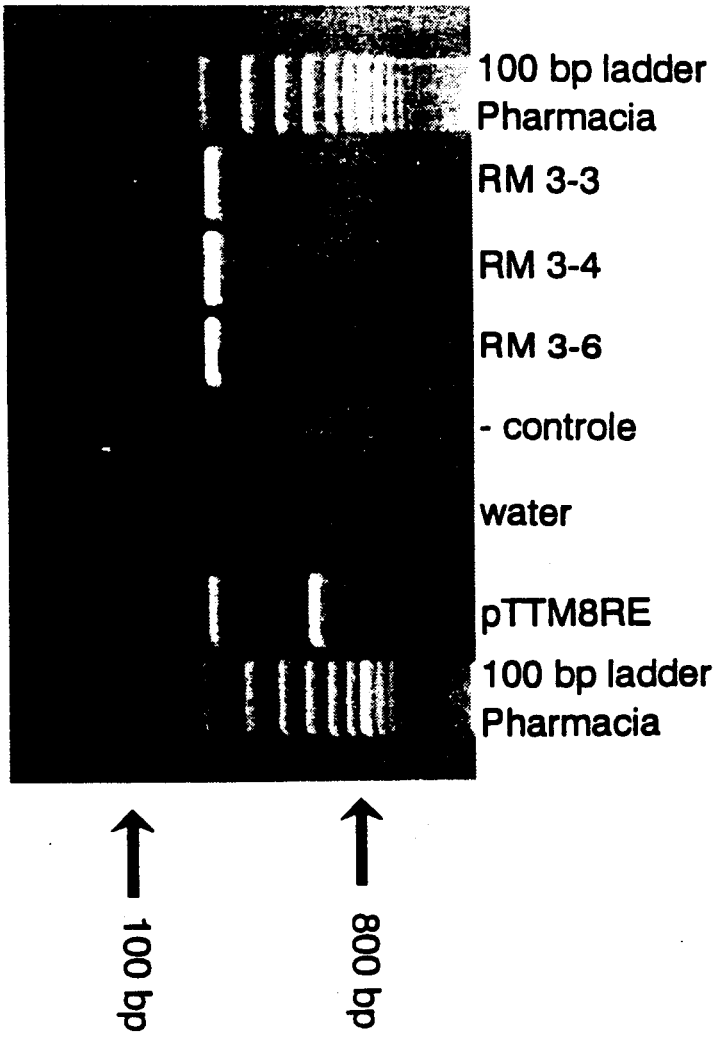
2.3.4 Results and conclusion

The results in the table indicate that for none of the transgenic plants described imperfect integration has occurred or sequences beyond the left border are integrated.

Transformant		External fragment MDB 74-76 (523bp)	Internal fragment MDB 74-75 (212bp)
T122	RM3-3	-	+
T123	RM3-4	-	+
T124	RM3-6	-	+

Position of the PCR primers to the Left Border

- position LB : from base 8480 - 8504
- position primer MDB 74 : from base 8040 -> 8058
- position primer MDB 75 : from base 8231 <- 8251
- position primer MDB 76 : from base 8542 <- 8562



2.4 EVALUATION OF THE EXPRESSION OF THE INTRODUCED GENES AND ANALYSIS OF THE POTENTIAL OCCURENCE OF CRYPTIC GENE EXPRESSION

Responsible : M. De Beuckeleer, Senior Researcher

2.4.1 Goals of the experiment

To demonstrate the expression of introduced transgenes in the male sterile progenies and to analyze the potential occurrence of cryptic gene expression.

2.4.2 Plant material

Molecular analysis has been performed on plants carrying the male sterility construct. Non-transgenic *Radicchio rosso* plants have been used as negative control.

Table 1: Plant material

Lines	Generation	Leaves Plant N° (Greenhouse)	Head and Roots Plant N° (Field)
RM 3-3	Tx	T13	T326
RM 3-4	Tx	T14	T327
RM 3-6	Tx	T17	T328
Control *Radicchio rosso*		T201	T325

2.3.3 Results and conclusions

Transgene expression

Bar
The detected bar mRNA levels in leaves vary between ±12.5 and ±75pg/µg total RNA. The bar mRNA levels in head tissue vary between ±10 and ±12.5pg/µg total RNA. In some root samples we detect low levels of bar mRNA (see tables 2.1, 2.2, and 2.3). The hybridization results are also presented in figure 1. Note that due to the different *bar* mRNA expression levels, the blots were exposed to X-Ray film for a different length of time. Another set of blots, with the normal dilution series ranging from 0.5pg up to 32pg, were also hybridized. The results of this set of blots was used to determine the detection limit stated in tables 2.1, 2.2, and 2.3.

Barnase
We couldn't detect any barnase mRNA signals whatsoever (detection limit 0.1pg/µg total RNA, see tables 2.1, 2.2, and 2.3. This was expected since the barnase is driven by the tapetum specific PTA29 promotor.

Neo
The detected neo mRNA levels vary between 0.1pg and 0.2pg/µg total leaf RNA and 0.4pg/µg total head RNA. In some root RNA samples we detect 1pg mRNA/µg total root RNA (see tables 2.1, 2.2, and 2.3).
The hybridization results are also presented in figure 2.

Cryptic gene expression

Bar* - *Barstar* - *Neo
Using sense RNA probes of the specified transgenes, we were unable to detect any hybridization signals whatsoever. Detection limits are indicated in tables 2.1, 2.2, and 2.3.

Right Border T-DNA (MDB168-169) - PSsuAra (MDB170-171)

The in vitro synthesized SP6 probes of both fragments were used together in a hybridization experiment (the same was done with the T7 probes). We were unable to detect any hybridization signals with both set of probes. Detection limits are indicated in tables 2.1, 2.2, and 2.3.

Left Border T-DNA (MDB159-160) - PTA29-PNos (MDB172-173)

Both SP6 probes were used together in the hybridization (the same was done with the T7 probes). We were unable to detect any hybridization signals. Detection limits are indicated in see tables 2.1, 2.2, and 2.3.
The hybridization results from MDB172-MDB173 and MDB159-MDB160 SP6 probes are presented in figure 3.

Table 2.1: Summary hybridization results *Radiccio rosso* transformants - Leaf samples

	Transgene expression (pg/µg total RNA)			Cryptic gene expression						
	bar	neo	barnase	bar	neo	barnase	MDB168-169 + MDB170-171		MDB159-160 + MDB172-173	
Total RNA	pGembar/SP6	pFM146/SP6	pVE113/SP6	pGembar/T7	pFM146/ T7	pVE113/T7	SP6	T7	SP6	T7
RM3-3 - T13 - A	50	0.2	-	-	-	-	-	-	-	-
RM3-3 - T13 - B	75	0.2	-	-	-	-	-	-	-	-
RM3-4 - T14 - A	50	0.2	-	-	-	-	-	-	-	-
RM3-4 - T14 - B	12.5	0.2	-	-	-	-	-	-	-	-
RM3-6 - T17 - A	50	0.1	-	-	-	-	-	-	-	-
RM3-6 - T17 - B	50	0.2	-	-	-	-	-	-	-	-
Control - T201	-	-	-	-	-	-	-	-	-	-
Detection limit (pg/µg total RNA)	0.1	0.1	0.1	0.2	0.1	0.2	0.1/0.2	0.1/0.1	0.1/0.1	0.1/0.2

-: no signal detectable

Table 2.2 : Summary hybridization results *Radiccio rosso* transformants - Head samples

