ELEMENTS OF THE S-GENE COMPLEX

II.* MUTATION AND COMPLEMENTATION AT THE S₁ LOCUS IN *NICOTIANA ALATA*

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1. INTRODUCTION

FOR a finer genetic analysis in higher plants, of a gene that has a multiple allelic series of the magnitude and complexity shown by *S* alleles, the two basic requirements of experimentation—a careful choice of material and an availability of sensitive techniques—assume a special significance. This has been well demonstrated by the mutation studies of the *S*-gene complex.

All attempts to produce spontaneous and X-ray induced mutations in the *S* alleles in *Oenothera organensis* (Lewis, 1951), *Prunus avium* (Lewis and Crowe, 1954), *Trifolium pratense* and *T. repens* (Pandey, 1956a) resulted only in the breakdown of self-incompatibility. The self-compatible mutations comprised three types: (1) those in which the pollen-reaction had changed leaving the stylar-reaction normal, (2) those in which the stylar-reaction had changed leaving the pollen-reaction normal, and (3) those in which both pollen- and stylar-reactions had changed simultaneously. No mutation was found from one to another functionally self-incompatible allele. The investigations so far have led to an appreciation of the complexity of the *S* locus (Lewis, 1960; Pandey, 1962) but have failed to reveal the mechanism by which the different classes and series of alleles were created.

A further problem arose when Brewbaker and Natrajan (1960) working with *Petunia inflata* produced pollen-part mutations that were all characterised by the presence of an additional *S*-bearing centric fragment in their chromosome complement. These plants, by virtue of the presence of the fragment, produced a certain proportion of pollen having two different *S* alleles. It is known that when two different *S* alleles occur in the same pollen, as in diploid pollen of autotetraploids, often there is a competitive interaction resulting in self-compatibility (Lewis, 1947; Pandey, 1956b). On the basis of their study in *P. inflata*, these authors suggested that pollen-part mutations in general could be explained as being due to competitive interaction, and thus challenged the mutation theory of the *S* gene.

* In the first of this series of articles (Elements of the *S*-gene complex, *Genet. Res., Camb.* (1964), 5, 397-409) the sub-title, I. The *S*_{F1} alleles in *Nicotiana*, was inadvertently omitted in printing.

Apart from the fact that stylar-part mutation found in other plants could not be explained on the basis of competitive interaction, a close examination of the genetical results of mutation studies on *O. organensis*, *P. avium*, *T. pratense* and *T. repens* showed that mutants in these species could not have the fragment and that the results obtained could be explained only on the basis of *S*-gene mutation (Lewis, 1961; Pandey, 1965). The association of the *S*-bearing centric fragment with the pollen-part mutation of the *S* gene, therefore, was a new phenomenon, and hence required a detailed investigation.

Two questions arise from the observations in *P. inflata*: (1) Is this phenomenon an exceptional case limited to this species, or is it common to a bigger group of species involving the genus or the whole family? (2) What is its significance in relation to *S* gene mutation? With these problems in mind a mutation study was undertaken by the present author in *Nicotiana alata* ($n = 9$), a diploid sister species of *P. inflata*, both belonging to the family Solanaceæ. True *S*-gene mutations do occur in *N. alata* as well as in other studied species (Pandey, 1965). The *S*-bearing centric chromosome fragment, similar to that found in *P. inflata*, was also present in most of the *N. alata* mutants. However, the results in *N. alata* suggested that the role of the fragment in relation to pollen-part mutation was more than that of competitive interaction. A second, significant but less apparent, role was the covering by the normal allele on the fragment, of the lethal effect usually associated with the mutated allele—a phenomenon of compensation genetically termed “complementation” (Fincham and Pateman, 1957; Giles *et al.*, 1957; Catcheside and Overton, 1958)—and thereby making the otherwise inviable mutant pollen viable.

The present article describes the studies in *N. alata* in detail and presents evidence in support of the author's hypothesis of a dual role of the fragment in producing the pollen-part mutation of the *S* gene. Evidence has been obtained not only for the true pollen-part *S*-gene mutation but also for the stylar-part mutation, the latter, in addition, providing another interesting case of complementation in higher plants. The present paper confirms the theory of the *S*-gene complex and its mutations.

2. NOTATIONS, MATERIALS AND METHODS

(i) Notations

Pollen-part mutants: In this study they include both types of self-compatible plants, those produced through competitive interaction and those which are due to genuine pollen-part *S*-gene mutation.

Examples of cytological description:

15 (5+, 2-)—Total 15 plants, 7 of which were cytologically examined, 5 had the fragment and 2 did not.

15 (5+)—Only 5 plants examined, all of which had the fragment.

15 (2-)—Only 2 plants examined and neither had the fragment.

15 (+)—All had the fragment.

The fragment is present in addition to the normal complement of chromosomes.

fr_1, fr_3	Centric chromosome fragment bearing the $S_1(fr_1)$ or the $S_3(fr_3)$ allele.	
S.I.	Self-incompatible.	S.C. Self-compatible.
C.I.	Cross-incompatible.	C.C. Cross-compatible.
Hom.	Homozygous for the S allele.	
Het.	Heterozygous for the S allele.	
C	Compatible cross.	I Incompatible cross.
+	Compatible cross.	— Incompatible cross.

(ii) Induction of mutations

Only one genotype, S_1S_3 , of *Nicotiana glauca*, extracted from the hybrids between plants grown from the seeds of two sources, Istituto ed Orto Botanico dell'Università, Modena, Italy, and Institut Pflanzenbau, Geisenheim, Rheingau, Germany, was used in this experiment. Two groups of S_1S_3 plants (9112—12 plants; 9115—13 plants) derived from the families of reciprocal crosses between unrelated plants were used separately for the induction of mutations. Seeds of *N. langsdorffii* plants, used in test-crosses of mutants, were obtained from Royal Botanic Gardens, Kew.

All plants were grown at the Crop Research Division, Lincoln, in an insect proof greenhouse with supplementary heating and lighting during winter, but otherwise under uncontrolled environmental conditions. Contamination in pollinations for detecting mutation was avoided by growing only the single genotype studied in an isolated section of the greenhouse.

To induce mutations 1000 r of X-rays were delivered at room temperature (230 K.V.P., 15 M.A., 1.1 mm. copper filter, distance 50 cm.) to whole inflorescences containing young buds, the oldest of which had been previously ascertained to be at the meiotic or premeiotic stage. (It is known from earlier experiments that X-rays applied to mature pollen grains are not effective in producing S allele mutant pollen (Lewis, 1949; Pandey, 1956a.) Mature pollen from X-rayed buds was applied to the stigmas of non-irradiated flowers. To prevent abscission of flowers or young capsules and induce swelling of the ovary 1 per cent. naphthalene-acetamide in lanolin paste was applied around the calyx of all flowers after pollination. This allowed the recovery of rare mutant seeds which normally would have been lost.

(iii) Estimation of pollen per pollinated stigma

To determine the mutation rate, the proportion of mutated pollen in the total number of pollen grains deposited on the stigma must be estimated. A mixture of absolute alcohol and chloroform (1:2 by volume) has a density in which pollen grains of *N. glauca* remain suspended. Excised stigmas of 4 pollinated flowers were vigorously shaken for 15 minutes in 12 ml. of this mixture. Five grain counts were made from five 0.5 ml. samples of each of the 4 suspensions.

(iv) Testing of mutants

(a) *Identification of the mutant type*.—All mutant plants produced by self-pollination with or without X-irradiation (X_1 plants) were test-crossed to determine which of the 4 kinds of mutations: (a) pollen-part (δ -Hom. or δ -Het.), (b) stylar-part (φ -Hom. or φ -Het.), (c) both pollen- and stylar-part (complete: δ -Hom.), and (d) "revertible" (R-Hom. or R-Het.), was involved in the production of the mutant seed.

The technique employed in the production of mutations is selective of those mutations which arise owing to a change in pollen specificity (a and c). Any mutation discovered which can be ascribed to a change in the stylar specificity alone (b) must be incidental, and must have been associated initially with a simultaneous change in pollen specificity. The latter must have reverted later to the normal state leaving the stylar part changed (Lewis and Crowe, 1953; Pandey, 1956a). Similarly, in the "revertible" group (d) which does not show a change either in pollen or stylar

specificity, the pollen part at least must have changed originally to give rise to the mutant seed, but later reverted to the normal state (Lewis, 1951).

All X_1 plants were selfed, and crossed reciprocally with the parental genotype and the two homozygous genotypes (S_1S_1 and S_3S_3) produced through bud-self-pollination of the latter (Pandey, 1963). The results of these test-crosses are given in table 1.

