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Further attempts to induce "egg transformation" using irradiated pollen *

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Summary. Numerous pollination treatments involving heavily irradiated (40–100 krad) pollen of diverse plant species failed to produce any clear cut "egg transformants" of the type reported by Pandey in *Nicotiana*. Genetic stocks of pea, rapeseed, and apple, bearing multiple Mendelian markers, were employed to detect any possible transformation events. For each plant species, an optimal level of irradiation was determined which would allow normal pollen tube growth leading to fertilization, but which would prevent the formation of normal hybrids due to the "pulverized" condition of the chromosomes contributed by the irradiated pollen. Pollination treatments included selfing, pollination with donor pollen mixed with self pollen, pollination with irradiated donor pollen mixed with self pollen, pollination with irradiated pollen followed by a delayed self pollination, and pollination with irradiated pollen by itself. None of these treatments produced clearly transformed seedlings. The total number of potential transformation events screened was in excess of 6,046 including 2,268 for pea, 3,309 for rapeseed, and 469 for apple. It is concluded that if egg transformation occurs outside of *Nicotiana* it is a rare event, and its frequent occurrence in *Nicotiana* must be, at best, an isolated phenomenon.

Key words: *Pisum* – *Brassica* – *Malus* – Pollen irradiation – Transformation

Introduction

Pandey (1975, 1978, 1980a) has reported the unusual genetic phenomenon of "egg transformation" in *Nicotiana*, wherein he employed heavily irradiated pollen as both a genetic donor and as the vehicle for delivering chromosome fragments to the egg. Pandey has reported very high transformation rates for incompatibility S alleles, flower color, and pollen color. He has proposed that such transformation can occur by either of two mechanisms. The first mechanism involves polyspermy, where more than one pollen tube enters the same ovule. When irradiated donor pollen enters the same ovule as non-irradiated self pollen, the situation arises where normal fertilization can occur in the presence of radiation-pulverized chromosome fragments from the donor pollen, leading to transformation events. The second mechanism involves apomictic development of seed. When irradiated pollen alone enters the ovule, its genetic "debris" may simultaneously induce parthogenetic embryogenesis, block the first zygotic division (inducing diploidy), and lead to transformation events. Pandey has claimed evidence for the occurrence of both mechanisms.

This approach to plant transformation is very attractive for the following reasons: 1) germline cells are being transformed, leading to normal embryogenesis and by-passing the problem of regeneration from callus tissue; 2) fertilization serves as a natural micro-injection process, whereby hundreds of ovules can be reliably "microinjected" by way of a simple hand pollination, leading to high rates of transformation; 3) donor genetic material might be used which need not be characterized and cloned on the molecular level, but would be selected on the genomic level, simply for its agriculturally-relevant phenotypic effects.

To investigate to what extent the egg transformation phenomenon can be extended to other species, and to better understand its underlying mechanisms, several plant species were selected for study. Extensive studies

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on tomato are reported elsewhere (Sanford et al. 1984). Pea was selected as a self-pollinating species with good multiple markers. Genetic stocks of rapeseed were selected for their short life cycle, known potential for apomictic seed development, and known occurrence of polyspermy. Two crabapple species were selected for study because of their apomictic reproductive mechanisms.

Materials and methods

Visible genetic markers were employed to detect transformation events. Multiple recessive stocks of pea, and rapeseed were used for distinguishing hybrid contaminants from true transformants, and for detecting any differential transformation rates for different marker genes. In crabapples, where only a single character was involved, pollen contamination was prevented by isolation of trees and careful bagging of crosses.

Preliminary experiments were conducted to find a proper range of radiation dosage for pollen treatment for each crop. Techniques included microscopic examination of in vitro pollen germination, fluorescence microscopic inspection of in vivo pollen germination and pollen tube penetration, and seed set after pollination. Irradiation treatment was carried out in the Gamma Cell Facility at Ward Laboratory at Cornell University. Dosage applied was manipulated by adjusting the distance between the samples and the Co^{60} radiation source. Pollen was treated for 1 h.

