FURTHER EVIDENCE FOR EGG TRANSFORMATION IN *NICOTIANA*

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Received 21.ix.79

SUMMARY

Transformation of the egg by irradiated pollen has now been achieved in all five *Nicotiana* species tested, using self-compatible as well as self-incompatible species as the female parent. The self-incompatibility gene (*S*) and flower colour and pollen colour genes have been transferred in this manner. In one species combination, *N. bonariensis* ♀ ($n=9$) × *N. glauca* ($n=12$) ♂ donor, the transformed progeny having an *S* allele from the donor parent, were fertile and fully maternal in general morphology and chromosome number. Sexual hybrids are morphologically distinct from both parents and are completely sterile. This rules out the possibility that any pollen may have escaped the lethal dose of radiation and achieved normal fertilisation.

Transformation may occur in association with parthenogenetic diploidy, or a second, normal fertilisation by unirradiated pollen. The transferred gene may be expressed in the first (T1) generation or may be suppressed until the following sexual generation (T2). The observations suggest that a major gene closely linked with the *S* locus promotes development of the *S* transformed egg into a viable diploid seed, thus aiding recovery of transformed progeny. On the reasonable assumption that the *S* locus is only one of several genes that are transformed together in one act of pseudofertilisation it is suggested that the *S* locus may be used as a selection marker in the practical application of egg transformation in plant improvement.

1. INTRODUCTION

REPORTS of the occurrence in flowering plants of genetic transformation induced by donor DNA are still a matter of controversy (Cocking, 1977; Lurquin, 1977; Hess, 1977; Pandey, 1978a). ("Transformation" is broadly defined in this context as the transfer of certain genes rather than the total complement of a donor nucleus.) Recently, transformation has been achieved in *Nicotiana alata* and *N. forgetiana*, by a new method which does not involve extraction of the donor DNA (Pandey, 1975a, b, 1976, 1978b). In this method pollen from the compatible donor species (*N. alata*) was treated with a high dose (100 Kr) of ionising radiation and mixed with live pollen of the self-incompatible female parent before pollination. The small number of progeny (T1) produced by this method resembled the female parent in all but a few characters which were derived from the irradiated donor. An independent observation that single genes can be transferred from irradiated pollen to T1 progeny has recently been made by Virk *et al.* (1977) for *N. rustica*. An hypothesis for the molecular processes involved in egg transformation has been proposed earlier (Pandey, 1976, 1978b). After the initial discovery, a new series of experiments was undertaken involving a broader range of materials in the genus *Nicotiana*. Results of these experiments are reported here.

2. MATERIALS AND METHODS

Description of plant materials used is given in table 1, which also includes bud-selfed segregation data for *N. forgetiana* plants used as donors and recipients. *N. alata*, *N. forgetiana* and *N. langsдорffii* are closely related, and normal crosses between them produce fully fertile hybrids. *N. bonariensis* and *N. glauca* are relatively distantly related. Normal crosses between them produce few viable seeds, and their relatively rare hybrids are completely sterile. Numbering sequences in the *S* allelic series of different species are independent. Different homozygous *S* genotypes, mostly used as testers, were produced through bud pollination aided by the application of stigmatic secretion to the stigma before pollination (Pandey, 1963). Sources of most of the seed materials have been given before (Pandey, 1969, 1973, 1977). Seed of *N. forgetiana* used in the present study was kindly supplied by Dr H. Takahashi, Faculty of Agriculture, Kyoto University, Japan.

TABLE 1

Plant materials and their characteristics

Species plant no.	Incompatibility <i>S</i> genotype	Flower colour	Pollen colour
<i>N. alata</i> * (<i>n</i> = 9)			
9151-2	$S_{F10}S_{F11}$	Homozygous white	Homozygous white
8541-3	S_3S_3	Homozygous red	Homozygous white
<i>N. forgetiana</i> † (<i>n</i> = 9)			
403-1	S_3S_5	Heterozygous coloured	Heterozygous blue
403-2	S_4S_5	Heterozygous coloured	Heterozygous blue
403-6	S_3S_5	Heterozygous coloured	Heterozygous blue
403-5	S_3S_6	Heterozygous coloured	Homozygous white
<i>N. bonariensis</i> (<i>n</i> = 9)			
9587-13	$S_{12}S_{13}$	Homozygous white	Homozygous white
<i>N. glauca</i> (<i>n</i> = 12)			
8468-10	S_2S_9	Homozygous red	Homozygous white
<i>N. langsдорffii</i> (<i>n</i> = 9)			
Self-compatible		Homozygous green	Homozygous blue

* *S*-homozygous testers $S_{F10}S_{F10}$, $S_{F11}S_{F11}$, S_1S_1 and S_2S_2 were homozygous for white flower colour and white pollen, with the exception of S_1S_1 which was heterozygous for red flower colour.

† *S*-homozygous tester $S_{F1}S_{F1}$ was homozygous for white flower and white pollen.

