Proc. Natl. Acad. Sci. USA
Vol. 92, pp. 12165–12169, December 1995
Plant Biology

Pollen selection: A transgenic reconstruction approach

(*in vitro* pollen maturation/hygromycin B/male gametophyte selection)

ALISHER TOURAEV, CHRISTINE S. FINK, EVA STÖGER, AND ERWIN HEBERLE-BORS*

Vienna Biocenter, Institute of Microbiology and Genetics, University of Vienna, Dr. Bohrgasse 9, A-1030 Vienna, Austria

Communicated by S. J. Peloquin, University of Wisconsin, Madison, WI, August 30, 1995

ABSTRACT **A transgenic reconstruction experiment has been performed to determine the feasibility of male gametophytic selection to enhance transmission of genes to the next sporophytic generation. For tobacco pollen from a transgenic plant containing a single hygromycin-resistance (hygromycin phosphotransferase, *hpt*-) gene under control of the *dc3* promoter, which is active in both sporophytic and gametophytic tissues, 3 days of *in vitro* maturation in hygromycin-containing medium was sufficient to result in a 50% reduction of germinating pollen, as expected for meiotic segregation of a single locus insert. Pollination of wild-type plants with the selected pollen yielded 100% transgenic offspring, as determined by the activity of the linked kanamycin-resistance gene—present within the same transferred T-DNA borders—under control of the *nos* promoter. This is direct proof that selection acting on male gametophytes can be a means to alter the frequency of genes in the progeny.**

The plant life cycle consists of two distinct phases, the diploid sporophytic and the haploid gametophytic phase. In angiosperms, the haploid phase is reduced, and the male and female gametophytes are enclosed in the respective sex organs of the sporophyte. Male and female gametophytes (i.e., pollen and embryo sacs) are formed from haploid spores, the direct products of meiosis, and the sporophytic phase starts again after the fusion of the two pollen-contained sperms with the egg and polar cells of the embryo sac in a double fertilization event. In spite of the large morphological and physiological differences, a large overlap of gene expression has been demonstrated between the sporophytic and gametophytic phase on the protein and mRNA level (for reviews, see refs. 1 and 2).

These data provided the theoretical basis for the early pollen selection experiments (for review, see ref. 2). The idea was that if such an overlap exists, it should be possible to exert selection pressures on pollen to increase the frequency of responsive genes in the subsequent sporophytic generation. Angiosperm pollen has some features that makes pollen selection a very attractive method of plant breeding: large population size, haploid nature of the cells, and independence from the maternal plant (3).

A variety of selection pressures such as drugs, heat, water, cold temperature, and other stresses have been applied to mature pollen just before pollination, and nonstressed plants have been pollinated with the selected pollen in anticipation of obtaining progeny in which an increased frequency of resistant individuals can be found (for review, see ref. 4). Despite a few positive reports (5–9), the feasibility of pollen selection is still under question. This is mainly due to the problem of discriminating between gametophytic and sporophytic effects on pollen development (resulting from pollen- and anther-expressed genes, respectively; refs. 2 and 9) and a lack of appropriate selection schemes. The developmental window open for selection, 1 or 2 days before anthesis to a few hours after germination, at a time when the pollen is preparing for desiccation or using preformed proteins and RNAs and internal pools of nutrients for germination, respectively, was simply too short to allow efficient selection.

This laboratory has developed (10) an *in vitro* system that allows the development of isolated microspores into mature fertile pollen. During this development, the bulk of gametophytic gene expression takes place (11). Thus, the *in vitro* system produces a developmental window for selection that is much larger than in the conventional pollen selection schemes. In this paper, we describe a transgenic reconstruction experiment for pollen selection using defined gene constructs in stably transformed plants and an antibiotic as a selective agent acting during *in vitro* pollen development. We show that selection of pollen during *in vitro* development is highly effective and that the selected gene, as identified by the activity of a closely linked marker gene that is active in the sporophytic phase, can be efficiently transmitted to the progeny.

METHODS

Plant Material. *Nicotiana tabacum* cv. Petite Havana SR1 plants were grown in a growth chamber at 25°C with a 16-h light/8-h dark regime. Transgenic tobacco plants containing *dc3–hgB* and 35S–*uidA* constructs were produced by leaf disk transformation using the disarmed *Agrobacterium tumefaciens* LBA4404 as described by Horsch *et al.* (12). Seeds of *dc3–uidA*, *lat52–uidA*, and *pa2–uidA* gene constructs containing plants were provided by T. L. Thomas (Texas A&M University, College Station), S. McCormick (U.S. Department of Agriculture, Agricultural Research Service, Plant Gene Expression Center, Albany, CA), and A. van Tunen (Centre for Plant Breeding and Reproduction Research, Wageningen, The Netherlands), respectively.