TABLE 1

Testing of X_1 mutants: Results of selfs and crosses with three tester plants and determination of the presence or absence of the fragment

Total No. of X_1 plants	Selfed	S_1S_3		S_1S_1		S_3S_3		Type of mutant	Plants examined for fragment	
		♂	♀	♂	♀	♂	♀		With	Without
62	+	—	+	—	+	—	+	Pollen-part heterozygous (δ -Het.)	60*	1
2	—	—	+	—	+	—	+	S.I. Pollen-part heterozygous (S.I. δ -Het.)	2	0
11	—	—	—	—	+	—	+	"Revertible" heterozygous (R-Het.)	7	4
2	—	+	—	—	—	+	+	"Revertible" or Styler-part homozygous (S_1S_1) (R-Hom. or δ -Hom.)	1	1
3	—	+	—	+	+	—	—	"Revertible" or Styler-part homozygous (S_3S_3) (R-Hom. or δ -Hom.)	3	0
2	+	+	+	+	+	—	+	Pollen-part or Pollen & Styler (complete) homozygous (S_3S_3) (δ -Hom. or δ -Hom.)	2	0
82	* There is one plant, 9160-44/3, which is partly compatible with S_1S_3 (δ).								75	6

As is evident from table 1, the R-Hom. and δ -Hom. mutants showed similar test-cross reactions and were thus indistinguishable at that stage. To distinguish between these alternatives, progenies of these mutants, when bud-self-pollinated and crossed as females with an unrelated genotype, were test-crossed with the normal homozygous genotype (δ) of the allele present in the mutant. The following types of progeny were expected (table 2):

TABLE 2

Test-crosses of the progeny to distinguish between R-Hom. and δ -Hom. mutants

Type of mutant	Bud-selfed-progeny		Progeny of cross with S_2S_2 (δ)	
	Without fragment	With fragment	Without fragment	With fragment
R-Hom. δ -Hom.	All C.I. 3 C.I. 1 C.C.	All C.I. 15 C.I. 1 C.C.	All C.I. 1 C.I. 1 C.C.	All C.I. 3 C.I. 1 C.C.

Similarly the ♂-Hom. and ♀-Hom. mutants were indistinguishable. Progenies of these mutants were tested in the same way as above (table 3):

TABLE 3

Test-crosses of the progeny to distinguish between ♂-Hom. and ♀-Hom. mutants

Type of mutant	Selfed-progeny		Progeny of cross with S_2S_2 (♂)	
	Without fragment	With fragment	Without fragment	With fragment
♂-Hom. ♀-Hom.	All C.I. 1 C.I. 1 C.C.	All C.I. All C.I.*	All C.I. 1 C.C. 1 C.C.	All C.I. 3 C.I. 1 C.C.

* It is assumed that the presence of the fragment is essential for the viability of the mutant pollen.

With the exception of the ♀-Hom. group, in the homozygous X_1 plants, the identity of the mutated allele was automatically revealed. In the heterozygous plants, however, as will be seen later, the presence of the S -bearing chromosome fragment usually obscured such determination. Nevertheless, the rare occurrence of homozygous plants in the progeny of some of these mutants was often indicative of the identity of the mutated allele.

(b) *Identification of the allele on the fragment.*—The heterozygous plants S_1S_3 with a S -bearing centric fragment could have any one of the four constitutions, $S_1S_3fr_3$, $S_1S_3fr_1$, $S_1S_1fr_3$, $S_3S_3fr_1$, depending on whether the normal complement is homozygous or heterozygous for S alleles and, in the latter case, which of the two alleles is present on the fragment. The exact genotypic constitution of the mutant, including the identity of the S allele located on the fragment, was determined by crossing the mutant as female with a new homozygous genotype (S_2S_2). The following genotypes were expected in the progeny:

Cross	Progeny genotypes
$S_1S_1fr_3 \times S_2S_2$	$S_1S_2 + S_1S_2fr_3$
$S_3S_3fr_1 \times S_2S_2$	$S_2S_3 + S_2S_3fr_1$
$S_1S_3fr_3 \times S_2S_2$	$S_1S_2 + S_2S_3 + S_1S_2fr_3 + S_2S_3fr_3$
$S_1S_3fr_1 \times S_2S_2$	$S_1S_2 + S_2S_3 + S_1S_2fr_1 + S_2S_3fr_1$

In the case of mutants which are homozygous for S alleles in the normal complement only two genotypes, a diallelic and a triallelic, will be found in the progeny. The diallelic genotype discloses the particular alleles in the complement and on the fragment. The mutants heterozygous for S alleles in the complement will yield three genotypes, two diallelic and a triallelic. One of the two diallelic genotypes will have a certain proportion of plants with the S -bearing fragment. A cytological study of some of the diallelic plants will identify the genotype with the fragment, thereby also distinguishing the allele on the fragment.

(v) Cytological techniques

Pollen fertility was ascertained by the stainability of the normal-sized pollen with aceto-carmin. Self- or cross-incompatibility as judged from fruit- and seed-set was, in doubtful cases, confirmed by pollen-tube growth studies. Cytological examinations were made of all *N. alata* $\times$ *N. langsdorffii* (♂) test-crosses which, owing to the female

parent having a considerably longer (3-4 times) style than the male parent, generally fail to set seed even in compatible combinations. In a compatible cross *N. langsdorffii* pollen-tubes may penetrate as much as twice the length of its own style in 48 hours, whereas in an incompatible cross they penetrate little more than the stigmatic region of the *N. alata* styles. The technique used for the pollen-tube growth study has been reported earlier (Pandey, 1964).

For chromosome studies meiotic squashes were prepared using Snow's staining technique (1963). Anthers were fixed in acetic-alcohol (1:3) to which a few drops (5 drops to 10 ml. of fixative) of a saturated solution of ferric chloride in 45 per cent. acetic acid were added. For mitotic studies bud styles were used. They were pretreated with 0.2 per cent. colchicine, fixed in Carnoy, stained in Feulgen and squashed in aceto-orcein.

3. RESULTS

(i) Mutation rate

In the present study mutation rate is expressed as the number of fully developed seeds produced per million pollen grains tested. This is a rough estimate of the true gene mutation rate, the calculation of which depended upon a number of extremely variable factors. The most important of these concerned (1) the number of pollen grains applied per stigma and (2) the time of S-gene mutation during microsporogenesis.

Variables influencing the number of pollen grains applied per stigma included differences in the size of the stigma from plant to plant; differences between persons doing the pollination (four persons were involved), and between individual pollinations, in applying a constant amount of pollen on each stigma; difference in pollen viability, which varied between 20 and 90 per cent. in the irradiated pollen, as compared with about 100 per cent. in the controls.

In the estimation of the number of pollen grains per stigma these variabilities were expressed to a very limited extent, chiefly for three reasons: only one person was involved in the pollen estimation; pollen and styles from only a few plants were used; and pollination was done only with non-irradiated pollen.

The number of mutant pollen grains produced in an anther was a function of (a) the number of independent mutations and (b) the time of individual mutations—the earlier a mutation occurred in microsporogenesis the greater the number of mutant pollen grains, all resulting from the same one mutation, produced in an anther. The time of individual mutation was the important variable factor in this experiment and depended on the stage of floral bud development at which the X-rays were applied. Since the whole inflorescence containing buds at various stages of development was treated at one time, almost every bud was expected to show a different rate of mutation.

An analysis of variance of the five $\frac{1}{2}$ c.c. samples per bottle gave:

Mean number of pollen grains per stigma = $17,400 \pm 570$.

Coefficient of variation of pollen grains per stigma = 6.0 per cent.

Owing to the relatively large size of the stigma, the small size of the pollen, and the abundance of stigmatic secretion, a considerably larger number of pollen grains was tested per pollination in *N. alata* than was possible in other species (approx. values in *Oenothera organensis*—5000, *Prunus avium*—1140 (Lewis, 1948); in *Trifolium pratense*—250, *T. repens*—360 (Pandey, 1956); in *Petunia inflata*—1800-4500 (Brewbaker and Shapiro, 1959)). Thus with a total of only 743 pollinations (459 with X-rays, 284 without X-rays) approximately 12.93 million pollen grains were tested, assuming all pollen grains applied to the stigma had an equal chance of fertilising ovules.

