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Attempted pollen-mediated plant transformation employing genomic donor DNA

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Summary. Experiments were conducted to test the validity of previous reports of pollen-mediated plant transformation utilizing genomic donor DNA. Multiple Mendelian markers were employed in *Zea mays* L. and *Lycopersicon esculentum* Mill. to detect transformation events. Pollen from multiple recessive (recipient) lines was incubated with genomic DNA from multiple dominant (donor) lines, under various conditions. Treated pollen was subsequently used for pollinations on multiple recessive females, and resulting seeds were screened for transformation events. Over 200 crosses were made in tomato, and over 80 crosses were made in corn. Over 600 resulting seedlings were tested in tomato and over 800 seeds were screened in corn. Because multiple markers were used, 4,937 potential transformation events were screened. No clear-cut transformation events were observed. Therefore, using well-defined multiple markers, we have been unable to confirm the earlier claims of high efficiency pollen-mediated transformation employing genomic donor DNA.

Key words: Transformation – Pollen – DNA – *Lycopersicon* – *Zea*

Introduction

It has been claimed that plants can be transformed by incubating pollen with donor genomic DNA, followed by normal pollination and seed production (Hess 1977, 1980). Flower color genes were reportedly transferred within and between *Petunia* species, at rates exceeding 0.3%. Such high rates of pollen-mediated transformation would have major implications for crop improvement. This is because it would then be possible to bypass the need for gene identification and cloning and for plant regeneration from protoplasts, which are major limitations of current plant transformation systems.

Transformation employing genomic DNA has previously been demonstrated in mammalian cell systems (Wigler et al. 1978). However, transformation rates in such DNA-mediated systems are normally very low, especially when employing genomic DNA. The high rates of transformation reported by Hess could only be explained if a second major determinant of transformation efficiency, such as DNA uptake into the cell, was highly favorable. Pollen may have a unique ability to take up DNA and other macromolecules, because pollen has a discontinuous plasma membrane when in its desiccated state. During hydration, pollen is believed to 'leak' macromolecules before the outer membrane reconstitutes itself. Hess et al. (1974) have previously claimed to have demonstrated the efficient uptake of protein and entire bacteriophage into hydrating *Petunia* pollen by this mechanism. However, such uptake studies have generally been found to be of questionable value, due to artifacts relating to external cellular binding and uptake of degradation products (Kool 1977; Behki and Lesley 1979; Tupý et al. 1980).

The genetic systems employed in the previous *Petunia* experiments were far from ideal, since varying degrees of flower color were expressed in "white" control plants as well as in "transformed" plants. Poorly defined genetic systems are not amenable to the conclusive demonstration of transformation. In order to obtain less equivocal results, we have tested the concept of genomic DNA-mediated pollen transformation in corn and tomato, which have multiple Mendelian markers genes showing discrete rather than continuous variation.

Table 1. Genetic stocks employed in pollen transformation experiments, including genotype and source

Stock	Genotype[a]	Source
Tomato		
'New Yorker'	wild type	Dr. R. W. Robinson
LA 159	a, e, mc, t, y, wf	Dr. C. M. Rick
76-1083	d, c, a, l, r, y	Dr. R. W. Robinson
Corn		
69-1484-3	A, A_2, C, C_2, R^r, Pr, B, Pl	Dr. R. J. Lambert
86-1430-10/1482-2	a, su, pr, y, A_2, C, R^r	Dr. R. J. Lambert
8-1346-1349	$a^{m(r)}/a^{m(r)}$	Dr. P. A. Peterson
8-10260-22/0301	a^{m-1}/a (plus En)	Dr. P. A. Peterson

[a] For explanation of tomato gene symbols, see Tomato Genetics Cooperative Report Vol. 30 (1980). For explanation of corn gene symbols, see Neuffer et al. (1968) and Peterson (1978)

