Theor Appl Genet (1984) 67:553–558

An attempt to induce "egg transformation" in *Lycopersicon esculentum* Mill. using irradiated pollen *

J. C. Sanford, Y. S. Chyi and B. I. Reisch

Department of Pomology and Viticulture, New York State Agricultural Experiment Station, Cornell University, Geneva, NY 14456, USA

Received August 23, 1983
Communicated by H. F. Linskens

Summary. Experiments were designed and carried out to investigate the possibility of inducing "egg transformation" in tomato, as described by Pandey in *Nicotiana* L. Pollinations were made, which included the following treatments: irradiated donor pollen, irradiated donor pollen mixed with normal self pollen, irradiated donor pollen followed by delayed self-pollination, and a simple pollen mixture of non-irradiated donor and self pollen. No transformants were found after screening 5,620 seedlings representing 22,300 potential transformation events. If egg transformation occurs, it would appear to be limited to species outside of *Lycopersicon esculentum* Mill.

Key words: *Lycopersicon* – Tomato – Pollen – Gamma radiation – Transformation

Introduction

It has been claimed that irradiated pollen can be used as a vector for plant-to-plant transformation. Pandey (1975, 1978, 1980a), in a series of studies using heavily irradiated pollen of *Nicotiana* species, observed the transfer of characters from radiation-"killed" donor pollen to non-hybrid, maternal progeny. Observed transformants were fertile, cytologically normal, and had maternal phenotypes except for those specific traits transferred from the donor. It was proposed that such maternal or non-hybrid progeny arose by two different mechanisms. In most cases, irradiated pollen was mixed with self-incompatible recipient pollen. In these cases, it was hypothesized that the irradiated pollen acted as "mentor" (allowing self-fertilization and seed set) and simultaneously provided a source of genetic debris – leading to transformation events (Pandey 1980b). However, in some cases gene transformation was observed when irradiated pollen was used alone, in which case it was believed that the irradiated pollen stimulated egg doubling and parthenogenesis, while again providing the genetic debris required for gene transfer (Pandey 1980a, b). This unorthodox genetic phenomenon was termed "egg transformation".

If valid, Pandey's approach to gene transfer would be highly attractive to plant breeders as an alternative to existing backcrossing methods, since this approach would require only one generation rather than the 5–6 generations usually needed for development of isogenic lines. Furthermore, because this approach would not involve a hybrid intermediary step, the problems of hybrid inviability or sterility could be avoided, allowing gene transfer across much wider taxonomic distances than normally possible. In some cases, Pandey's approach might also represent an attractive alternative to more sophisticated molecular approaches to gene transfer, since Pandey's method would be simple, inexpensive, would be based on whole-plant phenotype, and would not require the identification and cloning of relevant genes on the molecular level.

For the reasons stated above, we felt it was important to determine to what extent Pandey's findings could be extended to other plant species. We also wished to elucidate the mechanism underlying the phenomenon of "egg transformation". Tomato (*Lycopersicon esculentum* Mill.) was selected as an appropriate model system for studying this phenomenon and its mechanism.

* Approved by the Director of the New York State Agricultural Experiment Station for publication as Journal Paper No. 3537, August 5, 1983

Materials and methods

The following experiments were designed to employ the clearly defined genetic markers found in the tomato. Multiple recessive stocks were used as female recipients to 1) detect the transfer of genes from wild-type donors, 2) distinguish between transformants vs. hybrids arising from pollen contamination, and 3) detect differential transformation rates for different genetic markers.

Preliminary experiments were conducted first to determine the effects of different radiation dose levels on pollen viability, pollen tube growth rate, and fertilization ability. An appropriate dose range was then selected for pollen treatment. Basically 4 types of crosses were made, each containing several parental combinations. 1) Pollinations using only irradiated donor pollen were made to investigate the possibility of parthenogenesis induced by irradiated pollen, as a mechanism leading to transformation events. 2) Pollination mixtures containing self and irradiated donor pollen in various genotype combinations were used to test the possibility that irradiated pollen could contribute genetic material to the normal selfing process, either through pollen tube interactions or by polyspermy. 3) A series of mixed pollinations were made where the application of irradiated pollen and the application of self pollen were separated by −2 to 18 h. These crosses were used to determine the importance of simultaneous vs. sequential pollen tube arrival into the ovary. 4) Simple mixed pollinations involving non-irradiated donor pollen were also used, to test the possibility of spontaneous exchange of genetic material between different types of non-irradiated pollen.

