SEGREGATION IN NEW ALLOPOLYPLOIDS OF NICOTIANA.
I. COMPARISON OF 6x (N. TABACUM × TOMENTOSIFORMIS) AND
6x (N. TABACUM × OTOPHORA)

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GENETIC segregation in synthetic allopolyploids of the genus *Gossypium* has been described in several earlier papers by the author and his colleagues (see GERSTEL and PHILLIPS 1958, for a summary). We had found in that genus that allopolyploids produced from two very closely related species segregated like autopolyploids, i.e. they gave approximately 5:1 ratios for crosses of the duplex type ZZzz to recessive testers. In contrast, very little segregation occurred where the two parents of an amphiploid belonged to different sections of the genus; such amphiploids behaved like classical allopolyploids. From still other amphiploids intermediate ratios had been obtained. In some amphiploids the ratios differed somewhat from locus to locus, but their averages reflected in a quantitative fashion the taxonomic affinity of the two parent species which were combined in the particular amphiploid. Herein lay the main interest of the method, specially when the argument was inverted and the relationship between two species assessed quantitatively from the segregation frequencies of their amphiploid.

This paper commences a series of similar studies on segregation in synthetic allopolyploids of the genus *Nicotiana*.

MATERIALS AND METHODS

The three species of *Nicotiana* used were the diploid ($n = 12$) species *N. tomentosiformis* Goodsp., *N. otophora* Griseb. and the natural tetraploid *N. tabacum* L. Two accessions were employed of each of the diploids. The *N. tomentosiformis* accessions had been given to us by the late PROFESSOR R. E. CLAUSEN of the University of California and came originally from the locations Cañamina and Tajma in Bolivia; we also owe the Jujuy (Argentina) race of *N. otophora* to the courtesy of DR. CLAUSEN. A second accession of *N. otophora* had been obtained from U. S. Department of Agriculture sources. Though these accessions had been maintained for many years in the U. S. by close inbreeding, the Tajma race still segregated for the factor *Pb*, or purple bud, of which use was made as a marker; both *N. otophora* accessions were homozygous for purple bud. The symbol *Pb* will also be used for the *N. otophora* factor for convenience though homology of the purple bud factors from the two diploid species was not proven.

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Purple bud is unknown in *N. tabacum*; the character behaved as a dominant in interspecific hybrids.

The remaining markers employed were recessive mutants of *N. tabacum*; here also the use of identical symbols for the dominant genes derived from the wild species does not necessarily imply homology. The mutants coral (*co*), fasciated (*fs*), and white seedling (*ws*) were available in the otherwise, more or less, isogenic background of the race Red Russian and were obtained from DR. CLAUSEN's collection. Fasciated plants and white seedlings differ from some normal varieties by two recessive factors (CLAUSEN and CAMERON 1950 and unpublished), but single dominant genes from the diploid species cover the recessives completely, and the segregation of these single dominant alleles was studied. The marker white corolla (*wh*) came from the tobacco race known as Cuba White. Burley tobacco (varieties Ky 16 and By 21) is characterized by white stems and early yellowing leaves; it differs by two recessives ($\gamma b_1 \gamma b_2$) from most normal tobacco varieties (STINES 1959), but a single dominant gene from the diploids produces fully normal plants. Yellow green (*yg*) had originally been found by NOLLA (1934), but was employed here in the local variety "402" into which DR. T. J. MANN had transferred the *yg* allele.