Methods for collection, storage and irradiation of pollen, and for chromosome studies and the promotion of fruit set have been given previously (Pandey, 1979*a*). In all cases except where the self-compatible species *N. langsдорffii* was used as the female parent, the donor, irradiated pollen was mixed (1:1) with live, normal, self-incompatible pollen of the female parent before pollination. Normally, between 20 and 30 flowers were pollinated for each combination. In order to provide the best possible environment for germination and early growth, T1 seeds were sown on a sterile culture medium formulated by Butenko and Luneva (1966).

(i) *Progeny testing for gene transfer*

Plants of the T1 generation were tested for transfer of the *S* gene from the donor parent and, in order to determine whether self-fertilisation had

occurred, for the presence of one or both of the *S* alleles from the maternal parent. Incompatibility in test crosses which failed to produce seed was confirmed by examination of pollen tube growth (Pandey, 1969). In addition to the *S* gene, observations were made on transfer of the flower colour and pollen colour genes where applicable. Selfed and crossed T2 progenies were also tested for the presence of transferred genes.

3. RESULTS AND DISCUSSION

(i) Transformation of *N. alata* 9151-2 with irradiated pollen from *N. alata* 8541-3

Crosses and observations on T1 progeny are presented in table 2. Observations on test crossed and selfed T2 progeny are shown in table 3. When two different *N. alata* donor parents were used with the same *N. alata* female parent, one T1 progeny consistently showed gene transfers while the other progeny showed none, although both donor plants were members of the same selfed progeny. However, a study of the T2 progeny of the group showing no gene transfers revealed that seven out of eleven plants examined were in fact carrying transferred genes but these had not been expressed in the T1 generation. Five of them carried both transferred *S* and flower colour genes and one each carried a transferred *S* or flower colour gene only.

TABLE 2

Observations on T1 progeny of *N. alata* and *N. langsdorffii* obtained through the use of irradiated pollen of *N. alata*

Combination No.	Female parent	Irradiated, donor parent	Total no. of T1 plants studied	No. of plants with expressed <i>S</i> or flower colour gene transfer	No. of V.I. ¹ plants
(1) <i>N. alata</i>	9151-2	<i>N. alata</i> 8541-3	47 ²	0	9
(2) <i>N. alata</i> ³	9151-2	<i>N. alata</i> 8541-A	13	13	0
(3) <i>N. langsdorffii</i> N.1.(1) (self-compatible)		<i>N. alata</i> 8541-3	5 ⁴	5 ⁵	0

¹ Defined in the text.

² From a total of 62 plants grown from seeds arising from 9 pods.

³ Data from a previous experiment included for comparison (Pandey, 1975a, 1978b).

⁴ Arising from 4 pods.

⁵ In this combination there was some difficulty in the T1 generation in testing for the presence of transferred *S* genes owing to a very large difference in the stylar lengths of the two species concerned. Since the donor parent *N. alata* has flowers which are three times longer than those of the female parent *N. langsdorffii*, and since the self-pollen tubes of *N. alata* are normally inhibited about one-third of the distance down their style, it appeared that in T1 *N. langsdorffii* *N. alata* pollen tubes with an *S*₃ allele were often able to traverse the whole length of a style containing the transferred *S*₃ allele. However, the overall picture of pollen tube growth left no doubt of the presence of *S*₃ allele and incompatibility, which was further confirmed by the study of T2 generation of two plants (table 3).

A further difference between the two sets of progeny was the occurrence of 9 out of 47 T1 plants with the vegetative inflorescence (V.I.) character among the group showing no gene transfer, while no such plants occurred in the group in which gene transfer was observed, these plants occurring only in

TABLE 3
Observations on crossed and selfed T2 progeny of *N. alata* and *N. langsdorffii*