Pea

Pea stocks were supplied by Dr. G. A. Marx, Department of Seed and Vegetable Science, Agricultural Experiment Station, Geneva. A multiple recessive stock with 7 single-gene marker traits was used as the recipient, and a wild-type stock was used as the donor (Table 1).

Table 1. Genetic stocks used in these studies, their codes, and their genotypes

Species and stocks	Code	Genotype
Pea:		
A 79-390(5)	D	Wild type
B 80-233(8)	R	*a af i le r st tl*[a]
Rapeseeds:		
B. napus (PI323939)	D1	Wild type
B. campestris	D2	Wild type
B. campestris	R	*gr lem tu*[b]
Apples:		
Red foliage donors	D1	*Rr*[c]
M. sargenti	R1	*rr*
M. hupehensis	R2	*rr*

[a] See Blixt et al. (1978) for gene codes

[b] Green hypocotyl, lemon-colored flower, and tucked petal respectively; all single gene characters. (Hawk 1982; Hawk and Crowder 1978 b; Mutschler, personal communication)

[c] Several donors heterozygous for the single dominant gene for red foliage (Brown 1975) were used. These included the genotypes 'Liset', NY40-5, *M. purpurea aldenhemensis,* Pk-14, and 'Purple Wave'

Pea pollen was collected from ready-to-bloom flowers on the day of irradiation, and was used in the following four days. A 40–100 kr dosage was used, as this was found to produce pollen which was genetically inviable yet physiologically functional. Pollination treatments included selfing, pollination with donor pollen mixed with self pollen, pollination with irradiated donor pollen mixed with self pollen, pollination with irradiated donor pollen followed by a delayed self-pollination, and pollination with irradiated donor pollen by itself.

Peas were grown in an isolated plot in the field. Emasculation was done a day before pollination following the method described by Gritton (1980). For mixed pollination, the two types of pollen were mixed 1 : 1 by volume and were mixed between thumb and index finger before pollination. No further protection was given after pollination. Pods were harvested when mature. Seeds were extracted, air-dried, and scored for skin and cotyledon characters before planting.

Rapeseed

Triple recessive rapeseed stocks were synthesized by intercrossing single and double recessive stocks supplied by Dr. M. A. Mutschler, Department of Plant Breeding and Biometry, Cornell University, Ithaca; and Dr. J. A. Hawk, Department of Plant Science, University of Delaware, Newark. *B. campestris* stock had been selected for a short life cycle of 42–50 days (Hawk and Crowder 1978 a). A triple recessive population having good fertility and vigor was used as the recipient. It carried the recessive traits for green hypocotyl, lemon-colored petal, and tucked petal. Wild type *B. campestris* and *B. napus* were used as donors. *B. napus* is an amphidiploid species, which contains the genome of *B. campestris* as a genomic component and is sexually compatible to *B. campestris* (Downey et al. 1980). The wild type *B. napus* was supplied by the Regional Plant Introduction Station, Iowa State University, Ames.

Rapeseed pollen was found to lose viability rapidly after radiation treatment. Therefore, the pollen was collected from the newly opened flowers early on the day of irradiation, used as soon as possible after irradiation, and was always kept at 4 °C except during irradiation. The dosage applied was 80 kr, which was found to render the pollen genetically inviable yet physiologically functional. Pollination treatments included sibbing of the recipient plants, pollination with donors, pollination with irradiated donor pollen mixed with self pollen, and pollination with irradiated donor pollen by itself.

Rapeseed was grown in styrofoam trays. Donor and recipient stocks were kept in different greenhouses. Donors were sib-mated for two generations before use to ensure genotypic purity. Recipient plants were not emasculated for mixed pollination with irradiated pollen and self pollen. No further protection was applied to the recipient plants. Pods were harvested at maturity, dried, and then seeds were extracted and planted for screening.