Amphiploids were produced from F_1 seed of interspecific hybrids by treating emerging seedlings at room temperature with 0.1 percent aqueous colchicine solution for three hours. Positive results were achieved almost without fail; but in addition to the desired amphiploids the cultures usually contained unaffected plants, periclinal chimaeras with polyploid morphology but low fertility, offtypes (probably aneuploids) and monstrosities of various degrees (probably at least in part higher polyploids). All these were discarded and only plants of typical morphology, high percentage of filled pollen and good spontaneous seed set were used. These plants were presumably of the duplex genotype $ZZzz$, where the dominant alleles had been derived from the wild parent and the recessives from *N. tabacum*. Since the chromosome number was checked only rarely, it is quite possible that some of the amphiploids were numerically not fully balanced. Influence upon segregation from that source was minimized by testing several amphiploids of the same origin wherever possible. The segregation ratios were obtained by crossing the amphiploids with *N. tabacum* lines homozygous for the appropriate recessive marker. The observed segregation frequencies, therefore, reflect directly the gametic output of the amphiploid parents. An exception were the tests involving *Ws*. Since white seedling plants were inviable, amphiploids had to be synthesized from green heterozygotes (actually $ws_1 ws_1 Ws_2 ws_2$) and wild species. About one half of these amphiploids segregated and the other half did not; the latter were discarded. The crosses designed to test segregation were made between the segregating amphiploids (for short $Ws Ws ws ws$) and $Ws ws$ heterozygotes of *N. tabacum*. The observed segregation ratios were then corrected by dividing the total offspring minus twice the number of white seedlings by twice the number of white seedlings.

Segregation for the white seedling character was scored from seedlings germinating in Petri dishes. The other characters were scored from older plants.

RESULTS

6x (*N. tabacum* × *tomentosiformis*): In the first column of Table 1 the marker genes and in the second column the assigned plant numbers of the individuals employed are listed. In most cases some replication was available, either from two or more individual amphiploids, or from different accessions of *N. tomentosiformis* (third column) or from reciprocal crosses (fourth column). In the case of *Fs* no replication was obtained, and for *Wh* the data from three parents were not homogeneous ($P < .01$), and their summation is not justified. One would suspect that plant B 53-2 which gave an approximate 1:1 ratio was an unbalanced type, perhaps *Wh wh wh* instead of *Wh Wh wh wh*.

The over-all picture of the ratios (last column) is this: within each genotype the results are quite uniform since reciprocal testcrosses and the data from the two accessions of *N. tomentosiformis*, as available, gave similar results. Generally, the ratios are smaller than the tetrasomic 5:1 expected with random chromosome segregation; in most cases the ratios are even smaller than 3.7:1 which could result from random chromatid segregation, though only in one case(*) significantly so. Only the ratios for *Yg* were larger than 3.7:1.

In all of the progenies only two phenotypes could be separated with certainty. With incomplete dominance or pronounced dosage effects it might have been possible to distinguish between three classes from the crosses which were *ZZzz* × *zz* as had been possible in the *Gossypium* work quoted above. In the material segregating for *Fs* the 29 plants scored as having the recessive *fs* phenotype showed more or less extreme fasciation of the stem and had at least four carpels to each pistil; the remaining 87 plants had normal vegetative growth and usually the normal number of two carpels per pistil; a number of plants in this class had an occasional flower with three carpels. Three plants were intermediate; they could have been chromosomally unbalanced.

Presence or absence of anthocyanin coloration of the calyces and pedicels (*Pb* vs. *pb*), best studied in flower buds, was easily verified though the intensity of coloration was variable. Two of the families segregating for *Pb* segregated simultaneously for *Wh*. These are listed separately in Table 2. Instead of the expected two classes with white and pink corollas, three classes were found; among the *Pb* plants 12 had very pale pink flowers, but purely white ones were missing. Apparently the *Pb* factor produces a flush of color in the *wh* flowers. Listed in Table 2 is also the progeny from amphiploid B 53-2, which did not carry *Pb* and in the offspring of which no very pale flowered plants appeared.

Plants B 51-17 and B 55-15 were amphiploids from burley tobacco and *N. tomentosiformis* carrying *Pb*; the backcross populations to burley segregated simultaneously for *Pb* and *Yb*. Forty-eight of the total offspring were phenotypically *Yb Pb*, 20 were *yb pb*, one was *Yb pb* and two *Yb* plants did not reach the flowering stage. Thus the nonburley and purple bud factors are linked in *N. tomentosiformis*. CLAUSEN and CAMERON (1944) had previously shown by means of monosomic analysis that *yb₁* and a purple plant factor transferred from

TABLE 1

Segregation of 6x (N. tabacum × tomentosiformis)