Cross		Progeny ¹			No. of plants of different S genotypes in the progeny ²
Female parent	Tester	Segregation for donor S ₃ allele: No. of plants with S ₃ /without S ₃	Segregation for donor flower colour gene: No. of plants with / without white coloured fl. / or green fl.	Expression of tester S ₂ allele in the progeny: No. of plants with S ₂ / without S ₂ expression	
<i>N. alata</i> T1 747-6/2 × <i>N. alata</i> S ₂ S ₂		19/9	9/19	9/16	6 S ₂ S _{F11} , 15 S ₂ S ₃ , 4 S ₂ S ₃ S _{F11}
<i>N. alata</i> T1 747-6/3 × <i>N. alata</i> S ₂ S ₂		15/14	12/17	9/16	4 S ₂ S _{F10} , 2 S ₂ S _{F11} , 3 S ₂ S ₃ , 8 S ₂ S ₃ S _{F10} , 3 S ₂ S ₃ S _{F11}
<i>N. alata</i> T1 747-7/1 × <i>N. alata</i> S _{F11} S _{F11}		0/16	3/13	—/—	4 S ₂ S _{F10} , 1 S ₂ S _{F11} , 4 S ₂ S ₃ , 6 S ₂ S ₃ S _{F10} , 3 S ₂ S ₃ S _{F11}
<i>N. alata</i> T1 747-7/3 × <i>N. alata</i> S ₂ S ₂		13/7	13/7	9/9	
<i>N. alata</i> T1 747-7/5 (V.I.) × <i>N. alata</i> S ₂ S ₂		2/3	2/3	3/—	
<i>N. forgetiana</i> 403-6 × <i>N. alata</i> 747-7/5 (V.I.)		0/15	—/—	—/—	
<i>N. alata</i> T1 747-7/11 × <i>N. alata</i> S ₂ S ₂		8/12	6/14	7/13	
<i>N. alata</i> T1 747-7/6 × <i>N. alata</i> S ₁ S ₁		0/30	—/—	—/—	
<i>N. alata</i> T1 747-7/16 × <i>N. alata</i> S _{F10} S _{F10}		0/17	0/17	—/—	
<i>N. alata</i> T1 747-7/18 × <i>N. alata</i> S ₁ S ₁		0/20	—/—	—/—	
<i>N. alata</i> T1 755-5/2 × <i>N. alata</i> S _{F10} S _{F10}		0/19	0/19	—/—	
<i>N. alata</i> T1 747-7/4 Selfed (pseudo-self-compatible)		3/9	0/12	—/—	2 S _{F10} S _{F10} , 1 S _{F11} S _{F11} , 3 S _{F10} S _{F11} , 3 S ₃ S _{F10} S _{F11}
<i>N. langsdorffii</i> T1 755-12/1 Selfed		5 ³ /11	0/12	—/—	
<i>N. langsdorffii</i> T1 755-12/1 × <i>N. alata</i> S ₂ S ₂		13/6	3 ⁴ /16	0/19	
<i>N. langsdorffii</i> T1 755-12/2 Selfed		12 ³ /6	0/16	—/—	
<i>N. langsdorffii</i> T1 755-12/2 × <i>N. alata</i> S ₂ S ₂		15/8	6/17	9/13	

¹ All plants in a progeny were not always examined for the four characters studied, so there may be differences in totals regarding the different features concerned. This is true for other tables as well as for this one.

² Presence of S₂ allele assumed whether it was expressed or not; in some families not all plants tested.

³ The data may be biased against the presence of S₃ allele because owing to flower size difference *N. alata* S₃ pollen may reach the base of the *N. langsdorffii* style even in incompatible crosses where the style has the S₃ allele.

⁴ Selfed family grown from one such plant produced 7 plants with coloured flowers and 9 plants with green flowers.

the T2 generation (Pandey, 1978*b*) (table 2). (In V.I. plants which were otherwise normal in appearance, the flower production was suppressed and the floral shoot became vegetative, with tufts of small leaves in place of flower buds.)

Another type of gene suppression was evident among the T2 generation of plants (table 3). This was the sporadic lack of expression of the S_2 gene contributed by the tester pollen parent (S_2S_2). Normally all the progeny should express S_2 . But in this case 86 out of 132 plants examined, in seven separate families, did not express S_2 . (In the family ♀ *N. langsdorffii* T1 755-12/1 × *N. alata* S_2S_2 , none of the 19 plants showed expression of the S_2 allele.) Most, but not all, of these plants showed expression of the transferred gene S_3 . The question arises: Under certain circumstances does the presence of a transferred S allele suppress the action of other S alleles even when present in the *trans* configuration? Interallelic complementation involving the regulatory elements of the S complex has been previously reported in *Oenothera* and *Nicotiana* (Lewis, 1958; Pandey, 1967; Fincham and Sastry, 1974).

S allelic examination of five T2 progenies (table 3) confirmed the previously reported observation that the transferred S allele may occupy any of three proposed positions in the nucleus:

(1) Remain attached to the chromosome where it was first introduced, making this chromosome biallelic.

(2) The biallelic chromosome may lose the original maternal allele, giving a substituted monoallelic condition.

(3) The transferred gene may cross-over to the homologous chromosome thereby making that chromosome biallelic (Pandey, 1978*b*).

In four of the present crosses, where normal plants were used as males, and the nature of the male gametes was known, the S allelic constitution of the progeny provided reasonable evidence for the S gametic constitution of the female parent. The results, however, do not rule out the possibility that transferred chromatin may be attached at sites other than the homologous position on the chromosome.

It is also interesting to note that in the T2 progeny of the cross between the T1 V.I. plant 747-7/5 and a normal plant of *N. alata* or *N. forgetiana*, there is a reciprocal difference suggesting gametic selection. When the V.I. plant was used as the female parent two out of five plants in the T2 progeny contained the transferred S_3 allele while in the reciprocal cross, when the V.I. plant was used as the male parent, none of the 15 T2 plants contained the S_3 allele. Similar observations have been made before (Pandey, 1978*b*).