The tomato stocks used in this research were supplied by Dr. R. W. Robinson, Department of Seed and Vegetable Sciences, New York State Agricultural Experiment Station, Geneva; or by Dr. C. M. Rick, Tomato Genetics Cooperative Center, University of California, Davis. A diploid potato hybrid, US-W 5295.7, (producing n and 2n pollen) was supplied by Dr. R. E. Hanneman, Department of Horticulture, University of Wisconsin, Madison. Table 1 indicates the genetic stocks used and their genotypes.

Generally, the multiple recessive lines T1, T2, T3, T4, and T5 were used as female recipients. T4 is a tetraploid. Stock T5 and the four multiple dominant lines were used as donors. Among the multiple dominant lines, T6 is a diploid cultivar ('New Yorker'), T7 is a tetraploid cultivar ('San Marzano'), T8 is the botanical variety *L. esculentum* var. 'cerasiforme', and P1 is a diploid *Solanum tuberosum* clone producing n and 2n pollen.

Table 1. Genetic stocks used in tomato egg transformation studies, including genotype, ploidy, and source

Code	Stock	Genotype[a]	Ploidy	Source
T1	76-1803	a c d l r y	2x	NY
T2	LA 159	a e mc t u wf y	2x	Calif
T3	77-980	ah nv	2x	NY
T4	LA 793	a c d l r y	4x	Calif
T5	LA 986	aw bk d o p s Wo^m	2x	Calif
T6	'New Yorker'	wild type except u	2x	NY
T7	'San Marzano'	wild type except o	4x	Calif
T8	*cerasiforme*	wild type	2x	Calif
P1	*Solanum tuberosum*	wild type	2x	WI

[a] For explanation of gene symbols and the locations of genes, see Tomato Genetics Cooperative Report Vol. 30 (1980)

For pollen collection, newly-opened flowers were removed from the plants, usually on the day before irradiation, and pollen was extracted with an electric buzzer. Hands and tools were sterilized with 95% alcohol between handling of different stocks. Pollen was kept over desiccant in gelatin capsules at 4 °C until use, except during irradiation.

Gamma irradiation dosage was controlled by adjusting the distance of pollen samples from the Co^{60} radiation source, while using a constant exposure time of 1 h. Pollen was used within one week after irradiation. In vitro germination was employed to test pollen viability before use in pollination.

Preliminary studies on dosage effects involved light microscopic observation of in vitro pollen germination, and fluorescence microscopic observation (Kho and Baer 1968) of pollen tube growth rate in the style and subsequent penetration into ovules.

Flower buds were emasculated following the method described by Rick (1980), one day before pollination or right before pollination. In the case of mixed pollination, two types of pollen were combined in a 1 : 1 ratio by volume in a gelatin capsule, and were mixed with an electric buzzer for 2 min before pollination.

All progeny were classified for seedling markers, and 5% of the progeny of each family were transplanted to pots and were additionally screened for flower and fruit markers. Where tables refer to the "number of genes tested", the number of seedling traits screened is indicated, but it can be inferred that 5% of such progeny were also screened for flower and fruit characters, where applicable.

Results

Preliminary studies showed that doses up to 200 kr of gamma-radiation reduced pollen tube growth rate but not percent germination. Penetration of pollen tubes into ovules was observed even in the 200 kr treatment, yet no seed set resulted from pollen treatments exceeding 10 kr. Parthenocarpic fruits were harvested from treatments of up to 400 kr. The progenies obtained from crossing with pollen irradiated with 10 kr or below were all hybrids, and segregated for all marker traits in F_2 and F_3 generations.

A dose range of 60–130 kr was chosen for pollen radiation treatment based on the results from preliminary experiments. The results of transformation-inducing crosses were pooled and are summarized in Tables 2–5, according to the type of pollination made. A total of 5,620 seedlings from 2,096 pollinations were screened, but not a single transformant was found. Because multiple markers were employed, the seedlings were screened for more than 22,300 potential transformation events.

Where irradiated pollen was utilized alone for pollination, parthenocarpic fruits were common (Table 2). However, only 7 progeny were produced by this treatment, all coming from a single fruit. All seven were maternal in phenotype and bred true for all characters screened. This fruit was obviously contaminated with self-pollen.