Character	Plant no. of hexaploid	Tomentosiformis strain in hexaploid	Hexaploid as	Phenotype of progeny		Ratio
				Dominant	Recessive	
<i>Fs</i>	C 114-8	Cañamina	♂	87	29	3.0:1
<i>Pb</i>	B 48-11	Tajma	♀	66	18	
	B 53-1	Tajma	♀	30	10	
	B 53-4	Tajma	♀	51	16	
				147	44	3.3:1
	B 48-11	Tajma	♂	39	17	
	B 51-17	Tajma	♂	34	17	
	B 53-4	Tajma	♂	284	67	
	B 54-5	Tajma	♂	4	0	
	B 54-12	Tajma	♂	6	9	
	B 55-15	Tajma	♂	14	4	
				381	114	3.3:1
<i>Wh</i>	B 53-1	Tajma	♀	32	5	
	B 53-2	Tajma	♀	26	30	
	B 53-4	Tajma	♀	56	11	
				(114	46	2.5:1)
	C 117-2	Cañamina	♂	226	97	2.3:1*
<i>Ws</i>	C 121-3	Cañamina	♀	762	95	3.5:1†
	C 121-3	Cañamina	♂	2481	309	3.5:1‡
<i>Yb</i>	A 25-A	Cañamina	♀	143	49	
	A 25-D	Cañamina	♀	171	51	
				314	100	3.1:1
	A 25-A	Cañamina	♂	70	19	
	A 25-D	Cañamina	♂	86	34	
				156	53	2.9:1
	B 51-17	Tajma	♂	36	16	
	B 55-15	Tajma	♂	15	4	
				51	20	2.6:1
<i>Yg</i>	C 120-4	Cañamina	♀	107	23	4.7:1
	B 54-5	Tajma	♀	198	45	4.4:1

* Highly significant minus deviation from 3.7:1.

† Ratios corrected since a *Ws ws* tester had to be used.

‡ One subfamily gave a heterogeneous 186:8 (or 11.1:1 corrected) ratio and was omitted from the total.

TABLE 2

Simultaneous segregation for Pb and Wh in 6x (N. tabacum × tomentosiformis)

Hexaploid parent (♀)	Corolla color	Bud color					
		Purple			Green		
		Pink	Very pale	White	Pink	Very pale	White
B 53-1*		26	2	0	6	0	3
B 53-2†		Not segregating for <i>Pb</i>			26	0	30
B 53-4		41	10	0	15	0	1

* Two additional plants were scored *Pb* and one *pb*, but came into bloom too late in the season for the corolla color to be recognized.

† This family is listed here because it shows that in the absence of *Pb* no very pale flowers are obtained.

the related species *N. tomentosa* were both located in the same tobacco chromosome.

6x (*N. tabacum* × *otophora*): Segregation ratios for crosses from this combination were in each case larger than in the progenies from 6x (*N. tabacum* × *tomentosiformis*) scored for similar loci (Table 3). This is the only generalization which can be drawn from the data presented in the last column. The ratios for different loci cover a wide range from 3.5:1 to 14.8:1, and the results from replications, where available, are not in as good an agreement as for 6x (*N. tabacum* × *tomentosiformis*). Further work with the 6x *N. tabacum* × *otophora* combinations is needed in order to clear up the reasons for the lack of uniformity of the results.

Several points are of interest. As in the preceding chapter, *Yg* gave the widest ratio of all the loci which were studied. With respect to the *Co* and *Ws* genes somatic instability was observed. Among the offspring of plant B 22-3 which segregated for *Co* 12 segregants had self-coral flowers. One hundred and nineteen of the 128 plants scored as carmine had variegated carmine-coral flowers while in eight no variegation could be detected. The extent of variegation differed from occasional small coral spots on carmine background (never *vice versa*) to heavy sectoring. It is not impossible that the eight self-carmine plants also fell into the variegated category and that the spots were too infrequent and too small to be observed with the naked eye, though numerous flowers were studied from each of these plants. One additional plant was not scored for variegation since it bloomed late in the season, but it could be classified as carmine from its flower buds.

