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"Egg transformation" induced by irradiated pollen in *Nicotiana:* critical appraisal of Chyi and Sanford's observations

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Summary. This note strongly refutes the suggestion by Chyi and Sanford (1985) that "egg transformation" by the use of lethally irradiated pollen in *Nicotiana* reported by the present author could be explained on the basis of mutation, contamination or other causes, and suggests that their conclusion is based on faulty experimentation and interpretation. It also shows that, contrary to these authors' assertions, their results, in fact, contain certain significant demonstrations of egg transformation supporting the present author's work.

Key words: *Nicotiana* – Egg transformation

Introduction

In a recent report Chyi and Sanford (1985) claimed that evidence for the phenomenon of egg transformation by lethally irradiated pollen in *Nicotiana* (Pandey 1975, 1980) could have resulted from mutation, contamination or other causes. In rebuttal I would like to comment on the following points with reference to supposed sources of error in my experiments and possible re-interpretation of those of Chyi and Sanford.

Pollen contamination and repeatability of results

Chyi and Sanford's experiments were carried out in an uncontrolled environment which apparently did not preclude cross-pollination by insects. The authors state that "in 1982 experiments, no special measures were taken to prevent possible cross-pollen contamination". In 1983 and 1984, flowers were protected by partially covering them with gelatin capsules. The uncontrolled conditions permitting contamination meant that the results were likely to be unreliable and inconsistent and, also since the presumably environmentally sensitive rare incidence of parthenogenetic diploidy involved in egg transformation was highly variable from year to year, unrepeatable. In contrast, all experiments of the present author were carried out in a regularly fumigated insect-proof greenhouse, having controlled heating and lighting conditions. Since self-incompatible *Nicotiana* is strictly insect pollinated, there is no chance of any seed set on fully self-incompatible plants grown under these conditions. Thus while pollen contamination may have been a source of error in Chyi and Stanford's work, it was certainly not so in the present author's work.

Interpretation

Chyi and Sanford found four groups of results ("Cases") which could be classed as transformation, but which were ascribed to mutation, contamination or other causes.

Case 4

This group of results could not have resulted from contamination, and is therefore pivotal to understanding these authors' experimental techniques and interpretation. It involves interspecific pollination between self-compatible (S.F.) *N. langsdorffii* (S_fS_f) as the female parent and self-incompatible (S.I.) *N. alata* (S_3S_3) as the irradiated male, donor parent. Irradiated *N. alata* pollen was mixed with unirradiated *N. langsdorffii* pollen before pollination. This resulted in two plants which were undoubtedly *N. langsdorffii* and yet were S.I. They were heterozygous for the *S* allele, having a S.I. (S_I) allele and a S.F. (S_f) allele. Chyi and Sanford explain these plants as the results of spontaneous mutations from a S_f allele to a S_I allele. There are several elements in this assumption which are untenable.

Firstly, these authors cited hearsay evidence (Kostoff 1943; Dr. Sisson, personal communication) of the "presumed" rise of S.I. *N. langsdorffii* plants by mutation. But no such authenticated case is on record. Extensive studies by the present author have shown that *N. langsdorffii* is truly S.C., apart from introgression, there being no authenticated case of a S_I allele occurring in the pure species (Pandey 1977). Indeed, siblings of *N. langsdorffii* material grown by Chyi and Sanford themselves were all S.C.

Secondly, a mutational origin of a S_I allele from a S_f allele is extremely unlikely on theoretical grounds. While mutational production of a new S_I allele from another S_I allele (Ramulu 1982) or a S.C. allele from a S.I. allele (de Nettancourt 1977) has been reported, there has been no authenticated case of an origin of a normal multiallelic type of S.I. allele from a S.C. allele. The rise of S.I. alleles from S.C. alleles is considered by

several authors to have occurred only once early in the evolution of angiosperms (de Nettancourt 1977).

