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Attempted "egg transformation" in *Zea mays* L., using irradiated pollen *

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Summary. Experiments were conducted to determine if "egg transformation" could be achieved in *Zea mays* L. as described by Pandey in *Nicotiana* L. Multiple recessive and multiple dominant marker stocks were employed, as well as a tester and a donor line for the "En" transposable element. Recipient tester females were pollinated with dominant donor pollen, which was applied in several treatment combinations. The pollination treatments included: 1) pollen irradiated at 20, 30, 40, 80, and 100 Krad; 2) pollen irradiated with the same doses, mixed with non-irradiated recipient pollen; 3) pollen irradiated at 80 Krad, followed by self pollination delayed 18 h; 4) non-irradiated donor pollen mixed with non-irradiated recipient pollen. Zero seed were produced from 100 pollinations with irradiated pollen. There were 258 pollinations made with irradiated donor plus self pollen mixtures, producing over 21,300 seed. Of these seed, 3 were unexpected. One was clearly from pollen contamination, one was clearly derived from a pre-meiotic mutation, and the third occurred as a mutant sector in the seed's endosperm. There were 56 pollinations with non-irradiated pollen mixtures, producing over 5,000 seed. Among these seed, there were 7 unexpected seed. Three of these were clear-cut cases of heterofertilization. Four progeny were dominant for all seed and seedling markers except one endosperm marker. These cases appear to represent spontaneous recessive endosperm mutations. More than 59,000 potential transformation events were screened producing only 6 apparent mutations. It is concluded that if egg transformation occurs in *Zea mays*, it is a very rare event, and is not likely to be useful in corn improvement.

Key words: *Zea mays* – Corn – Pollen – Irradiation – Transformation

Introduction

It has been claimed by Pandey (1975, 1978) that irradiated pollen can be used as a vehicle for plant-to-plant transformation. Pandey (1980a, b), has called this phenomenon "egg transformation". The mechanism proposed for this involves injection into the egg of pulverized donor DNA from the irradiated pollen tube (Grant et al. 1980), – followed by embryogenesis and seed development. The egg may develop parthenogenically (Pandey and Phung 1982), or may be fertilized by a second, non-irradiated pollen tube. This would appear to be a very attractive mechanism for gene transfer, however, it has not yet been demonstrated in any plant species outside of *Nicotiana*. The purpose of the research was to determine if egg transformation could be induced in corn.

Materials and methods

The following experiments were designed to employ the clearly defined classical genetic markers found in corn. The transposable element 'En' was also employed, because of its known ability to insert randomly in the corn genome and be expressed. The genetic stocks used in this research, including genotypes and sources, are listed in Table 1. Multiple recessive stocks were used as female recipients to: 1) detect the transfer of genes from multiple dominant donors, 2) distinguish trans-

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Table 1. Genetic stocks of corn used in egg transformation studies, including code, stock number, genotype, and source

Code	Stock no.	Genotype[a]	Source
L1	78-788-2×	*a su pr y gl wx* bm_2 *lg j g*	Maize Stock Center[b]
L2	66 $\frac{1430\text{-}10}{1482\text{-}2}$	*a su pr y gl wx* A_2 *C R*	Maize Stock Center
D	69-1484-3×	*A* A_2 *C* C_2 R^r *Pr B Pl*	Maize Stock Center
T	8 1346-1349	$a^{m(r)}/a^{m(r)}$	P. A. Peterson[c]
S	810260-22/0301	$a^{m\text{-}1}/a$ (plus En)	P. A. Peterson

[a] For explanation of gene symbols see Maize Genetics Cooperative Newsletter
[b] Dr. R. J. Lambert, Department of Agronomy, Univ. of Illinois, Urbana, IL
[c] Dr. P. A. Peterson, Agronomy Department, Iowa State Univ., Ames, IO

formants from hybrids arising from pollen contamination, and 3) detect differential transformation rates for different genetic markers.

