GENETIC ANALYSIS OF ENGINEERED MALE STERILITY IN OILSEED RAPE

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INTRODUCTION

Development of hybrid oilseed rape varieties has been an important goal of breeding research with this crop for over a decade. Early studies have demonstrated the heterosis that could be tapped for further exploitation of its economic potential (Brandle and McVetty 1989). Over the years, a number of Cytoplasmic Male Sterility systems have been found in Brassica germplasm, other sources have been introduced from more distant relatives through protoplast fusion. Other groups have developed hybrid seed production procedures based on the use of self-incompatibility systems in the crop. Several years of study on these systems have exposed a number of weaknesses in most of them, including instability of either the male sterility or the restoration of fertility, yield depression as a side effect of the CMS gene, difficulties in following the introgression of the restorer genes into male lines, or low hybrid seed production. While considerable progress has been made, notably with the recent development of the Ogura CMS system, and the discovery of an effective restorer gene, there remains demand for a simple, highly effective male sterility system combining stability, absence of yield depression, excellent restoration of fertility and easy transfer from one germplasm to another.

We have used recombinant DNA technolgy to develop a male sterility gene encoded as a single dominant allele by the nuclear genome (NMS), and a nuclear encoded fertility restorer gene (RF), both tightly linked to easily selectable markers. The present report describes the development of the gene systems used, the production of male sterile and restorer plants in spring and winter oilseed rape, and the results of the primary evaluation of these plants.

MATERIALS AND METHODS

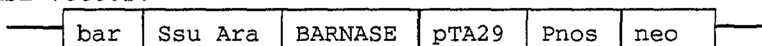
Development of Vectors with Male Sterility or Restorer Genes.

A gene expressed specifically in the tapetum cells of immature anthers was isolated from tobacco. Its promoter region was used to construct chimeric genes directing the synthesis of several enzymes that interfere with vital functions of the cell(Mariani et al. 1990). The two male sterility inducing gene constructs used in further work contained the RNAses RNAse T1 from Aspergillus oryzae and BARNASE from Bacillus amyloliquefaciens. The fertility restorer gene construct contained the gene BARSTAR from B. amyloliquefaciens, which encodes a protein inhibitor of BARNASE. Each of these chimeric constructs was built into a vector containing the marker genes neo (coding for Neomycin Phosphotransferase II, conferring resistance to the antibiotic

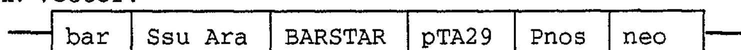
Kanamycin) and bar (coding for Phosphinotricin Acetyl Transferase, conferring resistance to the herbicide phosphinotricin) (Figure 1).

Figure 1. vectors used in transformation experiments

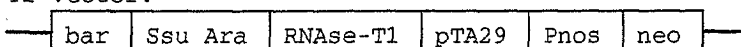
BARNASE-vector:



BARSTAR-vector:



RNAse-T1-vector:



Production of Male Sterile and Restorer Oilseed Rape Plants.

The vectors containing the male sterility and restorer genes were inserted into the genome of the spring oilseed rape variety Drakkar by Agrobacterium tumefaciens mediated gene transfer (De Block et al. 1989). Shoots regenerated on a selective medium containing 75 mg/l Kanamycin were transferred to the greenhouse.

Primary Evaluation of Transformed Plants.

All plants that were transferred to the greenhouse were first analysed for the presence of the chimeric genes and the number of insertions by Southern Blot analysis. Plants containing at least one copy of the gene were allowed to flower. Candidate male sterile plants were closely monitored for reduced pollen production. Candidate restorer plants containing the BARSTAR gene were crossed with those plants containing the BARNASE gene that proved to be completely male sterile. Male sterile plants containing either the RNAse T1 gene or the BARNASE gene were reproduced by cross pollination with Drakkar. Candidate restorer plants containing the BARSTAR gene were selfed.