Table 4 gives the comparative results of self-pollination with irradiated and non-irradiated pollen. In the 74 capsules set from irradiated pollen, the number of seeds per capsule varied from 1 to 14 (number of seeds per capsule—number of capsules):

1—29; 2—8; 3—11; 4—7; 5—6; 6—3; 7—2;
8—3; 9—1; 10—1; 11—1; 12—1; 13—0; 14—1.

(In a compatible cross approx. 1000 seeds/capsule.) Thus half the number of capsules contained either one or two seeds. All the six capsules produced from non-irradiated pollen contained only one seed each.

Using irradiated pollen increased the seed-set per flower pollinated over 30-fold. In the two different groups of S_1S_3 plants, 9112 and 9115, derived from the reciprocal crosses between two unrelated plants, the average seed-sets were remarkably close (0.54 and 0.53 per flower pollinated). Apparently there were no cytoplasmic or major genic factors influencing the mutation rate.

Seeds from individual capsules were usually sown separately. Of the 254 mutant seeds produced from 80 capsules sown, only about one-third (88; 34.6 per cent.) germinated. These eventually gave rise to 82 mature plants (X_1), the others died before flowering. Most of the X_1 plants were highly fertile, only two plants having below 50 per cent. pollen fertility (74—75 to 98 per cent.; 6—52 to 69 per cent.; 1—30 per cent.; 1—19 per cent.). Table 5 gives an account of the mutant seedlings according to their origin from individual capsules.

(ii) Types of mutations

All X_1 plants were crossed with *N. langsdorffii* pollen to see if any of these had an S_{FI} type of mutated allele, which when present in the style rejects this pollen (as opposed to a normal S_I allele which accepts it) (Pandey, 1964). Pollen-tube studies showed that all such crosses were compatible and, therefore, no S_{FI} type of mutation had occurred.

Table 1 gives the results of other test-crosses made with X_1 plants to determine the type of mutation involved. A majority of the 82 X_1 plants (75; 91.5 per cent.) were heterozygous for S alleles, and most of these (62) had a changed pollen specificity resulting in self-compatibility (δ -Het.). There were two plants, apparently belonging

TABLE 4
Production of mutant seeds: Results of pollinations with irradiated and non-irradiated pollen

Groups	No. of flowers pollinated	No. of capsules set	Total No. of seeds	Seeds/Capsule	Seeds/Flower pollinated	Seeds/Million pollen grains
<i>With X-rays</i>						
9112	350	59	190	3.22	0.54	31.19
9115	109	15	58	3.86	0.53	30.57
Total	459	74	248	3.35	0.54	
<i>Without X-rays</i>						
9112	116	3	3	1.00	0.03	1.49
9115	168	3	3	1.00	0.02	1.03
Total	284	6	6	1.0	0.02	
Grand total	743	80	254	Approx. 12.93 million pollen grains tested		

TABLE 5
Account of the mutant seedlings according to their origin from individual capsules

Serial no. of capsule	No. of seeds in individual capsule	No. of plants obtained	No. of different types of mutants, and cytology					
			♂-Het.	S.I. ♂-Het.	R-Het.	♂-Hom.	R-Hom.	♀-Hom.
<i>With X-rays</i>								
1	4	1	...	1(+)	2(+)	...	...	...
2	8	3	1(+)	...	...	...	...	1(+) S_3S_3
5	6	1	...	...	1(-)	...	...	1(-) S_1S_1
6	4	2	...	...	1(-)	...	...	...
8	3	1	...	...	...	...	1(+) S_3S_3	...
9	3	3	2(+)	...	...	...	...	...
14	5	1	1(+)	...	1(+)	...	...	...
15	7	5	4(+)	...	1(+)	...	...	...
17	5	2	1(+)	...	...	...	...	...
19*	6+3	1	1(+)	...	...	...	...	...
20*	3+1	1	1(+)	...	...	...	...	...
21	2	1	...	...	...	...	...	...
23*	4+1	3	3(+)	...	...	...	...	1(+) S_3S_3
24*	12+5	2	2(+)	...	...	...	...	...
32	3	2	2(+)	...	...	...	...	...
37	6	2	1(+)	...	1(-)	...	...	...
39*	4+4+3+2	2	2(+)	...	...	...	...	...
40*	8+5	3	3(+)	...	...	...	...	...
43	2	1	1(+)	...	...	...	...	...
44*	8+5+3+1+1	7	7(+)	...	...	...	...	...
45	1	1	1(+)	...	1(+)	...	...	...
46	9	2	1(+)	...	...	...	...	...
47	2	1	1(+)	...	...	...	...	...
48	5	4	4(+)	...	...	...	...	...
51	1	1	...	...	...	...	...	...
52*	14+11+1	15	13(11+, 1-, 1?)	...	...	...	1(+) S_3S_3	...
55	7	1	1(+)	...	1(+)	...	...	...
58	3	1	1(+)	...	...	...	1(+) S_3S_3	...
63	1	1	...	...	...	...	...	...
64	2	2	1(+)	...	...	...	...	...
65	10	7	5(+)	1(+)	2(1+, 1-)	...	...	...
<i>Without X-rays</i>								
28	1	1	1(+)	...	...	...	...	...
59	1	1	1(+)	...	...	...	...	...

* In these cases seeds from 2 or more capsules were sown together.

to this group, whose pollen was compatible on the parent genotype but which were themselves self-incompatible (S.I. ♂-Het.). (One of these, 9160-1/1, was the sole seedling obtained from a capsule; whereas the other, 9161-14/2, was one of two seedlings from a capsule, the other seedling being a normal ♂-Het. (cf. table 5)). The plant 9160-1/1 became self-compatible later in the same growing season and has remained so in the subsequent growths for three years; the plant 9161-14/2 has maintained its self-incompatibility.

There were seven plants which were reciprocally incompatible with the parent genotype and therefore had their specificity unchanged in both pollen and style (R-Het.). Seven X_1 plants were homozygous for S alleles, five of which (two S_1S_1 , three S_3S_3) were R-Hom. or ♀-Hom. There were, however, two homozygous plants in which at least the pollen specificity, or possibly both pollen and stylar specificities, had changed (♂-Hom. ($S_3S_3\sigma$) or ♀-Hom. ($S_3S_3\phi$)). It can be seen from table 5 that different types of mutants, with or without the fragment, can occur in plants obtained from seeds of the same capsule.

(iii) Cytology of mutants

With the exception of one plant which died early, all X_1 plants were examined cytologically, particularly for the presence or absence of a centric chromosome fragment (cf. table 1). All X_1 plants, whether having an additional fragment or not, apparently had the normal complement of nine pairs of chromosomes. Apart from one plant all ♂-Het. mutants had a fragment. In the R-Het. group four out of eleven and in the R-Hom. group one out of five had no fragment. Both ♂-Hom. mutants had the fragment.

At meiosis the fragment usually divided at the first anaphase (plate I, figs. 3 and 4); at the second anaphase it was often seen lagging between groups of chromosomes. Among the selfed progeny of X_1 plants with the fragment, plants were found which had two fragments (tables 6 and 11). These fragments at meiosis often showed loose pairing. Occasionally pairing between the fragment and the apparently homologous bivalent was seen at the first metaphase (plate I, figs. 1 and 2).

The size of the fragment varied from plant to plant ranging from very small to a size where it was indistinguishable from a normal chromosome (for figs. see Pandey, 1965). The configuration of the lagging divided fragment at the first meiotic anaphase was generally of the "rod"-type (as opposed to "V"- and "J"-types) (plate I, fig. 3). This indicated that the S -bearing chromosome was one of the three sub-terminal chromosomes found in this species (Avery, 1939; Goodspeed, 1954), and that the variation in the size of the fragment was related to the comparative distance from the centromere at which the breakage occurred in the long arm. Since two of the three sub-terminal chromosomes in *N. alata* are satellited and none of the fragments showed any evidence of a satellite, it is reasonable to assume

that the third subterminal chromosome, without a satellite, is the S -bearing chromosome giving rise to the fragments (plate I, figs. 8 and 9).

(iv) *Progeny of mutants*

(A) ♂-Het. with fragment

(a) *Selfed progeny*.—Table 6 gives the results of selfed progeny of 38 ♂-Het. X_1 mutants. The following points emerge from these observations:

- (1) Although the S.C. plants predominate (over seven times S.I.), there are S.I. (49) as well as S.C. (385) plants in the progeny.
- (2) Of the eight S.I. plants examined cytologically, four did not have the fragment; all the six S.C. plants examined had the fragment. One of the S.C. plants had two fragments owing presumably to both gametes contributing a fragment each.
- (3) Although the vast majority (401) of plants were heterozygous, there were a considerable number (33) which were homozygous.
- (4) Among the homozygous plants 27 were S.C. and six S.I. Excluding the exceptional family 7596, of the seven S.C. plants examined four had the fragment (one plant of the fam. 7512 had two fragments). In one family (9178) where two of the four S.C. homozygous plants were examined, one had the fragment and the other did not.
- (5) The exceptional family 7596 comprised 15 plants all of which were self-compatible to varying degrees and homozygous for the S_1 allele, and none of which had the fragment. (All plants had a low degree of pollen fertility: 10—50 to 60 per cent.; 3—30 to 40 per cent.; 2—15 to 20 per cent.)