All transformed plants were kept in the isolated growth chambers at 25°C with a 16-h light/8-h dark regime and used, after cutting them back, for two flowering periods. The original transformants were selfed to produce homozygous lines. Their homozygous nature was verified by producing 100% transformed offspring in backcrosses with wild-type plants.

Pollen Culture. Isolation, maturation, and germination of microspores and midbinucleate pollen were performed essentially as described by Benito Moreno *et al.* (10). The conditions for maturation and germination have, however, been slightly modified. After isolation, uninucleate microspores were first cultured for 3 days in medium T1 [0.5 M sucrose/3 mM glutamine/lactalbumin hydrolysate (10 mg/ml)/10 mM KNO_3/1 mM $Ca(NO_3)_2$/1 mM $MgSO_4$/0.16 mM H_3BO_3/1 mM uridine/0.5 mM cytidine/1 mM sodium phosphate, pH 7; ref. 13], a modification of medium MR26 (10). Then, the cultures were diluted with an equal amount of medium AM-GLU [MS macro and micro salts (13)/0.5 M sucrose/3 mM glutamine, pH 7 (10)] and after one more day finally diluted

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviation: GUS, β-glucuronidase.

*To whom reprint requests should be addressed.

with an equal amount of medium P (1.17 M sucrose/0.1 M L-proline; ref. 14). A highly homogeneous starting population for *in vitro* maturation was obtained from midbinucleate pollen by Percoll density gradient centrifugation (15). The two-step gradient (50/60% Percoll diluted in 1 M mannitol) was repeated twice and resulted in a population of midbinucleate pollen, which was matured in medium AMGLU for 3 days. *In vitro*-matured microspores and midbinucleate pollen were allowed to germinate in medium GQ [0.3 M sucrose/casein hydrolysate (1 g/liter)/1 mM KNO_3/0.3 mM $Ca(NO_3)_2$/0.8 mM $MgSO_4$/1.6 mM H_3BO_3/2.5 mM Mes, pH 5.9/2 μM quercetin; ref. 16]. In these modified conditions, high germination frequencies could be achieved. In microspore cultures, 65% and, in cultures of midbinucleate pollen, >90% of the starting pollen population germinated after 7 h and 3.5 h, respectively, incubation in medium GQ.

Plasmids and Cloning Strategy. Two binary vectors of *A. tumefaciens* that are derivates of pBIN19 (17) were used. The pBIPA2 plasmid contained the β-glucuronidase (GUS) (*uidA*) coding sequence under control of the pollen-specific *pa2* promotor (18). The *uidA* gene in the pBIHPA2 plasmid was replaced with the *hpt* (hygromycin B phosphotransferase) coding sequence under control of the *pa2* promotor. Both plasmids contained an additional marker, the neomycin phosphotransferase II gene (*nptII*) under control of the regulatory sequences of the nopaline synthase gene (*nos*), conferring resistance to kanamycin. The pBIHDC3 plasmid was constructed by replacing the *pa2* promotor of pBIHPA2 with the *dc3* promotor (19, 20) from carrot (DNA of the *dc3* promotor originated from the SF16 plasmid, provided by T. Thomas). Cloning was performed by blunt-end ligation as described by Sambrook *et al.* (21).

GUS Assays. Fluorimetrical and histochemical GUS assays were carried out as described by Jefferson (22) and Stöger *et al.* (23). Under the conditions used, no endogenous GUS activity was detected in either assay (ref. 24 and unpublished data). Histochemical assays with *in vivo* pollen were performed after isolating them from the surrounding anther tissues (25).

***In Situ* Pollination with *in Vitro*-Matured Pollen.** Mature flowers just before anthesis were emasculated 1 day before pollination. The *in vitro*-matured pollen was washed several times in medium GQ and then transferred onto stigmas in a 3-μl droplet. To prevent cross pollination, all the other buds in the climate chamber were removed before they could open. Mature seed pods were collected after 4–5 weeks.

