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Expression of Engineered Nuclear Male Sterility in *Brassica napus*

Genetics, Morphology, Cytology, and Sensitivity to Temperature

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A dominant genetic male sterility trait obtained through transformation in rapeseed (*Brassica napus*) was studied in the progenies of 11 transformed plants. The gene conferring the male sterility consists of a ribonuclease gene under the control of a tapetum-specific promoter. Two ribonuclease genes, *RNase T1* and *barnase*, were used. The chimaeric ribonuclease gene was linked to the bialophos-resistance gene, which confers resistance to the herbicide phosphinotricine (PPT). The resistance to the herbicide was used as a dominant marker for the male sterility trait. The study presented here concerns three aspects of this engineered male sterility: genetics correlated with the segregation of the T-DNA in the progenies; expression of the male sterility in relation to the morphology and cytology of the androecium; and stability of the engineered male sterility under different culture conditions. Correct segregation, 50% male-sterile, PPT-resistant plants, and 50% male-fertile, susceptible plants were observed in the progeny of seven transformants. The most prominent morphological change in the male-sterile flowers was a noticeable reduction in the length of the stamen filament. The first disturbances of microsporogenesis were observed from the free microspore stage and were followed by a simultaneous degeneration of microspore and tapetal cell content. At anthesis, the sterile anthers contained only empty exines. In some cases, reversion to fertility of male-sterile plants has been observed. Both ribonuclease genes are susceptible to instability. Instability of the *RNase T1*-male sterility trait increased at temperatures higher than 25°C. Our results do not allow us to confirm this observation for the *barnase* male-sterile plants. However, the male-sterile plants of the progeny of two independent *RNase T1* transformants were stably male sterile under all conditions studied.

The selection of more productive rapeseed (*Brassica napus*) varieties by the creation of F_1 hybrids has been for several years the major goal of breeders. Hybrids have been demonstrated to be much superior to inbred lines with respect to yield and vigor (Lefort-Buson et al., 1987; Brandle and McVetty, 1989). Because rapeseed is about 30% allogamous and 70% autogamous (Rives, 1957), it is necessary to devise a system for pollination control to produce the F_1 hybrids. Male sterility seems to be the most reliable system to prevent self-pollination. Many researchers have concentrated on the establishment of a CMS system, until now recognized as the most efficient tool in the production of hybrids in other species. Several systems of CMS are available, but until recently none has proven completely satisfactory. In many cases, as in the *nap* and *pol* CMS system, male sterility was found to be unstable under different climatic conditions (Fan and Stefansson, 1986). Partial female sterility of the male-sterile plants (Pellan-Delourme and Renard, 1987), or partial female sterility of the restorer (Pellan-Delourme and Renard, 1988) in the *ogu* CMS system, has made the systems inapplicable in practice. In some cases, these problems have been overcome; recently, improvement of female fertility of the restorers in the radish system led to the development of a workable CMS system (Delourme et al., 1991).

Genetic male sterility has also been studied (Takagi, 1970; Li et al., 1988), but the absence of marker genes does not permit the sorting of male-sterile or -fertile plants in the progeny. To this end, a new system of genetic male sterility has been devised (Mariani et al., 1990) using genetic engineering and *Agrobacterium tumefaciens*-mediated gene transfer techniques. Two genes coding for ribonucleases were transferred independently, *RNase-T1* from *Aspergillus oryzae* (Quaas et al., 1988) and *barnase* from *Bacillus amyloliquefaciens* (Hartley, 1988, 1989). These genes, under the control of the TA29 promoter (Mariani et al., 1990), are expressed only in the tapetum of the anther and induce male sterility by destruction of the tapetal cells. Both ribonuclease genes were linked to the *bar* gene (Murakami et al., 1986), which confers resistance to the herbicide PPT, to obtain a marker for the male sterility. Consequently, the transformed plants are male sterile and PPT resistant. Application of PPT permits elimination of male-fertile, PPT-susceptible plants in the field and assures the production of 100% F_1 hybrid seeds on the male-sterile plants. In this work, we have analyzed the segregation of the transferred genes and the stability of the male sterility trait under different culture conditions. Floral morphology and microsporogenesis of male-sterile plants have been investigated in detail in a comparative study with wild-type plants.

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Abbreviations: *bar* gene, bialophos-resistance gene; CMS, cytoplasmic male sterility; PPT, phosphinotricine; SEM, scanning electron microscopy; T-DNA, transfer DNA.

MATERIALS AND METHODS

Plant Materials

The spring double-low rapeseed (*Brassica napus*) variety Drakkar was transformed according to the procedure of De Block et al. (1989). The study on male sterility has been performed on the first generation progeny (T_1) obtained from a back-cross of 11 primary transformants (T_0) with fertile Drakkar plants. Seven primary transformants (T_0-1 through T_0-7) carried the *TA29-RNase T1* construct, whereas four (T_0-8 through T_0-11) carried the *TA29-barnase* construct. Wild-type (untransformed, male fertile) Drakkar plants were used as controls.

Culture Conditions

Seedlings were allowed to grow for 3 weeks in the greenhouse followed by 3 weeks of vernalization at 4°C in a culture room, 8 h/4 h day/night. Every stock of plantlets was then transferred to the greenhouse for 1 week, during which a PPT test was performed. Thereafter, plantlets were grown either in the greenhouse or under controlled temperature conditions, as specified.

PPT Test

When the plants had five well-developed leaves, a solution containing 1% PPT and 0.1% SDS was applied on the fifth leaf counting from the top of each plant. After 3 d, susceptible plants show a white necrosis on both faces of the leaf. PPT-resistant plants show a light necrosis at the superior face of the leaf as well as a hardening at the point where the PPT solution was applied.

Southern Blot Analysis

Southern blot analysis was performed on the T_0 plants according to commonly used molecular techniques (Maniatis et al., 1982; Dellaporta et al., 1983). DNA was digested with *Hind*III, fractionated on a 0.8% agarose gel, transferred to nylon Hybond-N filters, and hybridized with DNA labeled by the multiple priming method. Two probes were used consecutively: RB-3'T7, to detect the presence and the copy number of the *bar* gene, and LB-3'OCS, to detect the presence and the copy number of the ribonuclease genes (*RNase T1* or *barnase*) (Fig. 1). In case of multiple insertions (more than two T-DNA inserts), the number of the hybridizing fragments does not necessarily correspond to the number of genes.