Apples

The apple stocks used for this study are listed in Table 1. Plant material was supplied by Dr. J. N. Cummins in the Department of Pomology and Viticulture, Agricultural Experiment Station, Geneva. Two highly apomictic crabapples, *Malus hupehensis* and *M. sargenti*, (Schmidt 1970, 1974; Brown 1975) were used as recipients. The single gene character of red foliage was employed as the genetic marker. Five red-foliage crabapples carrying the anthocyanin pigmentation gene in a heterozygous condition were used as donors. Seedlings were

screened for the red-foliage marker, general morphology, and in some cases for isozyme markers.

Apple branches of the donor stocks were taken into the greenhouse to force blooming. Apple pollen was collected one day before irradiation and was used in the following four days. Radiation dosage applied was 80 kr, as with rapeseed. Pollen was kept in gelatin capsules over desiccant at 4 °C except during irradiation.

Pollination treatments included pollination with only irradiated donor pollen, pollination with donor pollen, and selfing (except for *M. hupehensis* which does not produce pollen). On both recipients, all the buds and blooms on a labelled branch were emasculated and pollinated at one time. Control pollination with non-irradiated donor pollen was done one week after pollination with irradiated pollen, and on a different section of the trees. The *M. hupehensis* tree was isolated in a greenhouse. The branches on the field-grown *M. sargenti* tree were covered with paper bags for protection. Bags were put on before blooming and left on until styles withered (three weeks after pollination) except during emasculation and pollination. Fruits were harvested when mature. Seeds were extracted and stratified for three months before planting.

Seedlings from the crosses on *M. hupehensis* were analyzed for isozyme patterns, to determine their possible apomictic origin. Young leaf tissue was used. The electrophoretic and histochemical methods used were those of Weeden (1983).

Results

Pea

Pea pollen irradiated with 100 kr and higher dosage of γ-ray germinates well, but tube growth rate is severely retarded so that no penetration into the ovule is observed under the fluorescence microscope 48 h after pollination. However, penetration into the ovule is observed for pollen treated with doses of 80 kr and below.

The results of transformation crosses on pea are summarized in Table 2. Some of the seedlings were grown in the field and these died before flowering, so only five characters out of seven were screened. The remaining seeds were grown in the greenhouse and were screened for all seven marker traits. All seedlings had phenotypes as would be expected, with two exceptions. In the treatment involving irradiated donor mixed with normal self pollen (*iD*+R, in Table 2), seven seedlings from a single pod were of hybrid type. They were dominant for all seven traits involved, segregated normally in progeny tests, and clearly represent pollen contamination. Another five seeds that were first classified as round seed coat (R_) produced seedlings which were multiple recessive, and bred true for recessive seed type. We believe these "round" seeds were simply phenotypically misclassified, since intermediate phenotypes occasionally arise for this character.

Tallying the total number of pea seedlings and the number of traits screened for each seedling, 2,268 potential transformation events were screened, with no clear transformants being observed.

Rapeseed

Both *B. napus* and *B. campestris* pollen irradiated with up to 200 kr γ-ray were found to germinate nearly 100% and the tubes penetrated to the ovules within 24 h after pollination. The amount of seed set, however, decreased as the dosage increased. Pollen receiving a dosage of 12.5 kr and below produced hybrids and some matromorphs after pollination, with the F_2 progenies of the hybrids segregating normally for genetic markers. Pollen treated with 25 kr or higher resulted in only two matromorphic seedlings. One of these was nearly sterile, and was assumed haploid, but died before a chromosome count could be made.

The results of transformation crosses made with rapeseed stock is summarized in Table 3. A total number of 1,103 seedlings were screened for three genetic traits. No transformants were observed. Three hybrid plants from the cross (R×D1) and three from the cross (R×D2) were bud-pollinated for progeny testing. The F_2 progeny segregated for the three characters involved as expected.

