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The development of a nuclear male sterility system in wheat. Expression of the *barnase* gene under the control of tapetum specific promoters

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Abstract Nuclear male sterility within *Triticum aestivum* is considered as the ideal basis for the development of a hybridization system for wheat. We engineered nuclear male sterility in wheat by introducing the *barnase* gene under the control of tapetum-specific promoters derived from corn and rice. A biolistic-mediated transformation method, based on the use of the poly(ADP-ribose)polymerase inhibitor niacinamide, was set up which enriched for low-copy integrations (1–3 copies). Most of these copies were not linked and segregated in the next generation.

Key words Wheat · *Triticum aestivum* · Transformation · Nuclear male sterility · DNA-integration

Introduction

Over the last 50 years, different cytoplasmic male-sterile (CMS) and nuclear male sterile (NMS) systems and chemical hybridizing agents have been developed and evaluated for the production of hybrid wheat (Pickett 1993) . However, all the present systems have serious handicaps in restoration or in obtaining, under different environmental conditions, a high degree of male sterility in all parts of the female parent (Pickett 1993). Complete sterility is a primary requirement because incomplete sterility leads to self-fertilization of the female parent which results in an unacceptable contamination of the F_1-hybrid seed. The ideal hybridization system would be a reliable NMS system whose establishment could be achieved within *Triticum aestivum*, so avoiding time-consuming crosses and possible genetic drift.

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In the present study, a first step in the development of an NMS system in wheat by means of genetic engineering is presented. Genes containing the *barnase* gene, under the control of tapetum-specific promoters derived from corn and rice, were introduced into wheat and their expression at the T_0, B_1 and B_2 generations were studied. The use of tapetum-specific promoter-*barnase* constructs to produce NMS plants has already been successfully applied to tobacco, rapeseed and corn (Mariani et al. 1990; Williams 1993). Expression of the *barnase* gene at specific stages of anther development destroys the tapetum, so preventing normal pollen development. Depending on the time the *barnase* gene is expressed, i.e. early or late microspore stage, either no pollen or else only sterile pollen is produced.

To achieve NMS in wheat, a transformation system which regenerates with a high efficiency transformants with 'elite' alleles would be desirable. This means that each transformant would have a simple integration pattern of the delivered DNA, a low-copy number and no rearrangements. Ideally, insertion of DNA would be at sites in the genome which do not interfere with the tapetum-specific expression of the transgene (no position effects). Non-specific expression of the *barnase* gene in non-target tissues, such as leaf or root, will interfere with the fitness of the transgenic plant (De Block 1993).

In the first part of this paper a biolistic-based transformation system for wheat will be described that meets the above requirements. In the second part, the behaviour of three tapetum-specific promoter-*barnase* constructs in wheat are studied.

Materials and methods

Plasmids

Three plasmids were used, each of which contained the *barnase* gene under the control of a different tapetum-specific promoter: one

derived from corn, *pca55* (see Fig. 1) (PGS patent publication WO 92/13956), and two from rice, *pE1* and *pT72* (PGS patent publication WO 92/13957). All three contained *p35S : bar* as a selectable marker gene (De Block 1990).

Media

The media are summarized in Table 1.

Wheat transformation

T. aestivum cv Pavon, a spring variety, was grown in the greenhouse. Developing seeds, white-greenish with white semi-liquid endosperm, were harvested and sterilized in 70% ethanol, 1–2 min, followed by a 1.3% NaOCl solution containing 0.1% Tween 20 for 15 min. Immature embryos of about 1 mm in size, were placed, scutellum up and embryogenic axis down, on callus-inducing medium (CI-medium). The material was incubated at 26–27°C for 3 weeks in the dark. After 3 weeks, the embryogenic parts of the calli were isolated and subcultured for 2 weeks on callus-propagation medium (CP-medium) at 24°C in the light (10 μEinstein $s^{-1} m^{-2}$) with a photoperiod of 16 h light and 8 h dark.

Four days before bombardment, the calli were cleaned by removing the non-morphogenic (non-embryogenic or non-meristematic) parts. Calli were then divided into ± 2-mm pieces and placed in a ± 0.5-mm-thick circle (2.5 cm diameter) at the centre of a 9-cm Petri dish containing CP-medium to which 1–2 mM of niacinamide had been added.