The sixteen different donor-recipient combinations tested for mixed pollination using self and irradiated donor pollen involved eight 2x–2x, three 2x–4x, two 4x–2x, one 4x–4x, two inter-varietal and two inter-specific combinations (Table 3). No transformants were recovered. Two seedlings were unexpected, one from a 2x–2x, the other from a 4x–4x combination. They were both dominant in all characters screened. The 2x–2x seedling segregated for all traits upon selfing, whereas the 4x–4x seedling was sterile. These two seedlings clearly represented recipient/donor hybrids arising from pollen contamination.

Double pollinations separated by different time intervals were tested using two recipients, T1 and T2, with the donor T6 (Table 4). No transformants were found among resulting seedlings. Four unexpected seedlings were found, which are dominant for all traits and segregated normally upon selfing. These were clearly contaminants.

Mixed pollinations involving non-irradiated donor and self pollen failed to produce any unexpected progeny (Table 5). The two expected classes of progeny were produced using a diploid donor, i.e. maternal-type seedlings and hybrids. No recombinants were found. Only maternal-type seedlings were seen in the progeny from mixed pollinations using the tetraploid donor T7. Sample progeny were selfed and failed to produce unexpected segregation in the F_2 generation.

Table 2. Crosses involving different donor-recipient combinations, using only irradiated donor pollen. Numbers of pollinations, fruit set, seed set, and seedlings produced

Treatment[a]	No. pollinations	No. fruits	No. seeds	No. seedlings
T1×*iT1*	20	1	0	
T1×*iT6*	32	2	0	
T1×*iT7*	51	1	0	
T1×*iT8*	20	0		
T1×*iP1*	19	0		
T2×*iT2*	20	14	0	
T2×*iT6*	19	1	10	7[b]
T2×*iT7*	7	1	0	
T2×*iT8*	5	0		
T2×*iP1*	5	0		
T3×*iT6*	23	3	0	
T4×*iT6*	28	0		
T4×*iT7*	7	0		

[a] For genotype codes see Table 1. *i* means irradiated
[b] All seven seedlings were maternal in phenotype for 7 markers, suggesting maternal self-contamination

Discussion

Transformation of higher plants has been an active area of study for several years. Most recent work has emphasized the use of plant protoplasts as recipient cells for various gene vectors such as plasmids, plant

Table 3. Crosses involving different donor-recipient combinations, using irradiated donor pollen mixed with self pollen. Numbers of genes tested, pollinations, fruits, seeds, seedlings, and non-maternal seedlings

Treatment[a]	No. genes tested[b]	No. pollinations	No. fruits	No. seeds	No. seedlings	No. non-maternal
T1×[*iT4*+T1]	1	34	25	626	322	0
T1×[*iT5*+T1]	5	44	8	56	23	0
T1×[*iT6*+T1]	4	310	33	503	438	0
T1×[*iT7*+T1]	4	139	9	90	84	0
T1×[*iT8*+T1]	4	77	10	156	142	0
T1×[*iP1*+T1]	4	107	10	147	98	0
T2×[*iT6*+T2]	7	277	70	912	790	1[c]
T2×[*iT7*+T2]	4	85	48	1282	862	0
T2×[*iT8*+T2]	4	65	11	222	151	0
T2×[*iP1*+T2]	4	14	4	32	12	0
T3×[*iT6*+T3]	2	29	2	23	18	0
T4×[*iT2*+T4]	4	9	2	29	4	0
T4×[*iT6*+T4]	4	82	14	164	74	0
T4×[*iT7*+T4]	4	27	1	44	18	1[d]
T6×[*iT1*+T6]	4	20	9	329	118	0
T6×[*iT2*+T6]	4	20	11	660	350	0

[a] For genotype codes see Table 1. [ ] indicates mixed pollination, *i* means irradiated
[b] Represents minimum number of markers tested, i.e. number of seedling traits tested. Roughly 5% of all progeny were also observed for flower and fruit markers (not shown)
[c] A normal hybrid between donor and recipient types
[d] A hybrid-plant, vigorous but sterile

Table 4. Crosses employing irradiated donor pollen followed by delayed self-pollination (hours between pollinations indicated in parentheses). Numbers of genes tested, pollinations, fruits, seeds, seedlings, and non-maternal seedlings