	Transgene expression (pg/µg total RNA)			Cryptic gene expression						
	bar	neo	barnase	bar	neo	barnase	MDB168-169 + MDB170-171		MDB159-160 + MDB172-173	
Total RNA	pGembar/SP6	pFM146/SP6	pVE113/SP6	pGembar/T7	pFM146/T7	pVE113/T7	SP6	T7	SP6	T7
RM3-3 - T326 - A	12.5	0.4	-	-	-	-	-	-	-	-
RM3-3 - T326 - B	10	0.4	-	-	-	-	-	-	-	-
RM3-4 - T327 - A	10	0.4	-	-	-	-	-	-	-	
RM3-4 - T327 - B	10	0.4	-	-	-	-	-	-	-	-
RM3-6 - T328 - A	10	0.4	-	-	-	-	-	-	-	-
RM3-6 - T328 - B	12.5	0.4	-	-	-	-	-	-	-	-
Control - T325	-	-	-	-	-	-	-	-	-	-
Detection limit (pg/µg total RNA)	0.1	0.1	0.1	0.2	0.1	0.2	0.1/0.2	0.1/0.1	0.1/0.1	0.1/0.2

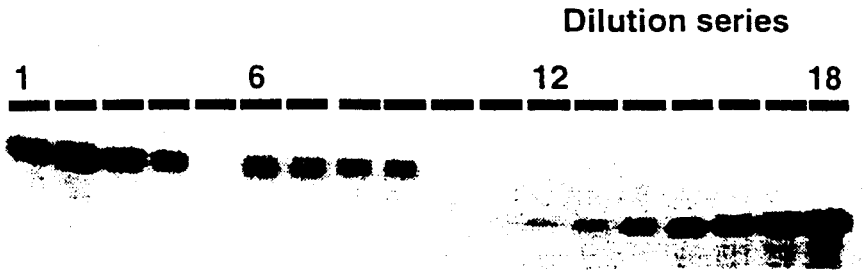
-: no signal detectable

Table 2.3: Summary hybridization results *Radiccio rosso* transformants - Root samples

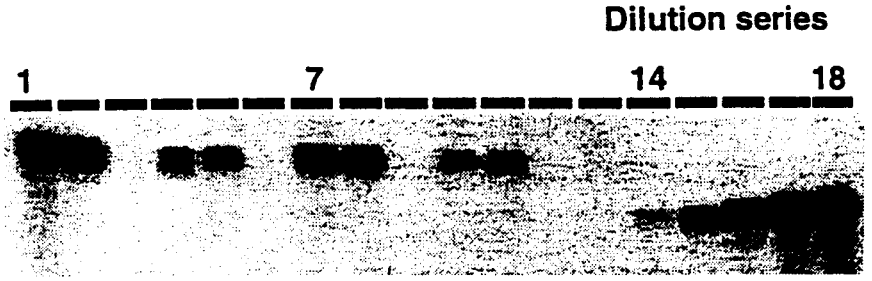
	Transgene expression (pg/µg total RNA)			Cryptic gene expression						
	bar	neo	barnase	bar	neo	barnase	MDB168-169 + MDB170-171		MDB159-160 + MDB172-173	
Total RNA	pGembar/SP6	pFM146/SP6	pVE113/SP6	pGembar/T7	pFM146/T7	pVE113/T7	SP6	T7	SP6	T7
RM3-3 - T326 - A	0.1	-	-	-	-	-	-	-	-	-
RM3-3 - T326 - B	-	-	-	-	-	-	-	-	-	-
RM3-4 - T327 - A	0.2	1	-	-	-	-	-	-	-	-
RM3-4 - T327 - B	-	-	-	-	-	-	-	-	-	-
RM3-6 - T328 - A	-	1	-	-	-	-	-	-	-	-
RM3-6 - T328 - B	2	1	-	-	-	-	-	-	-	-
Control - T325	-	-	-	-	-	-	-	-	-	-
Detection limit (pg/µg total RNA)	0.1	0.1	0.1	0.2	0.1	0.2	0.1/0.2	0.1/0.1	0.1/0.1	0.1/0.2

-: no signal detectable

Transgene expression



Loading sequence: see Gel A (page 123)



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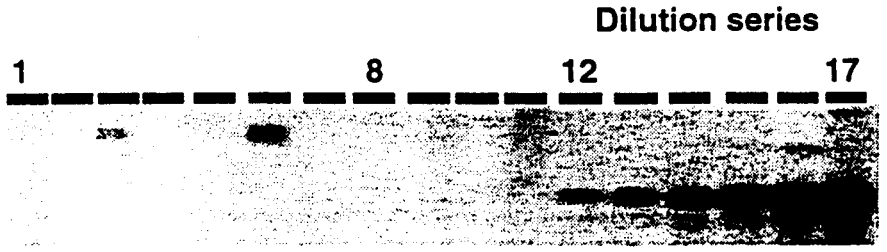
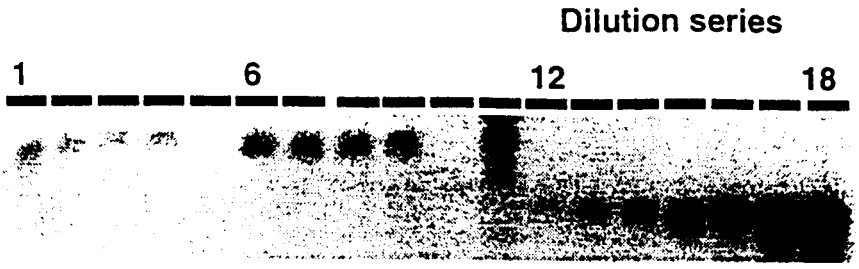


Loading sequence: see Gel C (page 123)

Figure 1 : *Bar* mRNA hybridization results

Transgene expression



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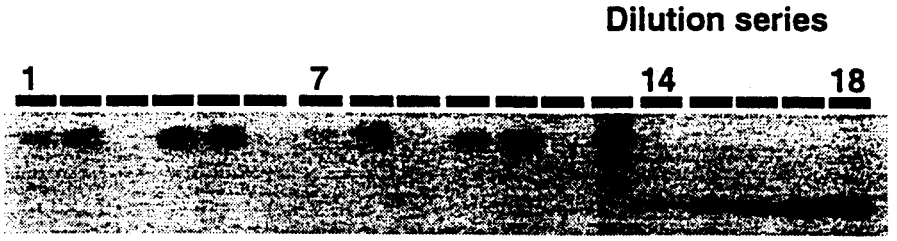

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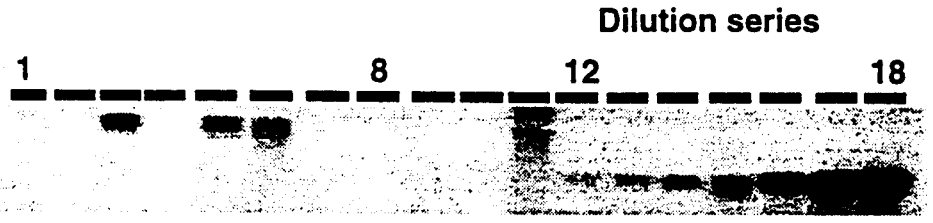