Morphological and Cytological Characterization of the Male-Sterile Plants

All the data presented here refer to plants grown in the greenhouse. Male-sterile plants have been studied in comparison with the fertile control.

Floral Morphology

The male-sterile phenotype is generally reflected in floral modifications (Takagi, 1970; Shiga, 1976; Bartkowiak-Broda et al., 1979). To detect possible morphological differences between sterile and fertile flowers, measures of floral pieces were taken on the youngest open flower situated on the principal stem of four sterile plants per progeny and on four fertile control plants. The floral parts subjected to measurement were: petal length and width; anther and filament length of the longest stamen; and anther and filament length of the shortest stamen. Diameter and length of the first two buds before flower dehiscence were also measured. All the data obtained were analyzed with a statistical test. When the *F* test was significant, the averages were compared by the Newmans-Keuls test at P = 5%.

Observations by SEM

Male-sterile and male-fertile (control) flowers were compared by SEM. Pieces or entire flowers were fixed, dehydrated by the critical point method, and then observed after gold sputtering (Hayat, 1978). This technique also made it possible to examine the content of the stamen in both male-fertile and male-sterile flowers.

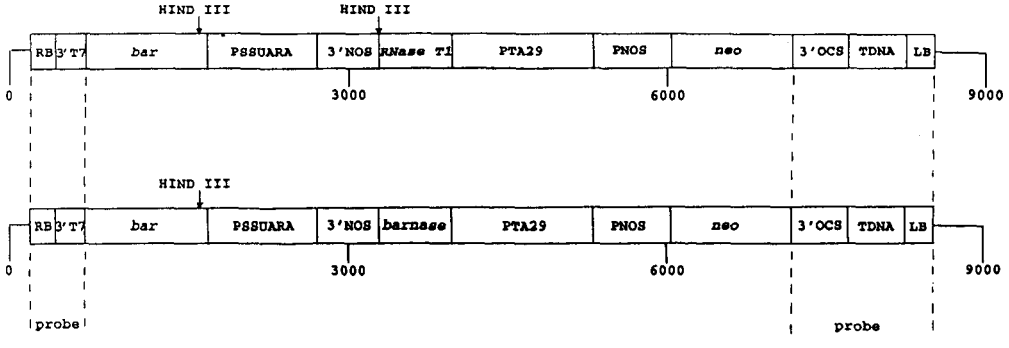

Figure 1. Maps of plasmid constructions. RB and LB, Right border and left border, respectively; 3'T7, 3' end of T-DNA gene 7 from *A. tumefaciens*; *bar*, *bar* gene (Murakami et al., 1986) coding for the PPT acetyl transferase; PSSUARA, promoter of the Rubisco small subunit gene from *Arabidopsis thaliana* (controls the *bar* gene expression); 3'NOS, 3' end of the nopaline synthetase gene from *A. tumefaciens*; *Rnase T1*, ribonuclease gene from *A. oryzae* (Quaas et al., 1988); *barnase*, ribonuclease gene from *B. amyloliquefaciens* (Hartley, 1988, 1989); PTA29, tapetum-specific promoter from *Nicotiana tabacum* (Goldberg, 1988) (controls the ribonuclease gene expression); PNOS, nopaline synthetase gene promoter from *A. tumefaciens* (controls the *neo* gene expression); *neo*, gene from *Klebsiella pneumonia* coding for the phosphotransferase that confers resistance to the kanamycin; 3'OCS, 3' end of the octopine synthetase gene from *A. tumefaciens*.

Microsporogenesis

The Alexander stain (Alexander, 1969) was used to determine rapidly the stage at which microsporogenesis stops in the male-sterile plants. The stamens or small buds were crushed between the slide and the coverslip in a drop of Alexander stain and observed by light microscopy directly or after a night at 30°C. For light microscopy of embedded material, anthers at different stages of development from male-sterile plants or fertile anthers of Drakkar line plants were fixed in glutaraldehyde (2.5%, w/v) for 14 h at 14°C and postfixed in osmium tetroxide (1%, w/v) for 2 h (both in 0.1 M cacodylate buffer, pH 7.2). After the dehydration through an ethanol series and propylene oxide, the samples were embedded in epoxy resin. Semi-thin sections (1 μm thick) from each sample were stained with toluidine blue and mounted in resin for light microscopy (Lynn, 1965).

Stability of the Male Sterility

Stability of male sterility was studied by visual observations of the stamens of the male-sterile plants over 2 weeks under different temperature conditions in a culture room. The temperature conditions used for this test were 30°C day/25°C night, 20°C day/15°C night, or 15°C day/10°C night. The day period was 8 h, and the night period was 4 h. The light intensity was 26,000 lux. Each experiment was performed in the same culture room to preserve the same light conditions. Consequently, the studies at different temperatures took place at successive times. After 3 weeks in the vernalization room, the plantlets were placed in the greenhouse for 1 week for the application of the herbicide. For each temperature test, approximately 20 PPT-resistant plants were transferred to the culture room along with 4 susceptible plants for each progeny and 4 control plants. The plantlets were positioned at random in the culture room. Records were taken every 2 d. Alexander stain was used to monitor the presence of pollen and its fertility or sterility. At flowering, anther dehiscence with pollen release was recorded.

In addition, the stability of male sterility was checked in the greenhouse (around 23°C day and 17°C night) as well as in the field.

RESULTS

bar Gene and Ribonuclease Gene Copy Number in the T_0 Plants

The results of the segregation analysis of the sterility and herbicide-resistance traits were correlated with the results of the molecular analysis of the primary transformants (T_0). The segregation observed in the progenies T_1-1, T_1-2, T_1-4, T_1-5, T_1-9, T_1-10, and T_1-11 is consistent with the presence of one functional locus in the corresponding T_0 plants; the *bar* gene and the ribonuclease gene are linked and the progenies consist of 50% male-sterile/resistant plants and 50% male-fertile/susceptible plants. The Southern blot results confirm the segregation data in all but one case, T_0-2. In this case, the Southern blot analysis of the primary transformant indicates the presence of two or more copies of the T-DNA cassette instead of one.