Materials and methods

The tomato genetic stocks for these experiments were supplied by Dr. R. W. Robinson, Horticultural Sciences, New York State Agricultural Experiment Station, Geneva, and by Dr. C. M. Rick, Tomato Genetics Cooperative Center, University of California, Davis. Corn stocks were obtained from Dr. R. J. Lambert, Maize Stock Center, Dept. of Agronomy, University of Illinois, Urbana, and from Dr. P. A. Peterson, Agronomy Department, Iowa State University, Ames. The nature of these genetic materials is described in Table 1. The tomato cultivar 'New Yorker' was employed as a wild-type DNA donor. Two multiple recessive lines of tomato were used as DNA recipients. A multiple dominant corn line was used as a DNA donor, while a corresponding multiple recessive line was used as the DNA recipient. A corn line carrying the transposable element 'En' was also used as a DNA donor, with an "En-tester" line used as DNA recipient. The 'En' transposable element was specifically chosen as a marker for use in transformation experiments because of its known ability to randomly insert into the corn genome and be expressed as a clear-cut seed marker (Peterson 1978).

DNA was isolated from the corn and tomato DNA donors using modified protocols of Hamilton et al. (1972), and Zimmer and Newton (1982). Young leaves from one-month-old plants were soaked in ether and homogenized in a Waring blender with extraction buffer (0.3 M sucrose; 50 mM Tris-HCl, pH 8.8, 5.0 mM $MgCl_2$). The homogenate was then filtered through 10 layers of cheesecloth, and centrifuged for 8 min at 250 g. Nuclei were lysed in lysis buffer (50 mM Tris-HCl, pH 8.0, 20 mM EDTA; 1% Sarkosyl), and the lysate was treated with RNase and Pronase, followed by phenol, chloroform, and ether extractions. Resulting DNA was ethanol precipitated. Quantity and purity of the nucleic acid were determined using spectrophotometric readings. The molecular weight of the DNA was determined by agarose electrophoresis, using lambda phage Hind III restriction fragments as molecular weight standards. By these criteria we had relatively pure DNA (260 : 280 absorbance ratios ranging from 1.6–1.7), with high molecular weights (exclusion above the 23.1 kb molecular weight standard), and with less than 1% RNA contamination.

DNA incubation methods involved hydration and soaking of recipient pollen in pollen germination medium (300 ppm Ca $(NO_3)_2$, 100 ppm H_3BO_3; with 9–14.5% sucrose depending on genotype) with and without poly-l-ornithine (PLO) as a DNA protectant and uptake enhancer (Hughes et al. 1979; Hasnain et al. 1980). The germination medium contained DNA concentrations ranging from zero (control) to 580 μg/ml. The solution was continuously aerated during incubations, which lasted from 5 to 40 min.

Following incubation, recipient tomato pollen was collected on a millipore filter, scraped off with a spatula, and applied to the stigmas of emasculated recipient flowers. Treated corn pollen was sprayed onto bagged silks of emasculated greenhouse-grown corn plants using an air brush (Paasche Co., Type H). Following such pollinations, plastic bags were placed over recipient flowers, to prevent subsequent pollen dessication.

Resulting corn seed was scored directly for seed markers, and corn and tomato seeds were planted in the greenhouse and field for the purpose of scoring of seedling and plant characters. Where appropriate, resulting plants were selfed to observe F_2 seed marker segregations in corn.

Results

Preliminary experiments involving pollinations with pre-incubated pollen indicated that pollen germination was greatly affected by incubation conditions. Pollen was found to bind to the stigma best by using an incubation solution with a neutral pH, and using short incubation times (less than 1 h for tomato, less than 10 min for corn). Relatively high germination rates were found only when humidity was kept high and, in the case of corn, when the pollen was applied with an air brush. High PLO concentrations (above 50 μg/ml) resulted in the precipitation of tomato DNA, but did not reduce tomato pollen viability. Corn pollen viability was lost at even the lowest PLO concentrations tested.