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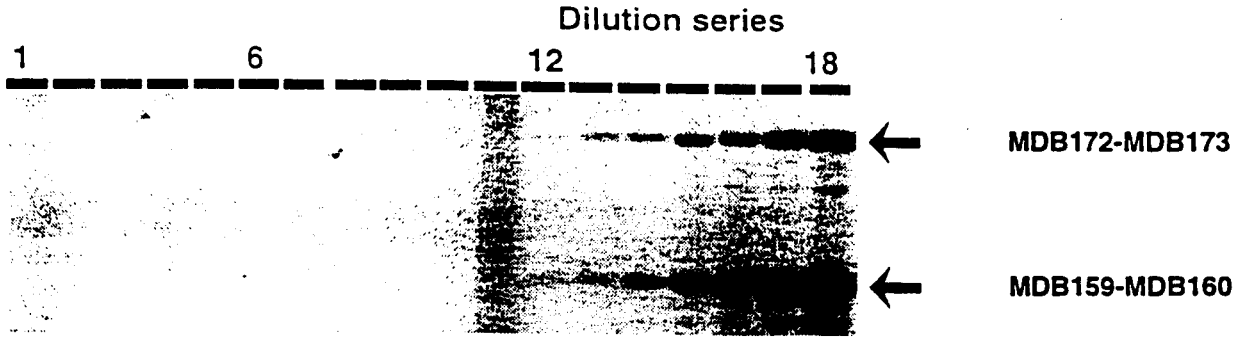
Figure 2 : *Neo* mRNA hybridization results

Cryptic gene expression



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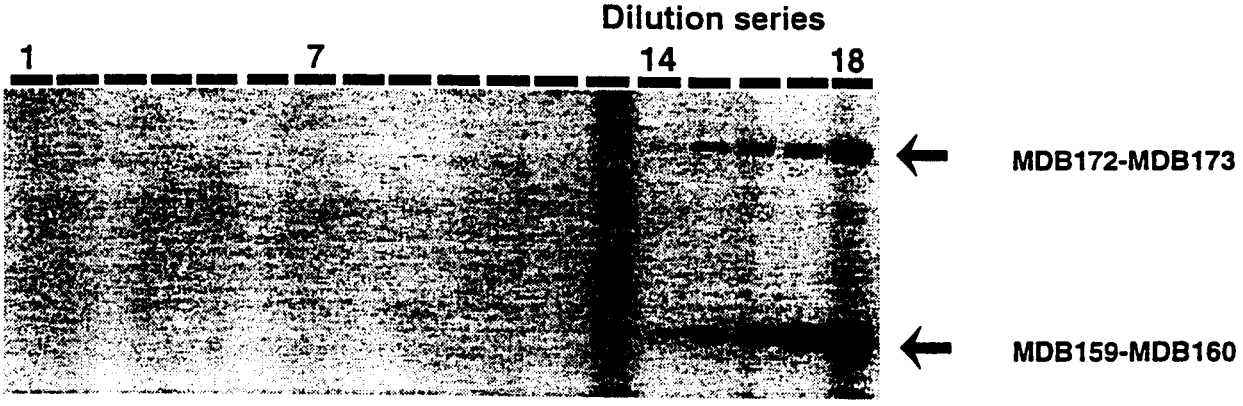

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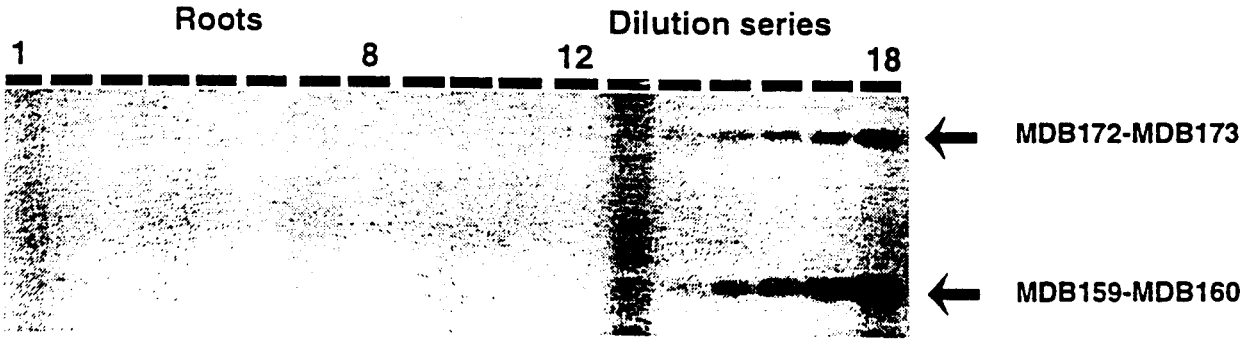


Loading sequence: see Gel C (page 123)

Figure 3 : MDB172-MDB173 and MDB159-MDB160 SP6 hybridization results

LOADINGSEQUENCES

Gel A:	Line	Plant N°	Tissue
1.	RM3-3	T13 - A	Leaf
2.	RM3-3	T13 - B	Leaf
3.	RM3-4	T14 - A	Leaf
4.	RM3-4	T14 - B	Leaf
5.	Control	T201	Leaf
6.	RM3-3	T326 - A	Head
7.	RM3-3	T326 - B	Head
8.	RM3-4	T327 - A	Head
9.	RM3-4	T327 - B	Head
10	Control	T325	Head
11.	MW marker (0.16 - 1.77 Kb RNA ladder, Life Technologies Inc.)		
12.-18.	Control RNA dilution series (*in vitro* synthesized RNA complementary to the probe used): 0.5pg - 1pg - 2pg - 4pg - 8pg - 16pg and 32pg. In the case of the *bar*-T7 transcript: 8pg - 16pg - 32pg - 64pg - 128pg - 256pg and 512pg. These control RNA samples are complemented with 5µg control leaf RNA.		

Gel B:	Line	Plant N°	Tissue
1.	RM3-6	T17 - A	Leaf
2.	RM3-6	T17 - B	Leaf
3.	Control	T201	Leaf
4.	RM3-6	T328 - A	Head
5.	RM3-6	T328 - B	Head
6.	Control	T325	Head
7.-12.	(samples wich do not relate to this demand)		
13.	MW marker (0.16 - 1.77 Kb RNA ladder, Life Technologies Inc.)		
14.-18.	Control RNA dilution series (*in vitro* synthesized RNA complementary to the probe used): 0.5pg - 1pg - 2pg - 4pg - 8pg. In the case of the *bar*-T7 transcript: 8pg - 32pg - 128pg - 256pg and 512pg. These control RNA samples are complemented with 5µg control leaf RNA.		

Gel C:	Line	Plant N°	Tissue
1.	RM3-3	T326 - A	Roots
2.	RM3-3	T326 - B	Roots
3.	RM3-4	T327 - A	Roots
4.	RM3-4	T327 - B	Roots
5.	RM3-6	T328 - A	Roots
6.	RM3-6	T328 - B	Roots
7.	Control	T325	Roots
8.-10.	(samples wich do not relate to this demand)		
11.	MW marker (0.16 - 1.77 Kb RNA ladder, Life Technologies Inc.)		
12.-18.	Control RNA dilution series (*in vitro* synthesized RNA complementary to the probe used): 0.5pg - 1pg - 2pg - 4pg - 8pg - 16pg and 32pg. In the case of the *bar*-T7 transcript: 8pg - 16pg - 32pg - 64pg - 128pg and 256pg (17 lanes in total). These control RNA samples are complemented with 5µg control leaf RNA.		