(ii) *Transformation of N. langsdorffii with irradiated pollen from N. alata alone*

In this experiment irradiated donor pollen from *N. alata* 8541-3 (S_3S_3) was not mixed with living pollen of the self-compatible female parent. All five T1 plants examined appeared to carry a transferred S_3 gene (table 2), and this was subsequently confirmed by a study of T2 progenies from two T1 plants (table 3). Tests among the crossed and selfed T2 progenies showed that S and flower colour genes were transferred in both the T1 plants tested although no evidence for transferred flower colour genes had appeared among the T1 plants themselves.

The results for *N. langsdorffii* show that parthenogenetic diploidy and gametic transformation may occur without the aid of the mixed live pollen, although fewer viable seeds are produced.

(iii) *Transformation of N. alata 9151-2 with irradiated pollen from N. forgetiana 403-1, 403-2 or 403-6*

The observations on T1 plants obtained are given in table 4. Of a total of 209 T1 plants tested 109 showed *S* gene transfer. Of these 3 showed transfer of flower colour + *S* gene, 11, pollen colour + *S* gene, and one showed transfer of all three characters, flower colour, pollen colour and *S* gene. There were also 7 plants with transfer of flower colour alone and one where only pollen colour was transferred. Pollinations with only two of the three donor parents gave rise to V.I. plants. Apparently *S* allelic constitution *per se* was not responsible for production of V.I. plants since, of the two donor plants, 403-1 and 403-6, having the same constitution (S_3S_5), one produced V.I. plants and the other did not. Another point to note is that the appearance of V.I. plants in the T1 progeny was not necessarily associated with lack of expression of transferred genes, as was the case in the earlier experiment (table 2). In this experiment pollination with two donor plants gave V.I. plants as well as normal plants with expressed transferred genes.

Thirty T2 progenies (excluding two V.I. families), comprising 20 in which the transferred *S* gene was expressed in the T1 generation and 10 in which there was no indication of *S* transfer, were studied (table 5). There was one family in the latter group (fa. 971) where there were transfers of the *S* gene and pollen colour gene which were not expressed in the T1 generation but expressed in the T2 generation. The T1 plant giving rise to this family belonged to the class of plants arising from the donor 403-1 which produced some V.I. plants. Six T2 families showed transfer of the pollen colour gene, none of which were identified in the T1 generation. In addition, ten T2 families showed transfer of the flower colour gene of which only one was expressed in the T1 generation. Thus, of the fifteen instances where a character appeared in the T2 generation but not in the T1 generation, with the exception of three (pollen colour, fams. 959, 962; flower colour, fa. 1070; donor 403-6), all had originated in experiments where plants 403-1 or 403-2 were used as donors, and which had also given rise to V.I. plants. Normal crosses between the original female parent (*N. alata* 9151-2) and the tester used in these crosses (*N. forgetiana* $S_{F1}S_{F1}$) have not produced, among 32 plants grown, a single plant having coloured flowers or coloured pollen. Another significant point is that in two families, 972 and 964 (table 5), almost all plants (32 out of 33) contained the transferred *S* gene. In the previous study also, there were a number of families showing strong gametic selection in favour of *S* gene transfer (Pandey, 1975*b*, 1978*b*). In all cases where the transferred genes were expressed in the T1 generation, they were also expressed in the T2 generation.

(iv) *Transformation of N. forgetiana 403-5 with irradiated pollen from N. alata 9151-2*

In this combination no V.I. plants were produced in the T1 progeny (table 4). Of a total of 42 T1 plants tested, 17 showed transfer of the S_{F10}

TABLE 4

Observations on T1 progeny of *N. alata*, *N. forgetiana* and *N. bonariensis* obtained through the use of irradiated pollen from a species other than the female parent

Female parent	Donor irradiated parent	Total no. of T1 progeny [†]	Number of V.I. plants	Total no. tested	T1 progeny tested for gene transfer		
					No. of plants with expressed S and/or flower or pollen colour gene transfer (heterozygous or homozygous for maternal S alleles)	No. of plants with no tested gene transfer (heterozygous or homozygous for maternal S alleles)	No. of plants with no tested gene transfer (heterozygous or homozygous for maternal S alleles)
<i>N. alata</i> 9151-2 (S _{F10} S _{F11})	<i>N. forgetiana</i> 403-1 (S ₃ S ₅)	53 (9)	13	38	23 (19 het. + 4 hom.); S ₅ , 2 also fl. col.; 1 also fl. and pol. col.; and 1 poll. col. alone	14 (3 het. + 11 hom.)	
<i>N. alata</i> 9151-2 (S _{F10} S _{F11})	<i>N. forgetiana</i> 403-2 (S ₄ S ₅)	103 (11)	11	84	38 (30 het. + 7 hom.); S ₅ ; and 7 fl. col. alone	39* (2 het. + 33 hom.)	
<i>N. alata</i> 9151-2 (S _{F10} S _{F11})	<i>N. forgetiana</i> 403-6 (S ₃ S ₅)	92 (12)	0	88	49 (38 het. + 11 hom.); S ₅ , 1 also fl. col.	39 (6 het. + 33 hom.)	
<i>N. forgetiana</i> 403-5 (S ₃ S ₆)	<i>N. alata</i> 9151-2 (S _{F10} S _{F11})	43 (9)	0	42	17 (4 het. + 13 hom.); S _{F10} , 11 also poll. col.	25* (7 het. + 15 hom.)	
<i>N. bonariensis</i> 9587-13 (S ₁₂ S ₁₃)	<i>N. glauca</i> 8468-10 (S ₂ S ₉)	11 (5)	0	11	7 (1 het. + 6 hom.); S ₉	4 (2 het. + 2 hom.)	