Preliminary experiments were conducted to determine the appropriate radiation dose range to employ. Pollen was irradiated with 2.5, 5, 10, 20, 30, 40, 80 and 100 Krad gamma radiation. Gamma irradiation dosage was controlled by adjusting the distance of the samples from the CO^{60} radiation source, while using a constant exposure time of 1 h. A dose range was selected which was sufficient to 'pulverize' the pollen's DNA, precluding any chance of hybridization, while preserving the pollen's physiological ability to germinate and grow through stylar tissue. This was based on in vitro germination rates, in vivo growth rates in the style as assayed by fluorescent microscopy (Kho and Baer 1968), and seed set.

Five experiments were conducted. The first two experiments were conducted in 1981 and involved the same multiple dominant donor, but used two different multiple recessive recipient lines. In these experiments, there were four pollination treatments: 1) pollination with irradiated donor pollen' (80 Krad), 2) pollination with irradiated donor pollen mixed with self pollen, 3) pollination with irradiated donor pollen followed by self pollination delayed 18 h, and 4) pollination with a simple mixture of non-irradiated donor and self pollen.

The second two experiments were conducted in 1982, and largely repeated the 1981 experiments. However, pollination treatment No. 2 was modified and expanded to involve a range of radiation doses from 20–40 Krad, instead of 80 Krad. The pollination treatment involving a second delayed pollination was not included in these experiments.

The fifth experiment involved the En tester and En donor. En is an autonomous transposable element capable of activating a responsive allele ($a^{m(r)}$), thereby changing a white seed phenotype to a mottled-purple seed phenotype (Peterson 1978). The pollination treatments included: 1) irradiated pollen (20, 30, 40 Krad), 2) irradiated pollen (20, 30, 40 Krad) mixed with self pollen, and 3) self pollination.

In all five experiments, seed were harvested and scored for endosperm markers, some seed of each treatment were planted out for observation of seedling traits, and samples from each treatment were selfed to observed F_2 endosperm traits. Seed with unexpected phenotypes were planted in jiffy mix in the greenhouse, were grown, and were selfed.

Tassels were cut the evening before pollination, and stored as cut stems in water at 15 °C. Early the next morning pollen was collected by allowing anthers to dehisce under IR light. Collected pollen was stored before, during, and after radiation over a 40% $Ca(NO_3)_2$ solution at 1–4 °C (roughly 80% humidity). Pollen mixture was achieved by heavily pollinating with irradiated donor pollen, followed by heavy pollination with nonirradiated self pollen. Pollen was irradiated the same morning as collected, and used for pollination that afternoon. Pollen contamination was controlled by cutting silks before sunrise, careful pollination techniques, and physical isolation of crossing plots.

Results

Preliminary experiments indicated that radiation doses exceeding 10 Krad prevented hybrid seed set, and doses between 2.5 and 10 Krad produce hybrid seed with extremely low viability. A dose of 100 Krad was found to be essentially lethal to corn pollen, resulting in germination percentages and tube growth rates of nearly zero. A dose of 80 Krad was strongly inhibitory for pollen tube growth, while doses of 40, 30, and 20 Krad resulted in viable pollen with tube growth rates approaching normal growth rates. These results are in agreement with Pfahler (1971). On the basis of these results, we chose to use the maximum level of radiation capable of producing physiologically viable pollen (80 Krad), for our 1981 experiments. Based upon the negative results obtained in 1981, we used lower dose levels in 1982 (20, 30, and 40 Krad).

Table 2 summarizes the results from our first set of crosses. Irradiated pollen produced no seeds, indicating that the mechanism of induced diploid parthenogenesis as described by Pandey was not present. Irradiated pollen mixed with self pollen produced over 1,400 seed, all of which were maternal selfs except one which was a multiple dominant clearly arising from pollen contamination. Delaying self pollination until 18 h after use of irradiated pollen produced over 600 maternal-type seeds.