The Biolistic PDS-1000/He device of BIO-RAD was used for bombardment. The preparation of the microcarriers (0.4–1.2 μm) and the coating of the microcarriers with DNA was carried out essentially as advised by the manufacturer. The Petri dishes were placed at level two (from bottom). The bombardment was done at 1550 psi.

A few hours after bombardment, the calli were placed on selective CP-medium containing 2.5 mg/l of glufosinate, and incubated for 2 weeks at 24°C in the light (20–30 μEinstein $s^{-1} m^{-2}$, photoperiod of 16 h light and 8 h dark). After 2 weeks, the calli were transferred to the same medium. After 2 more weeks, the growing parts of the calli were transferred to regeneration medium (RE-medium) containing 2.5 mg/l of glufosinate. The morphogenetic parts of the calli were transferred every 2 weeks to fresh RE-medium (2–4 transfers). Calli with regenerated shoots were transferred to rooting medium (RO-medium) containing 2.5 mg/l of glufosinate. After 2–4 weeks, the rooted shoots were separated from the calli and from each other and transferred to P0-medium, containing 2.5 mg/l of glufosinate. Well-developed plants were transferred to soil.

Cross pollinations

The male-sterile plants were crossed by cutting the top of the florets with a scissors, after which they were pollinated with the pollen of untransformed control plants.

Test for phosphinothricin resistance

The functional expression of the *bar* gene in the transformed plants and their progenies was analysed by means of the ammonium multi-well assay as described by De Block et al. (1995). Glufosinate concentrations between 2 and 10 mg/l in 50 mM of potassium phosphate buffer, pH 5.8, were employed.

Southern hybridizations

Total genomic DNA was extracted from leaf tissue according to the CTAB method (Lassner et al. 1989). Genomic DNAs were digested with *Eco*RV, *Nco*I, and *Nde*I if the transgenic plants contained respectively *pca55 : barnase-p35S : bar*, *pE1 : barnase-p35S : bar*, and *pT72 : barnase-p35S : bar*. Digested DNAs were fractionated on a 1% agarose gels and transferred to Hybond-N^+ membranes (Amersham, UK) using the vacuum-blotting system (VacuGene XL, Pharmacia, Sweden, Uppsala). Pre-hybridizations and hybridizations of the Hybond-N^+ membranes were mainly done as advised by the manufacturer (Amersham, UK), except that 6 × SSC at 65°C was used. DNA fragments containing the *barnase* (478-bp) (Mariani et al. 1992), or *bar* (546 bp) (De Block et al. 1987) coding sequences were employed as probes. The probes were labelled with ^{32}P by random-primed DNA labelling. The hybridized membranes were washed three times for 30 min at 65°C with respectively 6 × SSC, 2 × SSC, and 0.1 × SSC solutions containing 0.1% SDS.

Microscopy

Florets between the time of terminal-spikelet formation until the emergence of the ear were harvested and fixed in 10% buffered

Table 1 Plant tissue-culture media

Medium	Application	Composition[a]
CI	Callus induction from immature embroys	MS medium[b] + 3% sucrose; 40 mg/l adenine · SO_4; 0.5 g/l MES pH 5.8; 0.5% agarose; 0.5 mg/l thiamine · HCl; 0.5–2.5 mg/l $CuSO_4 \cdot 5H_2O$[c]; 25 mg/l acetylsalicylic acid; 2 mg/l 2,4D
CP	Propagation of embryogenic callus	MS medium + 3% sucrose; 40 mg/l adenine · SO_4; 0.5 g/l MES pH 5.8; 0.5% agarose; 0.5 mg/l thiamine · HCl; 0.5 mg/l $CuSO_4 \cdot 5H_2O$; 1 mg/l 2,4D
RE	Shoot regeneration	MS medium + 3% sucrose; 40 mg/l adenine · SO_4; 0.5 g/l MES pH 5.8; 0.5% agarose; 0.5 mg/l thiamine · HCl; 0.5 mg/l $CuSO_4 \cdot 5H_2O$; 1 mg/l zeatine; 1 mg/l IAA
RO	Root regeneration	Half concentrated MS medium + 3% sucrose; 40 mg/l adenine · SO_4; 0.5 g/l MES pH 5.8; 0.5% agarose; 0.5 mg/l thiamine · HCl; 50 mg/l myo-inositol; 0.25 mg/l pyridoxine · HCl; 0.25 mg/l nicotinic acid; 0.5 mg/l $CuSO_4 \cdot 5H_2O$
PO	Outgrowth of rooted shoots	Half-concentrated MS medium + 1.5% sucrose; pH 5.8; 0.7% agar; 0.5 mg/l thiamine · HCl; 50 mg/l myo-inositol; 0.25 mg/l pyridoxine · HCl; 0.25 mg/l nicotinic acid; 0.5 mg/l $CuSO_4 \cdot 5H_2O$