If competitive interaction were the sole cause of the pollen-part mutations all selfed progeny of the mutants should be (1) S.C., (2) heterozygous and (3) should normally contain at least one fragment. No homozygous plants should occur in the progeny, *e.g.*:

$$S_1S_3fr_3 \text{ selfed } \longrightarrow S_1S_1fr_3 + S_1S_1fr_3fr_3 + S_1S_3fr_3 + S_1S_3fr_3fr_3.$$

The results show that none of these expectations is realised. The appearance in the progeny of the S.C. homozygous plants with or without the fragment shows beyond doubt that true mutations of the S gene are involved. The fact that there were S.C. homozygous plants without a free fragment, however, did not exclude the possibility that in these plants such a duplicate fragment was still present but had been incorporated in the normal genome.

In the case of the exceptional family 7596 in which the fragment was present in the parent plant but absent in all its progeny it is possible that the inflorescence of the parent plant was a chimæra having certain sectors of tissue with the free fragment and others in

TABLE 6—*The behaviour of selfed progeny of 38 pollen-part mutants*

Mutant plant selfed	Family no.	Total no. of plants in family	Heterozygous plants cross-incompatible with $S_2S_3(\delta)$		Homozygous plants cross-incompatible with only $S_1S_1(\delta)$ or $S_2S_3(\delta)$			
			S.C.	S.I.	S.C.	S.I.	S.C.	S.I.
9160-2/1	7517	11	9	$4(1+, 1-)$	...	...	...	...
9160-9/1*	7519	8	16	...	...	...	...	...
9160-9/3	7509	16	7	$1(-)$	...	...	$1(+)$	...
9160-15/1	7506	9	9	...	...	...	...	...
9160-15/4	7522	10	11	...	...	...	...	...
9160-15/5	7552	11	11	...	...	...	...	...
9160-17/1	7510	11	6	$1(-)$	...	...	$1(-)$	...
9160-23/1	7511	8	9	$1(-)$	...	...	...	...
9160-23/2	9178	13	10(1+)	...	$4(1+, 1-)$	...	...	...
9160-23/3	9179	10	2	$6(1-)$	...	...	...	...
9160-24/1*	7555	8	3	...	...	...	...	...
9160-24/2	9180	4	13	...	...	...	...	$1(-)$
9160-28/1	9181	14	7	...	...	1	...	...
9160-32/2	7527	8	11	...	...	...	...	...
9160-37/2	7528	11	13	...	...	...	...	...
9160-39/1	9182	13	10	...	...	...	...	...
9160-39/2	7529	10	11	...	...	...	...	...
9160-40/1	9183	13	12	...	...	...	...	...
9160-40/3	9185	11	10(1+)**	$1(-)$	...	...	...	...
9160-43/1	7530	14	13	2	...	...	...	...
9160-44/1	9186	14	8	...	$1(+)$	...	...	...
9160-44/2	7507	9	5	6	...	...	...	...
9160-44/4	7505	13	7	$3(1+)$	...	2	...	...
9160-44/5	7531	10	11	...	...	...	...	...
9160-44/6	7532	14	11	...	...	...	...	...
9160-44/7	7508	12	11	...	...	...	...	...
9160-45/1	3104	13	12	1	...	...	...	...
9160-46/1	3105	15	15	...	...	...	...	...
9160-48/3	9187	14	13(1+)	$1(+)$	...	...	...	...
9161-2/1***	9188	12	11	...	1	...	...	...
9161-2/2	9189	16	16	...	...	...	...	...
9161-2/4	7539	14	14	...	...	...	...	...
9161-2/5*	9190	12	6	6	...	...	...	...
9161-2/10	7512	15	14(1+)	...	$1(+)**$	...	...	...
9161-2/12	7541	5	4	$1(-)$	...	...	...	...
9161-2/15	9193	9	9	...	...	...	...	...
9161-15/3	7514	9	7	...	...	...	...	...
9161-15/6	7596	15	...	...	$15(-)$	1	...	...

* These mutants were only partly self-compatible; ** these plants had two fragments; *** with the exception of this mutant which was not studied cytologically, all others, studied here, had the fragment.

which the fragment was either translocated to one of the chromosomes of the genome or was lost. This would explain the absence of the free fragment in the female as well as the male gametes. Nevertheless, the possibility exists that in this plant the mutant allele somehow, at least partly, regained its viability and therefore did not need the fragment for its functional self-compatibility. The heterozygous parent plant of this family must have the S_3 -bearing fragment and may have the constitution $S_1S_1\delta fr_3$ ($S_1\delta$ -pollen-part mutated in the allele S_1).

(b) *Crossed progeny* (S_2S_2 as δ).—Results of F_1 progeny of crosses of three X_1 plants with S_2S_2 (δ) are given in table 7. The presence of triallelic plants in the progeny shows that the fragment does, in fact, bear the S allele. The genotypic constitution of the mutants revealed by the progeny tests shows that the fragment may bear either of the two alleles (see Materials and Methods).

(B) δ -Het. without fragment

(a) *Genetics of the progeny*.—Although one of the δ -Het. mutants (9161-2/9) did not have the fragment, there is the possibility that such a duplicate segment might have been incorporated in the normal genome. To test this, the selfed and crossed (S_2S_2 as δ ; S_3S_{F10} as φ) progenies of the mutant plant (S_1S_3) were studied:

Cross	No. of plants of different genotypes in the progeny (fragment)
S_1S_3 selfed	→ 20 S_1S_3 (4-)
$S_1S_3 \times S_2S_2$	→ 8 S_2S_3 (1-) + 10 $S_1S_2S_3$ (1-)
$S_3S_{F10} \times S_1S_3$	→ 31 S_1S_3 (1-) + 10 $S_1S_3S_{F10}$ (1-) + 2 S_3S_3

The results show that two types of gametes, S_3 and S_1S_3 , were produced by the mutant plant and, therefore, confirm the hypothesis that a duplicate chromosome segment bearing the S locus is incorporated into the normal genome and is linked with a normal S -bearing chromosome. The presence of two homozygous S_3S_3 plants in the progeny of the cross with S_3S_{F10} (φ) again shows that a mutant allele may occasionally go through the pollen without a duplication.

(b) *Cytology*.—Meiotic studies of the mutant and its progeny showed an almost regular presence of nine bivalents at MI and a frequent occurrence of a dicentric bridge and fragment at AI. At AII, a number of cells were observed to have a bridge, but no fragment, between two groups of chromosomes (plate I, figs. 5, 6 and 7). The regular presence of nine II at MI would suggest that duplication involving only the homologous chromosomes is involved, which is in agreement with the above observation of only two types of gametes being produced by the mutant plant. If this is true, then the occurrence of a dicentric bridge and fragment at AI would suggest that the duplication is of the "reverse" type; the bridge at AII being the result of an unbroken dicentric chromosome at AI.

TABLE 7
The behaviour of selfed (bud-pollination) and crossed progeny of the two
S.I. ♂-Het. mutants, 9161-14/2 and 9160-1/1

Cross	Family	Total no. of plants	Progeny genotypes														
			Heterozygous S_1S_3						Homozygous S_3S_3						Homozygous S_1S_1		
			S.I.	S.C.	Crossed with $S_1S_3^*$				S.I.	S.C.	Crossed with S_2S_2				S.I.	S.C.	Crossed with S_1S_3
					Non-parental		Mutant parent				Non-parental	Mutant parent					
					♂	♀	♂	♀				♂	♀	♂			
9161-14/2 selfed (bud-pollination) Crossed with 9161-14/2 (♂) A with different (♂) B non-parental 9160-1/1 (♂) C parental S_1S_3 (♀) { S_1S_1 (♂) 9160-1/1 (♂) { S_2S_2 (♀) crossed with S_3S_3 (♀) 9160-1/1 (♀) × 9161-14/2 (♂)	7563 7550 3156 7599 3103 3155 3174 3175 3162	14 25 28 23 18 13 22 26 28	13(7+) ... 4(2-) 1(+) 19 26(2-) 21	... 24(11+) 23(6+) 21(2+) 18 11(1+) 3(1+) ... 6	I I I I I I I I I	I C C** I C C I C I	I I I	I(-) I(-) I(-)		C C C	I I I	I(-) I(-) I(-)					

* In crosses with S_1S_3 all plants of a particular genotypic group, whether S.I. or S.C. behaved, alike, unless mentioned to the contrary. ** Only S.C. plants were cross-compatible, the S.I. plants were cross-incompatible.