* Not all plants were tested for S alleles.

† Figures in parentheses indicate number of capsules involved.

TABLE 5

Observations on T2 progeny of *N. alata* and *N. forgetiana* (♀ T1 plants × ♂ Normal *N. forgetiana* S_{F1}S_{F1})

Original pollination	Cross ♀ T1 <i>N. alata</i> with following transferred, and expressed, gene(s) from the donor	T2 progeny			
		Family no.	S gene: No. of plants with and without transferred allele with S ₅ / without S ₃ or S ₅	Flower colour: No. of plants, Col./White	Pollen colour: No. of plants, Blue/White
♀ parent <i>N. alata</i> 9151-2 (S _{F10} S _{F11})	S ₅	972 ¹	19/1	3/17	1/19
	S ₅	1073	7/5	2/10	0/12
	None	963	0/19	0/19	0/19
♂-irradiated donor <i>N. forgetiana</i> 403-1 (S ₃ S ₅)	None	970	0/19	0/19	1/18
	None	971	6/14	0/20	4/16
	None	973	0/2	0/2	0/2
♀ parent <i>N. alata</i> 9151-2 (S _{F10} /S _{F11})			with S ₅ / without S ₄ or S ₅		
	S ₅	964	12 ² /0	0/12	0/12
	S ₅	966	4/2	0/6	0/12
	S ₅	1064	4/3	2/5	0/7
	S ₅	1071	12/7	2/17	2/17
	S ₅	1072	7/11	8/10	0/18
♂-irradiated donor <i>N. forgetiana</i> 403-2 (S ₄ S ₅)	S ₅ and fl. col.	1075	8/6	10/4	0/14
	S ₅	1078	5/11	1/15	0/16
	V.I.	1076	4/4	1/7	0/8
	V.I. ³	1077	4/11	6/9	0/15
	None	1067	0/12	0/12	0/12
	None	1074	0/14	0/14	0/14
♀ parent <i>N. alata</i> 9151-2 (S _{F10} /S _{F11})			with S ₅ / without S ₃ or S ₅		
	S ₅	954	9/11	0/20	0/20
	S ₅	955	5/4	0/9	0/9
	S ₅	956	11/7	0/18	0/18
	S ₅	957	2/1	0/3	0/3
	S ₅	958	9/4	0/13	0/13
	S ₅	959	9/8	0/17	1/16
	S ₅	960	5/2	0/7	0/7
	S ₅	962	11/9	0/20	6/14
	S ₅	968	3/7	0/10	0/10
♂-irradiated donor <i>N. forgetiana</i> 403-6 (S ₃ S ₅)	S ₅	1068	5/8	0/13	0/13
	S ₅	1070	8/6	4/10	0/14
	None	961	0/20	0/20	0/20
	None	967	0/5	0/5	0/5
	None	977	0/11	0/11	0/11
	None	1069	0/15	0/15	0/15
			with S _{F10} / without S _{F10} or S _{F11}		
	S _{F10} ⁴	969	2/2	—	0/4
	S _{F10}	974	7/10	—	—
	S _{F10} ⁴	978	1/1	—	0/2
♀ parent <i>N. forgetiana</i> 403-5 (S ₃ S ₅)	S _{F10}	979	10/9	—	—
	S _{F10} ⁴	985	13/5	—	0/18
	S _{F10} ⁴	986	10/7	—	0/17
	S _{F10}				

TABLE 5 (cont.)

δ -irradiated donor	S_{F10}	987	10/10	—	—
<i>N. alata</i> 9151-2	None	975	0/3	—	—
($S_{F10}S_{F11}$)	None	976	0/19	—	—
	None	980	0/20	—	—
	None	981	0/15	—	—
	None	982	0/6	—	—
	None	983	0/6	—	—
	None	984	0/18	—	—

¹ The T1 plant did not express transferred flower colour or pollen colour genes, only the S_5 gene. T2 progeny showed transfer of all three characters. Among the segregants there was one plant, the only one in the family, which had transferred flower colour gene but no S or pollen colour gene; one plant with transferred flower colour gene and S gene but no pollen colour gene; and one plant with all three, flower colour, pollen colour and S genes. Similarly, there were five other T2 families (962, 970, 971, 1071, 1078) showing transferred characters which were not expressed in the respective T1 plants.

² Extreme gametic selection, all plants having the transferred allele S_5 .