Table 2. The results of crosses in pea, with numbers of pollinations, fruits, seeds, seedlings, and non-maternal progeny

Treatment[a]	No. genes tested	No. pollinations	No. fruit	No. seeds	No. seedlings	No. non-maternal
R×R	–	30	29	112	80	0
R×*iD*	7	11	1	2	2	0
R×[*iD*+R]	5	226	97	392	243	7[b]
R×[*iD*+(4)R]	5	42	21	93	47	0
R×[D+R]	5	91	28	126	124	46[b]

[a] *i*: irradiated; D: donor; R: recipient; see Table 1 for genotypes; [ ]: mixed pollination; ( ): hours delayed for the 2nd pollination

[b] Wild type seedlings

Table 3. Results of crosses in *Brassica campestris,* with numbers of genes tested, pollinations, fruits, seeds, seedlings, and non-maternal seedlings

Treatment[a]	No. genes tested	No. pollinations	No. fruit	No. seeds	No. seedlings	No. non-maternal
R×R (sib)	–	31	23	99	87	0
R×D2	–	10	8	26	26	26[b]
R×[*iD2*+R]	3	446	272	998	809	0
R×[*iD2*+R]	3	143	81	340	294	0

[a] *i*: irradiated; D2: *Brassica campestris*; D1: *B. napus*; R: triple recessive *B. campestris*; [ ]: mixed pollination
[b] Wild type seedlings

Table 4. The results of crosses in apomictic crabapples, with numbers of pollinations, fruits, seeds, seedlings, and red-foliage progeny

Treatment[a]	No. pollinations	No. fruits	No. seeds	No. seedlings	No. red-foliage
R1×*iR1*	42	0	0	0	0
R1×*iD2*	475	24	60	11	0
R1×D1	187	144	564	203	14
R2×*iD1*	629	443	1601	458	1[b]
R2×D1	57	41	156	29	1

[a] *i*: irradiated, see Table 1 for stock codes; D1: red-foliage donors, including 5 stocks
[b] See text for description of this seedling

Apple

Apple pollen gave no less than 95% germination after irradiation with 80 kr γ-ray. The irradiated pollen tubes were tested in vitro and were no shorter than those of non-irradiated pollen. Even after 15 days storage, irradiated pollen had 45–50% germination in vitro. After pollination with irradiated pollen, several stylar samples were examined under a fluorescence microscope. Pollen tubes were found to traverse the entire stylar distance.

The results from crosses on the two apple recipients are shown in Table 4. Fourteen red-foliage seedlings were obtained from *M. sargenti* pollinated with normal pollen of red-foliage donors, but no such seedlings were obtained from crosses with the irradiated donors or self pollen. Two red-foliage seedlings were obtained from the *M. hupehensis* (HP) tree. One from pollination with normal NY40-5 pollen, the other from irradiated 'Liset' (LI). Electrophoretic analysis was conducted to determine if the red-foliage seedling from the irradiated cross was matromorphic except for foliage color. The red-foliage seedling from the cross HP×iLI, 2 apomictic siblings, and the original female plant, were analyzed for 11 isozymes. The two siblings differed in only one enzyme (PGI). The red-foliage seedling showed difference from its siblings for 6 isozymes. The red-foliage seedling also differed from the female and the apomictic sibs morphologically, and was assumed to be a hybrid contaminant seedling. The non-apomictic origin of this seedling was supported by chloroplast counts, which indicated an elevated ploidy level, as expected in a hybrid.

Discussion and conclusion

The proposed mechanism for "egg transformation" in *Nicotiana* species was that the irradiated donor pollen delivered its genetic material in the form of chromatin fragments to the egg, leading to genetic transformation events (Grant et al. 1980).

In the case where only irradiated pollen was used (on the self-compatible *N. langsdorffii*), the genetic debris were believed to stimulate the mitosis of the egg nucleus without cytokinesis, resulting in a diploid egg cell. DNA fragment(s) were incorporated into the egg genome during DNA replication, resulting in a transformed egg cell. The egg cell then, stimulated by this "pseudofertilization" underwent parthenogenetic embryogenesis (Pandey 1980b, 1983a). The genetic debris might have also stimulated the autonomous development of endosperm, perhaps by something like the proposed embryo growth-promoting gene linked to transformed mark-

ers (Pandey 1978, 1983a). This is the so-called "parthenogenetic diploidization" mechanism.