If the duplicate segment is represented by the letters $b c d$, the present form of duplication ($a b c d d' c' b' e$), which appears to have been acquired from the homologous chromosome, would be of an intermediate type between the "reverse tandem" ($a b c d d c b e$), which is a duplication of a segment within a chromosome, and "displaced" duplication, which is acquired from a non-homologous chromosome. The occurrence of such a duplication may underline the phenomenon of unequal crossing-over taking place either within or in the proximity of a gene complex (Dunn, 1956; Swanson, 1957; Pandey, 1959).

(C) *Self-incompatible ♂-Het.*

In order to understand the nature of the change in the S allele reaction in the two S.I. ♂-Het. plants (9160-1/1 and 9161-19/2), which were cross-compatible on the styles of the parent plant and yet were themselves self-incompatible, progeny of these plants were studied when:

- (1) one, 9161-14/2, is bud-self-pollinated,
- (2) both are crossed as males with two or three different non-parental normal S_1S_3 plants, and with genotypes S_1S_1 and S_3S_3 ,
- (3) the two are intercrossed (cf. table 7);
- (4) 9160-1/1 is reciprocally crossed with S_1S_{F10} and S_3S_{F10} , and 9161-14/2 (♂) is crossed with S_3S_{F10} (table 8); and
- (5) 9160-1/1 is reciprocally crossed with S_2S_2 , and 9161-14/2 (♀) is crossed with S_1S_2 and S_2S_2 (table 9).

The plant 9160-1/1 which became self-compatible later in the first season and has remained so since, behaved as any other normal pollen-part mutant, in its progeny. Progenies of crosses with non-parental S_1S_3 (♀) were, as expected, generally all self-compatible. Progenies of other crosses in which the male parent had at least one allele that was not present in the female parent were, again as expected, largely self-incompatible. Thus the temporary self-incompatibility of this plant can probably be attributed to physiological causes.

The plant 9161-14/2, on the other hand, has remained consistently self-incompatible and the behaviour of its progeny confirmed the permanent nature of its unusual specificity. The bud-selfed progeny were, like the mutant parent, all self-incompatible, and except for one plant, all heterozygous. The latter (Het.) were also reciprocally incompatible with the mutant parent and a non-parental S_1S_3 . However, when the mutant plant was involved in crosses with other plants, the progeny largely behaved as would be expected of the progeny of a normal pollen-part mutant. In crosses, as males, with two non-parental S_1S_3 (♀) the progenies were self-compatible; in crosses, as females, with three other genotypes, self-compatible plants, as expected on the pollen-part mutation basis, were again recovered.

TABLE 8
The behaviour of crossed progeny of S.I. ♂-Het. mutants: S₁S₁F₁₀ (♂) and S₃S₃F₁₀ (♂ and ♀)

Cross	Family	Total no. of plants	Progeny genotypes							
			S ₁ S ₃		S ₁ S ₃ F ₁₀		S ₁ S ₃ S ₃ F ₁₀		S ₃ S ₃ F ₁₀	
			S.I.	S.C.	S.I.	S.C.	S.I.	S.C.	S.I.	S.C.
S ₃ S ₃ F ₁₀ × 9161-14/2 (♂)	3157	9	4(2-)	...	3	...	1	...	1(-)	...
S ₃ S ₃ F ₁₀ × 9160-1/1 (♂)	3178	20	10	...	8	...	2	...	...	...
9160-1/1 × S ₃ S ₃ F ₁₀ (♂)	3176	22	5(2*-)	...	1	...	13	3(2+)	...	...
S ₁ S ₃ F ₁₀ × 9160-1/1 (♂)	3179	20	8	1	...	...	...	...	9	2
9160-1/1 × S ₁ S ₃ F ₁₀ (♂)	3180	20	...	...	10	...	...	1	7(4-)	2

* These two plants, in test-crosses, show partial female-part mutation in the allele S₁(S₁[♀]).

TABLE 9
The behaviour of crossed progeny of S.I. ♂-Het. mutants: S₁S₂ (♂) and S₂S₂ (♂ and ♀)

Cross	Family	Total no. of plants	Progeny genotypes						Constitution of the mutant plant
			S ₁ S ₂		S ₂ S ₃		S ₁ S ₂ S ₃		
			S.I.	S.C.	S.I.	S.C.	S.I.	S.C.	
9160-1/1 × S ₂ S ₂ (♂)	3181	21	6	...	13	...	...	2	...
S ₂ S ₂ × 9160-1/1 (♂)	3177	18	6	...	11	...	1	...	...
9161-14/2 × S ₁ S ₂ (♂)	3158	26	6(2-)	...	6(1-)	6(1+)	...	7(1+)	S ₁ S ₃ fr ₃
9161-14/2 × S ₂ S ₂ (♂)	3159	8	1	...	3(1+)	...	...	4(2+)	S ₁ S ₃ fr ₃

From the above results it appears that the unusual behaviour of 9161-14/2 is due to its own peculiar background of cytoplasmic and/or genotypic constitution and is expressed only on the female side. For, not only was the mutant plant self-incompatible but the *self-compatible* progeny of crosses with S_1S_3 (♀) were still *incompatible* when backcrossed to the styles of the mutant parent. This behaviour is passed on, in a more intensified way, to its bud-selfed progeny, which were not only self-incompatible but also reciprocally incompatible with parental (9161-14/2) as well as normal non-parental S_1S_3 .

The situation in 9161-14/2 (and to a lesser extent also in 9160-1/1) is probably a form of inbreeding depression which is lowering its physiological efficiency below a certain threshold, necessary for the proper functioning of the incompatibility reaction. It is interesting to note here that it is the stylar function which is disturbed first, leaving the pollen function normal. In the next selfed-generation, however, both pollen and stylar functions are affected. An intensification of self-incompatibility in the selfed-progeny of partly self-compatible strains, of a normally self-incompatible species, has been recorded in several species and has generally been attributed to the physiological condition associated with inbreeding depression (Pandey, 1959, 1960).

Another possible explanation assumes that the unique behaviour of these mutants is probably indicative of a new class of mutations—of a “constructive” nature—which can rarely be identified by our experimental techniques, but which, nevertheless, occurring in small successive steps may be the real basis of the creation of new self-incompatible alleles from old ones (Pandey, 1959; Fisher, 1961). Although this latter hypothesis cannot be entirely ruled out, the data presented here seem to fit the simple inbreeding depression explanation quite satisfactorily.

The detailed study of the above crosses also revealed the following points of interest:

(1) The presence of triallelic genotypes in the crosses confirmed the earlier conclusion that the fragment bore the S locus (cf. tables 8 and 9).

(2) In selfed and crossed progeny (different non-parental S_1S_3 (♀)) of the two mutant plants infrequently individuals were recovered which were homozygous for the S allele (cf. table 7, families 3155, 3156, 7550, 7563, 7599). These were, with only one exception, all S.I. The only homozygous plant which was self-compatible also had the fragment (7599). These observations again support the view that true ♂-part S -gene mutations are involved and that the presence of the fragment is essential for the functioning of the mutant gene. The presence of rare S.I. homozygous and heterozygous plants suggests that occasionally the mutant gene goes through the pollen without the fragment, giving rise to seedlings which remain self-incompatible.

(3) The presence of homozygous S_3S_3 plants without the fragment in crosses of the plant 9161-14/2 (7550, 3156) would further suggest

that in this plant S_3 is the mutant allele. Similarly, it can be concluded that S_1 is the mutant allele in the plant 9160-1/1 (7599, 3155).

(4) From the crossed families (table 9, 3158, 3159) of the plant 9161-14/2 (S_1S_2 and S_2S_2 as ♂) it can be concluded that in this mutant the fragment bears the S_3 allele. The results in the progeny of 9160-1/1 (table 7, 7599; table 8, 3176), however, show that in the gametes of this mutant the fragment may bear either the S_1 or the S_3 allele.

The observation in the mutant 9160-1/1 would be explicable on the assumption that there was occasional pairing between the fragment and the normal homologous S -bearing chromosomes. This could, through crossing over, produce a proportion of fragments which would bear an allele different from that present on the fragment in the parent mutant.