A simple mixture of non-irradiated donor and self pollen produced 532 seeds including a mixture of selfs and hybrids. One of the multiple recessive seeds gave rise to a multiple dominant seedling, clearly indicating heterofertilization. Because all seed were screened for

Table 2. Summary of some 1981 corn crosses involving a multiple recessive female (L1) and a multiple dominant male donor (D); with cross type and numbers of pollinations, recessive and dominant seeds, and recessive and dominant seedlings

Cross type[a]	Pollinations	Recessive seed[b]	Dominant seed	Recessive seedlings[c]	Dominant seedlings
L1 × D(80)	5	0	0		
L1 × [D(80) + L1]	23	1,400+	1[d]	209	1[d]
L1 × [D(80) + L1*d*]	14	600+	0	174	0
L1 × [D + L1]	6	357	175	273[e]	150

[a] L1 and D genotypes given in Table 1, brackets indicate mixed pollination, number in parenthesis indicates radiation dosage in Krad, *d* postscript indicates second pollination was delayed 18 h
[b] No. of seed approximated. All seed screened for two endosperm markers – *y, su*
[c] A limited amount of seed was grown out and screened for two seedling markers – *j* and *lg*
[d] A single multiple dominant seed/seedling (pollen contamination)
[e] One multiple recessive seedling from a multiple dominant seed type (heterofertilization)

Table 3. Summary of some 1981 corn crosses involving a multiple recessive female (L2) and a multiple dominant male donor (D); with cross type and numbers of pollinations, recessive and dominant seeds, and recessive and dominant seedlings

Cross type[a]	Pollinations	Recessive seed[b]	Dominant seed	Recessive seedlings[c]	Dominant seedlings
L2 × D(80)	10				
L2 × [D)80) + L2*d*]	9	450+	0	112	0
L2 × [D(80) + L2*d*]	4	150+	0	77	0
L2 × [D + L2]	3	137[d]	178	50[e]	86[f]

[a] L2 and D genotypes given in Table 1, brackets indicate mixed pollination, numbers in parentheses indicate radiation dosage in Krad, *d* postscript indicates second pollination was delayed 18 h
[b] No. of seed approximated. All seed screened for three endosperm markers – *y, su, a*
[c] A limited amount of seed was grown out for screening two seedling markers – *B* and *Pl*
[d] Two intermediate seed types: plump white and shrivelled purple, (both seeds were inviable)
[e] One multiple recessive seedling from a multiple dominant seed type (heterofertilization)
[f] One multiple dominant seedling from a multiple recessive seed phenotype (heterofertilization)

two endosperm characters (*y* and *su*) and all 607 seedlings were screened for two characters (*lg* and *j*), more than 3,000 potential transformation events were screened. However, no transformants were observed.

Table 3 summarizes the results of our second set of crosses, involving a different tester and different genes. Pollination with irradiated pollen produced no seed. Pollination with irradiated donor pollen mixed with self pollen produced over 450 seed, all of which were maternal-type. Pollination with irradiated pollen followed by delayed self pollination produced more than 150 seed, all of which were maternal. Pollination with an non-irradiated pollen mixture produced 315 seed, including a mixture of maternal selfs, hybrids, and four unexpected seed. Two of the unexpected seed were clear cases of heterofertilization. The two other unexpected seeds combined recessive and dominant endosperm characters, as would be expected from transformation or mutation. Unfortunately both of these seed proved inviable. Because three endosperm traits (*y, su, a*) were screened in the seed, as well as two seedling traits (*B, Pl*) in the 325 seedlings, more than 1,900 potential transformation events were screened. Two off-types were observed.