Seed Germination Tests. Surface-sterilized seeds (NaOCl, 1% active chlorine) were placed on medium MS (13) with and without kanamycin (50 μg/ml) and germinated at 25°C under continuous light. The frequency of kanamycin-resistant and -sensitive seedlings was determined after 3 weeks, when resistant seedlings formed secondary leaves.

Isolation of Plant DNA and Southern Blot Analysis. Southern blot analysis was performed with commonly used molecular techniques (21). Digested DNA was separated on a 0.8% agarose gel, transferred to nylon Hybond-N filters, and hybridized with the DNA probe, randomly labeled with digoxigenin (DNA labeling kit, Boehringer Mannheim).

RESULTS

The *dc3* Promoter Is a Strong Pollen-Expressed Promoter. We studied several promoters for their expression during pollen development (i.e., the *lat52, pa2*, and *dc3* promoters) fused with the *uidA* gene in stably transformed homozygous plants. The *dc3* gene is a group III *lea* class gene of *Daucus carota* (19). *lat52*, isolated from an anther-specific cDNA library of tomato, was found to be active in pollen and, at a low level, in endosperm (26, 27). The pollen-specific promoter *pa2* is the distal part of the promoter region of *chiA*, one of the two chalcone flavanone isomerase genes of petunia (18).

None of the promoters showed remarkable activity at stage 1 (Fig. 1). The *pa2* promoter started to be active in stage 2 and highest activity was found in stage 6 (Fig. 1*A*). Expression of the *lat52* promoter also started in stage 2 (Fig. 1*B*) but maximum expression was measured in stage 5. The *dc3* promoter started to be active also in stage 2 and, with kinetics similar to the *pa2* promoter, turned out to be the strongest of all, with a maximum activity of 15,000 pmol per min per mg of protein at stage 7 (Fig. 1*C*). Mature seeds of the same plants yielded an averaged GUS activity of 18,500 pmol per min per mg of protein (data not shown). The strength and temporal regulation of GUS activity by the *pa2, lat52*, and *dc3* promoters during *in vitro* maturation closely paralleled the results obtained from *in vivo* pollen, although the maxima were somewhat lower *in vitro* (Fig. 1). The patterns of expression were confirmed by histochemical assays on isolated microspores and pollen from each transformant (data not shown).

Inhibition of Pollen Development and Germination by Hygromycin B. Several antibiotics (hygromycin B, kanamycin, and methotrexate) were tested for their effect on *in vitro* pollen development (data not shown). Hygromycin B was found to be an effective agent to inhibit maturation of isolated microspores (Table 1) and midbinucleate pollen (Table 2). The effect of hygromycin B was stronger the earlier it was added to the cultures. Concentrations between 200 and 250 μg/ml completely inhibited germination, even when added to microspores