In most of Pandey's crosses, a mixture of irradiated compatible donor pollen and normal self pollen were applied to self-incompatible species. The irradiated pollen served both as a vector to deliver its pulverized genetic material for egg transformation and as a "mentor pollen" to allow the self pollen to fertilize the egg and polar nuclei. The second normal fertilization by nuclei from self pollen thus helped to recover the transformed egg (Pandey 1980b, 1983a). The chimeric zygote formed in this way would go through a developmental competition in embryogenesis between the two genotypes and resulted in either a solid transformant due to the better competing ability of the heterozygous transformed genotype (Pandey 1978), or a chimera with both genotypes expressing in different sections of the plant (Pandey 1983a).

Each of the proposed mechanisms playing a role in "egg transformation" are all known to exist in *Nicotiana*. Self-incompatibility was first found in *Nicotiana* (East and Mangelsdorf 1925). The mentor effect of overcoming self-incompatibility by irradiated pollen in *Nicotiana* was known in the last decade (Pandey 1979), and it was in mentor pollen studies where "egg transformation" was first observed (Pandey 1975). Apomixis in *Nicotiana* has long been known to exist (Goodspeed 1954), and parthenogenesis has been demonstrated more recently (Pandey and Phung 1982).

Most plant species do not have all of the mechanisms listed above. Therefore, methods independent of them would have to be developed to make the "egg transformation" widely applicable. Pandey (1981a, 1983a) has proposed sequential fertilization which might be a widely applicable approach, and has claimed to have demonstrated this approach with an animal, chicken (Pandey and Patchell 1982).

Pea is not known to have apomictic means of reproduction (Nygren 1967), and does not have a self-incompatible reaction (Gritton 1980). It is therefore a good material for studying the idea of "egg transformation" as induced by irradiated pollen and recovered by a second normal fertilization with self pollen. Irradiated donor pollen was used for pollination alone to see if "pseudofertilization" could induce parthenogenetic progeny, and if any such progeny were transformed. Mixed pollinations involving irradiated donor pollen mixed with normal self pollen were made to determine if polyspermy events could result in a normal fertilization coupled with transformation due to genetic debris from the irradiated donor pollen. Pollinations with irradiated donor pollen followed by normal self pollinations 4 hours later were designed to give the irradiated pollen a head start with the hope that ovules would remain more receptive to a second fertilization if first "pseudofertilized" by irradiated pollen (Pandey 1983a). Mixed pollinations with non-irradiated donor and self pollen were made to explore the possibility of genetic exchange between unrelated gametophytes that were growing side by side in the styles (Stroun 1964).

The only unexpected progenies obtained from the pea crosses were the seven hybrid-type plants listed in Table 2. The dominant phenotype for all seven genes in the F_1 with normal F_2 segregation indicates these were the result of contamination. No genuine transformants were obtained. A more comprehensive study conducted on non-apomictic, self-compatible tomato has also produced negative results (Sanford et al. 1984).

These results suggested that "egg transformation" cannot be extended to self-compatible, non-apomictic species like tomato or pea, using the mechanism proposed by Pandey (Pandey 1981b). No seed set of apomictic origin could be induced by pollination with irradiated pollen. Neither could recombinant-type seedlings be found in the progeny from mixed pollination with donor (with or without irradiation) and self pollen. The absence of either apomixis or polyspermy may contribute to the failure to recover transformants. In other words, apomixis and/or polyspermy are prerequisites for "egg transformation".

Matromorphic plants have long been reported and studied in *Brassica* (Downey et al. 1980). The mechanism for producing matromorphic seedlings could be either parthenogenetic development of an unreduced egg or the spontaneous chromosome doubling and embryogenic development of reduced female gametes (Eenink 1976; Heyn 1977). Haploid seedlings were also known to be produced from interspecific crosses (Prakash 1973). Autonomous development of endosperm in unfertilized egg sac has been observed (Mackiewiez 1973). *B. campestris* has a sporophytic type of self-incompatibility which is controlled by a multiallelic single gene (Sareen and Kakar 1975; Zuberi et al. 1981). Besides having self-incompatibility and parthenogenic potential, *B. campestris* has been observed to have a high frequency of polyspermy. These characters make *B. campestris* a good candidate for "egg transformation" by Pandey's hypothesis.