Thirdly, a S_I allele and a S_f allele are usually independent in action, so that the heterozygous $S_I S_f$ plant is S.C. (Pandey 1964). In order to explain the self-incompatibility of the two heterozygous S.I. *N. langsdorffii* plants, Chyi and Sanford suggest that the two newly arisen S_I alleles were "fully dominant" (in pollen as well as style). But, contrasting with sporophytic incompatibility having dominance, gametophytic incompatibility in *Nicotiana* is characterised by complete absence of dominance both in the pollen and style (East and Mangelsdorff 1925). Thus dominance could not explain self-incompatibility in *N. langsdorffii*. It is also to be noted that it was *N. alata* pollen which was irradiated not *N. langsdorffii* pollen, so the two mutations in *N. langsdorffii* in their limited experimental material had to be spontaneous! It must also be emphasized that the two S.I. plants arose only in the transformation experiment and not in the control which were all normal self-compatible. The suggested mutational origin of S_I alleles in *N. langsdorffii* by Chyi and Sanford is thus totally unjustified.

I would like to suggest another possible explanation for Chyi and Sanford's results. They grew plants of *N. alata* S_{F10} S_{F11} from seed supplied by the present author. These alleles, when present in the style, have the additional property of rejecting S_f pollen from S.C. *N. langsdorffii* (Anderson and de Winton 1931; Pandey 1964). If, instead of using S_3S_3 material these authors mistakenly used irradiated donor pollen from $S_{F10}S_{F11}$, (or from $S_{F4}S_{F6}$, which has similar properties to that of $S_{F10}S_{F11}$) egg-transformed *N. langsdorffii*, plants arising from such pollinations might produce two kinds of plants, $S_{F10}S_f$ and $S_{F11}S_f$. These would be S.I. and intercompatible. The self-incompatibility in the transformants would be due to the fact that the S_{F10} and S_{F11} alleles in the styles will reject not only S_{F10} and S_{F11} pollen respectively but also the S_f pollen. There is no dominance.

Furthermore, these workers found in their transformation experiment only two different S alleles, both of the same highly specific S_{FI} type, as might be expected. It is extremely unlikely that two, not one, different S_I alleles, of the same special S_{FI} type, could spontaneously arise independently by mutation in a very small experimental population when none have ever been found in non-experimental material, including their own. Egg transformation is thus the only logical explanation for Case 4.

Case 3

If error in the irradiated material, combined with true egg transformation, explain the results in Case 4, then Case 3 is explicable on the same basis. In Case 3, a white-flowered *N. alata* plant was pollinated with irradiated pollen from a red-flowered *N. alata*. One of three resulting seedlings had pink flowers and an S allele different from that in the female parent. However, this new S allele was also different from that of the donor, yet the same as another S allele in their genetic stock. To explain the result the authors suggest contamination, or partial hybridisation resulting from a pollen grain which received less than a full dose of irradiation. They reject the possibility of cotransformation for flower colour and S allele, although it was shown to be fairly common in the present author's results (Pandey 1980). Linkage of one of the flower colour genes to the S locus (with an average 20% recombination), has been shown by Brieger and Mangelsdorf (1926), Anderson and de Winton (1931), Brieger (1935) and also by the present author. As in Case 4, egg transformation in association with error in the irradiated pollen used would explain the Case 3 results as well.

Case 2

This involved as female parent a white-flowered hybrid between S.I. *N. bonariensis* and S.F. *N. langsdorffii*. The irradiated male parent was an orange-flowered *N. glauca*. Out of 13 progeny three had pink flowers. Again the explanation of egg transformation is resisted by Chyi and Sanford in favour of alternative arguments of gene suppressor, interspecific hybrid segregation, genetic contamination and other remote possibilities.

Case 1

This involved four families in which a total of 16 white-flowered plants arose from intraspecific pollinations in *N. alata*, using white-flowered $S_{F4}S_{F6}$, $S_{F5}S_{F7}$ and $S_{F8}S_{F9}$ plants as female parents and red-flowered S_3S_{F10} plants as the irradiated male, donor parent. The irradiated pollen was mixed with normal, incompatible, self-pollen before pollination. Eleven of the 16 plants had an S allele from the irradiated donor parent. These results are very similar to the author's results but Chyi and Sanford reject egg transformation in favour of contamination from one white-flowered S_3S_{F10} plant which they found among their experimental plants.