The various treatments employed in these experiments, as well as the resulting seed set and the nature of the subsequent progeny are summarized in Tables 2 and 3. The 694 experimental tomato seedlings arising from DNA-treated pollen represented 4,047 potential transformation events, and were found to be normal in

Table 2. Data on tomato crosses attempting to demonstrate pollen-mediated transformation using genomic DNA. Shown are recipient lines, DNA and PLO concentrations, incubation times and numbers of pollinations, seedlings screened, potential transformation events, and off-types

Recipient line	DNA (μg/ml)	PLO (μg/ml)	Incubation time (min)	No. pollinations	No. seedlings screened	No. potential transformation events	No. off-types
76-1083	120	2.5	5	52	80	474	0
	150	0	5	20	149	849	0
	150	5	5	30	411	2,403	0
	150	0–50	40	36	0[a]	–	–
LA159	120–150	1–5	5	60	50	297	0
	186	0	40	4	4	24	0

[a] Prolonged incubation beyond 30 min often resulted in zero seed set

Table 3. Data on corn crosses attempting to demonstrate pollen-mediated transformation using genomic DNA. Shown are recipient lines, DNA concentrations, and number of pollinations, ears and seed set, potential transformation events, and off-type seeds

Recipient line	DNA (μg/ml)	Pollinations	No. seeds and seedlings screened	No. potential tranformation events	No. off-types
8-1346-1349	0 (Control)	6	1,200	0	0
	12	1	1	1	0
	50	8	76	76	0
	115	16	418	418	31[a]
	200	5	118	118	0
	250	18	103	103	0
	290	5	4	4	0
	438	4	30	30	0
	580	3	62	62	0
86-1430 -10/1482-2	132+264	23	26	78	0

[a] Off-type seeds were found on two ears that were treated with the same pollen/DNA solution. See text for explanation

all respects. The 838 experimental corn progeny, representing 890 potential transformation events, were also normal in all respects, with two exceptions. Two En-tester plants which were treated on the same day, with the same pollen/DNA mixture (Table 3) produced off-type (colored) seed, where only white kernels would be expected. The off-type seeds on these two ears were as follows: Ear No. 1–4 yellow, 3 yellow with purple spots, 6 white with purple spots, and 4 solid purple; Ear No. 2–7 yellow, 2 while with purple spots, and 5 solid purple. All ears treated as controls (6 crosses, 1,200 seed) were normal, including an ear from the same plant as Ear No. 1.

Discussion

The results of these experiments do not appear to support earlier claims of high-efficiency transformation by incubation of pollen with genomic donor DNA (Hess 1977, 1980). Our experiments did not attempt to precisely duplicate the earlier experiments because of the ambiguous expression of the genetic markers used in those experiments. However, *Lycopersicon esculentum* would appear to be a reasonable substitute for *Petunia,* since it is related to *Petunia,* has similar pollen biology, and has more numerous and better defined genetic markers. *Zea* is not closely related to *Petunia,* but also offers an excellent array of clearly defined markers, including multiple seed markers. The corn seed markers employed included a transposable element, 'En', which would appear ideal for detecting and achieving high transformation efficiency. This is because it is a DNA fragment known to insert randomly in the corn genome and which activates an unlinked marker gene already present in the tester line's genome (Peterson 1978). When activated, this marker ($a^{m\,(r)}$) produces

a purple mottled seed phenotype, which is very easily scored.

Upon first inspection, two ears from our experiments involving the 'En' transposable element appeared to have transformed seed. These ears had 20 out of 43 seed which displayed the chimeric aleurone layer which would indicate the presence of the 'En' gene, exactly as observed in test crosses. However, actual transformation was ruled out for three reasons: 1) the rate of transformation was much too high for these ears, relative to other ears treated similarly; 2) a second gene from the donor, '*Y*' (yellow endosperm), was observed on the same ears, strongly suggesting genetic contamination; and 3) all kernels from such ears produced sporophytes (from the zygotes) which genotypically matched the genotype of their endosperm. The significance of this last point requires some explanation. Since the embryo and the endosperm arise independently during the double fertilization process, their transformation should also occur independently. However, fertilization by normal donor pollen (i.e. pollen contamination) will nearly always produce embryo and endosperm within the same seed which bear the same genetic markers. We conclude that in spite of the great care which was taken to prevent pollen contamination in these experiments, a seed contaminant must have occurred within our pollen source population, which was the cause of the observed off-type progeny. This type of event reinforces the importance of using multiple markers, which have allowed us to distinguish between true transformants versus other anomalies.