³ The parent T1 V.I. plant showed flower colour gene transfer in the rare mutilated flowers, the T2 progeny (in this case, a result of cross $T1 \times N. alata S_2S_2$) showed the presence of transferred S_5 allele as well as flower colour gene.

⁴ T1 plant showed blue pollen colour, not present in either of the two parents.

gene, 11 of which also showed transfer of the pollen colour gene. Brieger (1935) has shown that pollen colour in *Nicotiana* is controlled by two independent complementary genes. In the present study, since both female parent and donor bred true for white pollen colour, the appearance of blue pollen colour in the T1 progeny was apparently due to transfer of a complementary gene from *N. alata*. This conclusion was further supported by the fact that none of the four T2 progenies from these plants contained plants with blue pollen (fams. 969, 978, 985, 986; table 5).

Since all 11 plants with pollen colour transfer also showed transfer of the S_{F10} gene, and the proportion (11/17) of these plants among the total T1 plants showing transfer of the S gene is very high, this suggests that the complementary gene for pollen colour may be linked with the S_{F10} locus. This is in agreement with the conclusion of Brieger (1935), that the S gene is linked with the basic anthocyanin colour factor C , and one of the factors for blue pollen colour, B_1 . The linkage between S and C is loose (20 per cent recombination) but that between C and B_1 is very close (0.5 per cent). Brieger also identified a flower colour factor R which is independent of this linkage group. The obvious close relationship between the transformation pattern and genetic linkage is interesting, and may be indicative of the size of individual transferred DNA molecules.

As regards transfer of the S gene, the behaviour of the 14 T2 progenies fully confirmed the observations on the T1 generation (table 5). The seven families in which the T1 parent showed transfer of the S_{F10} gene segregated for the S_{F10} gene as expected, the other seven families in which the T1 parent had shown no transfer of the S gene showed no evidence of S_{F10} in the progeny.

(v) *Transformation of N. bonariensis 9587-13 with irradiated pollen from N. glauca 8468-10*

Seven out of 11 T1 plants produced from this combination showed transfer of the S_2 allele from *N. glauca*. None showed transfer of the flower colour gene (table 4).

A total of 93 T2 plants grown from crosses of five T1 plants having transfer of the *S* gene gave rise to 11 diminutive, sterile (D.S.) plants in which the floral inflorescence was very much stunted and nearly all buds dropped before maturity (D.S./total No. of plants: 4/21, 3/20, 4/22, 1/20, 0/10) (fig. 1). In certain D.S. plants rare buds did reach maturity and produced nearly normal flowers. D.S. plants are comparable with V.I. plants arising in the T1 and T2 progenies of ♀ *N. alata* × *N. alata* donor and ♀ *N. alata* × *N. forgetiana* donor combinations.

(vi) Cytology of T1 plants

Six T1 plants from each of the five species combinations were examined for meiosis in the anthers. They revealed no significant chromosomal abnormalities. They all had the regular $2n = 18$ chromosomes of the female parents and no fragments. All plants had high pollen fertility (70–95 per cent).

The fact that in the combination *N. bonariensis* ♀ ($2n = 18$) × *N. glauca* donor ($2n = 24$) the T1 plants had the chromosome complement of the female parent ($2n = 18$) and not that of a hybrid ($2n = 21$), proved that no true fertilisation by a live male gamete had been involved in production of T1 embryos. True hybrids between these two species have been produced in our laboratory. They have never shown post-zygotic chromosome elimination or modification, their vegetative and floral characteristics are very distinct from either of the parents, and they are completely sterile. The transfer of the *S*₂ gene from the donor *N. glauca* to the recipient female parent *N. bonariensis* can, therefore, reasonably be attributed to gametic transformation of the egg as proposed. Indeed, the consistently maternal appearance of the T1 plants in all experiments showing gene transfer, including three interspecific combinations, leaves no doubt regarding the authenticity of the phenomenon of egg transformation. There is no other conceivable way by which such gene transfers between distinct species could occur without normal sexual union.

(vii) *S* gene complex and egg transformation

There are two intriguing aspects of the egg transformation phenomenon emerging from the present observations:

(a) *S* gene selection

There is a general specificity in favour of the *S* gene in transformation. Thus out of a total of 262 T1 plants examined in the five combinations summarised in table 4, 133, approximately 50 per cent, showed *S* gene transfer, whereas only 11 showed flower colour and 20, pollen colour gene transfers. Significantly, all 5, rare, T1 plants produced through parthenogenetic diploidy in *N. langsdorffii* showed *S* gene transfers.

(b) *S* allele selection

There is also absolute allelic specificity regarding *S* gene transfer. Without exception, all of the 110 T1 *alata* plants derived from the three

FIG. 1.—Normal (left) and Diminutive Sterile (D.S.) (right) plants of *Nicotiana bonariensis*. D.S. plants appeared in the T₂ generation of the S gene transformants in the combination ♀ *N. bonariensis* × *N. glauca* donor.

donor *forgetiana* plants, two having the constitution S_3S_5 and one S_4S_5 , showed transfer of the S_5 allele only. None of the T1 plants showed transfer of the sister alleles S_3 or S_4 . Similarly, in all 17 cases of S gene transfer to *forgetiana* from *alata* ($S_{F10}S_{F11}$), it was the S_{F10} allele only which was transferred, there being no transfer of the sister allele S_{F11} (table 4).