Variegation was also observed among the *Ws* material from plants D 134-4 and D 134-6. Ninety-eight seedlings were albinos; among the 1887 seedlings scored as green 12 were found with larger or smaller sectors on their cotyledons lacking chlorophyll (Figure 1). Five of these plants were grown to a later stage; they continued to produce variegated leaves. The seedlings with self-green cotyledons and the albinos were discarded after scoring at the cotyledonary stage. Among the green progeny of plant C 107-13 no variegation was observed on the cotyledons. The green seedlings of one subfamily were transferred to the field;

TABLE 3

Segregation of 6x (N. tabacum × otophora)

Character	Plant no. of hexaploid	Otophora strain in hexaploid	Hexaploid as	Phenotype of progeny		Ratio
				Dominant	Recessive	
<i>Co</i>	B 22-3	Jujuy	♂	128	12	10.7:1
<i>Fs</i>	B 32-2	USDA	♀	308	60	5.1:1
	B 32-2	USDA	♂	195	56	3.5:1
<i>Pb</i>	B 32-2	USDA	♀	329	39	8.4:1
	A 5-D	USDA	♂	68	14	
	A 9-E	USDA	♂	72	9	
	B 32-2	USDA	♂	202	54	
				342	77	4.4:1
	B 22-3	Jujuy	♂	108	32	
	B 23-3	Jujuy	♂	17	4	
<i>Ws</i>	C 107-13	Jujuy	♂	165	30	
				290	66	4.4:1
	D 134-4	USDA	♀	126	3	
	D 134-6	USDA	♀	1761	95	
				1887	98	9.1:1†
	C 107-13	Jujuy	♂	644	44	6.8:1†
<i>Yb</i>	A 5-D	USDA	♀	159	19	8.4:1
<i>Yg</i>	A 9-C	USDA	♀	260	24	
	A 9-D	USDA	♀	239	12	
	A 9-E	USDA	♀	286	17	
				785	53	14.8:1

† Ratios corrected since a *Ws ws* tester had to be used.

of 200 survivors two were found to be chlorophyll variegants. Thus somatic instability with respect to chlorophyll occurred in derivatives of amphiploids with *N. otophora* from both Jujuy and U.S.D.A. accessions.

DISCUSSION

N. tabacum is the classic example of a natural amphiploid. The mode of its origin was demonstrated in 1928 by GOODSPEED and CLAUSEN, who showed that one of the diploid ancestors came from the section *Tomentosae* to which both *N. tomentosiformis* and *N. otophora* belong. For short, we may designate these as the T species and the corresponding subgenome of tobacco as the T genome. It is quite likely that none of the now living species of that taxonomic group is identical with the parent that once went into ancestral *N. tabacum*, but perhaps it is not entirely futile to attempt to designate the modern species closest to that

FIGURE 1.—Seedlings showing instability of the chlorophyll factor from *N. otophora* (Approx. 24X).

ancestor. In the literature various arguments can be found favoring *N. tomentosiformis* over *N. otophora* or *vice versa*. Support for *N. otophora* comes from two observations. This species forms with *N. sylvestris* an amphiploid which is both male and female fertile, at least to some degree. *N. sylvestris* is believed by CLAUSEN and GOODSPEED to represent the other ancestral genome ("S") of tobacco. Amphiploids between other species of the Tomentosae and *N. sylvestris* are completely female sterile; the nature of these sterilities has been analyzed, and it has been found that relatively simple complementary gene systems are involved in each case (GREENLEAF 1942; AR-RUSHDI 1956). Secondly, only *N. otophora* and *N. sylvestris* share at present an extended geographic range between Salta and Sucre on the eastern slopes of the Andes; the other Tomentosae occur further north where *N. sylvestris* has not been found (see maps in GOODSPEED 1954). On the debit side *N. otophora* possesses, besides other morphological peculiarities, strongly reflexed corolla lobes (Figure 3a) which are retained in the

FIGURE 2.—Plant raised from a seedling of the type shown in Figure 1 with continuing instability.

amphiploid *N. sylvestris* $\times$ *otophora* (Figure 3b) but cannot be found in tobacco (Figure 3c). In contrast the floral morphology of *N. tomentosiformis* (Figure 3d) is much more like that of tobacco, and the *N. tomentosiformis* $\times$ *sylvestris* amphiploid resembles *N. tabacum* closely.