2.5 EVALUATION OF THE EXPRESSION AND INHERITANCE OF THE NEO GENE BY A NPTII ENZYMATIC ASSAY

Responsible : A. Van Vliet, Researcher

2.5.1 Goals of the experiment

The NPTII enzymatic assay was used to evaluate the expression and inheritance of the *neo* gene of a number of generations.

2.5.2 Plant material

Analysis has been performed on plants carrying the male sterility construct. Non-transgenic Radicchio rosso plants have been used as negative control.

2.5.3 Methods

The assay is based on the electrophoretic separation of the NPTII protein from plant endogenous phosphorylating enzymes by a non-denaturing gel electrophoresis and detection of its enzymatic activity by in situ phosphorylation of kanamycin in the presence of radioactively labeled ATP. Both kanamycin and (γ-^{32}P)ATP acting as substrates are embedded in an agarose gel placed on the polyacrylamide gel containing the separated proteins. After the enzymatic reaction, the phosphorylated kanamycin is transferred to P81 phosphocellulose ion exchange paper and the radiolabelled kanamycin is visualized by autoradiography. An overview of the different steps in the enzymatic assay are described. Details of the different steps and solutions used are described in the mentioned references (Reiss et al., 1984. The EMBO Journal, 3, 3317-3322. Reynaerts et al.,1987. In : Gelvin, S.B., Schilperoort, R.A. (eds). Plant molecular biology manual. Kluwer, Dordrecht, The Netherlands, Sect. A9, p 1-16).

Extraction of plant material

100 mg fresh plant tissue is homogenized in extraction buffer on ice and transferred into a microcentrifuge tube. The cell debris is pelleted by centrifugation in an Eppendorf centrifuge. The supernatans is transferred to another tube and the protein concentration in the crude extract is determined by Biorad assay with bovine serum albumine as standard. Protein concentration in all samples is adjusted with extraction buffer.

Enzymatic assay

Loading buffer is added to an aliquot of 100 µg total protein which is separated by electrophoresis through a 10% non-denaturing

polyacrylamide gel. The gel is washed in distilled water and equilibrated in reaction buffer. The gel is transferred onto a glass plate and overlaid with a 1% agarose gel containing 30 µg/ml kanamycin sulphate and 100 µCi (γ-^{32}P)ATP in reaction buffer. After incubation, the gel sandwich is covered with a sheet of Whatman P81 phosphocellulose paper. Upon incubation, the phosphorylated kanamycin is bound to the P81 paper. Subsequently, the P81 paper is washed, dried and exposed to a X-ray film.

2.5.4 Results and conclusion

		heads			leaves		
		mg[a] prot/mL . extract	µg NPTII /mL extract	µg NPTII/mg prot	mg[a] prot/mL extract	µg NPTII /mL extract	µg NPTII/mg prot
RM3-3 To T122	a				0.4	0.02	0.05
	b				0.5	0.02	0.04
RM3-3 Tx T326, T13	a	0.6	0.18	0.29	0.6	0.03	0.05
	b	0.6	0.09	0.14	0.6	0.03	0.05
RM3-4 To T123	a				0.5	0.02	0.04
	b				0.4	0.01	0.02
RM3-4 Tx T327, T14	a	0.6	0.12	0.23	0.5	0.04	0.09
	b	0.6	0.08	0.12	0.5	0.02	0.04
RM3-6 To T124	a				0.1	0.04	0.47
	b				0.4	0.02	0.05
RM3-6 Tx T328, T17	a	0.6	0.11	0.20	0.4	0.01	0.04
	b	0.5	0.12	0.22	0.6	0.02	0.03
CTRL red hearted chicory T325, T201	a	0.6	0.00	0.00	0.6	0.00	0.00
	b	0.6	0.00	0.00	0.5	0.00	0.00

a) protein concentration is measured with the microassay procedure of Biorad (Bradford method) with bovine serum albumin (BSA) as standard.

The numbers above indicate that the *neo* gene is inherited and stably expressed over a number of generations.

2.6 QUANTIFICATION OF PHOSPHINOTRICIN-ACETYL-TRANSFERASE IN PLANT EXTRACTS BY A SPECTROPHOTOMETRIC PAT ASSAY

Responsible : A. Van Vliet, Researcher

2.6.1 Goals of the experiment

Quantification of the amount of phosphinothricin-acetyl-transferase (PAT) in extracts from leaves and heads of male sterile Radicchio rosso plants.

2.6.2 Plant material

Analysis has been performed on plants carrying the male sterility construct. Non-transgenic Radicchio rosso plants have been used as negative control.

2.6.3 Methods

The spectrophotometric PAT assay was largely based on the protocol as described by D'Halluin et al. (1992) (Methods in enzymology, 216, 415-427).
After PAT catalyzed acetylation of phosphinothricin (PPT) at the expense of acetyl coenzyme A (AcCoA) the free sulfhydryl group of coenzyme A (CoA) can react with the Ellman's reagent (5,5' Dithiobis(2-nitrobenzoic acid) (DTNB). During this reaction a yellow colored product is formed which can be followed in time and which is a measure for the PAT activity.

Solutions :
- 0.4 mg DTNB/ml 100mM Tris/Cl pH 7.5
- 9.75 mg PPT/ml miQ
- 20.2 mg AcCoA/ml miQ

Measurement :
968 µl DTNB
2 µl PPT
20 µl AcCoA
10 µl sample

Optical density (OD) is measured at 412 nm, 25°C against reference cuvette containing all solutions except sample. 1 Unit is defined as the increase of 1 OD at 412 nm/min at 25°C.

2.6.4 Results and conclusion

		heads			leaves		
		mg[a)] prot/mL . extract	PAT U/mL extract	µg PAT/mg prot	mg[b)] prot/mL extract	PAT U/mL extract	µg PAT/mg prot
RM3-3 To T122	a				1.8	0.7	2.4
	b				1.2	1.2	5.9
RM3-3 Tx T326, T13	a	1.4	0.4	1.6	2.1	2.0	5.5
	b	2.0	0.1	0.2	1.8	3.5	11.2
RM3-4 To T123	a				2.4	1.8	4.2
	b				1.6	1.4	5.3
RM3-4 Tx T327, T14	a	2.2	0.4	1.0	1.8	0.9	2.9
	b	1.9	0.4	1.2	2.0	1.9	5.6
RM3-6 To T 124	a				2.2	0.5	1.2
	b				1.2	0.2	0.8
RM3-6 Tx T328, T17	a	1.5	0.2	0.8	1.2	1.5	7.5
	b	0.9	0.2	1.5	1.3	3.2	14.7
CTRL red hearted chicory T325, T201	a	1.9	0.2	0.7	1.4	0.0	0.0
	b	2.0	0.1	0.3	0.9	0.0	0.0

a) protein concentration is measured with the microassay procedure of Biorad (Bradford method) with bovine serum albumin (BSA) as standard.
b) protein concentration is measured with the Dc protein assay of Biorad (Lowry method) with BSA as standard.
c) µg PAT is based on an estimated specific activity of 170 U/mg PAT.

The results clearly indicate that the *bar* gene is inherited and stably expressed over a number of generations.