[a] The compounds listed after agarose or agar were filter sterilized and added after autoclaving
[b] Murashige and Skoog basal medium with the macro- and micro-nutrients, and vitamins, as described (Murashige and Skoog 1962)
[c] Purnhauser and Gyulai (1993)

neutral formalin containing 0.25% glutaraldehyde. The embedding was done in hydroxyethylmethacrylate using the Histo-resin embedding kit (Jung, Leica, Heidelberg, Germany); 3-μm sections were stained with 0.05% toluidine blue O and examined with a Leitz Dialux 22 microscope.

Results

Biolistic-mediated transformation: the use of niacinamide

Biolistics is a powerful tool in plant transformation and has been successfully applied in the transformation of wheat (Vasil et al. 1993; Weeks et al. 1993; Becker et al. 1994; Nehra et al. 1994). However, transgenic plants generated by this method often have very complicated integration patterns of the delivered DNA (high copy number and rearrangements) (De Block 1993). Although several variables in the pre-treatment of the starting material (period of subculture, growth at lower temperatures) and the delivery of the DNA (amount of DNA, helium pressure, and target distance) were evaluated, none of these influenced the integration pattern (data not shown).

The nuclear enzyme poly(ADP-ribose)polymerase (PARP) is involved in DNA repair (de Murcia et al. 1994), cell division (Eki 1994), apoptosis (Monti et al. 1994), recombination (Waldman and Waldman 1990), and several other processes in animal cells. Although poly(ADP-ribose)polymerase is found in plants (Lepiniec et al. 1995) not much is known about its function. For *Arabidopsis* and tobacco, Puchta et al. (1995) showed that the addition of PARP-inhibitors in the plant growth medium enhanced intra-chromosomal homologous recombination. In animal tissue culture, inhibition of PARP results in a reduced illegitimate recombination and an enhanced intra-chromosomal homologous recombination (Waldman and Waldman 1990). Because illegitimate recombination is the mechanism by which extrachromosomal DNA inserts into the chromosome, the influence of PARP on transformation was studied. Embryogenic callus tissue was incubated for 4 days before transformation on callus propagation medium containing 2 mM of the PARP-inhibitor niacinamide (Sims et al. 1982). The same amount of calli was incubated on niacinamide-free control medium. The transformation was done with DNA containing the construct *pT72 : barnase-p35S : bar*. The results of three independent experiments are summarized in Table 2. In addition to the fact that only very few transformants were obtained if no niacinamide pre-treatment had been applied (see lower), the transgenic plants obtained from the niacinamide pre-treatment experiments contained only a limited number of integrated copies of the transgenes.

The transgenic plants have simple integration patterns of the transgenes

In total seven transformation experiments with the constructs *pT72 : barnase-p35S : bar*, *pE1 : barnase-p35S : bar*, and *pca55 : barnase-p35S : bar*, using 1–2 mM of niacinamide in the pre-treatment medium, were done. An average transformation frequency of between 1 and 2% was obtained, of which about 50% of the transgenic plants showed a sterile phenotype. In total 29 sterile plants were obtained (0.8%) and further analysed in the T_0 (primary transformants) (Table 3), B_1 ($T_0 \times$ Pavon) and B_2 ($B_1 \times$ Pavon) generations (Table 4). For the Southern analyses the total DNAs of the T_0-plants were hybridized with both the *barnase* and *bar* genes (Fig. 1). The results of the Southern hybridizations are summarized in Table 3. About 75% of the transformants contained between 1 and 3 copies of the transgenes. It has to be emphasized that if multiple copies of the transgenes were inserted, very few tandems were found. Moreover, segregation analyses of the B_1 and B_2 generations showed that the different integration sites were often not linked (see below).