Table 4 summarizes the results from our third set of crosses, in our second season. Even at lower doses (20, 30, 40 Krad), pollination with irradiated pollen produced no seed. Pollinations with irradiated pollen (20, 30, 40, 80 Krad) mixed with self pollen produced over 5,800 seed, all of which were maternal-type. Pollination with a non-irradiated mixture of donor and self pollen produced over 2,200 seed, including a mixture of selfs, hybrids, and a single unexpected seed. The unexpected seed was a shrunken purple seed. This seed produced a purple hybrid seedling with normal F_2 segregation. This indicates that the endosperm was probably also hybrid, and that the purple endosperm mutated from *su/su/Su* to *su/su/su*. Over 19,000 potential trans-

Table 4. Summary of some 1982 corn crosses involving a multiple recessive fermale (L1) and a variably irradiated multiple dominant male donor (D); with cross type and numbers of pollinations, seeds, unexpected seeds, seedlings, and unexpected seedlings

Cross type[a]	Pollinations	Seed[b]	Unexpected seed	Seedlings tested[c]	Unexpected seedlings
L1 × D(20 – 80)	38	0			
L1 × [D(20) + L1]	19	700+	0	145	0
L1 × [D(30) + L1]	46	4,000+	0	784	0
L1 × [D(40) + L1]	23	400+	0	74	0
L1 × [D(80) + L1]	12	700+	0	95	0
L1 × [D + L1]	20	2,200+	1[d]	831	0

[a] L1 and D genotypes are given in Table 1, brackets indicate mixed pollination, numbers in parenthesis indicate radiation dosage in Krad
[b] Nos of seed approximated. All seed screened for two endosperm markers – *y, su*
[c] A limited amount of seed was grown out and screened for two seedling markers – *j* and *lg*
[d] A shrunken purple seed, producing a normal purple hybrid seedling

Table 5. Summary of some 1982 corn crosses involving a multiple recessive female (L2) and a multiple dominant male donor (D). Cross type and numbers of pollinations, seeds, unexpected seeds, seedlings, and unexpected seedlings are shown

Cross type[a]	Pollinations	Seed[b]	Unexpected seed	Seedlings tested[c]	Unexpected seedlings
L2 × D(20 – 40)	37	0			
L2 × [D(20) + L2]	16	1,850+	0	302	0
L2 × [D(30) + L2]	55	5,900+	1[d]	2,148	1[d]
L2 × [D(40) + L2]	7	800+	0	194	0
L2 × [D + L2]	17	2,150+	1[e]	538	0

[a] L2 and D genotypes are given in Table 1, brackets indicate mixed pollination, numbers in parentheses indicate radiation dosage in Krad
[b] Nos of seeds approximated. All seed screened for three endosperm markers – *y, su, a*
[c] A limited amount of seed was grown out and screened for two seedling markers – *B* and *Pl*
[d] A plump white seed, where a shrivelled (*su*) white seed is expected. The resulting seedling was maternal for all characters but segregated for *Su*
[e] A plump white seed, where a shrivelled white seedling or a plump purple seed is expected. Seedling was purple hybrid

formation events were screened, and only one mutant was observed.

Table 5 summarizes the results from our fourth set of crosses. Pollinations with irradiated pollen produced no seed. Pollinations with irradiated pollen plus self pollen produced more than 8,550 maternal-type seed, with a single exception. One seed had plump white endosperm and produced a maternal-type seedling which segregated for *Su*. The probability that both the endosperm and the embryo were simultaneously transformed relative to *Su* is extremely remote, indicating that this off-type seed must have arisen by a mutation prior to gametophytic mitosis either in the pollen or the egg sac. Non-irradiated mixed pollination with donor and self pollen produced over 2,150 seed, including selfs, hybrids and one unexpected seed. This was a plump white seed, producing a purple hybrid seedling. This seed is best explained as a mutation of the hybrid endosperm from *aaA* to *aaa*. Over 31,000 potential transformation events were screened, producing only a single mutant.