2.7 STUDY ON THE OCCURENCE IN PLANT MATERIAL OF THE *AGROBACTERIUM TUMEFACIENS* STRAIN APPLIED IN TRANSFORMATION EXPERIMENTS

The transformants RM3-3, RM3-4 and RM3-6 were tested for absence of the *Agrobacterium tumefaciens* strain used in transformation.
In the BEJO laboratories the transformants were cultured *in vitro* on MS medium (Murashige and Skoog; 1962) without antibiotics at 25° C for three years; during this culture period no bacterial contamination occurred.
Both *in vitro* grown primary transformants and greenhouse grown plants from RM3-3; RM 3-4 and RM 3-6 were tested for presence of *Agrobacterium*; therefore 6 different lots were tested.
From 10 individual plants of each lot 10 leaves were taken for examination; leaves from the greenhouse were surface sterilized for 15 minutes with a 10 % solution of commercial bleach and rinsed three times with sterile water. Each leaf was cut in pieces of about 4 x 4 mm and per leaf 30 of these were placed on petri dishes with LB medium (Sambrook *et al*; 1989), sealed with Parafilm and incubated for 5 weeks at 29° C. Dishes were monitored for bacterial growth every week. On *in vitro* grown primary transformants no growth was detected. On one out of 300 explants from a greenhouse grown plant bacterial growth was detected. This single colony was transferred to LB medium + rifampicine (25 $mg.l^{-1}$) + spectinomycin (50 $mg.l^{-1}$). On this medium no growth occurred. Since Agrobacterium is rifampicin resistant and both plasmids harbour a gene for spectinomycin resistance; this explant was considered to be *Agrobacterium* free.

Seeds harvested from these plants were surface sterilized and monitored for the presence of Agrobacterium as described above; no bacterial growth was observed.
The overall conclusion is that the described plantmaterial is *Agrobacterium* free.

T. Murashige and F. Skoog. Physiol. Plant. (1962) 15: 473.

J. Sambrook, E.F. Fritsch and T. Maniatis. Molecular cloning; a laboratory manual. 2nd ed. Cold Spring Harbor Laboratories. 1989. Part III. Appendix A1.

2.8 VERIFICATION THAT THE TRANSFORMING DNA LACKS GENE SEQUENCES CONFERRING RESISTANCE TO STREPTOMYCIN/SPECTINOMYCIN (Sm/Sp).

Responsible: De Beuckeleer Marc, Senior Researcher

Goals of the experiment:

The aim of the experiment is to demonstrate, by means of PCR analysis, the absence of Sm/Sp gene sequences in the plant genomes of the *Radicchio rosso* lines RM3-3, RM3-4 and RM3-6.

Plant material:

PCR analysis has been performed on *Radicchio rosso* plants (lines RM3-3, RM3-4 and RM3-6) carrying the male sterility gene. A non-transgenic plant has been used as control.

Table: Plant material

Plant material	Notation
Radicchio Rosso transformed with pTTM8RE	
RM3-3 progeny: T_x (RM3-3)	T13 (leaves)
RM3-4 progeny: T_x(RM3-4)	T14 (leaves)
RM3-6 progeny: T_x(RM3-6)	T17 (leaves)
non-transgenic ***Radicchio rosso***	T201

Method:

Two primer-combinations were used to perform the PCR analysis (see table). The 5' portion and the 3' portion of the coding sequence of Sm/Sp are targeted in two separate PCR's. Primers targeting the barnase sequence are included to serve as an internal control for the transgenic plants.

Assigned positive and negative controls to a PCR run:

- As a DNA positive control (POS) we used genomic DNA prepared from a non-transgenic plant, supplemented with 1pg pTTM8RE plasmid DNA, linearized with EcoRV.
  Successful amplification of this positive control demonstrates that the PCR was run under conditions which allow for the satisfactory amplification of target sequences.
- A wildtype DNA control (NON) is a PCR in which the template DNA provided is genomic DNA prepared from a non-transgenic plant. When the expected result (no target PCR products) is observed this indicates that there is no detectable background amplification in genomic DNA sample known to contain the target sequence.

- A DNA negative control (NEG) is a PCR in which no DNA is added to the reaction. When the expected result (no PCR products) is observed this indicates that the PCR cocktail was not contaminated with target DNA.
- We also include primers MDB6 and MDB7 targeting barnase sequences in every cocktail. This primer-pair serves as an internal control in transgenic DNA samples and the DNA positive control (POS). A positive result from this primer-pair demonstrates that there is ample DNA of adequate quality in the genomic DNA preparation for a PCR product to be generated.

Table: Primers used

Primer	position in Sm/Sp	sequence (5'--->3')
MDB396	+1014 ---> +991	GAC.ATT.ATT.TGC.CGA.CTA.CCT.TGG
MDB397	+478 ---> +498	AAG.TCA.CCA.TTG.TTG.TGC.ACG
MDB398	+488 ---> + 470	CAA.TGG.TGA.CTT.CTA.CAG.CG
MDB400	+35 ---> +54	AAC.GCA.GCG.GTG.GTA.ACG.GC
Primer	**position in barnase**	**sequence (5' --->3')**
MDB6	+104 ---> +126	CTG.GGT.GGC.ATC.AAA.AGG.GAA.CC
MDB7	+264 ---> +241	TCC.GGT.CTG.AAT.TTC.TGA.AGC.CTG

Table: Primer-combinations

combinations	fragment lengths	target sequences
MDB400 - MDB398 MDB6 - MDB7	455bp 160bp	5' region coding sequence Sm/Sp barnase
MDB397 - MDB396 MDB6 - MDB7	536bp 160bp	3' region coding sequence Sm/Sp barnase

Polymerase Chain Reaction (PCR):

5µl of isolated plant DNA is used in a 50µl PCR reaction containing 10mM Tris-HCl (pH8.3); 50mM KCl; 1.5mM $MgCl_2$; 200µM of each dNTP; 1.25 units *Taq* DNA polymerase (Pharmacia); 10 pmoles of the oligonucleotide primers (primer-combination MDB400, MDB398, MDB6 and MDB7 or primer-combination MDB397, MDB396, MDB6 and MDB7).
A master mix of reagents (water, buffer, dNTP's, primers and enzyme) is prepared for all samples and then aliquoted to the individual samples. The reaction mixtures are overlayed with 50µl mineral oil and thermocycling is started.

Thermocycling profile:		4 min. at 95°C
	Followed by:	1 min. at 95°C
		1 min. at 57°C
		2 min. at 72°C
		For 5 cycles
	Followed by:	30 sec. at 92°C
		30 sec. at 57°C
		1 min. at 72°C
		For 22 cycles
	Followed by:	10 min. at 72°C

20µl of each PCR sample is separated on a 1.5% agarose gel. The 100bp ladder from Pharmacia is used as a molecular weight marker. Results are documented with the Foto/Analyst™ Visionary imaging system (CCD camera).

Data interpretation:

Data for transgenic plant DNA samples within a single PCR run and a single PCR cocktail will not be acceptable unless the DNA positive control (POS) shows the expected PCR products, and both the DNA negative control (NEG) and the wildtype DNA control (NON) are negative for PCR amplification.

Data transgenic plant DNA samples:

- lanes showing visible amounts of the expected size PCR product (when compared to the molecular weight marker) indicate that the corresponding plant from which the genomic template DNA was prepared has inherited the transgene being assayed for
- lanes not showing visible amounts of the expected size PCR product indicate that the corresponding plant from which the genomic DNA template was prepared has not inherited the sequence assayed for.

Results:

Loading order of the agarose gel (see figure 1).