The progeny of primary transformants was sown in the greenhouse. Two-three week old seedlings were sprayed with the commercial herbicide Basta (a.i. phosphinotricin, 200 g/l) at an application rate of 6 l/ha of the commercial product. From the segregation rates of the selfings of the candidate restorers, the backcrosses of the male steriles and the crosses between male sterile BARNASE plants and restorer plants, the number of active copies of the bar gene in each of the primary transformants was postulated and compared with the total number of inserts as previously determined by Southern Blot analysis. Plants surviving the Phosphinotricin treatment were transferred to greenhouse soil and grown to maturity. At flowering, phenotype with respect to male sterility was determined. Each plant was also sampled for Southern Blot analysis, and the results of the molecular analysis were compared with the segregation into sterile and fertile phenotypes as observed in the greenhouse.

Field Evaluation of Male Sterile Plants.

Four single copy male sterile transformed lines were multiplied by backcrossing with Drakkar for further evaluation in the field in 1990. They were field planted in a randomized block design, using four repeats. Plot size was 1.5 x 9.0 m. Seeds were sown at a density of 5 kg/ha (120 seeds/m2). The average time from sowing to first flower was determined within each plot to detect any possible differences between fertile and sterile plants of a segregating cross. At the start of flowering, a one square meter plot of each line was dug out from repeat two of the experiment for vegetative development evaluation. Shoot length, stem and root diameter, root and shoot weight were determined on fertile, sterile and retarded non-flowering plants were determined and compared. Stability of the expression of male sterility was monitored by daily examination of 50 individually labeled male sterile plants from each segregating cross. Fifty fertile and fifty male sterile plants from each cross were harvested individually at maturity to evaluate yield and yield components.

RESULTS

Production of Male Sterile and Restorer Plants.

All experiments yielded enough transformed plants to allow a comparison of the success rate of male sterility produced by RNAse T1 and BARNASE (Table 1). In oilseed rape both RNAses give comparable results.

Table 1. Success rate of transformation experiments

	RNAse T1	BARNASE	BARSTAR
# of plants regenerated	52	45	61
# of transformed plants	26	14	41
# of single copy plants	11	7	7
# of male sterile plants	22	12	N.A.

Primary Evaluation of Transformed Plants.

Careful observation of primary transformants showed that some plants displayed obvious morphological abnormalities. These plants were discarded from further analysis. Male sterile plants had slightly smaller flowers than control plants or plants transformed with the restorer gene. Average corolla length of male sterile plants was 1.24 cm, compared with 1.49 cm for control plants and plants transformed with the restorer gene. Some plants expressing the RNAse T1 or BARNASE gene were not totally male sterile, and produced fertile and sterile flowers, but many transformants maintained complete male sterility over the whole flowering period, displaying shriveled anthers that were either completely empty or contained very small numbers of empty exine shells (Denis 1990). All male sterile plants were multiplied by hand pollination with Drakkar pollen. No obvious differences in

seed set between individual transformants and with self pollinated control Drakkar plants were observed.

Five independent transformants expressing the BARNASE gene were used as female parents for the evaluation of 7 candidate restorer plants, who expressed the BARSTAR gene, coding for a protein inhibitor of BARNASE. In total, 19 crosses produced seed. Progeny was sprayed with Basta and showed the expected 3:1 segregation for Basta resistance if both parents contained a single copy of the BARNASE, resp. the BARSTAR gene. Backcrosses of BARNASE plants with Drakkar pollen showed the expected 1:1 segregation (Table 2).

Table 2. Segregation for Basta resistance

	BACKCROSS	BARNASE X BARSTAR
SEGREGATION R/S	257/246	501/156
Chi2	0.240 (N.S.)	0.552 (N.S.)

The segregation for fertile vs. male sterile of the remaining plants showed that most crosses between BARNASE and BARSTAR lines induced full restoration of fertility (Table 3). The phenotype of each plant was compared with the result of Southern Blot analysis for the presence of the two genes. It was shown that in those crosses where phenotype segregation indicated restoration of fertility, all plants containing both the BARNASE and BARSTAR genes were fully fertile.