Table 6 summarizes the results from our last set of crosses, involving the En transposable element. Pollination with irradiated pollen produced no seed. Pollination with irradiated donor pollen mixed with self pollen produced 4,350 seed, one of which was unexpected. This seed had a small purple mottled sector in the endosperm, and produced a seedling having all white F_2 seed. A very similar seed was produced on an open pollinated ear of the En tester, producing a seedling with all white F_2 seed. These cases appear to be spontaneous endosperm mutations.

Table 6. Summary of 1982 corn crosses involving an En-tester female (T) and an En-source male (S); with cross type and numbers of pollinations, seeds, unexpected seed, seedlings tested, and unexpected seedlings

Cross type[a]	Pollinations	Seed[b]	Unexpected seed	Seedlings tested[c]	Unexpected seedlings
T×S(20–40)	10	0			
T×[S(20)+T]	10	1,800+	0	0	0
T×[S(30)+T]	10	150+	0	0	0
T×[S(40)+T]	10	2,400+	1[d]	15	0
T (open pollinated)	10	5,000+	1[d]	15	0

[a] T and S genotypes given in Table 1, brackets indicate mixed pollination, numbers in parentheses indicate radiation dose in Krad
[b] Number of seed approximated, all seed screened for 'En' seed phenotype
[c] A limited amount of seed was grown out and selfed, and scored for presence of 'En' phenotype
[d] White seed phenotype with small purple mottled sector. Produced normal seedling with white F_2 seed phenotype

Discussion

These experiments were designed to detect relatively high rates of transformation, of the type reported by Pandey (1975, 1978, 1980a). Ideally, transformation events should be verified using recipient stocks having a deletion at the locus of interest. This eliminates any possible confusion between true transformations and back-mutations. However, in this research it was believed that if Pandey's work could be extended to corn, the transformation rate would be much higher than could be explained by back-mutation. It was anticipated that multiple recessive recipient stocks would be much more valuable than a recipient stock with a deletion, allowing us to screen multiple loci, (since Pandey has argued that certain linkage groups may be preferentially transmitted). The multiple recessive stocks were also important in allowing the easy differentiation between true transformants and pollen contaminants. Very low levels of pollen contamination are largely unavoidable, and are usually much more likely to be confounded with Pandey-style transformation than is back-mutation. Lastly, multiple recessive stocks allowed unambiguous detection of heterofertilization events, which could be confused with transformation or mutation events in the endosperm. It might be added that if egg transformation rates are so low as to be easily confused with back-mutations, then this method of gene transfer would have virtually no practical value to plant breeders.

The results of these experiments clearly reveal that irradiated corn pollen does not have the ability to induce parthenogenic egg development (Tables 2–6). Since the occurrence of either parthenogenesis or polyspermy are prerequisite for Pandey's style of egg transformation, most subsequent crosses involved copollinations intended to maximize polyspermy.

Pollinations involving irradiated donor pollen and non-irradiated self pollen produced over 21,300 seed, but only three of these were unexpected. One of these unexpected seed was from pollen contamination (Table 2), one was clearly a back-mutation which occurred sometime before gametophytic mitosis (Table 5), and one appeared as an endosperm sector – probably arising from a back-mutation in that tissue (Table 6). No likely cases of egg transformation were found. The endosperm sector would appear to be the only conceivable candidate for being a transformant, however a similar sector was observed in open pollinated seed.

Mixed pollinations involving non-irradiated donor and self pollen were made, to serve as supplemental controls. These crosses were also made to test the claims of Stroun (1964), who has reported that mixed pollination can produce single progeny which combine characters from both pollen parents – which might suggest Pandey-type transformation. This treatment actually resulted in more off-type seed than did use of irradiated pollen, producing seven unexpected seed, among only 5,000 seed. However, three of these unexpected seed/seedling phenotypes were clear examples of heterofertilization. Of the remaining seed, two seed were produced having plump and white endosperm, and two seed were produced having shrunken and purple endosperm. Such seed combine the characters of both pollen parents. Unfortunately, one plump white and one shrunken purple seed were inviable. The one plump white and one shrunken purple seed which were viable both produced normal hybrid seedlings. This suggests that the endosperm was also hybrid and that recessive mutations had occurred at the *Su* and *R* loci. Alternatively, both seed were from heterofertilizations, with non-hybrid endosperm, where the dominant allele might have been replaced by a recessive allele by a

substitution transformation event. This would constitute a set of extremely unlikely events.