The barnase constructs confer male sterility, which is stably maintained in the B_1 and B_2 generations

Male-sterile plants were generated with all three constructs. In Fig. 2 it is shown that the anthers of the

Table 2 Influence of niacinamide pre-treatment on transformation efficiency and integration of the delivered DNA[a]

Pre-treatment	Total number of calli bombarded[b]	Number of transformants obtained	Southern analyses Copy number ***barnase*** and *bar*				
			1 copy	2 copies	3 copies	4 copies	5 copies
None	1556	4	**0** 0	**0** 0	**0** 0	**0** 0	**4** 4
4 days, 2 mM niacinamide	1653	15	**1** 2	**4** 4	**7** 4	**3** 2	**0** 3

[a] The numbers represent the total of three experiments
[b] Calli were bombarded with DNA of the construct *pT72 : barnase-p35S : bar*

Table 3 Results of the transformation experiments with the *tapetum-specific promoter : barnase-p35S : bar* constructs and Southern analyses of the transformants

Construct	Total number of calli bombarded	Total number of transgenic plants	Southern analyses Copy number of ***barnase*** and *bar*				
			1 copy	2 copies	3 copies	4 copies	5 copies
pE1 : barnase-*p35S : bar*	1225[a]	8	**0** 1	**5** 3	**1** 2	**0** 0	**2** 2
pT72 : barnase *-p35S : bar*	1653[b]	15	**1** 2	**4** 4	**7** 5	**3** 3	**0** 1
pca55 : barnase *-p35S : bar*	800[c]	6	**2** 2	**0** 1	**3** 3	**1** 0	**0** 0
Total	3678	29	**3** 5	**9** 8	**11** 10	**4** 3	**2** 3

[a] Total of two experiments; pre-treatment 4 days, 2 mM niacinamide
[b] Total of three experiments; pre-treatment 4 days, 2 mM niacinamide
[c] Total of one experiment; pre-treatment 4 days, 1 mM niacinamide

Table 4 Linkage of *barnase* gene copies in transgenic wheat lines[a]

barnase construct	Segregation analyses		
	Lines studied	Lines with linked integrations	Lines with unlinked integrations
pE1 : barnase	2	0	2[b]
pT72 : barnase	6	0	5[b] + 1[c]
pca55 : barnase	3	1	2[b]
Total	11	1	10

[a] Only two- and three-copy lines from Table 3 were analysed
[b] All lines containing the different segregated copies were sterile and phosphinothricin resistant
[c] Two-copy line of which the total progeny, containing lines with both copies, or one of the two copies, was fertile

male-sterile florets have completely degenerated. The swollen ovary and the absence of lodicules in the opened male-sterile floret, as seen in Fig. 2 C and D, have also been described for CMS flowers (Pickett 1993). In normal fertile wheat, pollination mainly takes place within the unopened flower. However, when no pollination occurred, as in the male-sterile flowers, the lodicules start to swell, which causes the lemma and the palea to separate. The swelling of the lodicules leads to the 'first opening' of the floret lasting for only a short time. At the end of the first opening the lodicules collapse, after which the pistil starts to expand. This causes the floret to open again (Fig. 2 B), exposing the stigma for several days during which cross-pollination may occur.

Microscopic observations on plastic-embedded and sectioned florets show the degeneration of the tapetum after meiosis and the complete degeneration of the microspores in the male-sterile florets (Fig. 2 E and F).

The sterile T_0-plants and their progeny set seeds only if cross-pollinations were done (see Materials and methods). Seed-set by selfing was never observed.

The behaviour of 14 transgenic lines, containing 1–3 copies of the *barnase* gene, was studied in more detail in the B_1 and B_2 generations. Segregation of the different copies occurred in 10 out of the 11 two- and three- copy lines (Table 4, Fig. 1). Except for one line (Table 4), all the different copies could confer complete male sterility on their own. The vegetative phenotype of the sterile progenies was, under greenhouse conditions, indistinguishable from that of the fertile control plants. Also, no delay in flowering time could be observed.