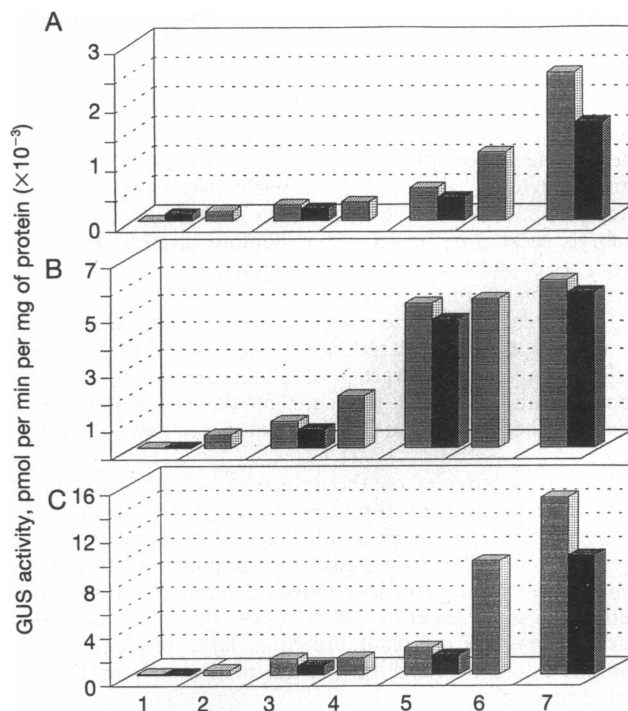

FIG. 1. GUS activity during pollen development. (*A*) *pa2* promoter. (*B*) *lat52* promoter. (*C*) *dc3* promoter. Solid bars, activity in pollen cultivated *in vitro*; shaded bars, *in vivo* development. Each value is the average of measurements from pollen of four plants. Pollen of several developmental stages (stage 1, microspores containing a single nucleus; stage 2, first pollen mitosis completed and generative cell still attached to intine; stage 3, generative cell detached from the wall, no vacuole anymore; stage 4, begin of starch accumulation; stage 5, binucleate starch-enriched pollen from colorless flowers that had already reached their full length; stage 6, flowers with pink corolla tips containing mature pollen in closed anthers; stage 7, dry pollen) was isolated from flower buds of transgenic plants and fluorimetrically tested for GUS activity. From *in vitro* cultures, samples for measurement of GUS activity were collected after 0, 2, 4, and 6 days, corresponding to microspores (stage 1), midbinucleate (stage 3), late binucleate (stage 4/5), and mature pollen (stage 6/7). Note, values for enzyme activity are drawn to different scales.

Table 1. Effect of hygromycin B on *in vitro* maturation and germination of tobacco microspores

Hygromycin B, μg/ml	Germination frequency, %	
	3 days	4 days
0	20.0	20.0
50	2.0	6.5
100	0.3	4.0
150	0.04	1.8
200	0.01	1.2

Hygromycin B at various concentrations was added to microspores in maturation medium after 3 days and 4 days of incubation. Pollen, cultured *in vitro* for 6 days, was germinated in germination medium GQ for 9–10 h. Data from one representative experiment are shown.

Table 2. Effect of hygromycin B on maturation and germination of midbinucleate tobacco pollen

Hygromycin B, μg/ml	Germination frequency, %		
	0 h	24 h	48 h
0		54.6 ± 1.1	
25	13.0 ± 1.6	43.4 ± 0.8	50.7 ± 6.9
50	3.1 ± 1.0	36.1 ± 0.2	38.0 ± 0.5
100	0	5.6 ± 1.3	36.6 ± 0.4
200	0	0.8 ± 0.1	34.6 ± 1.7
250	0	0	28.2 ± 1.0

Hygromycin B at various concentrations was added to maturation medium after 0, 24, or 48 h, and after 72 h, the matured pollen was germinated in hygromycin-free germination medium GQ for 4–5 h.

after 3 days of culture or to midbinucleate pollen after 1 day of culture. After further 4 days of culture for microspore and 2 days of culture for midbinucleate pollen, clear phenotypic differences between normal *in vitro*-matured pollen in a medium without hygromycin and pollen after maturation in a hygromycin-containing medium were observed (Fig. 2). Hygromycin-treated pollen grains accumulated dark granules, most likely deviant starch grains, and did not germinate, while nontreated pollen grains contained light starch grains and germinated at high frequency in the quercetin-containing germination medium GQ (data not shown).

***In Vitro* Selection of Pollen from a Stably Transformed Plant.** Since the *dc3* promoter is active in both the sporophytic and male gametophytic development, it is an example for overlapping gene expression in the two generations of the higher plant life cycle. It is also a very strong promoter, as found in this study. Therefore, the *dc3* promoter was chosen for the *in vitro* pollen selection experiments.

Several independent transgenic tobacco plants, harboring within the same transferred T-DNA borders, the *hpt* gene under control of the *dc3* promoter and the *nptII* gene under the control of the *nos* promoter, were produced by leaf disk transformation with *A. tumefaciens* (12). Only plants with single-locus inserts were used for pollen cultures, as shown by Southern blot analysis (two out of seven transformants) of the transferred T-DNA (data not shown) and Mendelian segregation of the dominant *nptII* marker gene in the F_1 progeny (Table 3).

Midbinucleate pollen was isolated from the plants with single inserts and cultured in AMGLU medium. After 1 day, hygromycin B was added to the pollen cultures (final concentration, 200 μg/ml). After two more days of culture, the pollen suspension was washed twice and transferred to hygromycin B-free germination medium GQ. About 50% of the pollen had the morphology of normal mature pollen and germinated (Fig. 3 *A* and *B*), while pollen after *in vitro* maturation in a hygromycin-free medium germinated with nearly 100% frequency (Fig. 3 *C* and *D*). Wild-type pollen, matured in the presence of hygromycin B did not have the morphology of normal mature pollen and did not germinate at all after transfer to medium GQ (Fig. 3 *E* and *F*), while without hygromycin selection again nearly 100% of the pollen matured and germinated (Fig. 3 *G* and *H*).