The segregation analysis of T_1-3, T_1-6, T_1-7, and T_1-8 indicates that there is more than one locus with the *bar* and/or the ribonuclease genes in the corresponding T_0 plants. Both segregation analysis and Southern blot data indicate that plant T_0-6 carries three loci each containing the *bar* and ribonuclease genes. In the case of plant T_0-8, segregation analysis suggests the presence of two loci with the ribonuclease and *bar* genes within the primary transformants, whereas Southern analysis indicates at least three T-DNA insertions. The segregation analysis of the T_0-3 and T_0-7 plants gives 75% herbicide-resistant plants but only 50% male-sterile plants. The excess of 25% herbicide-resistant plants is correlated with the presence of male-fertile but herbicide-resistant plants. This result suggests the presence of two loci in the primary transformants: one containing the *bar* and the ribonuclease genes and the second containing only the *bar* gene. One *bar* gene is linked to the ribonuclease. However, the Southern blot analysis revealed the presence of at least two copies of each gene in the T_0-3 and T_0-7 plants.

Morphological and Cytological Characterization of Male-Sterile Plants

T_1 sterile plants can be recognized even before flowering by the smaller size of their buds relative to those of fertile plants. At flowering, male sterility in the T_1 progenies is manifested by a reduction of the stamen size in comparison with the fertile control.

Analysis of Floral Morphology

Statistical analysis (Newmans-Keuls test at P = 5%) of data resulting from the measurement of various morphological parameters (see "Materials and Methods") showed significant differences between the sterile plants and the control with respect to bud diameter, filament length of the stamen, and petal length.

The diameters of the first and the second buds of the sterile plants (0.23–0.26 cm) were smaller than those of the control (0.29 cm). This explains why it is possible to recognize the sterile plants before anthesis.

The most prominent change in the male-sterile flowers was a noticeable reduction of the length of the stamen filament. The length was reduced by a factor of 2.5 for the longest stamens and 3 for the shortest stamens.

The last parameter measured was the petal length, which is shorter in sterile flowers (1.08–1.28 cm) than in the control plants (1.40 cm).

SEM Observations

Prominent differences in filament length were observed between fertile and sterile flowers (Fig. 2). However, the gynoecium and nectary did not show any differences in fertile versus sterile lines, and the number and the shape of the stomata situated at the nectary top were identical in the two kinds of flowers (data not shown).

Microsporogenesis

The Alexander test revealed the presence of green colored, empty exines in the sterile anthers at maturity (Fig. 3). This was in strong contrast with fertile pollen grains, in which the cytoplasm and nuclei were stained by fuchsin (Fig. 3). The empty exines were tricolpate and had normal columellas and a clearly visible tectum when analyzed by SEM (Fig. 2H), which implies that the arrest of the microsporogenesis occurs after meiosis and the tetrad stage. The Alexander stain technique also showed that sterile anthers in many cases lack cell