Since no transformants were observed from a total of 4,937 potential transformation events screened, the transformation rate by this method, in these species, with these markers, must be less than 0.02%. This would not appear to be consistent with the transformation frequency of greater than 0.3% observed by Hess (1980). This difference might be explained by the difference in species, the difference in markers employed, or by differences in incubation conditions (the exact incubation conditions used by Hess were not appropriate for tomato and corn pollen). However, another explanation for Hess' results might involve the "blushing" phenomenon in *Petunia* which is recognized by breeders (personal communication). For unknown reasons, white petunia lines occasionally produce progeny with varying degrees of red "blush". The blushing phenotype would appear to be quite similar to the "transformed" phenotypes described by Hess. Given that "transformants" were identified on the basis of subjective scores for a trait having continuous rather than discrete variation, there would appear to exist the possibility of an unintentional bias in assigning numerical scores to treatments versus controls.

The experiments of Hess must be considered inconclusive. Our own experiments indicate that pollen-mediated transformation involving genomic DNA does not result in the high rates of transformation required to make this approach practical for crop improvement.

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References

Behki RM, Lesley SM (1979) Uptake of exogenous DNA by carrot protoplasts. In Vitro 15:851–856

Cocking EC (1977) Genetic modification of plant cells: a reappraisal. Nature 266:13–14

Hamilton RH, Kunsch U, Temperli A (1972) Simple rapid procedures for isolation of tobacco leaf nuclei. Anal Biochem 49:48–57

Hasnain SE, Ganesan K, Upadhyaya KC (1980) Mechanism of enhancement of DNA-uptake by polycations: effects of polycations on DNA, DNase and plasma membrane. Ind J Exp Bot 18:1230–1232

Hess D, Gresshoff PM, Fielitz U, Gleiss D (1974) Uptake of protein and bacteriophage into swelling and germinating pollen of *Petunia hybrida*. Z Pflanzenphysiol 74:371–376

Hess D (1977) Cell modification by DNA uptake. In: Reinert J, Bajaj YPS (eds) Applied and fundamental aspects of plant cell, tissue, and organ culture. Springer, Berlin Heidelberg New York, pp 506–577

Hess D (1980) Investigations on the intra- and interspecific transfer of anthocyanin genes using pollen as vectors. Z Pflanzenphysiol 98:321–337

Hughes BG, White FG, Smith MA (1979) Fate of bacterial plasmid DNA during uptake by barley and tobacco protoplasts. 2. Protection by poly-l-ornithine. Plant Sci Lett 14:303–310

Kool AJ (1977) An autoradiography study of binding and uptake of DNA by cultures soybean cells and protoplasts. Genen Phaenen 19:61–63

Neuffer MG, Jones L, Zuber MS (1968) The mutants of maize. Crop Science Society of America, Madison Wis, p 74

Peterson PA (1978) Controlling elements: the induction of mutability at the A_2 and C loci in maize. In: Walden DB (ed) Maize breeding and genetics. Wiley and Sons, New York, pp 601–631

Tupy J, Hrabetova E, Capkova-Balatkova V (1980) Interaction of microbial DNA with germinating pollen of *Nicotiana tabacum* in Culture. Physiol Veg 18:677–687

Wigler M, Pellicer A, Silverstein S, Axel R (1978) Biochemical transfer of single copy eucaryotic genes using total cellular DNA as donor. Cell 14:725–731

Zimmer EA, Newton KJ (1982) A simple method for the isolation of high molecular weight DNA from individual maize seedlings and tissues. In: Sheridan WF (ed) Maize for biological research. University Press, Grand Forks ND, pp 165–168