Thus, distribution and fertility point towards *N. otophora* as the closer relation of tobacco while morphology decidedly favors *N. tomentosiformis*. It is of course possible, as AR-RUSHDI (1956) suggested, that early hybridization between the various T species combined in a single segregant form the various features which could have made it a successful ancestor of tobacco. Since, thus far, hybridization and introgression between T species has not been demonstrated, AR-RUSHDI's viewpoint must be considered speculative for the time being. It may be noted, however, that GOODSPEED (1954, p. 365) mentions the possibility that in the origin of *N. tomentosiformis* hybridization of *N. tomentosa* and *N. otophora* may have been involved.

Does the segregation of artificial polyploids which in *Gossypium* fairly faithfully reflected the relation between the combined species provide any clue to discriminate between *N. tomentosiformis* and *N. otophora* as the closer modern relative of tobacco? Quite consistently *N. tabacum* $\times$ *tomentosiformis* amphiploids gave smaller segregation ratios than the *N. otophora* combinations. This consistency points towards a greater homology of *N. tomentosiformis* chromosomes with those of the T subgenome of tobacco. But certain difficulties were

FIGURE 3.—Flowers of (a) *N. otophora*, (b) amphiploid *N. sylvestris* $\times$ *otophora* (first made by R. E. CLAUSEN), (c) *N. tabacum*, (d) *N. tomentosiformis* (Approx. natural size).

encountered in the *Nicotiana* work which were absent from the previous studies with *Gossypium*, and until these are fully understood judgment must be withheld.

In $6x$ (*N. tabacum* $\times$ *tomentosiformis*) all observed ratios were smaller than the 5:1 gametic output characteristic of autopolyploids. This could perhaps be ascribed to chromatid, as opposed to chromosome segregation. However, the chromatid segregation ratio of 3.7:1 is a limiting value obtained only under special conditions, one of which is great distance between centromere and locus on the chromosome (see review by LITTLE 1945). It is, therefore, surprising to find such low segregation ratios for almost all the loci which were studied. For most segregations in Table 1 the observed ratios were below those obtainable from chromatid segregation and in the starred case even significantly so. These results bring to mind the example of SKALINSKA (1935) who obtained a large excess of recessives from a selfed amphiploid *Aquilegia chrysantha* $\times$ *flabellata*. Instead of the 35:1 ratio expected from selfing of an autotetraploid, SKALINSKA observed an almost exact 3:1 ratio of 125 blue to 43 white flowering plants. That author reached the conclusion that at the first meiotic anaphase the two chromosomes bearing *C* (colored) always disjoined from those with *c* (white) resulting in only two kinds of gametes (*CC* and *cc*) giving in turn a 3:1 F_2 ratio. This phenomenon seems to resemble the "affinity" recently postulated by MICHIE (1953) and WALLACE (1958) for chromosomes of interspecific and interracial

hybrids of mice. WALLACE proposed that at first meiotic division centromeres of the same ancestral origin in such hybrids tend to travel to the same pole. The difference consists in that in the mouse material non-homologous chromosomes showed affinity, whereas in the *Aquilegia* amphiploid homologues of common origin were postulated to go to the same meiotic pole.

SKALINSKA's main argument was a negative one, namely that numerical irregularities in chromosome distribution were excluded as a cause of her results because all the progeny plants which she had studied possessed the full tetraploid chromosome number of 28. In the *Nicotiana* amphiploids described in this paper affinity may play a role, but it is not unlikely that genetic instability, either chromosomal or mutational, is a factor responsible for an increase of the frequency of recessive offspring by transforming potential *Aa* gametes into *aa* or *a*.