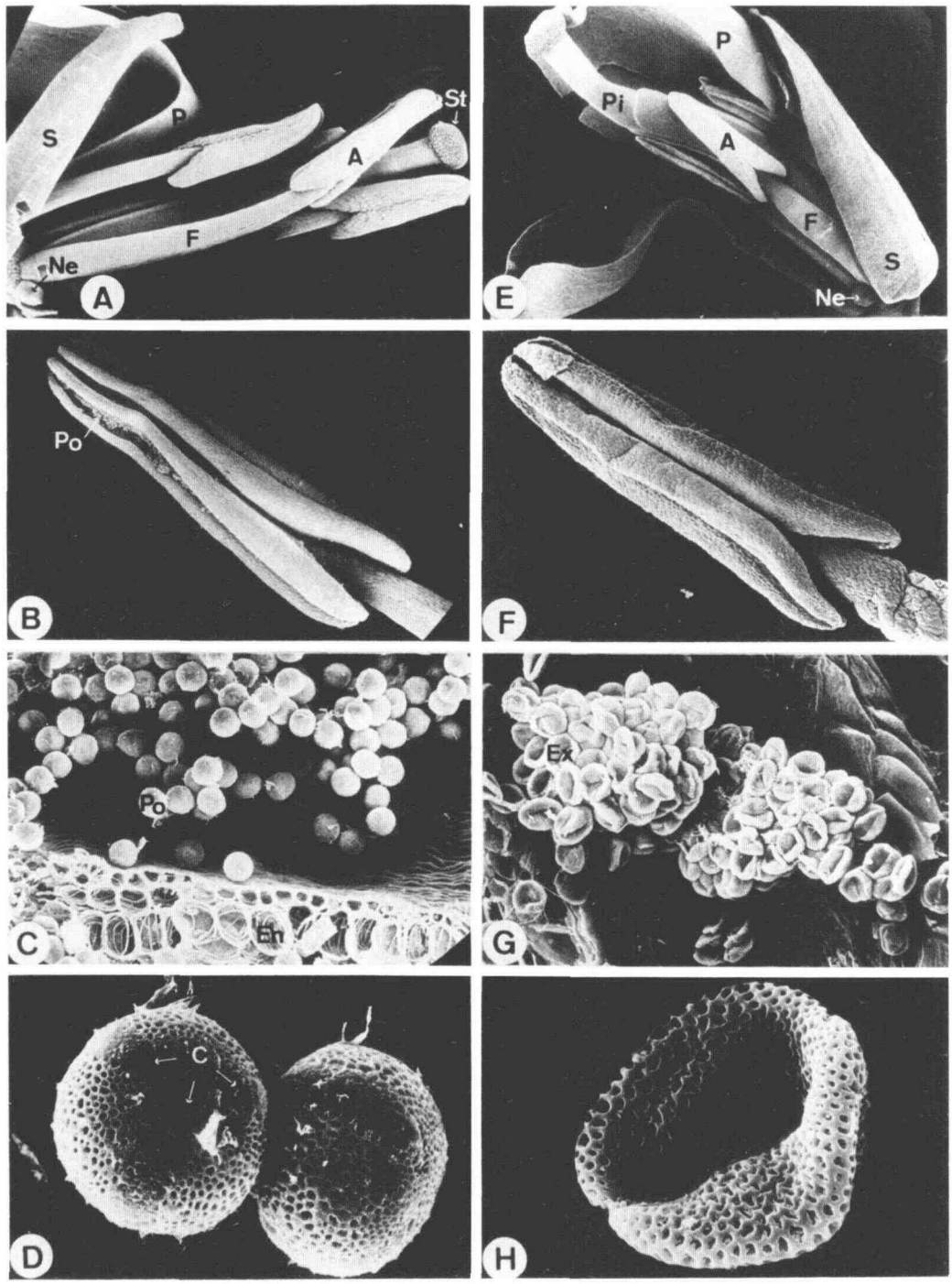

Figure 2. Selected ion micrographs. A, B, C, and D: Fertile control. E, F, G, and H: T_1-2 sterile plants. A, Flower showing the androecium. Notice the length of the stamen filament. ×7. B, Dehiscent anther with visible pollen. ×18. C, Swollen pollen grains, released from the locule. Wall thickenings are prominent in the endothecium. ×180. D, Two mature pollen grains, with three colpi. The relief of the tectum is attenuated by the tryphine. ×1390. E, A male-sterile flower. Notice the reduced length of the pistil filament. ×7. F, Indehiscent male sterile anther. ×23. G, Collapsed empty pollen grains. ×280. H, Empty exine from sterile pollen grain, devoid of tryphine in the tectal cavities. ×2540. A, Anther, C, colpus; En, endothecium; Ex, exine; F, filament; Ne, nectary; P, petal; Po, pollen grain; S, sepal; St, stigma.

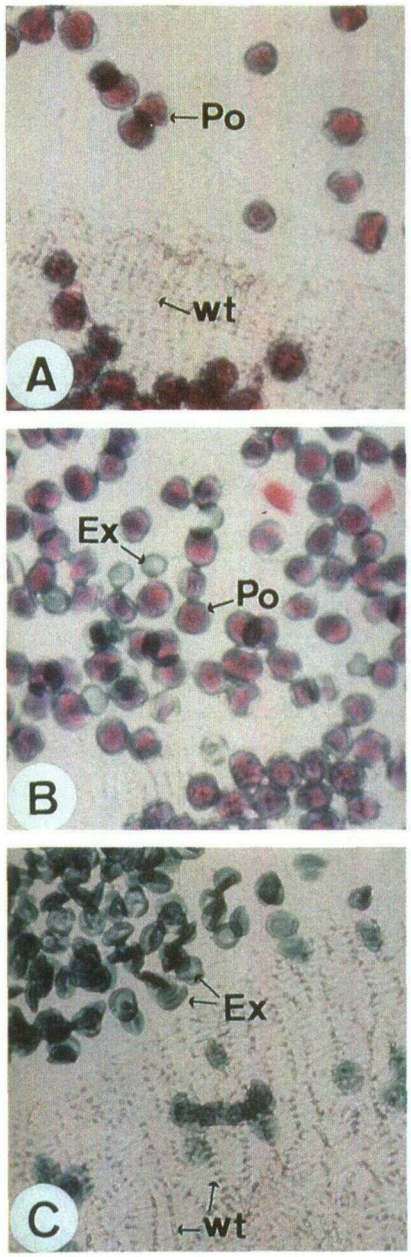

Figure 3. Alexander stain. The malachite green of the stain colors the exine of the microspores and the pollen grains. The glacial acetic acid permits the fuchsin to color red the cytoplasm of the microspores and the pollen grains. A, T_1-2 male-fertile, PPT-susceptible plant (greenhouse). Wall thickenings are visible in the subepidermal layer corresponding to the endothecium. B, T_1-1 PPT-resistant but unstable male-sterile plant (culture room 30°C). The majority of the pollen grains have a red cytoplasm after Alexander stain. C, T_1-2 male-sterile, PPT-resistant plant (greenhouse). The green empty exines are flat. Wall thickenings at the base of the sterile anther are also shown. Ex, Exine; Po, pollen grain; wt, wall thickenings of the endothecium.

wall thickenings in the subepidermal layer corresponding to the endothecium. In a few cases, however, thickening was found at the base of some anthers (Fig. 3C).

The results obtained by light microscopy were confirmed by comparing the fertile control (Fig. 2, A, B, C, and D) and sterile plants (line T_1-2 in Fig. 2, E, F, G, and H) by SEM. The transgenic plants are characterized by reduced filament length (Fig. 2E), indehiscence of the anthers (which had to be broken with a needle to visualize their locule content; Fig. 2F), and heaps of empty, flattened exines, devoid of tryphine at the time of anthesis (Fig. 2, G and H).

Light microscopy of epoxy-embedded samples sectioned and stained with toluidine blue provided more precise data on the arrest of the microsporogenesis. Figure 4 shows views of microsporogenesis in the control plants (A–H) compared with T_1-4 male-sterile plants (A′–H′). No significant difference between the two types of plant could be detected during the early stages of anther and microspore development. It was only when microspores reached their vacuolate, polarized stage that the tapetal cells showed deviation from their normal behavior. Their ability to be stained was heterogeneous; some of them were clearer than those of the control (Fig. 4D′). The high content of lipid globules that is characteristic of normally senescing tapetal cells of fertile Brassicaceae (Fig. 4, E and F) was found in the tapetum of neither T_1-4 (Fig. 4, E′ and F′) nor T_1-2 (data not shown). At the same time, the microspores lost their cytoplasm and the single nucleus by progressive lysis (Fig. 4, E′ and F′). No haploid mitosis could be observed. Only flattened exines devoid of tryphine were found in each of the four collapsed locules of the shriveled anther (Fig. 4, G′ and H′). No evidence of normal dehiscence could be observed; a functional endothecium was absent and no rupture of the interlocule septum was apparent. Thus, the androecia of these plants (T_1-4 and T_1-2) were sterile.

Stability of the Male Sterility

To study the stability of the engineered male-sterility trait, plants from the different progenies were tested under different temperature conditions in culture rooms, the greenhouse, and the field. The results of these tests are summarized in Table I.