A triple recessive stock of *B. campestris* was pollinated with irradiated donor pollen alone to induce matromorphic transformants, and pollinated with a mixture of irradiated donor and normal self pollens to explore the possibility of mentor pollen effect on sporophytic self-incompatibility as a mechanism for "egg transformation".

The results of the transformation-inducing pollinations were negative (Table 3). Preliminary experiments (not shown) did not indicate any mentor effects in this sporophytically self-incompatible material. Therefore, irradiated donor pollen was mixed with maternal-type sib pollen. Such mixed pollinations produced 1,103 seedlings, all of which were maternal type. Several hundred pollinations made with irradiated donor pollen alone (not shown) produced a very low rate of apparently apomictic seedlings. Of the four apparent apomicts recovered, all were very weak, and two died prematurely. None of these apomicts appeared to be transformants. The very low rate of recovery of apomictic seedlings from this material would make it nearly impossible to detect rare transformation events.

The apple stocks employed are strongly apomictic (Schmidt 1970, 1974; Brown 1975). These species therefore produce predominantly matromorphic seedlings, and are therefore suitable for testing the idea of transforming an apomictic embryo with genetic debris from

irradiated pollen. Flowers in various developmental stage from bud to full bloom were used for pollinations, such that pollen tubes reached ovules at various stages of development. However, of 469 seedlings from transformation-inducing crosses, only one had the donor color trait (Table 4). This appeared to be a hybrid contaminant, based on morphology, electrophoretic data, and ploidy level as determined by chloroplast counts.

Electrophoretic analysis indicated that only one enzymatic difference (PGI) was observed between the two apomictic sibling controls, reflecting their matromorphic nature. The isozyme patterns of the red-foliage seedling were different from those of the apomictic siblings in 6 out of 11 cases, suggesting a non-maternal origin of this seedling. The leaf morphology of this seedling was also different from the female and its siblings. The vigor of this seedling was much inferior to that of its siblings and open-pollinated seedlings. The vigor of this seedling has been too poor to allow a chromosome count, however, chloroplast counts indicated an elevated ploidy above that expected of a matromorphic triploid.

These experiments on pea, rapeseed and apple, and our experiments on tomato (Sanford et al. 1984) and corn (Sanford et al. 1984), cast doubt on the extension of "egg transformation" to non-*Nicotiana* plants. Other researchers have also been unable to confirm "egg transformation" by irradiated pollen in *Nicotiana* and other genera (Engvild 1981; Brock 1982).

There have been several reports of employing irradiated pollen to achieve "differential gene transfer" (Jinks et al. 1981; Caligari et al. 1981; Powell et al. 1983; Snape et al. 1983; Pandey 1983b). These reports must not be confused with Pandey's reports of "egg transformation", because they represent completely different phenomena. The authors of these papers have not claimed they are finding transformation (although some of these reports have been interpreted in that light). "Egg transformation" employs a high level of irradiation which pulverizes the pollen chromosomes (thereby making hybrid progeny impossible), while differential gene transfer involves sub-lethal levels of irradiation leading to hybrid progeny which are mostly normal. "Egg transformation" is reported to produce maternal progeny with occasional dominant paternal (donor) traits, while differential gene transfer produces hybrid progeny, with mutants occasionally expressing recessive maternal traits. In differential gene transfer experiments, subsequent generations indicate distorted segregation ratios, presumably due to differential transmission of the radiation-damaged paternal chromosomes. It should be clear that mutant F_1 hybrid progeny and distorted F_2 segregations do not constitute evidence of transformation. Furthermore, Zamir (1983), and our own lab (unpublished), have only found rare and subtle distortions of segregation ratios in sub-lethal irradiation pollen studies in tomato and corn. Regardless of the possible practical merit of using sub-lethal levels of irradiation to accelerate the back-crossing process, all of these types of studies involve hybrid progeny and none can be interpreted as being supportive of Pandey's claims regarding egg transformation.

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