An aliquot of the pollen from these experiments was used for *in situ* pollination of wild-type plants. No seed set was obtained after pollination with pollen from wild-type plants that had been matured in the presence of hygromycin B, whereas normal seed set, with >1000 seeds per pod, was obtained after pollination with nonselected wild-type pollen (Table 3). Evidently, in a seed germination test for kanamycin resistance, these seeds were kanamycin-sensitive. With seeds produced by pollination with hygromycin-selected pollen from the *dc3–hpt-* and *nos–nptII*-transformed plants, seed set was also normal and all offspring were resistant in the germination test on kanamycin-containing medium. This stringent selection was obtained in several experiments. After pollination with unselected pollen from transformed plants, 50% of the seedlings were resistant, which is the expected percentage for a Mendelian segregation in a backcross.

DISCUSSION

We demonstrate directly that selection acting on developing male gametophytes can be used to increase the frequency of genes in the progeny. Development of isolated immature

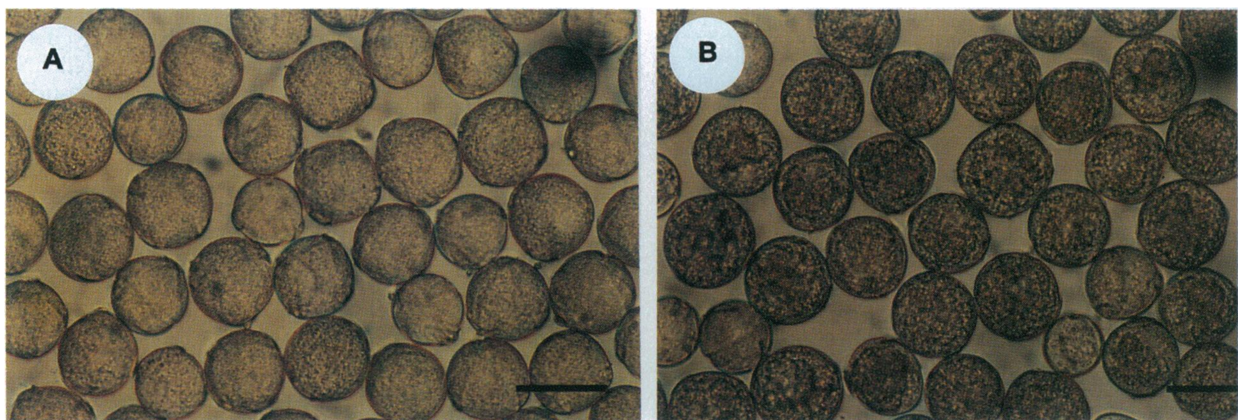

FIG. 2. Effect of hygromycin B on *in vitro*-matured *N. tabacum* pollen. (*A*) *In vitro*-matured pollen showing normal morphology in medium without hygromycin B. (*B*) *In vitro*-matured pollen showing abnormal development in medium with hygromycin B (200 μg/ml). In all cases midbinucleate pollen was matured in the presence or absence of hygromycin B in the maturation medium AMGLU for 3 days. Hygromycin B was added to the medium 10 h after culture initiation (Bars = 30 μm.)