What could be the basis of such a close relationship between the S gene and transformants and such extreme allelic specificity in gene transfers? Recently it has been proposed (Pandey, 1979a) that a number of embryo growth-promoting genes of varying potency may be present in the genome and in particular one such gene of a relatively high potency may be linked with the S locus. Thus all 5 rare parthenogenetic seedlings obtained through pseudofertilisation by irradiated pollen alone in *N. langsdorffii* showed S gene transformation. This S -linked gene may have a number of allelic forms, some competent, others incompetent in the promotion of embryo growth. Only those S alleles which are associated with the competent allele will be effective in promoting normal embryo growth and recovery of the seed with specific S allele transformation.

Although an overwhelming proportion of transformed plants contained the S gene transfer, either alone or in association with transferred flower or pollen colour genes, there was a small proportion of transformants which had no S gene transfer (table 4). All T1 plants showing flower colour transformation alone have appeared in combinations where there was a mixed pollination and seed was usually produced through a second normal fertilisation, the result of a weak mentor pollen effect on slower growing self pollen (table 4, and Pandey, 1978b). Thus in these combinations, presumably ovules which had been partly stimulated as a result of transformation by the embryo growth-promoting gene, either linked with the flower colour gene or independently but simultaneously transferred, were rescued by the second, normal fertilisation. Even those T1 plants which did not show transfer of any of the three genes studied may, hence, have contained transferred partly potent embryo growth-promoting genes which allowed their embryos to be rescued by the second fertilisation. The use of irradiated pollen, in certain cases combined with the mentor pollen effect, could thus act as a selection sieve to identify genes promoting embryo growth after pseudofertilisation. It may then be possible to combine several such genes and synthesise genotypes which would have a tendency to produce a high frequency of parthenogenetic diploids.

(viii) *Mentor pollen effect*

S allelic tests on T1 plants in the present series of experiments (table 6) showed that self-fertilisation as a result of a *weak* mentor pollen effect was quite common in most *Nicotiana* plants used as female parents. More than 50 per cent of the T1 plants tested had identifiable gene transfers (table 4). Thus, it would appear that a large proportion of T1 plants resulted from two fertilisations, first by irradiated mentor pollen causing egg transformation, and then later by self-pollen. After pollination with a mixture of irradiated compatible pollen and normal, live, incompatible pollen, three types of seed development are possible:

(1) There may be no mentor pollen effect to overcome the incompatibility of the live pollen. In this case any seeds produced would arise by

TABLE 6

Observations on T1 plants showing the extent of self-fertilisation as opposed to parthenogenetic diploidy¹

Pollination		T1 plants		
		Total	No. homozygous for maternal S alleles	No. heterozygous for maternal S alleles
Female parent	Irradiated pollen parent			
<i>N. alata</i> 9151-2 $S_{F10}S_{F11}$	× self-pollen + <i>N. alata</i> 8541-A S_1S_3	13	13	0
<i>N. alata</i> 9151-2 $S_{F10}S_{F11}$	× self-pollen + <i>N. alata</i> 8541-3 S_3S_3	31	23	8
<i>N. alata</i> 9151-2 $S_{F10}S_{F11}$	× self-pollen + <i>N. forgetiana</i> 403-1 S_3S_5	37	15	22
<i>N. alata</i> 9151-2 $S_{F10}S_{F11}$	× self-pollen + <i>N. forgetiana</i> 403-2 S_4S_5	72	40	32
<i>N. alata</i> 9151-2 $S_{F10}S_{F11}$	× self-pollen + <i>N. forgetiana</i> 403-6 S_3S_5	88	44	44
<i>N. forgetiana</i> 403-5 S_3S_6	× self-pollen + <i>N. alata</i> 9151-2 $S_{F10}S_{F11}$	38	28	10
<i>N. bonariensis</i> 9587-13 $S_{12}S_{13}$	× self-pollen + <i>N. glauca</i> 8468-10 S_2S_9	11	8	3

¹ Families having only S homozygous plants arose from parthenogenetic diploidy (the first combination in the table); families having both S homozygous and heterozygous plants resulted from selfed seed but may also have plants arisen from parthenogenetic diploidy (all other combinations).

parthenogenetic diploidy after pseudofertilisation by irradiated pollen alone, and would be likely to have arisen by egg transformation and selective stimulation ((2), table 2).