Treatment[a]	No. genes tested[b]	No. pollinations	No. fruits	No. seeds	No. seedlings	No. non-maternal
T1×[*iT6*+(−2)T1]	4	20	5	41	16	0
T1×[*iT6*+(1)T1]	4	9	2	37	34	0
T1×[*iT6*+(2)T1]	4	36	3	67	48	0
T1×[*iT6*+(3)T1]	4	55	4	101	70	0
T1×[*iT6*+(4)T1]	6	19	2	21	20	2[c]
T1×[*iT6*+(5)T1]	6	19	4	33	32	2[c]
T1×[*iT6*+(6)T1]	4	25	3	29	20	0
T1×[*iT6*+(18)T1]	0	20	0			
T2×[*iT6*+(1)T2]	4	10	4	30	14	0
T2×[*iT6*+(3)T2]	4	27	18	514	397	0
T2×[*iT6*+(4)T2]	4	10	6	91	74	0
T2×[*iT6*+(6)T2]	4	10	7	108	69	0

[a] For genotype codes see Table 1. *i* means irradiated
[b] Represents minimum number of markers tested, i.e. number of seedling traits tested. Roughly 5% of all progeny were also observed for flower and fruit markers (not shown)
[c] Normal hybrids between donor and recipient types

Table 5. Simple mixed pollinations using non-irradiated donor pollen and self pollen. Numbers of genes tested, pollinations, fruits, seeds, seedlings, and unexpected seedlings

Treatment[a]	No. genes tested[b]	No. pollinations	No. fruits	No. seeds	No. seedlings	No. unexpected
T1×[T6+T1]	4	99	22	727	674	0
T2×[T6+T2]	4	22	14	440	433	0
T1×[T7+T1]	4	56	9	113	49	0
T2×[T7+T2]	4	16	8	184	159	0

[a] For genotype codes see Table 1. [ ] indicates mixed pollination
[b] Represents minimum number of markers tested, i.e. number of seedling traits tested. Roughly 5% of all progeny were also observed for flower and fruit markers (not shown)

organelles, viruses, bacteria, and artificial lipid vesicles (Kado and Kleinhof 1980; Fujiwara 1982; Ahmad et al. 1983). Microinjection of foreign DNA into the egg or embryo has also been proposed (Germeraad 1976; Soyfer et al. 1976; Turbin et al. 1975), although no significant result has been reported (Gordon 1983).

Reports by Pandey of extremely high frequencies of "egg transformation" in *Nicotiana* would appear to have no precedent, yet appear credible in that a variety of plant reproductive mechanisms do exist, which in combination, would yield the observed results. The level of irradiation employed by Pandey does indeed "pulverize" pollen chromosomes into fragments of chromatin (Grant et al. 1980). Such highly irradiated pollen does indeed function, producing a pollen tube and "fertilizing" the egg sac – i.e., "micro-injecting" its DNA fragments into the egg sac (Vassileva-Dryanovska 1966). Two different pollen tubes can enter the same egg sac (Sprague 1929, 1932), which could allow self-fertilization of the embryo and endosperm within the same embryo-sac which was previously "micro-injected" by the irradiated donor pollen. It is therefore not unreasonable that this series of events could lead to transformation events.

However, *Nicotiana* is less than an ideal model system to study this phenomenon, because there are relatively few genetic markers, and the markers that occur have relatively ambiguous inheritance and expression. It is for this reason that we decided to conduct this research using tomato. The results of this research reveal two things.

Firstly, caution is necessary in interpreting these types of experiments. Rare pollen contamination events are usually inevitable, and can easily be mistaken for transformants, if a good multiple marker system is not employed. As can be seen in Tables 3–5, all six un-

expected progeny from mixed pollinations were proven by their multiple markers to be hybrids arising from stray pollen from the donor. Likewise, the fact that all seven maternal-type progeny in Table 2 arose from a single fruit strongly indicates that these progeny were due to some stray self pollen, and were not diploid apomicts. Further caution is called for, even when multiple markers suggest that transformants are occurring at a high rate. In a series of crosses (not shown), a high number of apparent transformants appeared in the progeny. However, it was later found that a back mutation had occured in the female line, and some of the parental female plants were segregating for the gene in question.