Observation of the male-sterile plants every 2 d revealed that a reversion to fertility occurred in some T_1 offspring under particular growth conditions. The first symptom of instability of male sterility was the elongation of the filament. At this stage, crushing of the anthers or the use of Alexander stain indicated the presence of pollen in some samples. At later stages, stamen dehiscence with pollen release could be detected in some plants. Three different conditions of temperature were chosen to test the plants in growth rooms: 15, 20, and 30°C.

Stamen elongation was observed at 15°C day/10°C night in one plant of the T_1-4 and T_1-8 series and in four plants of the T_1-10 series. However, the Alexander stain showed no normal pollen grains in the anthers of these plants at this temperature.

Under temperature conditions of 20°C day/15°C night, anther dehiscence with pollen release was detected in two plants of the T_1-1 progeny, although Alexander stain showed pollen grains also present in one plant of the series T_1-8 and T_1-10.

When the test was carried out at 30°C day/25°C night, the humidity increased noticeably. This caused a failure in flow-

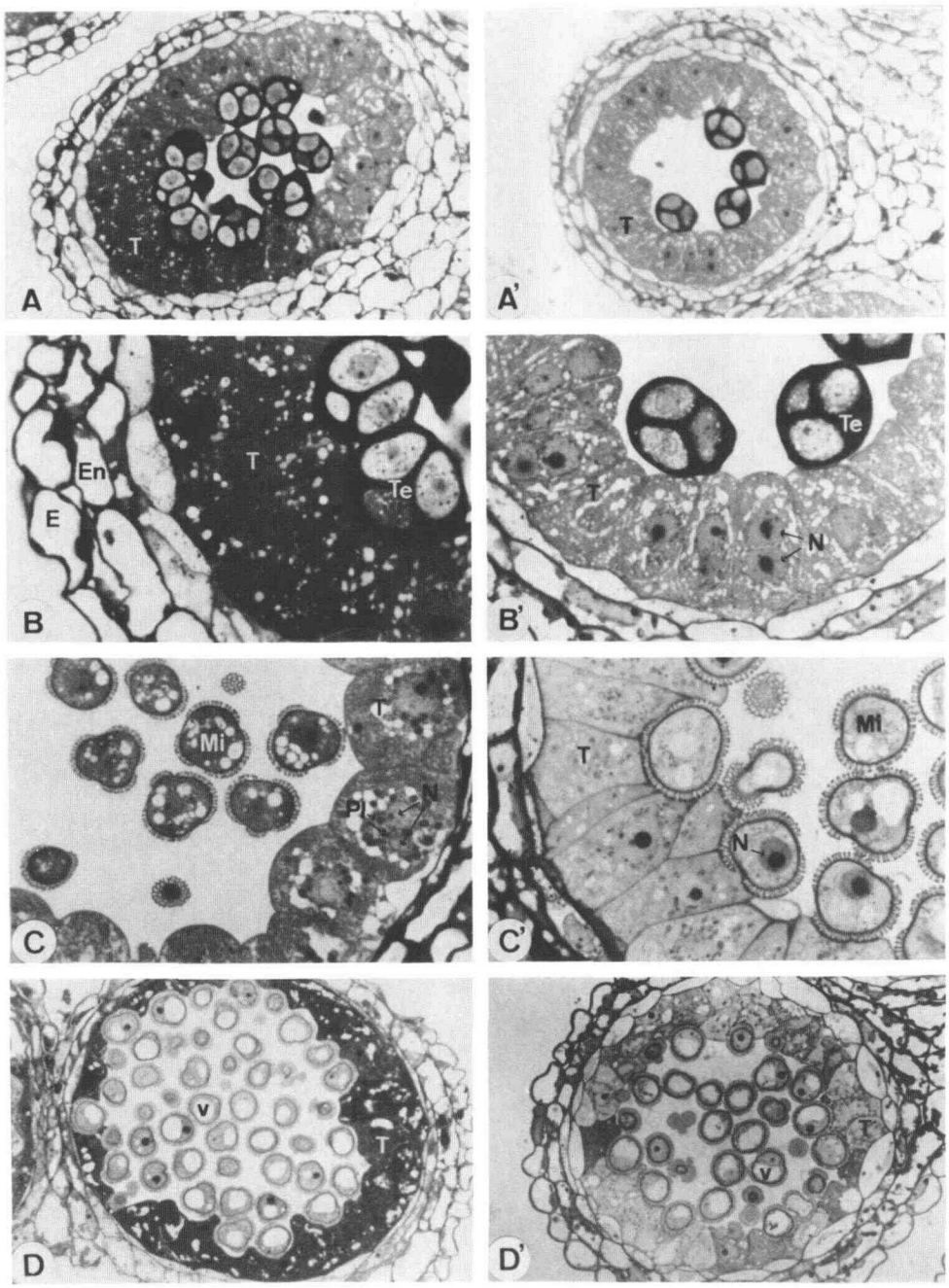

Figure 4 (Complete legend appears on facing page).

ering and in dehiscence of the anthers in most plants. Thus, pollen release could not be observed. Consequently, only Alexander stain could be used to detect instability of the male-sterile trait. The results indicated that only male-sterile plants of the series T_1-2 and T_1-4 did not contain any viable pollen in their anthers. After 2 weeks at 30/25°C, plants were transferred to the greenhouse and the observations were carried out under greenhouse conditions (23/17°C). In the greenhouse, the herbicide-resistant plants that showed normal pollen grains upon staining finally showed anther dehiscence with pollen release. However, after 14 d in the greenhouse at lower temperature, these same plants produced new flowers, which were male sterile. In the plants carrying the *TA29-barnase* gene (T_1-8–T_1-11), instability was assayed only using the Alexander stain. Pollen grains were observed in the anthers of only one plant in the series T_1-9 and T_1-10 and two plants of the T_1-11 offspring.