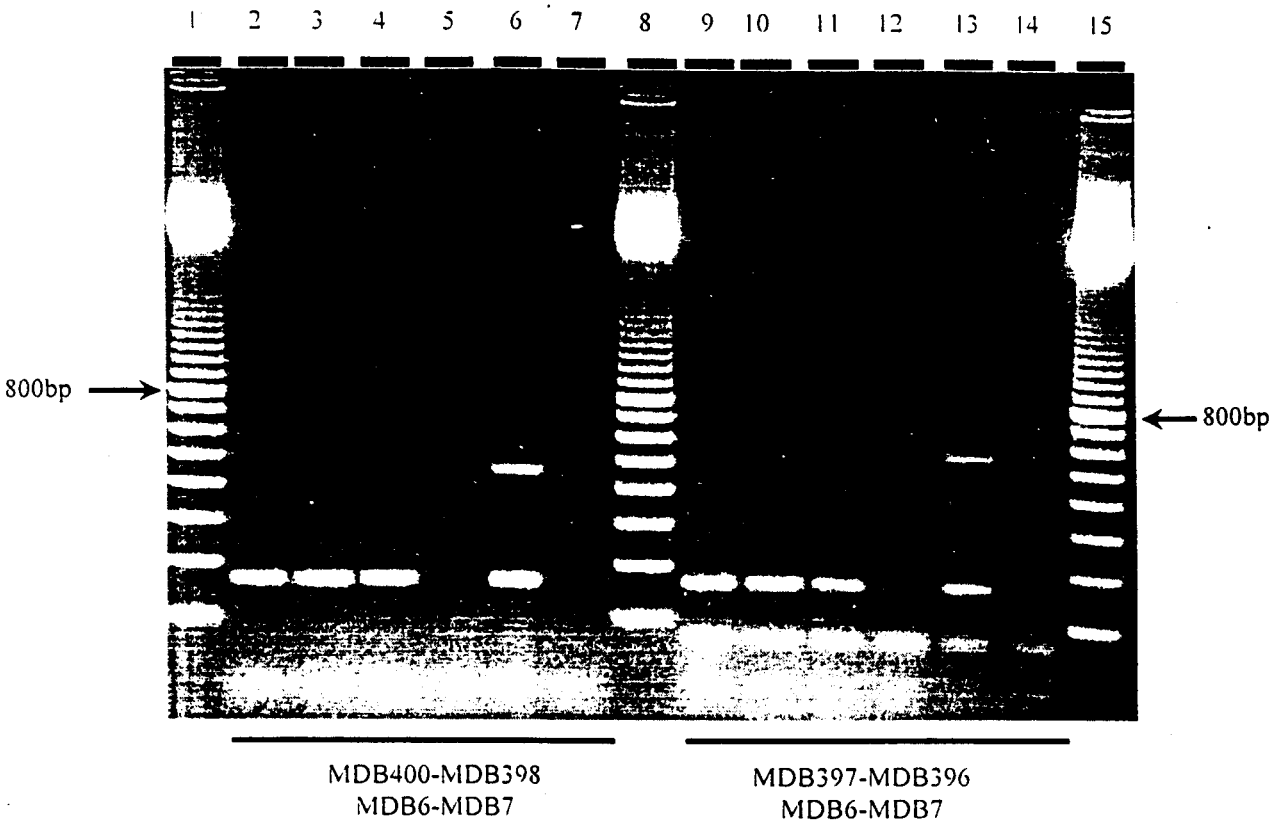
1.	MW marker (100bp ladder PHARMACIA)	
2.	RM3-3 (T13)	MDB400-MDB398-MDB6-MDB7
3.	RM3-4 (T14)	
4.	RM3-6 (T17)	
5.	wildtype (T201)(NON)	
6.	wildtype (T201) + pTTM8RE (POS)	
7.	water (NEG)	
8.	MW marker (100bp ladder PHARMACIA)	
9.	RM3-3 (T13)	MDB397-MDB396-MDB6-MDB7
10.	RM3-4 (T14)	
11.	RM3-6 (T17)	
12.	wildtype (T201)(NON)	
13.	wildtype (T201) + pTTM8RE (POS)	
14.	water (NEG)	
15.	MW marker (100bp ladder PHARMACIA)	

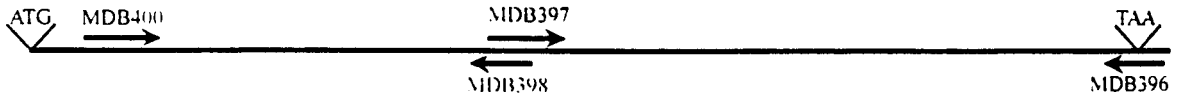
Conclusion:

The assigned positive and negative controls show the expected results for each PCR cocktail used. Obtained data for transgenic plant DNA samples can therefore be accepted (see figure 1).

The PCR analysis demonstrates that the Sm/Sp gene is not integrated in the transgenic *Radicchio rosso* lines RM3-3, RM3-4 and RM3-6.

Figure 1





graphical position of Sm/Sp primers used

Annex 6 SEGREGATION DATA OF RM3-3, RM3-4 AND RM3-6 AFTER TREATMENT WITH GLUFOSINAAT-AMMONIUM (BASTA®)

Segregation in juvenile stage compared to copy number of the inserted gene construct. The expected segregation for single copy plants is 1:1.
T = tolerant, S = sensitive, Bx = one or more generation through seed, Bo = original transformant.

PLANT CODE	SEEDLOT	SEGREGATION		COPY NUMBER	
		T	S	Bx	Bo
RM3-3*R3*R3	933581	25	25	1	1
RM3-3*R3	933605	196	287	1	1
RM3-3*Mar1-1	922927	18	14	1	1
RM3-3*609-3	933610	9	7	1	1
RM3-3*308-2	933611	4	7	1	1
RM3-3*Si5-2	933612	3	4	1	1
RM3-4*R3	933609	134	130	1	1
RM3-4*Mar1-1	922928	14	18	1	1
RM3-6*R3*R3	933583	23	32	1	2
RM3-6*R3	933620	176	186	1	2
RM3-6*R3*R3	933586	14	21	1	2
RM3-6*R3*Mar1-1	933598	9	13	1	2
RM3-6*R3*609-3	933599	14	14	1	2

Annex 7 SCHEMATIC OVERVIEW OF THE DEVELOPMENT OF THE PRODUCT FROM THE 1991- TILL THE 1996 SEASON

1991:

Starting material: An existing inbred line of Radicchio rosso.
Radicchio rosso line: R3.

Transformation work with the line R3 has been done at Plant Genetic Systems at Gent, Belgium.

1992:

February 4, 1992: Transportation of the R3-transformants from Plant Genetic Systems to the laboratory of Bejo Zaden BV.
RM3-3, RM3-4 and RM3-6, one plant each.
R = Radicchio rosso.
M = Transformed with the construct for male sterility.
3-3 = Transformant number 3 from inbred line number 3.
3-4 = Transformant number 4 from inbred line number 3.
3-6 = Transformant number 6 from inbred line number 3.

Within the laboratory of Bejo Zaden transformants were vegetatively propagated till the following number of plants were reached on march 18, 1992:
RM3-3: 4 plants
RM3-4: 6 plants
RM3-6: 7 plants
These plants were cold-treated to induce flowering and were transplanted in the greenhouse. Bolting started in may and the first flowers appeared in june. From this moment on, crosses were made for maintenance/seedpropagation of the male sterile lines and for the production of experimental hybrids. Also, observations were done to check male sterility and behaviour of flies and honey bees which were used for pollination.