The second, and more important point revealed by this research is that if such transformation occurs in tomato, it must either occur at an extremely low rate compared to the work of Pandey, or such transformation does not affect the 19 markers we studied.

The results shown in Table 2 indicate that induced diploid parthenogenesis, as described by Pandey in *Nicotiana* (Pandey and Phung 1982), does not occur in tomato. Irradiated tomato pollen does not induce egg doubling or parthenogenic development. The large number of donor/recipient combinations tested in Table 2 suggests that the failure to induce parthenogenesis was not merely due to the use of a recalcitrant female or an ineffective pollinator.

The results shown in Table 3 indicate that mixing self-pollen with the irradiated pollen provides seed set (thereby eliminating the need for parthenogenic development), but still fails to produce transformants. Pandey has argued that the efficiency of transformation can be greatly influenced by the choice of either the female or male (Pandey 1980a). Therefore, many donor/recipient combinations were tried. A diploid potato clone was also used as a donor with the hope that some genes affecting morphology would be transferred. However, no unusual phenotypes were observed. Two tetraploids, a donor and a recipient, were also included in the cross combinations, in the hope that endosperm ploidy balance (Lin 1975; Nishiyama and Yabuno 1978; Johnston 1980; Johnston et al. 1980) might be improved where unusual double fertilization events might be occurring. Such ploidy variation did not improve our success.

Since polyspermy is assumed to be a prerequisite for transformation in non-apomictic tomato, the simultaneous arrival of both irradiated donor and self pollen to the ovule may be a prerequisite for such polyspermy. By slightly altering the timing of pollination, the less competitive pollen in a mixture might be given enough of a head-start so as to reach the ovary at the same time as the faster pollen. Alternatively, it might be desirable to give the irradiated pollen a considerable head-start, such that all ovules are first pollinated by irradiated pollen. Egg sacs "fertilized" by irradiated pollen might then remain receptive to a second fertilization by viable self pollen. However, even by altering the timing of the second pollination from −2 to 18 h, no transformation resulted (Table 4).

Stroun (1964) reported that mixed pollination tomato crosses resulted in individual seedlings containing traits from both pollen parents. If valid, this report would suggest that genetic events not unlike those reported by Pandey could indeed occur in tomato. To test the validity of Stroun's claims, simple mixed pollinations, without irradiation, were tried in 4 combinations, using diploid and tetraploid donors. As shown in Table 5, 1,315 seedlings resulted, all of which were in the two expected classes, having either maternal or hybrid phenotypes. Because at least four markers were scored for each seedling, this represents over 5,260 potential transformation events. These results seem to strongly contradict the results of Stroun.

Our results are consistent with the results of Brock (1982) who tried to transfer genes from *L. pimpinellifolium* to *L. esculentum* with irradiated pollen. No successful recovery of gametic transformants was obtained after screening 847 seedlings.

Egg transformation does not seem to occur in *Lycopersicon* for any of numerous markers tested, while Pandey reports in *Nicotiana* very high levels of egg transformation for all three of the markers tested. This difference might be reconciled in part by the genetic differences between these two closely related genera. While *Nicotiana* is self-incompatible and has a predisposition toward parthenogenesis, the *Lycopersicon* stocks used were self-compatible and apparently have no parthenogenic potential. The very high rates of transformation reported by Pandey could only be explained if intense cell selection for transformants was occuring in style and/or ovary. The pollen/style incompatibility system could result in such cell selection. In Pandey's work, thousands of pollen grains and many hundreds of ovules were involved in each cross, with each cross producing only one or two seeds. Therefore it is possible to envision several possible selective mechanisms which might have allowed only those gametes or embryos which were transformed, to survive. In the tomato crosses reported in this paper, seed set was generally good, so no similar selective mechanism could be in operation.

The reports of Pandey remain an enigma, but it is clear that his results can not be expected to be applicable to *Lycopersicon esculentum.*

Acknowledgements. The authors wish to thank Dr. H. C. Aderhold in the Department of Nuclear Engineering at Cornell University for use of the Gamma Cell irradiation

facilities. We would also like to thank Dr. R. W. Robinson for his valuable advice, and Mr. Marc Baase and Ms. Nancy Adams for their technical assistance. This research was supported, in part, by a grant from Procter and Gamble Co., which is gratefully acknowledged.

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