(2) There may be a *weak* mentor pollen effect, allowing a few incompatible, live pollen tubes to grow slowly and eventually reach the embryo-sac, after pseudofertilisation by compatible irradiated pollen has already occurred. Most of the ovules would have been "sterilised" by a preceding pseudofertilisation but some, presumably owing to transformations involving embryo growth-promoting genes, might be capable of accepting a second fertilisation to produce a normal diploid seed. In this case only a small number of seeds are set, and the weak mentor pollen effect is beneficial for it enhances the recovery of eggs transformed for embryo growth-promoting genes, particularly those which are insufficiently stimulated and might otherwise not be recovered. However, with the rise in the number of seeds through self-fertilisations, the proportion of T1 plants with S gene transfer declines (table 4).

(3) There may be a highly efficient mentor pollen effect, making the incompatible pollen behave as compatible (Pandey, 1978c, 1979b). In this case the live, now compatible, pollen may compete successfully with the irradiated compatible pollen and reach the ovules first. Normally fertilised embryo-sacs would not allow a second fertilisation by the later arriving irradiated pollen, and would thus produce only normal seeds without transformation. Any comparatively slow-growing transformed embryo that might have resulted from an initial pseudofertilisation would be disadvantaged by the large numbers of vigorously growing normal embryos in the same ovary. Under such circumstances, although a large number of seeds would be produced these would be unlikely to contain transformations. Thus, the mentor pollen effect can be an aid to the practical

application of egg transformation only where it is relatively weak, allowing only a few normal, second fertilisations to occur. The fact that in the present experiments the efficiency of mentor pollen effect has generally been of this order has been a significant factor in the elucidation of the relationship between mentor pollen effect and egg transformation.

(ix) *Use of the S locus as a selection marker in egg transformation by irradiated pollen*

It is obvious from the above observations that the use of a self-incompatible donor, providing an *S* locus, with which a major gene promoting embryo growth is closely linked, has been the key to success of the egg transformation work. When the recipient and donor are both self-incompatible, recovery of transformed seeds can be enhanced with the aid of a second, normal fertilisation by self-pollen, facilitated by the mentor pollen effect.

Since the transferred gene in many cases is not expressed in the T1 generation but only in the following sexual generation, it becomes necessary that T2 progenies be examined from each T1 plant. This may become an impractical proposition if the number of T1 seeds is too large and the prospect of recovering transformed seedlings from them too low. Any technique which ensures that all the T1 seedlings obtained have in fact undergone genetic transformation would be advantageous. Combinations in which pollen of a self-incompatible species is used as a donor and there is no mixture of pollen from the self-incompatible female parent, offer this possibility. All such T1 seedlings should be the result of parthenogenetic diploidy and would be expected to have a transferred *S* gene. It is reasonable to assume that in any transformed seed the transferred *S* locus would be only one of many genes transferred together in one act of pseudofertilisation. The ability of a growth factor linked with the *S* locus to rescue transformed embryos is therefore highly significant. The *S* locus may thus be employed as a selection marker in egg transformation where the donor is self-incompatible. This feature is even more significant for combinations where the female, recipient, parent is self-compatible, for in such cases this is the only way transformed viable seed could be obtained. The use of embryo and ovule culture may boost the number of T1 progeny recoverable by this technique.

4. CONCLUSIONS AND FUTURE PROSPECTS

There are two main limitations to the wide practical application of egg transformation at present. One is the necessity for donor pollen to be able to germinate, grow and discharge its contents within the recipient pistil. In nature growth of foreign pollen is normally hampered by pollen-pistil incompatibility, particularly in distantly related plants. However, certain recent observations suggest that for appropriate combinations of female and pollen parents the "mentor pollen effect" may make egg transformation feasible in situations where normal sexual hybridisation is impossible (Pandey, 1979b). Alternatively, in certain favourable materials *in vitro* fertilisation, or direct injection of a suspension of donor pollen into the

ovary, combined with ovary or ovule culture techniques, may overcome pollen–style incompatibility.

Another limitation to egg transformation is the relatively rare development of seeds through parthenogenetic diploidy after pseudofertilisation by irradiated pollen. However, the finding that certain *S* alleles may be linked with a major gene which promotes growth of the *S* transformed eggs into viable embryos is highly significant. It would allow the possibility, in a combination where the donor is self-incompatible, for the *S* locus to be used as a selection marker to rescue transformed embryos which otherwise would not be recovered. Application of hormones and embryo culture may also be helpful in promoting parthenogenesis and recovering transformed seed.

It is possible that the technique might be used to convert a presently self-compatible species into a self-incompatible one by incorporating *S* alleles from related self-incompatible species of the same genus. With regard to plant improvement in general, egg transformation offers a potentially rapid method for transferring specific genes without recurrent back-crossing. By breaking down linkage relationships the technique may allow selective transfer of desirable genes alone from relatively distantly related species. However, there is still much work to be done in developing this technique, and in elucidating the molecular and physiological mechanisms which underlie this phenomenon.

Acknowledgements.—I thank Mr G. B. Petterson and Miss Janet Plummer for able technical assistance, and Mr R. J. Trott, Physicist, Palmerston North Hospital, for his help in the irradiation of pollen.

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