In parallel to the culture room tests, the stability of male sterility in the progenies of T_1-1 to T_1-11 was also tested under continuous greenhouse conditions (23°C day/17°C

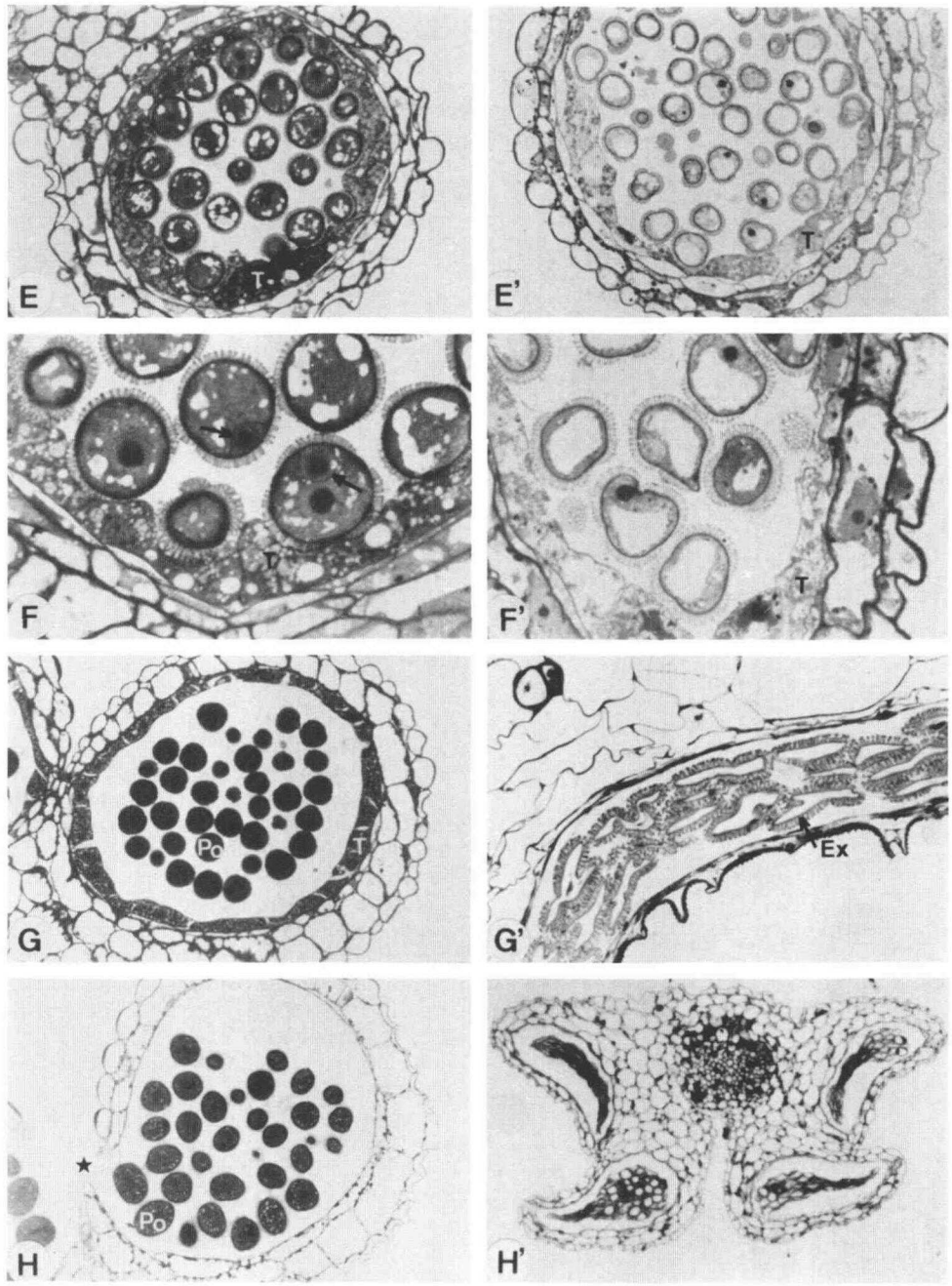

Figure 4. A, B, C, D, E, F, G, and H: Fertile control. A′, B′, C′, D′, E′, F′, G′, H′: T_1-4 sterile plants. All figures are micrographs of semi-thin sections of epoxy-embedded samples, stained with toluidine blue. A and B (enlargement of A), Tetrad stage. Notice the thick and dense tapetum. A, ×285; B, ×720 (1.5–2 mm bud). C and D, Free microspores. In C, the black spots around the nuclei represent the plastids. C, ×720 (3 mm bud); D, ×285 (4 mm bud). E and F (enlargement of the upper part of E). Approximately the first haploid mitosis (arrow) (transition from microspore to pollen). The tapetum has an essentially lipidic content derived from plastids and cytoplasmic globules. E, ×285; F, ×720 (4.5–5.5 mm bud). G, Pollen grains staining heavily in a still closed microsporangium. A continuous, senescing, and lipidic tapetum surrounds the locule cavity. ×285 (6–6.5 mm bud). H, Mature pollen grains ready to be freed by the dehiscing mechanism. The interlocule septum is broken (star). Tapetum has disappeared. ×285 (at flower anthesis). A′ and B′ (enlargement of A′), Tetrad stage. Two nuclei are visible in some tapetal cells. A′, ×285; B′, ×720 (1.5–2 mm bud). C′, Free microspores. The tapetum is thick and looks normal. Plastids are visible around the nuclei. ×720 (3 mm bud). D′, Microspores at their vacuolate, polarized stage. Disorganization of the tapetum begins with different stainability of the tapetal cells. Lipidic accumulation is less pronounced than in the fertile control. ×285 (3.5 mm bud). E′ and F′, Concomitant abnormal lysis of microspores and tapetum. Microspores do not undergo haploid mitosis. E′, ×285 (4 mm bud); F′, ×720 (4.5 mm bud). G′, Only the sporopollenin material of the exines is present in the collapsed and indehiscent anther. ×720 (at flower anthesis). H′, Mature sterile anther. The exines are completely crushed and are represented by a compact mass in each locule of the anther. ×75 (at flower anthesis). E, Epidermis; En, endothecium; Ex, exine; Mi, microspore; N, nucleus; Pl, plastid; Po, pollen; T, tapetum; v, microspore vacuole.