Contamination seems a very unlikely explanation in this case for a number of reasons. Firstly, the four families concerned arose from single capsules giving 10, 2, 2, 2 plants respectively, whereas pollen contamination in an insect pollinated species, where pollen is usually dispersed in lumps, would normally produce considerably larger number of seeds. Secondly, it is extremely unlikely that four different families of very rare plants will independently give rise to contaminant plants from the same one pollen parent. Thirdly, the occurrence of one plant among 16 having the homozygous genotypic constitution $S_{F4}S_{F4}$ and three plants having the maternal genotype $S_{F4}S_{F6}$ suggests that a quarter of the progeny resulted from self fertilisation by the mixed self-pollen due to a very weak mentor pollen effect, and 11 resulted from pseudofertilisation by the irradiated pollen, leading to S transformation followed by parthenogenetic diploidy. Most importantly, contamination would not give maternal S homozygous and maternal S heterozygous plants. Again, egg transformation is the only logical explanation fully consistent with the facts.

Chyi and Sanford also discounted egg transformation because they did not find S-triallelic plants among the progenies of some of these plants. The above analysis of the plants suggested that all of them resulted from single fertilisations, either normal or pseudo, and that there were no plants arising from a second normal fertilisation by self-pollen subsequent to pseudofertilisation and egg transformation by the irradiated pollen. Since identifiable triallelic plants can only arise, and only in a small proportion at that, when there is a second normal fertilisation (Pandey 1980), a lack of triallelic plants among the progeny of certain transformants studied is only to be expected.

A significantly higher proportion (about 50%) of transformants in the present author's experiments (Pandey 1980) as compared to those of Chyi and Sanford was the result of a critically efficient mentor pollen effect in the materials used – leading to first pseudofertilisation by compatible, irradiated donor pollen causing egg transformation, followed by a second normal fertilisation by slower growing self-pollen which are able to complete growth owing to a slight mentor pollen effect – and no contamination. This helped in rescuing many egg transformants which would normally have aborted.

Significantly, of the five S alleles used as donors in different experiments, only the three (S_3, S_{F10} and S_{F11}) which

are known to be transferred through transformation (Pandey 1980) are actually obtained by Chyi and Sanford among their so-called exceptional plants.

Concluding comments

Cases 1 and 2 appear to be straightforward true results of egg transformation and Cases 3 and 4 appear to have resulted from egg transformation associated with error in the irradiated pollen. The present author supplied Drs Chyi and Sanford with seeds of certain *N. alata* material, not genetically labelled plants. There has evidently been a misidentification of *S* genotypes and errors in collection of pollen for irradiation added to possible problems of pollen contamination. These authors are too inclined to accept contamination or other causes as the basis for their "unexpected" results. They also appear to be prepared to assume anything but egg transformation as the basis for their results, even if the assumptions, as shown in Case 4, are against all reasonable evidence – experimental, theoretical and historical.

The question of interpretation posed by these authors' paper can be very simply resolved by making two pollinations ($S_{F10}S_{F10}$ and $S_{F11}S_{F11}$ *N. alata* plants as males) on each of the two self-incompatible *N. langsdorffii* plants (♀) that the authors claim occurred in their research progeny by spontaneous mutations and not by egg transformation. But repeated requests to Dr. Sanford by the present author for seeds of these two plants have been in vain, a very belated reply informs me that the two precious "spontaneous mutants" have been lost due to virus infection and they have no seed!

These authors have also argued against the suitability of *S* alleles as markers. Yet, studied under proper conditions and competently, they have been successfully used as marker genes for some of the most elegant investigations in plant genetics, involving species of *Oenothera, Nicotiana, Trifolium, Solanum, Lycopersicon, Petunia* and other genera (de Nettancourt 1977; Lawrence 1985).

In conclusion, contrary to these authors' assertions, and despite serious shortcomings in experimentation and interpretation, Chyi and Sanford's paper contains some significant demonstrations of egg transformation in *Nicotiana* which support the present author's work.

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Rebuttal

Dear Dr. Pandey,

Dr. Chyi and myself devoted 3 years of intensive research effort, in the sincere hopes of being able to confirm your claims of pollen-mediated transformation. In that time we worked with numerous, well-defined model plant systems, including extensive studies in corn, tomato, *Brassica,* and pea. In *Nicotiana* alone, we made roughly ten times as many "transformation pollinations" as you yourself have reported. We were very disappointed that we were unable to substantiate your claims. In fact, after 10 years, despite the wide notoriety of your claims of transformation by lethally-irradiated pollen, it appears no lab has been able to confirm or reproduce them. This has not been for lack of effort, since numerous labs have attempted to use your approach and have failed. Our own efforts were most extensive, hence we were willing to publish on this non-reproducibility. Given the very high rates of your reported "transformants", and the very simple nature of your protocol, it is hard to understand why these claims are not easily confirmed by ourselves or by others.