(D) R-Het.

Five R-Het. plants (3+, 2-) were bud-self-pollinated to determine whether the progeny showed any change in S specificity which was not evident in the X_1 plants. Results, given in table 10, show no evidence of any such change in the S specificity.

TABLE 10

The behaviour of selfed (bud-pollination) progeny of R-Het. mutants

Mutant plant bud-selfed	Family	Total no. of plants in family	Progeny genotypes		
			Heterozygous (S.I.) S_1S_3	Homozygous (S.I.)	
				S_1S_1	S_3S_3
9160-6/1 (-)	7598	25	11	11	3
9160-37/1 (-)	3106	13	11	2	...
9160-2/2 (+)	3108	8	7	1	...
9161-2/3 (+)	3130	12	6	1	5
9161-15/1 (+)	3128	12	8	2	2

If the first two families, without the fragment, are taken together, the observations fit the expected ratio of 1 Het.: 1 Hom. $X^2=0.947$, $P=0.50-0.30$. In the 3 families with the fragment, two types of ratios are possible, assuming that the fragments are always included in the gamete nuclei: (1) 11 Het.: 5 Hom.—if the normal chromosome complement is heterozygous for the S allele, e.g., $S_1S_3fr_3$ or $S_1S_3fr_1$; (2) 3 Het.: 1 Hom.—if the normal chromosome complement is homozygous for the S allele, e.g., $S_1S_1fr_3$ or $S_3S_3fr_1$. Taken the 3 families together the observations fit the expected ratio of 11 Het.: 5 Hom. $X^2=0.145$, $P=0.80-0.70$.

Nine R-Het. plants were also crossed with S_2S_2 (♂). Progenies (14-20 plants each) of four mutants which did not have the fragment, each comprised two diallelic genotypes (S_1S_2 and S_2S_3), and were all S.I. (9160-6/1 - 12+8, 9160-8/1 - 7+9; 9160-37/1 - 7+7; 9161-15/7 - 2+15). Since no triallelic plants were discovered in these progenies it is evident that these mutants, in fact, had no S -bearing duplicate

segment incorporated in the normal genome. Results from the progeny of five other plants, having the fragment, are given below:

Plant crossed with S_2S_2 (δ)	No. of plants in different genotypes			Constitution of the mutant
	S_1S_2	S_2S_3	$S_1S_2S_3$	
9160-2/2 $\longrightarrow$	8 (1+, 2-)	8 (1-)	4	$S_1S_3fr_1$
9160-17/2 $\longrightarrow$		9	3	$S_3S_3fr_1$
9160-44/3 $\longrightarrow$	8	4	1	$S_1S_3fr_1$ or $S_1S_3fr_3$
9161-2/3 $\longrightarrow$	8 (1+, 1-)	4	3	$S_1S_3fr_1$
9161-15/1 $\longrightarrow$	4 (1-)	11 (1+, 1-)	1 (+)	$S_1S_3fr_3$

About half ($\frac{5}{12}$) of the triallelic plants were partly or fully S.C. In 9160-44/3 and 9161-2/3 there were six (three S_1S_2 and three S_2S_3) and three (S_1S_2) diallelic plants respectively which were S.C. In the plant 9160-44/3 the presence of S.C. plants in both diallelic genotypes is unexpected and is in line with the unusual behaviour of the mother X_1 mutant, which was partly female-compatible with S_1S_3 (cf. table 1). It is likely that there was a partial female-part mutation involved in this mutant.

(E) δ -Hom. or ϕ -Hom.

The occurrence of homozygous X_1 plants, whether S.I. or S.C., and with or without the fragment, is significant for it is only in these plants that the problem of whether true gene mutations occur in *N. alata* can be tested unambiguously, without the implication of the competitive interaction.

The presence, among X_1 mutants, of two plants which were homozygous (S_3) and showed pollen-part mutation provides the conclusive proof for the occurrence of genuine mutation in the S gene. Selfed and crossed (S_2S_2 as δ) progeny of these plants were studied, firstly, to confirm the nature of the mutation and secondly, to distinguish between δ -Hom. and ϕ -Hom. mutations, both of which produce identical behaviour in X_1 plants. The results from these progenies are given in table 11.

The results clearly show that the two plants have the normal specificity of only one allele, S_3 , and no trace whatever of the S_1 allele. With regard to the type of mutation, however, it is likely that a complete (ϕ) mutation without the fragment is inviable in the female gamete, in which case it would be impossible to distinguish between the two alternatives (δ - or ϕ -mutation). Nevertheless, the results of crosses show that both mutants undoubtedly have at least a pollen-part mutation and each have only one type of allele, thus excluding the phenomenon of competitive interaction as an explanation for the mutation.

The fact that all plants of the selfed progeny examined cytologically

had the fragment and that 50 per cent. (four out of eight) of them had two fragments each, shows that the only functional pollen grains were those carrying the fragment. This confirms the hypothesis that the

TABLE 11
*The behaviour of selfed and crossed (S_2S_2 as δ) progeny of
 δ -Hom. or ϕ -Hom. mutants*

Plants	Total no. of plants in family	Selfed		Crossed with the genotypes as males		
		S.C.	S.I.	S_1S_1	S_2S_2	S_3S_3
Selfed						
9161-2/6 (+)	16	16(3+)*	0	+	...	—
9161-13/1 (+)	14	14(5+)**	0	+	...	—
Crossed with S_2S_2 (δ)						
9161-2/6	19	6(2+)	13(1—)	+	—	—
9161-13/1	20	8	12	+	—	—

* All 3 plants had 2 fragments each.

** One of these plants had 2 fragments.

presence of the fragment is essential for the recovery of the mutant pollen. Furthermore, since the mutant allele in the pollen produces self-compatibility even in the presence of a presumably normal allele borne on the fragment, it may be concluded that the mutant allele is dominant over the normal allele.

(F) *R-Hom. or ϕ -Hom.*

In order to distinguish between these two types of mutations five plants (3+, 2—) belonging to this group of mutants were crossed as females with S_1S_3 (except the plant 9161-1/1) and S_2S_2 genotypes, and one plant, 9161-1/1 (—), was bud-self-pollinated. The progeny were crossed with appropriate testers. The results are given in table 12.

All the progenies of the crosses with S_1S_3 (δ) were uniformly self-incompatible and also cross-incompatible with S_1S_1 (δ) and S_3S_3 (δ). This would suggest that the four X_1 plants thus studied were all R-Hom. and had no ϕ -part mutation. However, when the progenies of the same plants crossed with another genotype, S_2S_2 , were examined they clearly showed that two of the four plants had a ϕ -part mutation, which could not be detected either in the X_1 generation or in the progeny of the crosses with S_1S_3 . Progenies of these two plants, as expected on the basis of ϕ -part mutation, contained approximately 50 per cent. of plants which were self-compatible, and also cross-compatible as females with S_3S_3 (in the case of 9160-5/1) or S_1S_1 (9160-6/2).

One of the two ϕ -part mutants, 9160-5/1, which had the fragment, and a third plant, 9160-9/2, also having the fragment, produced a few

TABLE 12
The behaviour of selfed and crossed (S_1S_3 and S_2S_3 , (δ)) progeny of R -Hom. and φ -Hom. mutants

Mutant plant crossed	Total no. of plants in family	Self-compatible				Self-incompatible					
		No. of plants	Testers as ♂				No. of plants	Testers as ♂			
			S_1S_1		S_3S_3			S_1S_1		S_3S_3	
		C	I	C	I	C	I	C	I		
9160-5/1 (S_3S_3) (+) $\times S_1S_3$	15	0	...	...	...	15(4-)	0	15	0	15	
9160-9/2 (S_3S_3) (+) $\times S_1S_3$	13	0	...	...	...	13	0	13	0	13	
9160-21/1 (S_3S_3) (+) $\times S_1S_3$	15	0	...	...	...	15	0	15	0	15	
9160-6/2 (S_1S_1) (-) $\times S_1S_3$	11	0	...	...	...	11	0	11	0	11	
9161-1/1 (S_1S_1) (+) Bud-selfed	12	0	...	...	...	12	0	12	0	0	
9160-5/1 $\times S_3S_2$	13	13	...	...	9(2-)	7	...	...	0	7	
9160-9/2 $\times S_3S_2$	10	1	...	...	0	9	...	...	0	9	
9160-21/1 $\times S_3S_2$	8	0	...	...	...	8	...	...	0	8	
9160-6/2 $\times S_3S_2$	11	5	5	0	...	6	...	6	...	...	
9161-1/1 $\times S_2S_2$	19	0	...	...	...	19	0	19	...	...	

individuals in the progeny which too were self-compatible, but these were cross-incompatible as females with S_3S_3 . The self-compatibility of these plants was presumably the result of competitive interaction, owing to the presence of the fragment rather than to ♀-part mutation.