Discussion

The use of niacinamide in transformation

Transformants generated by biolistics are often characterized by multiple insertions of the delivered DNA. These insertions are often linked and/or organized in tandem. These complicated integration patterns are less frequent if PEG, electroporation, or *Agrobacterium tumefaciens* are used as the delivery system. In our experiments the high-copy number, the tandem integrations, and the genetic linkage of multiple insertions could be prevented by incubating the tissue for several days before biolistics on niacinamide-containing medium. About 75% of the transformed plants contained 1–3 copies. Most of these copies were not linked and segregated in the B_1 generation, leading to new one- and two-copy lines. From the 11 two- and three-copy lines studied, seven new one-copy lines and four new two-copy lines were generated. All of these lines were sterile and phosphinothricin resistant.

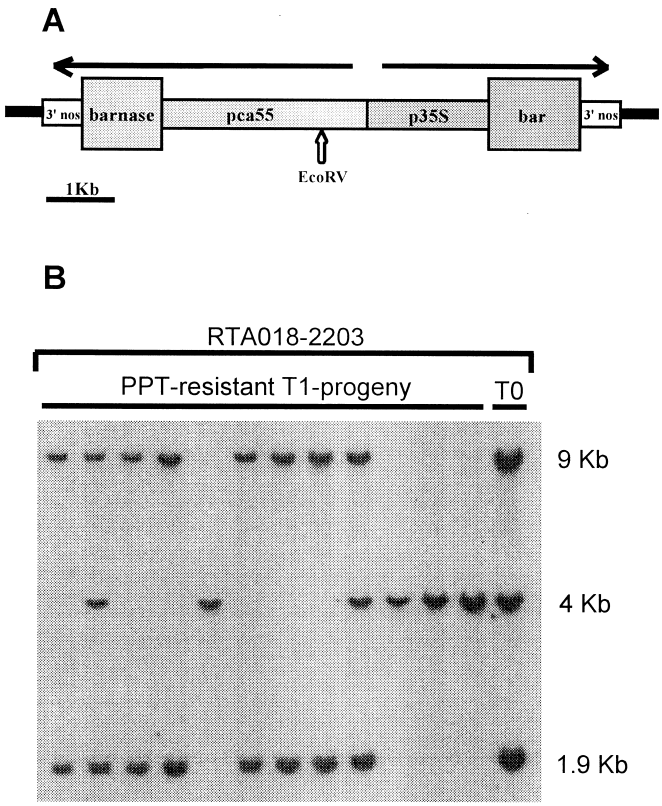

Fig. 1A, B Southern analysis of progeny from RTA018-2203. The transgenic plant line RTA018-2203, containing *pca55 : barnase-p35S : bar*, was pollinated with pollen derived from an untransformed control plant. The B_1-seeds obtained were germinated and the seedlings were tested for phosphinothricin resistance by means of the ammonium multi-well assay. **A** Schematic presentation of the *pca55 : barnase-p35S : bar* construct. **B** Genomic DNAs from the mother plant (T_0) and 12 phosphinothricin-resistant B_1-plants were digested with *Eco*RV, fractioned by electrophoresis, transferred to a nylon membrane and hybridized with a ^{32}P-labelled DNA probe containing the *barnase* coding sequence. The T_0-plant contained three bands (9 kb, 4 kb, and 1.9 kb) hybridizing with the *barnase* probe. These bands segregated in the B_1-progeny in one (4 kb) band and two (9-kb and 1.9-kb) bands