You have suggested that some of the rare anomalies we observed were true transformants, and that we were too biased or too naive to see this obvious fact. Since we spent three years searching for evidence of transformation by this method, it would seem any bias would be in the opposite direction. As we state in our paper, we have seen rare anomalies, but when we tested them, they always proved inconsistent with transformation, and they have not been reproducible. We also point out that even if these anomalies were transformants, their rare and sporatic occurrence would contradict your claims, and make the general approach unproductive. While these anomalies are largely inconsistent with transformation, we are not able in every case to explain their true origin. In some cases their origin seems genuinely interesting. In other cases they seem to have resulted from simple genetic contamination. We freely admit that we can make errors which

can lead to rare cases of contamination, but we must point out two things. Firstly, rare genetic contaminants cannot explain away the massive amount of negative data we have generated. Secondly, we have traced most of our genetic contamination to stocks supplied directly by your lab. Naturally, it would be preposterous to claim that any lab is free of errors or contamination. This is why reproducibility is essential in science.

I realize that you would like to believe that the 2 self-incompatible *N. langsdorffii* plants were really transformants. However, the gametophytic self-incompatibility system in plants is famous for producing various anomalies, such that I do not believe an explanation of transformation is compelling. More importantly, our results could only be consistent with transformation, when (you) assume there was gametic contamination by a particular parent in that particular cross. Obviously, by this type of logic, all things are possible. We were already aware of (the unlikely) explanation wherein an S_F allele might have been unintentionally transferred to *N. langsdorffii,* instead of S_3. In our paper, we ruled this out by showing that the S.I. plants were reciprocally compatible to normal *N. langsdorffii.* You go on to speculate that perhaps our observed reciprocal compatibility was only due to our "experimental error", or was due to multiple transferred alleles, etc. We must point out that there is no limit to the extent to which one can re-interpret data, if one is willing to assume different parents than what were used, and different realities from what were observed. By this method, one inevitably believes what one wishes to believe.

Your main argument seems to hinge on your absolute assertion that S. I. plants of *N. langsdorffii do not occur naturally,* (and since we observed such plants, we must have had transformation). This seems a rather bold assertion, since you admit you have only observed a few hundred plants of this species. Dr. V. A. Sisson (USDA Tobacco Research Lab, Oxford, North Carolina) and others, directly contradict your assertion. We have tested line 28 B from Dr. Sisson, which he indicates is a naturally self-incompatible accession of *N. langsdorffii.* We find it is true *N. langsdorffii,* it is strongly self-incompatible, and resists bud-pollination, just as we observed before.

A secondary point involves the question of linkage. We were wrong to state that flower color and the *S*-allele locus segregate independently. What we should have said was that based on our own data, and that of others (Brieger, J. Genet. 30:79), these traits are not tightly linked (as would be necessary to explain high rates of co-transformation of these genes). You may wish to contest other secondary points in our paper, but space only permits that I attempt to address the more important issues.

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Reply

In Chyi and Sanford's observations, *N. langsdorffii* results are the only ones which can be examined critically. Here, they are not only against all logic based on every reasonable evidence – experimental, theoretical and evolutionary – but they are also inconsistent with their own interpretation. In their reply they say "more importantly, our results could only be consistent with transformation, when (you) assume there was gametic contamination by a particular cross. Obviously, by this type of logic, all things are possible". Yet, they themselves have interpreted two of their four groups of results (Cases), which could be classed as transformation, on the basis of such contaminations. For example, in Case 1, they explained the whole class of plants to be likely results of contamination from one white-flowered S_3S_{F10} plant which they found among their experimental plants.

These authors seem to use contamination from a rare plant present for explanation when it suits them but object vehemently when the same observation is used for the case of *N. langsdorffii.* This is very typical of their approach to interpretation of their data.

Vast quantities of data produced under uncontrolled conditions do not make for reliable, scientific results, and worthwhile interpretation.

It should be noted that this very technique was used by the present author, in association with Patchell, in transferring genes in an animal, the chicken (Mol Gen Genet 62:295–300, 1983). A recent report in NATURE (D. Swinbanks, 321:720, 1986) informs that this technique has been successfully applied for transferring a gene in chicken commercially in Japan. No further proof is needed to show that the technique when applied suitably, and competently, is effective in gene transfer.

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