Observations to support this viewpoint have not yet been made with the critical *N. tabacum* $\times$ *tomentosiformis* amphiploids which gave the unusual ratios, but the literature on genetic instability in *Nicotiana* is fairly extensive. This was recently reviewed by MOAV and CAMERON (1960) who demonstrated such instability in combinations of *N. tabacum* and *N. plumbaginifolia*. Despite the distant relationship of these two species their amphiploid segregated extensively for a factor for which it was of duplex constitution. Since instability for this factor had been amply demonstrated by the authors, they attributed the appearance of recessives in the progeny to the same cause.

In addition to the unstable hybrids between distantly related species listed by MOAV and CAMERON (1960), SMITH and SAND (1957) described cases of flower color variegation in hybrid derivatives from closely related *N. langsdorffii* and *N. sanderae*. The unstable pentaploids derived from 6x (*N. tabacum* $\times$ *otophora*) mentioned in the present paper are additional examples of instability in hybrids from closely related parents. Studies in progress are expected to reveal whether there is a relation between the somatic instabilities found and the progeny ratios which were obtained.

The unusual but fairly consistent ratios from 6x (*N. tabacum* $\times$ *tomentosiformis*) do not represent the only problem calling for a solution. The ratios from the *N. tabacum* $\times$ *otophora* amphiploids were inconsistent and varied over a wide range from 3.5:1 for *Fs* to 10.7:1 for *Co* (P for homogeneity $< .001$) omitting from consideration the even larger ratio for *Yb* for reasons given below. This spread may reflect variable amounts of preferential pairing of different chromosomes but could also have other causes, as, e.g., differences in the degree of instability of the various markers. In this context it may be noted that the *Co* factor from *N. otophora* was somatically unstable in almost every plant of the pentaploid progeny whereas *Ws* from the same source was unstable only infrequently. Thus, differing degrees of instability do exist. Clearly, this complex of problems needs further clarification.

The ratios for *Yg* were larger than the remainder. In the case of 6x (*N. tabacum* $\times$ *tomentosiformis*) they were 4.7:1 and 4.4:1 for the two amphiploids synthesized from the races Cañamina and Tajma of *N. tomentosiformis*. These ratios were the largest ones listed in Table 1. Similarly, the ratio 14.8:1 obtained from a

N. tabacum $\times$ *otophora* amphiploid was the largest in Table 3. A comparable observation has also been made with respect to segregation for yellow green from 6x (*N. tabacum* $\times$ *sylvestris*) where a different subgenome was involved (GERSTEL and PHILLIPS 1958, and in prep.). The yellow green pentaploids in all these cases were throughout their lives conspicuously weaker than the normal sibs. In the original work on *Yg* segregation in *N. tabacum* NOLLA (1934) had obtained a highly significant deficiency of recessives in segregating populations. It is, thus, likely that the large segregation ratios in the pentaploid populations were due to a lessened viability of the yellow green segregants and not to a different behavior of the chromosomes carrying *Yg*.

SUMMARY

1. The original plan was to discover whether the chromosomes of *N. tomentosiformis* or those of *N. otophora* are more nearly homologous with one genome of *N. tabacum*.

2. The segregation ratios produced by the synthetic amphiploids *N. tabacum* $\times$ *tomentosiformis* and *N. tabacum* $\times$ *otophora* differed with 6x (*N. tabacum* $\times$ *tomentosiformis*) giving consistently the smaller ratios.

3. These results, taken at their face value, would favor *N. tomentosiformis* as the closer relation of tobacco, as far as chromosome homology is concerned, but judgment should be postponed because of the following complications:

4. Segregation ratios for 6x (*N. tabacum* $\times$ *tomentosiformis*) were always smaller than the 5:1 ratio expected for amphiploids synthesized from two very close relatives; in general the ratios were even smaller than the 3.7:1 ratio expected with chromatid segregation.

5. Segregation ratios for 6x (*N. tabacum* $\times$ *otophora*) varied over a wide range.

6. Only future work will indicate whether this variability is due to differences in the extent of differential affinity between chromosomes or of genetic instability.

7. Examples of somatic instability were described in the derivatives from 6x (*N. tabacum* $\times$ *otophora*).

8. The yellow green locus gave larger segregation ratios than any other. Reduced viability of yellow green seedlings was the probable cause.

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