That the two X_1 plants, 9160-5/1 and 9160-6/2, had a ♀-part mutation is undoubtedly true, for there is no other explanation possible for the behaviour of their progeny. The problem then arises: why was this ♀-part mutation expressed in the progeny of the crosses with S_2S_2 , but not in the progeny of the crosses with S_1S_3 ? The answer to this question probably lies in the phenomenon of complementation. The mutated alleles, S_3 in the plant 9160-5/1 and S_1 in the plant 9160-6/2, were complemented by a normal S_1 and S_3 allele respectively in the hybrid progeny, thus restoring the normal function of the mutant alleles, an observation similar to that made by Lewis (1958) in the pollen of *Oenothera*. Apparently, no complementation of the mutant alleles occurred in association with the allele S_2 .

There was a possibility that one or both of the remaining R-Hom. mutants might still have a ♀-part mutation that was masked by complementation, and was not revealed because of the very limited number of test crosses (only two, S_1S_3 and S_2S_2 (♂)) studied. Similarly some of the R-Het. mutants might fall into this category.

Another interesting characteristic of these ♀-part mutations was that they did not need the fragment for their viability. In the progeny of both mutants the S.C. plants, which had the ♀-part mutant allele, were also female compatible with the testers homozygous for the alleles of the respective mutants. This definitely excluded the possibility of the fragment in them. Two of these plants, examined cytologically, had no fragment, as expected.

The presence of the fragment, even though not necessary for viability, in one of the two mutants can be attributed to the fact that a simultaneous ♂-part mutation is essential if the ♀-part mutation is to be recovered at all. It has been shown that revertible mutations do occur either with or without the fragment.

4. DISCUSSION

(i) Significance of the duplicate fragment

(a) *S-gene mutation and complementation in the pollen*.—The present study shows that true *S*-gene pollen-part and stylar-part mutations occur in *N. alata* as well as in *Oenothera*, *Prunus* and *Trifolium*. It also suggests that in *N. alata* and *P. inflata* the pollen-part mutations of *S* alleles are lethal in the pollen unless associated with a duplication carrying a normal *S* locus. It has been proposed that the normal *S* allele on the fragment compensates for the loss (mutation) of a certain essential element, which is closely linked with the mutated element of the *S*-gene complex and usually mutates along with it, and thereby makes the inviable mutant pollen viable (Pandey, 1965).

There is one observation, however, which needs further explanation. On the above hypothesis, homozygous and heterozygous mutants should occur in equal proportions. This is not the case. Heterozygous mutants occur in overwhelming numbers. It seems likely that competitive interaction plays some part in this behaviour. In contrast to ♂-part mutants, many of which were fully self-compatible, the colchicine-induced autotetraploids of the genotype S_1S_3 were only partially self-compatible. This again showed that competitive interaction could not be the sole cause of the origin of ♂-part mutants. Although all of high pollen fertility, out of eleven tetraploid plants, four were completely S.I., another four set only rare capsules containing a very small number of seeds, and only three produced 80 per cent. or so capsule-set. However, even this last group had very much fewer seeds than were produced in other fully S.C. tetraploids (unpublished results). Nevertheless, in the diploid pollen of this genotype competitive interaction, producing partial self-compatibility, does occur. It is, therefore, reasonable to conclude that in the heterozygous mutant pollen, having a duplicate *S*-bearing fragment, competitive interaction will be an additional factor favouring self-compatibility. This will virtually lead to a selection of heterozygous pollen.

But for the fact that the competitive interaction between the two alleles in the present material was incomplete, no homozygous mutant plants at all would have been recovered, as was the case in *P. inflata*. This fortunate selection of genotype has allowed the author to discern between the two factors—genuine *S*-gene pollen-part mutation and competitive interaction—in *Nicotiana* mutants, whereas in *P. inflata*, where competitive interaction in the genotypes studied was absolute, only competitive interaction heterozygotes were produced. This secondary role of competitive interaction in producing ♂-part self-compatible mutants is further underlined by the occurrence of ♀-part mutations which do not need the fragment for viability, as is shown by the study of their progeny.

There seems little doubt that, *as far as the study of the true S-gene mutations is concerned*, competitive interaction has only a nuisance value and therefore must be avoided in future studies of this kind, through a careful selection of experimental material excluding this phenomenon.

The true role of the fragment with regard to *S*-gene mutation appears to be complementation, the normal allele on the fragment complementing the lethal mutant self-compatible allele in the normal genome, so that the latter's viability is restored. Complementation of a ♂-part mutant allele by a normal allele in the pollen, restoring the original specificity of the mutated allele has been reported earlier by Lewis (1958) in *Oenothera organensis*. In the present experiment, however, mutated pollen with a duplication showing *Oenothera*-type complementation would automatically be rejected. The only type of complementation which would be selected in the pollen would be that which restores the viability of otherwise inviable mutant pollen. That

complementation of various forms is common at the *S* locus is demonstrated by its occurrence in relation to both the male- and female-part mutations.

The failure by Davies and Wall (1961) to produce mutants of the *S* gene in one genotype of *Lycopersicon peruvianum* studied, may be attributed either to the lack in this species of a means of specific breakage producing *S*-bearing duplication, and/or to the occurrence, in this genotype, of the same type of complementation as was found in *Enothera*, thus making the mutant pollen ineffective in producing seed.

Complementation would seem to throw light on another problem: the apparent lack of ♂-part mutation in the homozygous *Petunia* genotypes (Brewbaker and Shapiro, 1959). There is a distinct possibility that the mutant pollen with the fragment giving rise to the ♂-Hom. plants may actually be *heterozygous* but that the mutant allele has lost both the pollen and stylar specificities and therefore is neutral in this respect, the different normal allele on the fragment together with the similar allele from the egg characterising the specificity of the resultant plant. If this is the case, then it would appear that the absence of pollen-part mutations in *S* homozygous plants may be due to complementation, owing to the presence on the fragment of the normal form of the mutant allele in the same pollen.

From the accumulated evidence at hand, it now seems plausible that in *Enothera*, *Trifolium* and *Prunus*, where *S* locus duplication is not necessary for the viability of the ♂-part mutation, we should be able to produce mutation in the *S* homozygous plants. On the other hand, in *Nicotiana* and *Petunia*, where this duplication is necessary, it may be unobtainable owing to complementation.

(b) *Evolution*.—The occurrence of a duplicate centric chromosome fragment allowing complementation that restores the viability of an otherwise inviable mutant pollen would appear to have definite evolutionary advantages. A case of this kind has been reported by Darlington and Janaki-Ammal (1945) in *Nicandra physaloides*, another Solanaceous species. *N. physaloides* is the solitary species of its genus or even tribe. This species has nine pairs of regular autosomes and an additional one or pair of isochromosomes apparently derived from the normal chromosomes. Individual plants may, therefore, have 19 or 20 chromosomes. The isochromosomes behave abnormally at meiosis, thus leading to the production of a certain proportion of grains that lack an isochromosome altogether. Although over 90 per cent. of the ripe pollen is good, the only pollen grain mitoses seen are those with 10 chromosomes. Evidently, pollen (but not the egg) lacking the isochromosome dies, presumably because the presence of the isochromosome is essential for pollen viability—a situation similar to that occurring in the *S* allele pollen-part *Nicotiana* mutants.

Darlington and Janaki-Ammal found that deficient individuals ($2n = 19$) of this species showed delayed germination which “might well confer an advantage of decisive value to a species like *N. physaloides*

which is an isolated relic". An additional advantage for the retention of the isochromosome in the first place, suggested from the present study, may lie in the phenomenon of complementation which probably makes the originally self-incompatible species (like some of the other Solanaceous species that have $n = 9$) self-compatible. For long-distance dispersal and successful colonisation self-compatibility appears to be an essential pre-requisite ("Baker's Law": Stebbins, 1957; Baker, 1955). This would be much more so for a relic like *N. physaloides*, which, in the case of long-distance dispersal, would be under severe strain of a sudden change in environment.