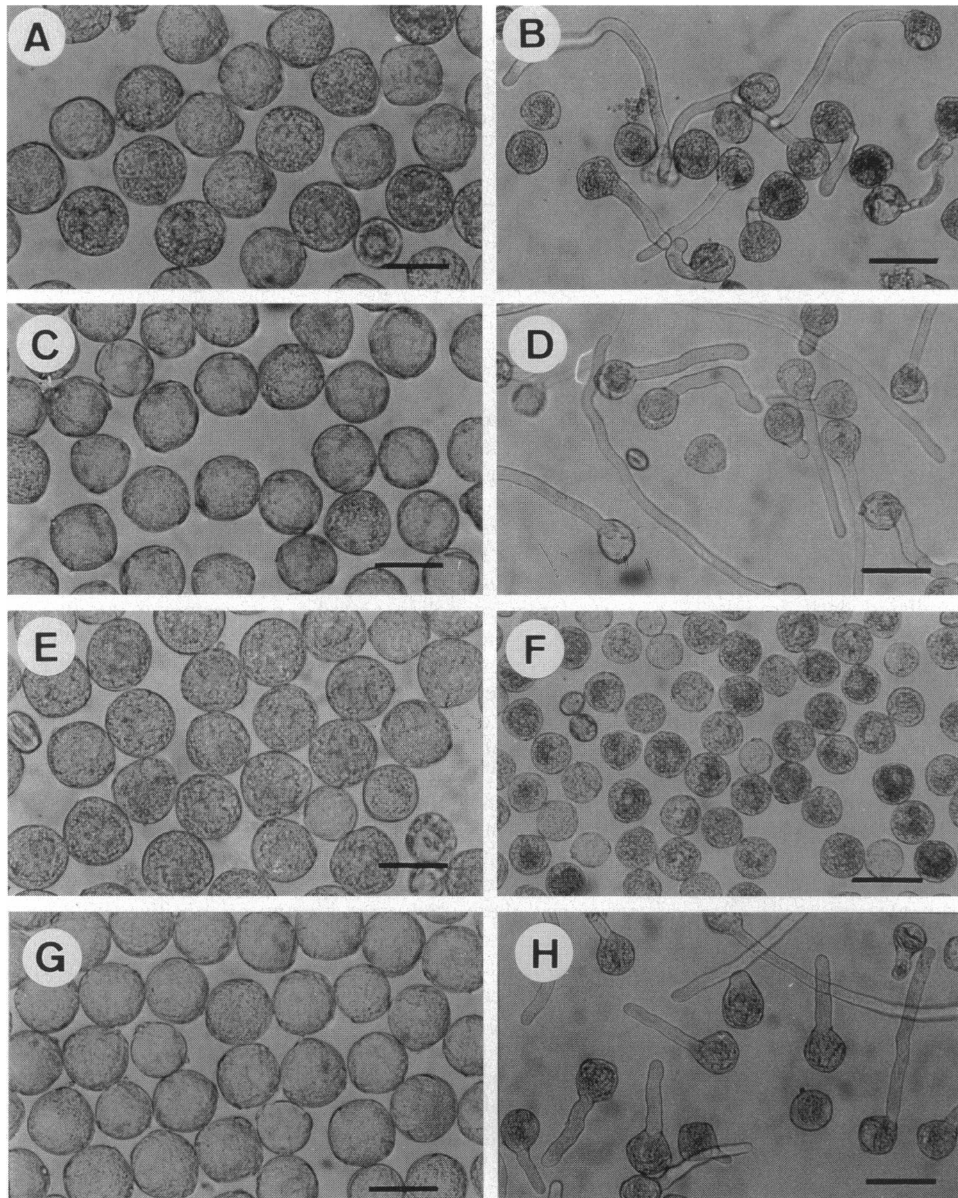

FIG. 3. *In vitro* maturation and germination of pollen from control wild-type and transgenic plants containing the *dc3–hpt* construct. Midbinucleate pollen was matured *in vitro* with or without hygromycin B in the medium for 3 days and allowed to germinate in medium GQ without hygromycin B for 5 h. (*A* and *B*) Maturation and germination, respectively, of pollen from transgenic plants after selection in hygromycin B (200 μl/ml)-containing medium. (*C* and *D*) Maturation and germination, respectively, of pollen from transgenic plant without selection (no hygromycin B in the medium). (*E* and *F*) Maturation and germination, respectively, of pollen from wild-type plants with selection in hygromycin B (200 μl/ml)-containing medium. (*G* and *H*) Maturation and germination, respectively, of pollen from wild-type plants without selection (no hygromycin B in the medium). (Bars = 30 μm.)

pollen from a plant transformed with a hygromycin-resistance gene under the control of the sporophyte- and gametophyte-

Table 3. Seedling analysis of pollen selection experiments

Crosses for seed set, pollen × pistil	Seedlings, no.		Phenotypic segregation, no. Kan^r:no. Kan^s
	Resistant	Sensitive	
T* × T	147	52	3:1
T* × W	125	132	1:1
W (+s) × W	NSS		
W (−s) × W	0	270	0:1
T (+s) × W	347	0	1:0
T (−s) × W	147	155	1:1