Table I. *Stability/instability of the male sterility in different growth conditions*

a, Instability estimated by Alexander stain; b, instability estimated by the observation of anther dehiscence with pollen release; nd, not determined; CR, culture room; G, greenhouse. Only the *TA29-RNase* T_1 plants were analyzed in the field trial.

Progenies	CR 15/10°C			CR 20/15°C			CR 30/25°C			G 23/17°C			Field		
	No. of Sterile Plants Analyzed	No. of Unstable Sterile Plants		No. of Sterile Plants Analyzed	No. of Unstable Sterile Plants		No. of Sterile Plants Analyzed	No. of Unstable Sterile Plants		No. of Sterile Plants Analyzed	No. of Unstable Sterile Plants		No. of Sterile Plants Analyzed	No. of Unstable Sterile Plants	
		a	b		a	b		a	b		a	b		a	b
T1-1	10	0	0	17	3	2	18	14	14	35	3	1	2	nd	1
T1-2	11	0	0	17	0	0	17	0	0	32	0	0	42	nd	0
T1-3	17	0	0	14	0	0	9	9	9	46	1	1	42	nd	32
T1-4	13	0	0	16	0	0	13	0	0	42	0	0	41	nd	0
T1-5	12	0	0	14	0	0	7	1	1	24	1	0	38	nd	5
T1-6	20	0	0	26	0	0	7	2	2	44	0	0	63	nd	13
T1-7	20	0	0	12	0	0	4	2	2	13	0	0	15	nd	2
T1-8	16	0	0	17	1	0	5	0	nd	35	0	0			
T1-9	14	0	0	7	0	0	2	1	nd	21	0	0			
T1-10	14	0	0	13	1	0	4	1	nd	20	2	2			
T1-11	13	0	0	16	0	0	12	2	nd	37	1	0			

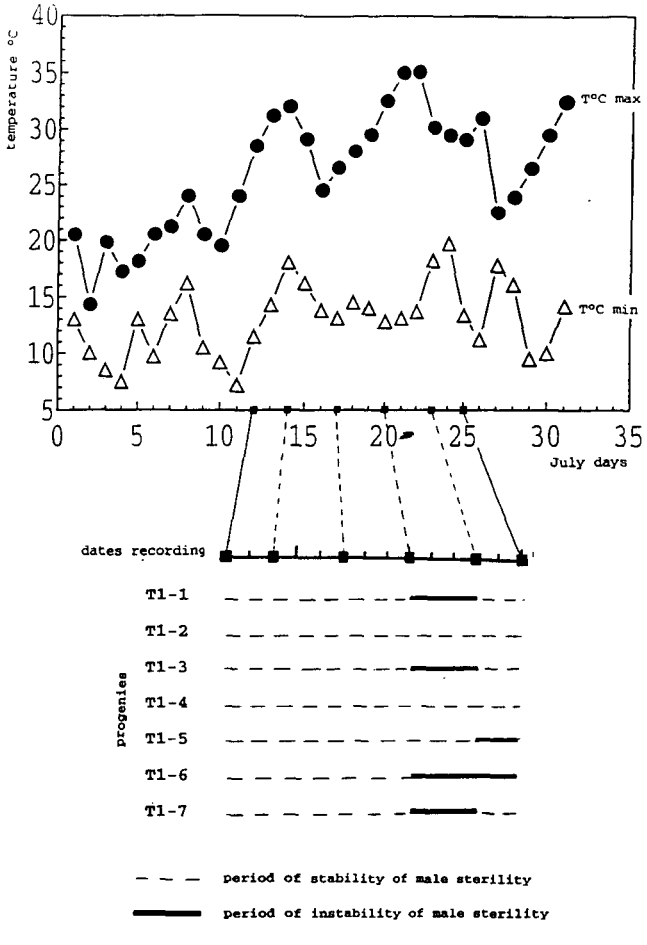

Figure 5. Influence of temperature on the stability of male sterility under field conditions.

night). One plant of the series T_1-1 and T_1-3, and two plants of the T_1-10 offspring showed anther dehiscence with pollen release in the greenhouse. One plant of the group T_1-5 and one plant of the group T_1-11 did not dehisce but showed normal pollen grains upon staining.

Only the plants carrying the *TA29-RNase T1* gene were studied in the field. The male-sterile plants of the series T_1-2 and T_1-4 were stable. Reversion to fertility, anther dehiscence, and pollen release were observed in all the other lines. The first unstable sterile plants were noticed between July 15 and July 20, 1990 (Fig. 5). The T_1-3 line was the most unstable; 32 of 42 male-sterile plants showed a reversion to fertility. However, after July 25, a return to total sterility was observed in the lines T_1-1, T_1-3, and T_1-7.

It is noteworthy that the instability in the 30°C day/25°C night culture room was observed after 10 d at these conditions. Similarly, in the field the instability occurred 7 d after a temperature peak of 30°C on July 13, 1990. Thus, the observations in the field were similar to those registered in the 30/25°C culture room; the same progenies were unstable and we observed the instability after a time lapse of 7 to 10 d.

DISCUSSION

The linkage of the *bar* gene to the male sterility trait allowed us to use the PPT test to estimate the number of T-DNA integrations in the primary transformants. Differences were observed between the results of the segregation and the copy number of the integrations as determined by Southern blot analysis. In addition, in some cases PPT-resistant and male-fertile plants were observed. Such observations could be explained by the position of particular inserts in the plant genome or by possible rearrangements that occurred in the course of insertion in the plant genome (Morota and Uchimiya, 1988). These events are not unusual when populations of primary transformants are examined. The probes used in our Southern analysis do not enable us to analyze the second possibility.