Table 1: Maintenance/seedpropagation:

Cross	Date	Male sterile: Yes/No	Behaviour flies and honey bees
RM3-3 * R3	July, 28 August, 11	Yes Yes	n.t.(not tested) +
RM3-4 * R3	June, 26 July, 15	Yes Yes	n.t. +
RM3-6 * R3	June, 12 June, 15	Yes Yes	n.t. +

An aditional test has been done to check the male sterility of the lines. In these tests plants of the male sterile lines have been isolated in cages and flowers have been visited by flies during flowering. At the end of the flowering season the plants were harvested and checked for the

number of seeds that have been produced. In all cases no seeds were produced at all. In conventional breeding these tests are normally used to check the male sterility.

Table 2: Experimental hybrids:

Cross	Date	Male sterile: Yes/No	Behaviour flies and honey bees
RM3-3 * Mar 1-1	July, 15	n.t.(not tested)	+
RM3-4 * Mar 1-1	June, 11 July, 15	Yes Yes	n.t. +

In september/october the seeds were harvested. In 1993 these seeds were sown for fieldrelease (B/NL/92/13) en for crosses in the greenhouse. Additional *in vitro* propagation has also be done to get sufficient plant material for greenhouse experiments in 1993.

1993:

Fieldrelease: Evaluation of male sterile lines and experimental hybrids at a trial field location at Bejo Zaden (B/NL/92/13). Seeds that were harvested in the greenhouse in 1992 were used for this fieldtrial.

Greenhouse:
- Phenotypic observations of the lines and experimental hybrids compare to the conventional plant material.
- Observations on male sterility.
- Behaviour of flies and honey bees which take care for pollination.
- Maintenance/propagation through seed.
- Pollination to obtain (experimental) hybrids.
- Back cross program.

Table 3: Maintenance/propagation through seed of male sterile lines together with phenotypical observations of male sterility and behaviour of flies and honey bees on several data.

Cross	Date	Male sterile: Yes/No	Behaviour flies and honey bees
RM3-3 * R3	June, 10	Yes	n.t.(not tested)
	June, 18	n.t.	+
	June, 25	n.t.	+
	July, 9	Yes	n.t.
RM3-4 * R3	June, 10	Yes	n.t.
	June, 18	n.t.	+
	June, 25	n.t.	+
RM3-6 * R3	June, 18	n.t.	+
	June, 25	n.t.	+
RM3-6 * R3	June, 10	Yes	n.t.
	June, 18	n.t.	+
	June, 25	n.t.	+
	July, 9	Yes	n.t.
RM3-6 * R3	June, 18	n.t.	+
	June, 25	n.t.	+
RM3-6 * R3	June, 10	Yes	n.t.
	June, 18	n.t.	+
	June, 25	n.t.	+

Table 4: (Experimental) hybrids of male sterile lines crossed with non-transgenic pollinator lines together with phenotypical observations of male sterility and behaviour of flies and honey bees on several data.

Cross	Date	Male sterile: Yes/No	Behaviour flies and honey bees
RM3-3 * Mar1-1	June, 18 June, 25	n.t.(not tested) n.t.	+ +
RM3-3 * 609-3	June, 18 June, 25	n.t. n.t.	+ +
RM3-3 * 308-2	June, 18 June, 25 July, 2	n.t. n.t. Yes	+ + n.t.
RM3-3 * Si 5-2	June, 18 June, 25	n.t. n.t.	+ +
RM3-3 * Ot1-1	June, 18 June, 25	n.t. n.t.	+ +
RM3-3 * Mar1-2	June, 18 June, 25	n.t. n.t.	+ +
RM3-6 * Mar1-1	June, 18 June, 25 July, 9	n.t. Yes Yes	+ + n.t.
RM3-6 * 609-3	June, 18 June, 25	n.t. Yes	+ +
RM3-6 * 308-2	June, 18 June, 25	n.t. Yes	+ +

Table 5: Back crosses of male sterile lines crossed with non-transgenic pollinator lines together with phenotypical observations of male sterility and behaviour of flies and honey bees on several data.

Cross	Date	Male sterile: Yes/No	Behaviour flies and honey bees
(RM3-3 * Marl-1) * Marl-1	June, 18 June, 25 July, 2 July, 9	Yes n.t.(not tested) Yes Yes	+ + n.t. n.t.
(RM3-3 * Marl-1) * Marl-2	June, 18 June, 25	Yes n.t.	+ +
(RM3-4 * Marl-1) * Marl-1	June, 18 June, 25 July, 9	Yes n.t. Yes	+ + n.t.
(RM3-4 * Marl-1) * Marl-2	June, 18 June, 25	Yes n.t.	+ +

1994:

Fieldrelease:
- Netherlands: Evaluation of transgenic lines and hybrids (B/NL/93/6) and registration-fieldtrials at the General Netherlands Inspection Service for Flower and Vegetable seeds, NAKG (B/NL/93/16) to test the hybrids.
- Belgium: Evaluation of hybrids (B/B/94/W4).
- France: Evaluation of hybrids (B/F/94/03/09).
- United Kingdom: Evaluation of hybrids (UK/94/R12/1).
- Italy: Experimental hybrid seed production (B/IT/93/7) and evaluation of hybrids (B/IT/94/04).

Greenhouse:
- Maintenance/seed propagation of male sterile lines.
- (Experimental)hybrids.
- Back crosses.

Maintenance/seed propagation of male sterile lines:

RM3-3 * R3
RM3-4 * R3
RM3-6 * R3

(Experimental)hybrids:

RM3-3 * Mar1-1
RM3-3 * 308-2
RM3-3 * 80-2
RM3-4 * Mar1-1
RM3-4 * 308-2
RM3-6 * Mar1-1
RM3-6 * 308-2
RM3-6 * Ot1-1
RM3-6 * Si 15-9

Back crosses:

(RM3-3 * 609-3) * 609-3
(RM3-3 * 308-2) * 308-2
(RM3-4 * Mar1-1 * Mar1-1) * Mar 1-1
(RM3-4 * Mar1-1 * Mar1-1) * Si 15-9
(RM3-4 * Mar1-2) * Mar1-2
(RM3-4 * Mar1-2) * Si 5-6
(RM3-6 * Mar1-1) * Mar1-2
(RM3-6 * Mar1-1) * Si 5-6
(RM3-6 * 609-3) * 609-3
(RM3-6 * 308-2) * 308-2

1995:

Fieldrelease:

- Netherlands: Evaluation of transgenic male sterile lines and hybrids (B/NL/94/23) and registration fieldtrials at the General Netherlands Inspection Service for Flower and Vegetable Seeds, NAKG, to test the hybrids (B/NL/93/16).
- Belgium: Evaluation of hybrids (B/B/95/V5B).
- France: Evaluation of hybrids (B/F/95/03/01).
- United Kingdom: Evaluation of hybrids (B/UK/95/R12/2).
- Italy: Hybrid seed production (B/IT/94/02) and evaluation of hybrids (B/IT/95/37).
- United States of America (California): Start with the first fieldtrial in november in Oceano, California, for evaluation of new hybrids.

Greenhouse:

Complete integration of the male sterile lines RM3-3, RM3-4 and RM3-6 in the breeding program. Continuation of maintenance and seed production of the male sterile lines, experimental hybrid crosses and back crosses.