Niacinamide is an inhibitor of poly(ADP-ribose)polymerase (PARP) (Sims et al. 1982). PARP is a nuclear enzyme that is bound to the DNA. This enzyme is activated by single- and double-stranded DNA breaks. Activated-PARP transfers the ADP-ribose moiety of NAD to the carboxyl group of glutamate in acceptor proteins. These acceptor proteins are nuclear-associated proteins like histones, topoisomerase I and II, and PARP itself (de Murcia and Ménissier de Murcia 1994). The automodified PARP has a lower affinity for nicked DNA and does not affect the DNA break, so that the repair enzymes can reach the DNA. Apart from the importance of PARP in DNA repair (de Murcia and Ménissier de Murcia 1994), the poly-ADP ribosylation seems to have an important function in very different cellular functions including: stress, illegitimate and homologous recombination, chromosome stability, cell differentiation, cell division and apoptosis (Waldman and Waldman 1990; de Murcia and Ménissier de Murcia 1994; Eki 1994; Monti et al. 1994). The function of PARP in recombination and cell division may explain the simple integration patterns. Inhibition of PARP reduces the illegitimate recombination (less integration) and enhances the intra-chromosomal homologous recombination (deleting newly formed tandem copies) (Waldman and Waldman 1990). Eki (1994) showed that PARP inhibits the human replicative DNA polymerases α, δ and ε. We also found an enrichment for cells blocked in the G_1 and/or G_2 phases when the tissues had been incubated for several days on niacinamide-containing medium (unpublished results). Kartzke et al. (1990) showed the importance of the cell cycle on the integration of PEG-delivered DNA. By analogy with the results described by these authors, it is likely that cells transformed by means of biolistics at the G_1- and/or G_2-phase also have more simple integration patterns. However, this cannot explain the higher transformation efficiencies obtained after niacinamide pre-treatment (Table 2). A possible explanation can be found in transformation experiments using constructs lacking the *barnase* gene (data not shown). Without miacinamide pre-treatments, transformation frequencies of 5–10% were obtained. However, with niacinamide pre-treatment, the transformation frequencies dropped to 1–2%. By analogy with the experiments using the *barnase* gene, these transgenic plants also had more simple integration patterns of the delivered DNA if niacinamide pre-treatment had been included. In addition to the effect of niacinamide on the integration, niacinamide in some way counteracts the negative effect of *barnase* on transformation.

Because the niacinamide treatments were done before DNA delivery and the integration of the DNA probably occurred only a few days later, niacinamide must have had a prolonged effect. When niacinamide treatment was postponed until after bombardment, the above described effects were not seen (data not shown). Probably the state of the plant material just before transformation is important and largely determines the mode of integration. In this way, the beneficial effect often seen when a niacinamide pre-treatment was included, completely disappeared or was even negative if the starting material was not in optimal condition. Experiments have been initiated to study the interaction between the physiological state of the plant before transformation and the DNA integration event.

The transgenic plants and their progenies are vegetatively normal and male sterile

Progeny analyses of the transgenic plants showed that, under greenhouse conditions, the expression of the