A similar purpose may be served in evolution by a duplicate translocation without an additional free centric fragment, as is shown in the present work by the stable behaviour of the δ -part mutant 9161-2/9, which regularly has 18 chromosomes in itself and in its selfed and crossed progeny. Although there is a small proportion of aberrant pollen produced, apparently only those pollen gametes function which have the necessary duplication. In a plant producing a large amount of pollen a slight decrease in the number of functional pollen grains would be a small price to pay for its survival, if this guarantees self-compatibility.

It is interesting to speculate whether the different patterns of supernumerary chromosomes found in many genetic cultures and varieties of certain species (Darlington and Upcott, 1941) have arisen under a similar selective force as is exemplified in *Nicotiana* and *Nicandra*. Such duplications would provide for the survival of a large number of "adaptive" mutations which would otherwise be inviable in the pollen.

Another point of interest about the S -bearing centric fragment in relation to evolution, is the ability of a species, through this mechanism, to alter its basic chromosome number. As shown from the present study this fragment, which may be denoted as a "derived chromosome", may vary greatly in size, thus suggesting that the S gene is located very close to the centromere. It may be so large, and so normal in behaviour, as to be indistinguishable from the usual S -bearing chromosome in the complement, and to a certain extent it may increase in number in future generations. In addition to producing self-compatibility the derived chromosome may thus provide an alternative evolutionary basis—short of euploidy which often has other undesirable consequences—in increasing the genetic content of the species. It is significant that *Nicotiana* and *Petunia* (at least in the one species of each genus studied), which have been shown to possess the mechanism for the duplication of the S -bearing chromosome, are two of the four genera of the family Solanaceae which have, in fact, an aneuploid series of basic chromosome numbers (*Nicotiana*: $X = 9, 10, 12$; *Petunia*: $X = 7, 9$). *Lycopersicon*, which appears to lack this ability, on the other hand, along with the other 25 genera of this family, has only one basic chromosome number ($X = 12$) (Darlington and Wylie, 1955).

(ii) *Complementation of the S locus*

Lewis (1958) was the first to discover complementation in the diploid *pollen* of induced autotetraploid *Oenothera*. As far as the author is aware, there has been no other report of the occurrence of this phenomenon in the higher plants. The present discovery of complementation, in styles as well as pollen of *N. alata*, underlines Lewis' finding and makes this observation of considerable theoretical interest.

In the present study almost all pollen-part *S*-gene mutants were studied in *Trans* configuration (the mutant and complementing alleles lying on different chromosomes of the homologous pair) and were necessarily of the *complementary* type. The occurrence of complementation in *Trans* configuration, as discovered at the *S* locus, is relatively uncommon in bacteria where it is generally observed in the *cis* configuration (the mutant and complementing alleles located on the same chromosome) (Benzer, 1957; Lewis, 1960). A regulator mutation which acts both in the *cis* and *Trans* positions and, like mutants of the *S* locus, is dominant to the wild type has been found in the "lactose system" of *Escherichia coli* (Willson *et al.*, 1964). Synthesis of hybrid enzyme polymers has been postulated to explain interallelic (*Trans*) complementation in *Neurospora* heterocaryons (Catcheside and Overton, 1958; Fincham, 1959).

The interallelic complementations in *E. coli* and in *N. alata* and other self-incompatible species (Lewis, 1960), in which the complex loci (or functional chromosomal regions) studied are composed of separate protein specificity cistron(s) ("structural" unit) and "regulator" cistron(s), have one aspect in common: both involve mutations of the regulator unit of the complex. Although more evidence is needed before a firm conclusion is reached it seems possible that in this type of gene complex the cistron concept, as defined by Benzer, is applicable only to the structural unit of the gene coding the *specificity* of a protein; complementation in this unit can occur only in the *cis* configuration. This concept, however, does not necessarily apply to the regulator unit of the complex governing the *production* or *activity* of the protein; complementation between different elements of a cistron in this unit, or possibly in other forms of gene complex generally, can occur in the *trans* as well as *cis* configuration.

Another unusual characteristic of complementation at the *S* locus is that it occurs between a mutant allele and a normal allele; in other studies it occurs between two mutant alleles. This again may be a property of mutants of regulator genes, as opposed to those of structural genes in which complementation is possible only between two mutant genes.

Complementation in stylar action is a new observation not recorded before. From the point of view of the evolution of self-incompatibility, complementation in stylar action is of special interest, for interaction between alleles in styles is not a common phenomenon in the gametophytic system of incompatibility. On the contrary, the absence of such

interactions is one of the important characteristics of this system, any irregularity in this condition leading to the breakdown of incompatibility. Yet interaction is found to occur between a normal and a mutant allele in the style.

Because of the diploid nature of the style, the selection, which operated in nature in the evolution of gametophytic incompatibility, eliminated all interactions of normal S alleles in the style. This effectively excluded dominance and competitive interaction from stylar action. Complementation, however, could not be selected against since it is not a property of the normal class (wild type) of alleles. This explanation of the occurrence of complementation is in accord with the pollen-interactions encountered in the diploid pollen of artificially synthesised autotetraploids. Thus, like dominance and competitive interactions, complementation is also one of the basic properties of genes and, since selection against such interactions is usually not possible in nature, may occur in any diploid cell that has one or both alleles (of a gene) of mutant origin.

A point worth considering with regard to the present observation of complementation in styles is that the mutant alleles were complemented by the sister allele of the parental genotype from which the mutant arose. (The mutant alleles $S_1\varphi$ and $S_3\varphi$, obtained from the genotype S_1S_3 were complemented by the normal S_3 and S_1 alleles respectively.) This may not be significant, because in *Oenothera* complementation was found between alleles which were not so related. Nevertheless, it would be of interest to see if, in *N. alata*, complementation of the mutated alleles is achieved by any other allele(s) besides those already observed.

5. SUMMARY

1. True S -gene mutations, similar to those found in *Oenothera*, *Prunus* and *Trifolium*, also occur in *N. alata*. But, in *N. alata* pollen-part mutations generally appear to be lethal, unless associated with an S -bearing duplication.

2. The results suggest that in the present material the function of the duplication is two-fold:

- (i) production of diallelic heterogenic pollen grains resulting in competitive interaction and self-compatibility, and
- (ii) complementation of the mutant allele, which would otherwise have been lethal in the pollen grain.

3. Further conclusive evidence of true S -gene mutation and complementation is provided by two stylar-part mutations which do not need the fragment for their viability. In these, as in the two ♂-Hom. mutants, competitive interaction as a basis of explanation is ruled out.

4. Proper genotypic selection of material in the investigation of

S-gene mutations is important to avoid confusion arising from competitive interaction. A detailed study of R-Hom. mutants, some of which have been shown to mask mutant alleles, is also necessary.

5. The role, in evolution, of duplicate centric fragments in the survival of mutant pollen through complementation is discussed. A naturally occurring case of *Nicandra physaloides*, exemplifying such an evolution, is cited.

6. Such derived chromosomes may have a further evolutionary role in increasing the basic chromosome number, and thus the total hereditary material, of the species without involving full ploidy.

7. The phenomenon of complementation is briefly discussed in relation to mutations of the *S* gene.

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Plate I

Meiotic and mitotic cells of N. alata mutants. × 2350

FIGS. 1-4. Meiotic cells showing the behaviour of the two fragments in a selfed-progeny of a ♂-Het. mutant, 9161-2/10, of *N. alata*.

FIG. 1.—The fragments which show loose pairing between themselves, also appear to show pairing with the apparently homologous bivalent.

FIG. 2.—The two fragments are unpaired.

FIG. 3.—The divided fragments lagging at AI.

FIG. 4.—The lagging divided fragments moving to the opposite poles at AI.

Figs. 1 and 2 also show a quadrivalent in each. This type of pairing often leads to abnormal AI division giving 8 and 10 chromosomes at the poles (fig. 3).

FIGS. 5-7. Meiotic cells in the ♂-Het. mutant 9161-2/9, which has no free fragment.

FIG. 5.—Normal 9 II at MI.

FIG. 6.—AI with a bridge and fragment.

FIG. 7.—AII with a bridge (but no fragment) between two groups of chromosomes.

FIGS. 8 and 9. Mitotic cells showing the morphology of the fragment in the ♂-Hom. mutants 9161-2/6 (fig. 8) and 9161-13/1 (fig. 9). Chromosomes are contracted differently in the two nuclei.

FIG. 8.—The fragment is without a constriction; the centromere is believed to be nearly at one end of the fragment, which probably includes little more than the short arm of the subterminal chromosome from which it arose.

FIG. 9.—The fragment, which shows a constriction (centromere), includes the short arm of the chromosome and a very small part (smaller than the short arm) of the long arm.

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