If all four of these unexpected seed arose by mutation, it would constitute a mutation rate of nearly 4×10^{-4}, which is higher than would be expected for spontaneous mutation. However, extraneous sperm entering the egg sac are known to be degraded (Vigfusson 1970), which must release large numbers of nucleotides and polynuceotides. Such DNA residues may themselves be mutagenic, which might explain the apparently elevated mutation rate in these types of experiments.

Even if all of the observed off-types in these experiments had been legitimate transformation events, the rate of transformation over all experiments would be less than 1.6×10^{-4}. This is dramatically different from transformation frequencies of greater than 50% as reported by Pandey. As already pointed out, the question of whether such rare events would represent mutations or transformations is somewhat irrelevant, since at these frequencies they would be of no practical value for plant improvement.

There are several possible reasons for our failure to induce "egg transformation" in corn. Since Pandey has indicated that the specific choice of parents or markers can affect success, it might be argued that we were unlucky in our choice of parental stocks. However, we employed seven markers in these experiments, and we conducted experiments with other recipient and donor lines (not shown) in addition to the lines described in Table 1. This would seem to represent a reasonable sampling of parents and markers.

It would make no sense to conduct Pandey-style experiments with corn at the same 100 Krad radiation dosage level as he used for *Nicotiana*. This is because corn pollen is physiologically killed at this dosage, and can not possibly deliver genetic material to the egg. However, we tested a range of radiation doses from 20–80 Krad. All of these doses pulverized the chromosomes sufficiently to prevent hybridization, while preserving varying degrees of pollen viability. Regardless of the dosage applied, no clear transformants were recovered.

It would be reasonable to assume that there was a significant rate of polyspermy in these experiments. The three observed cases of heterofertilization represent only a fraction of the actual polyspermy events. Typically, polyspermy events involve two pair of sperm being introduced into an egg sac. Even when those pair differ genetically, there is only a 50% chance that endosperm and embryo will be fertilized by genetically different sperm, which would be consequently detectable as heterofertilization. Where irradiated pollen was employed, presumably no heterofertilization events would be detectable, regardless of the rate of polyspermy, since no hybrid embryo or endosperm could develop. According to Sprague (1932), heterofertilization rates in corn can range from 1–25%, indicating polyspermy rates of 2–50%. Assuming a 2% rate of polyspermy in our experiments, at least 520 polyspermy events should have occurred. This assumes that irradiation had no effect on rates of polyspermy. According to Pandey, egg sacs pseudofertilized by irradiated sperm may remain receptive to second fertilizations – which should increase the rate of polyspermy. Likewise, irradiated pollen may have a greater tendency toward pollen-tube fusions in the style, which would, in effect, increase the rate of polyspermy.

Even if the rate of polyspermy could be made very high, it is reasonable to expect low transformation rates. This is because only crude genomic DNA is being introduced into the egg sac. A given gene is likely to be present in only a single dose, diluted by tens of thousands of other donor genes. The probabilities for transformation of a given gene under these circumstances become extremely remote. In this case, only where strong gametophytic or zygotic cell selection was occurring could transformants be recovered at a reasonable rate.

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 1983). 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.

It is concluded that "egg transformation" as proposed by Pandey, has little relevance to corn or corn breeding. These results, in conjunction with results in tomato (Sanford et al. 1984; Brock 1982), pea, rapeseed, and apple (Chyi et al. 1984), indicate that egg transfor-

mation using irradiated pollen is, at best, an isolated phenomenon.

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