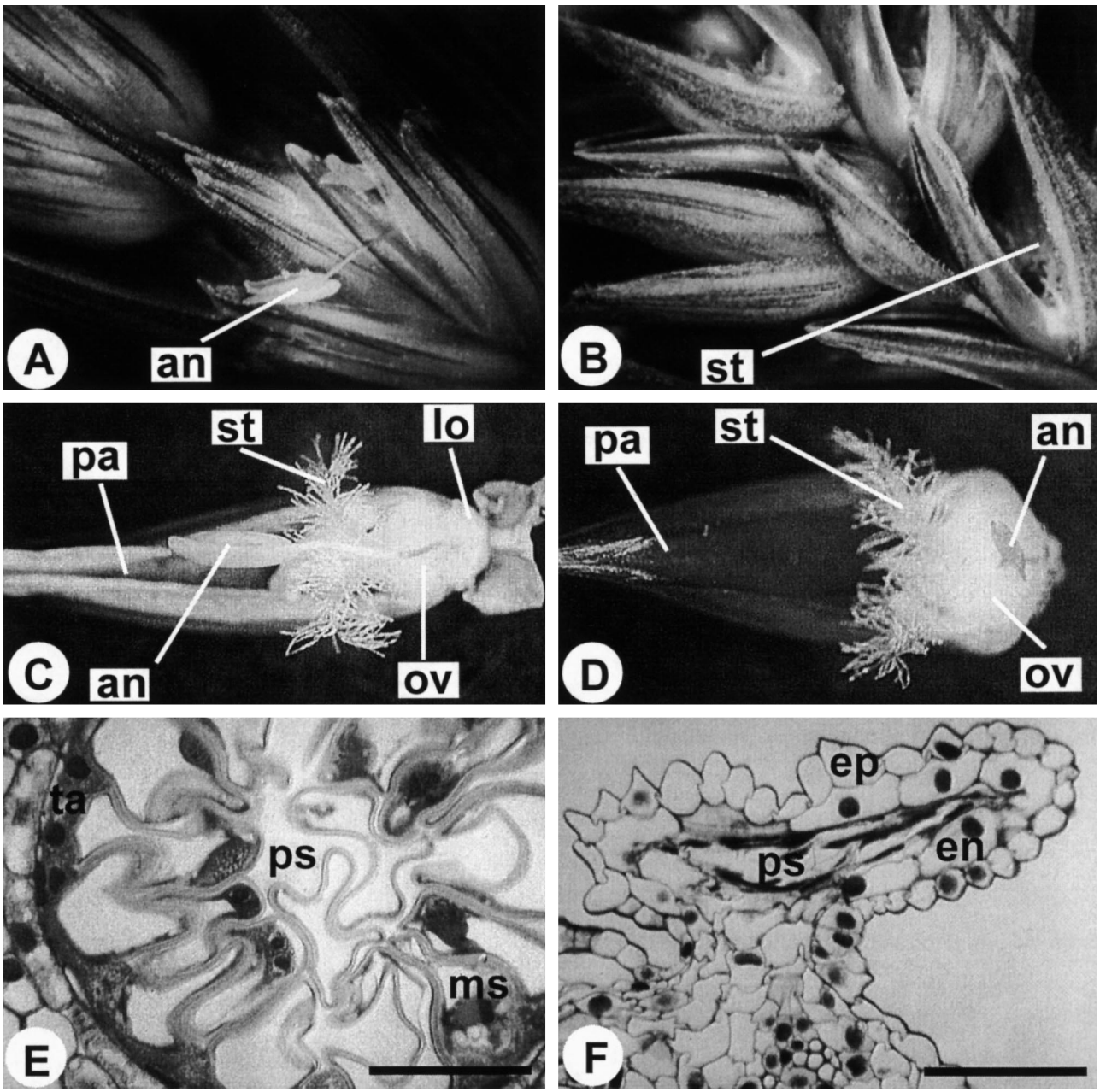

Fig. 2A–F Morphology of wheat florets from a male-fertile control plant and a transgenic male-sterile plant containing the *pE1 : barnase* gene. **A and B** spikelets from male-fertile (**A**) and male-sterile (**B**) plants. Notice the opened florets in the male-sterile plant, exposing the stigma to the environment. **C and D** florets from male-fertile (**C**), just before anthesis, and male-sterile (**D**), plants. The lemma of the florets has been removed. The degeneration of the anthers and the swelling of the ovarium in the male-sterile plants is clearly visible. **E and F** Normasky interference contrast photographs of anther cross-sections form male-fertile (at the vacuolated microspore stage) (**E**), and male-sterile plants. The bars represent 50 µm. *an* anther; *en* endothecium; *ep* epidermis; *lo* lodicule; *ms* microspore; *pa* palea; *ps* pollen sac; *ov* ovarium; *st* stigma; *ta* tapetum

tapetum specific promoter : barnase constructs did not affect the vegetative phenotype. The transgenic plants were vigorous, and had a normal height, leaf size, and tillering. Also, no delay in flowering compared with the control plants was observed.

Male-sterile flowers were obtained with all three *barnase* constructs. Microscopic studies confirmed the premature degeneration of the tapetum. No abnormalities could be observed in the other parts of the floret, confirming the tapetum-specific expression of the two rice promoters, *pE1* and *pT72*, and the corn promoter, *pca55*. Because no self-fertilization occurred in the male-sterile flowers, the unfertilized florets opened to allow cross-pollination. The separation of lemma and

palea was made possible by the swelling of the pistil. Until now, only the B_1 and B_2 generations have been studied. Except for one line, no instability of the male sterility was observed. A few lines (female) were backcrossed with the spring wheat variety Bobwhite (male). The male sterility was also stable in these hybrids (data not shown).

All the above observations were made under greenhouse conditions. In 1997 field trials will be carried out to study the behaviour of the one- and two-copy lines in the field.

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