Pollen from wild-type plants (W) and plants containing the *hpt* gene under control of the *dc3* promoter and the *nptII* gene under the control of the *nos* promoter (T-plants) was matured in the presence (+s) or absence (−s) of hygromycin B (200 μg/ml) and was then used to pollinate emasculated flowers of wild-type or transgenic tobacco plants. Seeds obtained were grown on kanamycin (50 μg/ml)-containing medium MS. T-plants with single inserts, used for *in vitro* selection experiments, were identified by selfing and backcrossing with wild type (*). Kan^r, kanamycin resistant; Kan^s, kanamycin sensitive; NSS, no seed set.

expressed *dc3* promoter and a directly linked kanamycin-resistance gene under control of the *nos* promoter, when cultured *in vitro* in the presence of hygromycin, resulted in a 50% reduction of germinating pollen, as expected for the meiotic segregation of microspores in a hemizygous plant containing the transgene at a single locus. Pollination of wild-type plants with the selected pollen yielded offspring that all expressed the linked kanamycin-resistance gene, which was used as a convenient marker to identify the transgenic offspring. In addition, biolistic cotransfer of physically unlinked transgenes for the hygromycin-resistance gene and the GUS reporter gene, both under the control of pollen-expressed promoters, to isolated immature wild-type pollen resulted, when cultured *in vitro* in the presence of the antibiotic, in the selection of mature germinating pollen that in a high frequency also expressed the cotransferred reporter gene (C.S.F., E.S., A.T., E.H.-B., unpublished results).

The time available for selection to take place *in vitro* was 3 (for midbinucleate pollen) to 6 (for microspores) days. These time periods are more or less identical with the time tobacco pollen *in vivo* requires to develop from these stages to maturity, i.e., germinability (10). The actual and indeed effective time period for selection could be, however, reduced to 2–3 days

since hygromycin was added to the cultures at a time when the *dc3* promoter is active, i.e., at a late stage of pollen development. To our knowledge, such a short time for efficient selection has not been recorded in the plant genetics literature.

The present work demonstrates—although in a reconstruction experiment—that indeed genes expressed in the developing male gametophytes control pollen fitness. However, the reconstruction experiment on the basis of antibiotic resistance clearly does not reflect a likely situation *in planta*. It remains to be seen which selective conditions operate in the anther or in the style to control pre- and postanthesis components of pollen fitness, respectively. That pollen competition does take place, at least during tube growth in the style, seems to be an established fact (see refs. 2 and 28) and was first shown by Correns (29), who found that pollen tubes of the dioecious *Melandrium album* carrying the Y chromosome grew more slowly than those carrying the X chromosome.

Apart from their relevance for genetics and pollen biology, the present results have a bearing on plant breeding. It has been shown that a toxin from *Drechslera maydis* prevented germination of pollen from maize plants susceptible to the disease but not of pollen from resistant plants (30), and compounds present in *Alternaria brassicicola* culture filtrates inhibited *Brassica* pollen germination (6). In very few cases (5, 30) has it been possible to make pollinations with selected pollen. Success has previously been limited because the selection procedure killed the pollen-producing plants (but not in ref. 5) or the window of selection was too narrow (but not in ref. 31). Both of these difficulties are overcome through the pollen selection during *in vitro* maturation. Thus we propose that pollen from F_1 hybrids between a cultivar and a resistance gene source of alien germ plasm be selected *in vitro* and used to backcross the cultivar. Fewer generations should be required to introgress the resistance trait to the cultivar. The *in vitro* maturation system has the further potential advantage that mutagenized pollen can be selected and resistances to additional compounds and also additional forms of previously characterized resistance can be obtained.

We thank Dr. T. L. Thomas (Texas A&M University), Dr. S. McCormick (U.S. Department of Agriculture, Agricultural Research Service, Plant Gene Expression Center), and Dr. A. van Tunen (Centre for Plant Breeding and Reproduction Research), for providing seeds of *ds3–uidA*-, *lat52–uidA*-, and *pa2–uidA*-containing transgenic plants, respectively. We acknowledge also Dr. V. Zarsky and Dr. O. Vicente for valuable suggestions, Dr. M. Dahl for help in the construction of the used plasmids, Mrs. F. Touraeva for technical assistance, Mrs. M. Braun-Mayer for plant care, and Dr. L. Bögre for art work. This work was supported by Grant P9174-BIO of the Austrian Science Foundation (FWF).