This engineered male sterility is characterized by a reduc-

tion of stamen filament as well as petal length, reduction of bud diameter, and anther indehiscence. The reduction of the filament length is common in male-sterile rapeseed plants (Theis and Robbelen, 1990), and certainly is correlated with the arrest of microsporogenesis. Filament length reduction and anther indehiscence were also observed by Gourret et al. (1992) in cybrids of *B. napus* with *ogu* cytoplasmic male sterility. In two naturally occurring male-sterile mutants discovered by W. Schuster and G. Rakow (unpublished data) and in the γ-ray-induced mutant of Takagi (1970), these features were also found. In contrast, sterile *TA29-RNase T1* and *TA29-barnase* tobacco anthers are dehiscent (Mariani et al., 1990). Petal modifications have been observed in other male-sterile rapeseed plants, such as the Bronowski male-sterile plants studied by Bartkowiak-Broda et al. (1979), which have flowers with narrow petals. In some cases, partial female sterility and lack of nectar secretion were also associated with male sterility (Pellan-Delourme and Renard, 1987). The presence of normal gynoecium and nectaries are an important requirement for a workable male-sterility system in rapeseed. In the case of the engineered male sterility, both the gynoecium and the nectaries are morphologically normal. The small diameter of the sterile buds is the result of the inhibition of the microsporogenesis, which prevents the swelling of the anthers. Anther indehiscence in the engineered male-sterility trait could be correlated with the absence of wall thickenings in the endothecium, which is implicated in the dehiscence of anther (Souvré et al., 1987). Similar observations were described by Gourret et al. (1992).

The arrest of microsporogenesis in the T_1-4 sterile anthers happens after the microspores are released from the callose. As a consequence of this arrest, a mass of flat exines accumulates in the mature anther locules. The cavities of the exines are devoid of tryphine, a complex of proteins and lipids (Souvré et al., 1987) of tapetal origin. Accumulation of flat exines has also been observed in other male-sterile lines of *B. napus*, including the two naturally occurring male-sterility lines found by W. Schuster and G. Rakow (unpublished data), the CMS line Raphanus and the male-sterility lines Janpol, NPZ, and A (Theis and Robbelen, 1990), and the *ogu* CMS (Gourret et al., 1992). The Alexander staining procedure allowed us to observe flat, empty exines in all of the T_1 male-sterile plants and to confirm that in all cases the abortion of pollen occurs after the tetrad stage. It has been shown that the higher frequency of pollen abortion takes place between the tetrad and the nonvacuolate microspore stages (reviewed by Laser and Lersten, 1972).

There is convincing evidence for the idea that lesions of the tapetum play an important part in the abortion of the pollen (Horner and Rogers, 1974; Horner, 1977; Filion and Christie, 1986; Gourret et al., 1987, 1992). A trophic role has been attributed to the tapetum (Vasil, 1967; Heslop-Harrison, 1968; Echlin, 1971; Dickinson, 1973; Mascarenhas, 1975); it produces a number of proteins and other substances that either aid in pollen development or become components of the pollen outer wall (Esau, 1977; Knox, 1979).

In the engineered male sterility studied here, the disruption of the microsporogenesis is correlated with the activity of the chimeric *TA29-RNase* gene. *TA29* mRNA accumulates early in the normal development of the tobacco anther (Goldberg, 1988; Koltunow et al., 1990). The peak of the mRNA accumulation is after the release of the microspores from the callose, after which the level decays prior to anther dehiscence and pollen grain release. Mariani et al. (1990) observed a dramatic decrease of tapetal-specific mRNA level in the sterile tobacco anthers of plants carrying the *TA29-RNase* gene. Presumably, tapetum protein synthesis either in tobacco or in rapeseed is disrupted because of the activity of the exoribonuclease; therefore, the tapetum cannot play its role in relation to the microspores, leading to the degeneration of the microspores.

The study of the behavior of the male-sterile plants revealed instability of the sterility for some T_1 progeny plants under some growth conditions. Our analysis of the plants carrying the *TA29-RNAse T1* gene showed that only plants T_1-2 and T_1-4 were stable under all growth conditions investigated in this work. Our data are insufficient to draw accurate conclusions concerning the stability of the introduced trait in plants carrying the *TA29-barnase* gene. Furthermore, studies under a wider variety of growth conditions will be necessary. One further caveat concerns our use of the Alexander staining technique. Although it is useful as a rapid test for sterility, since Salako (1970) calculated a correlation of 0.85 between the fertility test and the Alexander stain, the fertility of the pollen produced by unstable male sterile plants should be further investigated with other techniques (Heslop-Harrison et al., 1984; Coleman and Goff, 1985) and breeding experiments.

The observations in the field are similar to those registered in the 30/25°C culture room; the same lines were unstable and the instability was observed after a lapse of time of 7 to 10 d. It is interesting that Fan and Stefansson (1986) have observed that the same instability occurs in the *nap* and *pol* CMS systems under similar growth conditions. Reversion to fertility occurs in the *nap* and *pol* CMS plants 7 to 10 d after increase of temperature. In contrast, a reversion to fertility at low temperatures (11–16°C) was observed in the two naturally occurring male-sterility lines discovered by W. Schuster and G. Rakow (unpublished data). However, two of seven *TA29-RNase T1* lines were stable under all culture conditions investigated in this work. In addition, the segregation of the two loci in these lines gives the expected 50% PPT-susceptible, fertile plants and 50% PPT-resistant, sterile plants. Although anther dehiscence with pollen release has not been observed in the *TA29-barnase* sterile plants, Alexander stain showed viable pollen at least one time in each *TA29-barnase* line. These plants have never been tested in the field.

To date, we are unable to discern where the cause of instability lies; whether the environmental factors influence promoter activity or destabilize the ribonuclease protein. However, it is possible to obtain plants stably expressing a nuclear male sterility gene using gene transfer, although a population of plants must be screened to select truly stable lines. The use of this nuclear male sterility system leads to the creation of F_1 hybrids with 50% male-sterile plants and 50% male-fertile plants. This system can be used directly; in fact, it has been shown that only 10% of male-fertile plants in a mixed variety are enough to assure pollination (Renard and Mesquida, 1987). Transfer of the ribonuclease gene to other genotypes of *B. napus* can be accomplished in a backcross program.

In parallel with this work, it has been shown that male-sterile plants carrying the *barnase* gene can be restored to fertility. Mariani et al. (1992) have engineered a restorer line carrying the *barstar* gene, encoding a specific inhibitor for *barnase*. In many crop plants in which the harvest product is the seed, the existence of restorer lines makes it possible to obtain a 100% fertile F_1 hybrid.

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