1996:

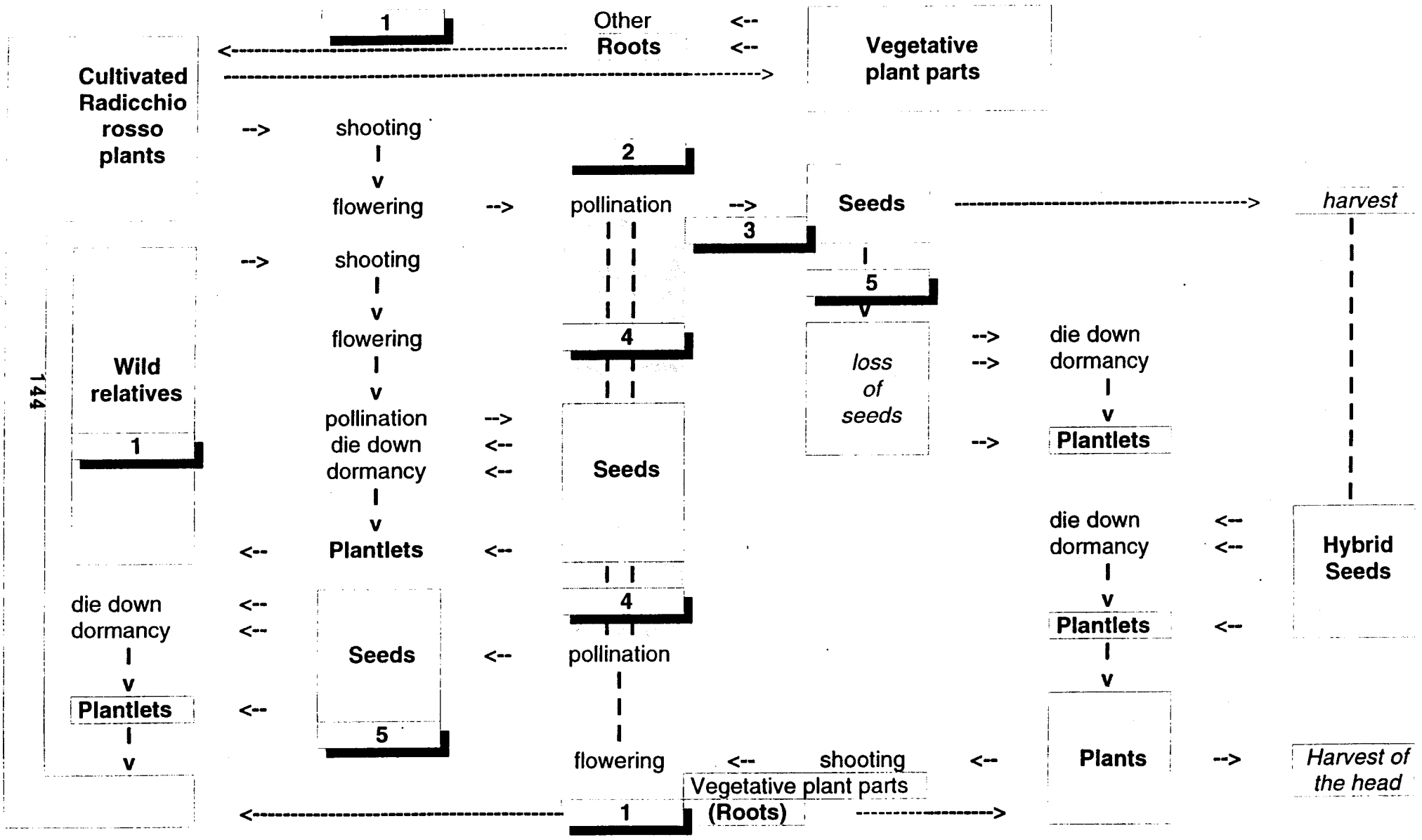
Fieldrelease:

- Netherlands: Evaluation of transgenic male sterile lines and hybrids (B/NL/94/23A) and registration fieldtrials at the General Netherlands Inspection Service for Flower and Vegetable Seeds, NAKG, to test the hybrids (B/NL/93/16A). Consent has been given by the Commission of the European Communities to Bejo Zaden for placing on the market the male sterile Radicchio rosso lines RM3-3, RM3-4 and RM3-6 and all plant material that has been derived from these lines by conventional breeding.

- Belgium: Evaluation of hybrids (B/B/96/V5C).
- France: Evaluation of hybrids (B/F/96/04/01).
- United Kingdom: Evaluation of hybrids (B/UK/95/R12/2).
- Italy: Hybrid seed production (B/IT/95/43). On a small scale seeds of four new hybrids were produced on commercial base. In total the production was 40, 31, 25 and 42 kilograms.
In another fieldtrial evaluation of 73 new experimental hybrids took place (B/IT/96/39).
- United States of America (California): In 1996 out of 18 experimental hybrid crosses three were very promissing for the spring time, namely V10581, V10609 and V10667.

Annex 8 PATHWAY OF THE PRODUCT AFTER RELEASE INTO THE ENVIRONMENT

The agricultural ecosystem : Hybrid seed production and crop cultivation



1

Survival and dispersal by means of the roots is not a general phenomenon, altough it is known that regrowth out of root pieces can take place. Temperature, eventual stress conditions or susceptibility to sprouting might influence regrowth out of roots.
Generally, roots will not survive longer dan one year, due to frost sensitivity. Yet, this depends strongly on the climatical conditions in the area where the vegetable is grown.
In those areas where the product is grown, after seeds or vegetables have been harvested the soil will be cultivated. Through these treatments roots are unlikely to survive.
Possible seedlings/volunteers are treated as weeds. They will be treated by a herbicide application or a mechanical cultivation.

2
3

Fecundation essentially occurs by entomological cross pollination. Self pollination is rather exceptional.
The success of pollination principally depends on the number of flowers on a plant, their shape and colour (in relation of insect visits), amount of donor pollen, the compatibility / incompatibility of the pollinated flower, the number and the activity of pollinating insects.
During flowering, a higher pollen production level is observed with warm and dry wether
Wind pollination does not occur.

4

Vegetable chicory crops can exchange genetic information with other lines and species of the same family (Cichorium) by cross polination.
It is possible that cross-pollinations occur with other, non modified vegetable chicory plants grown in the proximity or with wild relatives which possibly emerge in the surroundings of the cultivation areas. However, no dispersal can be expected by the male sterile plants - of which the uncapability of producing pollen already has been explained. Accordingly, transfer of the genetic material of the GMO towards wild relatives is unlikely to happen, the opposite is possible, but the probability is rather small, taking in account

1. the low rate of appearance of wild relatives in the seed production area;
2. the fact that through cultivation of the crop flowering essentially not occurs.

5

Dispersal of seeds is essentially restricted to the immediate surroundings of the mother-plant since seeds stay in the torus after flowering. Only when the plant is completely decomposed, seeds will be released. The environmental parameters wich influence the conservation of seeds are temperature, frost and humidity.

Annex 9 CHEMICAL ANALYSES OF FREEZE-DRIED MATURE HEADS.

In januari 1994 this was carried out with freeze-dried mature heads of the male sterile line RM3-6 (S 805) compare to the wild type R3 (S 804) harvested from the fieldtrial in october 1993.

In april 1995 this was carried out with freeze-dried mature heads of the male sterile lines RM3-3 (T 326) and RM3-4 (T 327) compare to the wild type R3 (T 325) harvested from the fieldtrial in october 1994.

The analysis were performed at the Rallis Agrochemical Research Station at Bangalore, India. These facilities have GLP-status, given by the german Bundes Gesundheits Ambt and since last year also by the FDA.