1. Mascarenhas, J. P. (1990) *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **41,** 317–338.
2. Ottaviano, E. & Mulcahy, D. L. (1989) *Adv. Genet.* **26,** 1–64.
3. Ottaviano, E., Sari-Gorla, M., Frova, C. & Pe, M. E. (1986) in *Biotechnology and Ecology of Pollen*, eds. Mulcahy, D., Bergamini Mulcahy, G. & Ottaviano, E. (Springer, New York), pp. 35–42.
4. Evans, D. E., Singh, M. B. & Knox, R. B. (1990) in *Microspores: Evolution and Ontogeny*, eds. Blackmore, S. & Knox, R. B. (Academic, Orlando, FL), pp. 310–338.
5. Searcy, K. B. & Mulcahy, D. L. (1985) *Am. J. Bot.* **72,** 1700–1706.
6. Hodgkin, T. & MacDonald, M. V. (1986) *New Phytol.* **104,** 631–636.
7. Hodgkin, T. (1990) *Sex. Plant Reprod.* **3,** 116–120.
8. Sari-Gorla, M., Ferrario, S., Gianfranceschi, L. & Villa, M. (1992) in *Angiosperm Pollen and Ovules*, eds. Ottaviano, E., Mulcahy, D. L., Sari-Gorla, M. & Bergamini-Mulcahy, G. (Springer, New York), pp. 364–369.
9. Sari-Gorla, M., Ferrario, S., Frascaroli, E., Frova, C., Landi, P. & Villa, M. (1994) *Theor. Appl. Genet.* **88,** 812–817.
10. Benito Moreno, R. M., Macke, F., Alwen, A. & Heberle-Bors, E. (1988) *Planta* **176,** 145–148.
11. Mascarenhas, J. P. (1992) *Int. Rev. Cytol.* **140,** 3–18.
12. Horsch, R. B., Fry, J. E., Hoffmann, N. L., Eichholtz, D., Rogers, S. G. & Fraley, R. T. (1985) *Science* **227,** 1229–1231.
13. Murashige, T. & Skoog F. (1962) *Physiol. Plant.* **15,** 473–497.
14. Tupy, J., Rihova, L. & Zarsky, V. (1991) *Sex. Plant Reprod.* **4,** 284–287.
15. Kyo, M. & Harada, H. (1986) *Planta* **168,** 427–432.
16. Ylstra, B., Touraev, A., Benito Moreno, R. M., Stöger, E., van Tunen, A. J., Vicente, O., Mol, J. N. M. & Heberle-Bors, E. (1992) *Plant Physiol.* **100,** 902–907.
17. Bevan, M. (1984) *Nucleic Acids Res.* **12,** 8711–8721.
18. van Tunen, A. J., Hartman, S. A., Mur, L. A. & Mol, J. N. M. (1989) *Plant Mol. Biol.* **12,** 539–551.
19. Seffens, W. S., Almoguera, C., Wilde, H. D., von der Haar, R. A. & Thomas, T. L. (1990) *Dev. Genet.* **11,** 65–76.
20. Vivekaneda, J., Drew, M. & Thomas, T. L. (1993) *Plant Physiol.* **101,** 576–581.
21. Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor Lab. Press, Cold Spring Harbor, NY), 2nd Ed.
22. Jefferson, R. A. (1988) *Plant Mol. Biol. Reptr.* **5,** 387–405.
23. Stöger, E., Benito Moreno, R. M., Ylstra, B., Vicente, O. & Heberle-Bors, E. (1992) *Transgenic Res.* **1,** 71–78.
24. Alwen, A., Benito Moreno, R. M., Vicente, O. & Heberle-Bors, E. (1992) *Transgenic Res.* **1,** 63–70.
25. Mascarenhas, J. P. & Hamilton, D. A. (1992) *Plant J.* **2,** 405–408.
26. Twell, D., Wing, R. A., Yamaguchi, J. & McCormick, S. (1989) *Mol. Gen. Genet.* **217,** 240–245.
27. Twell, D., Yamaguchi, J. & McCormick, S. (1990) *Development (Cambridge, U.K.)* **109,** 705–713.
28. Stephenson, A. G. & Bertin, R. J. (1983) in *Pollination Biology*, ed. Real, L. (Academic, Orlando, FL), pp. 109–149.
29. Correns C. (1927) *Hereditas* **9,** 33–44.
30. Laughnan, J. R. & Gabay, S. J. (1973) *Crop Sci.* **43,** 681–684.
31. Sari-Gorla, M. E., Ottaviano, E., Frascaroli, E. & Landi, P. (1989) *Sex. Plant Reprod.* **2,** 65–68.