Table 3. Fenotype of BARNASE X BARSTAR crosses

PARENTS	DR	R1-1	R1-2	R1-3	R1-4	R1-5	R1-6	R1-7
S2-1	-	+	-	n.d.	n.d.	+	+	+
S2-2	-	-	n.d.	n.d.	-	-	n.d.	n.d.
S2-3	-	n.d.	n.d.	n.d.	+	n.d.	+	n.d.
S2-4	-	n.d.	+	+	+	n.d.	+	n.d.
S2-5	-	n.d.	+	n.d.	+	+	+	+

(+) : restorer active
(-) : restorer not active
(n.d.) : combination not tested
(DR) : Drakkar

Field Evaluation of Male Sterile Plants.

Time between sowing and first flower in each segregating cross between a male sterile line and Drakkar was equal to control plots of pure Drakkar. Male sterile plants in all crosses were to some extent delayed in flowering. There were large differences between lines, with S1-1 and S1-4 being significantly different, and S1-2 and S1-3 not significantly different from control fertile plants. These observations indicated a delay in vegetative development of the male sterile plants in some crosses and not in others. The results of measurements of vegetative development confirmed this. In

lines S1-1 and S1-4 the average development of the male sterile plants was such that they were effectively outcompeted by the fertile plants in the segregating populations. At the time of measurement most of them had not reached the flowering stage and their genotype could only be confirmed by testing them for Basta resistance. They were discarded from further analysis. In lines S1-2 and S1-3 there were enough flowering plants of each genotype to analyse quantitative growth differences. Line S1-3 shows no difference between male sterile and fertile plants for all the parameters tested, while in line S1-2 the measurements gave significantly different results.

The very different behaviour of male sterile segregants in the different tested lines made yield measurement on a plot scale meaningless, since it led to large differences in density of plants contributing to harvest as well as to very different proportions of total yield produced on the male sterile plants. Therefore it was decided to label in each segregating population 50 randomly chosen male sterile and 50 fertile plants. They were harvested individually and yield components were measured (Table 4).

Table 4. Yield components

	# PODS/PLANT	# SEEDS/POD	SEED/PLANT (g)
DRAKKAR	87	21	4.50
S1-1 FERT.	95	24	5.81
STER.	47	18	2.10
S1-2 FERT.	84	14	2.00
STER.	36	14	1.28
S1-3 FERT.	56	24	3.63
STER.	65	19	3.04
S1-4 FERT.	113	20	6.55
STER.	46	12	1.44

Line S1-3 showed no significant differences in individual plant yield between male sterile and fertile plants. These results confirmed other observations indicating that this line was probably the most suitable for further use.

DISCUSSION

We demonstrated that it is possible to produce stable male sterile plants using recombinant DNA technology, as well as methods to restore fertility of the male sterile lines. The results indicate that Agrobacterium mediated transformation of oilseed rape delivers a sufficient number of primary transformants to allow selection of lines having the ideal combination of properties: excellent performance for the trait of interest combined with total absence of negative side effects on the agronomic qualities of the crop. The linkage of the male sterility and restorer genes to powerful selectable markers make this system uniquely suited for introgression in

other germplasm, since it is sufficient to screen for the herbicide resistance during each backcrossing step to ensure transfer of the male sterility or restorer gene.

Alternatively, it is possible to introduce the genes in other germplasm by new transformation experiments. The choice will be determined by factors such as relatedness between source and target germplasm, time available for backcrossing vs. new transformation, relative cost of development of the two methods, etc. The unusual genetics of the system, consisting of a monogenic dominant nuclear encoded male sterility gene, restored by a dominant nuclear encoded restorer gene, each linked to a powerful selectable marker, allows us to envisage different seed production schemes for commercial production of hybrid oilseed rape varieties. Current research focuses on further demonstration of the stability of the system, both with regard to environmental influences (climate) and genetic background of the varieties carrying the trait.

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