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Cytological studies of interspecific somatic hybrids in *Nicotiana*

Rong-Fong Lin and Chi-Chang Chen[1]
Department of Botany, National Taiwan University, Taipei, Taiwan, Republic of China

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Abstract. Fusion of protoplasts of haploid *Nicotiana plumbaginifolia* (P), *N. sylvestris* (S), and *N. otophora* (O) resulted in the production of interspecific somatic hybrids of various genome constitutions. Polyploidy, aneuploidy, and chromosome structural aberrations were common in the somatic hybrids. An unusual chromosome change observed in this study was centric fusion of the telocentric or acrocentric chromosomes of *N. plumbaginifolia* in somatic hybrids of PPO and PSO genome constitutions. The chromosomes of haploid plants of the three parental species formed predominantly univalents at metaphase I, indicating absence of homology among the chromosomes within the genomes. Analyses of chromosome associations at diakinesis and/or metaphase I in the somatic hybrids suggest that there is little or no homology among the genomes of the three species. The potential applications of the somatic hybrids in genetic research are discussed.

Key words: Chromosome association; Centric fusion; Interspecific somatic hybridization; *Nicotiana*; Phylogeny.

Introduction

Although analyses of meiotic chromosome associations in interspecific hybrids have provided much information concerning phylogenetic relationships of the species in *Nicotiana* (Kostoff, 1943; Goodspeed, 1954), the data obtained so far have been incomplete because of interspecific cross-incompatibility. Somatic hybridization through protoplast fusion is an effective means of overcoming this problem (for a review see Harms, 1985). However, in previous studies, almost without exception the protoplasts used for fusion have been from diploids or polyploids; the resultant hybrids therefore contained double or multiple genomes of parental species. These hybrids are of little value for investigation of genome relationships because the homologous chromosomes of each parent tend to pair preferentially.

Nicotiana plumbaginifolia Viviani ($2n=2x=20$), *N. sylvestris* Spegazzini & Comes ($2n=2x=24$), and *N. otophora* Grisebach ($2n=2x=24$) have been considered the most likely candidates for model species in somatic cell genetics (Bourgin *et al.*, 1979). The first two species are in section *Alatae*, while the third species is in *Tomentosae* (Goodspeed, 1954). Interspecific hybrids between *N. sylvestris* and *N. otophora* have been obtained from sexual crosses and chromosome associations in the hybrids have been studied (Goodspeed, 1954; Takenaka, 1956). *N. plumbaginifolia* and *N. sylvestris* are sexually incompatible (Christoff, 1928) but can be crossed asexually through protoplast fusion (Gleba *et al.*, 1987; Chae and Choi, 1987; Famelaer *et al.*, 1989). However, homology of the genomes of these two species has not been investigated. No sexual and somatic hybrids between *N. plumbaginifolia* and *N. otophora* have been reported.

Haploid plants of these three *Nicotiana* species have been produced from anther culture (Chen *et al.*, 1985), and somatic hybridization in the genus has now become a routine procedure. The objectives of this study were to obtain interspecific hybrids between

[1]Corresponding author.

these species through fusion of haploid protoplasts, and to study meiotic chromosome associations in the resultant hybrids.

Materials and Methods

Plant Material

Seeds of *Nicotiana plumbaginifolia, N. sylvestris*, and *N. otophora* were supplied by the U. S. Department of Agriculture, Beltsville, MD. Haploid plants of these species were obtained from anther culture (Chen *et al.*, 1985) and maintained *in vitro* according to the method of Negrutiu and Mousseau (1980).

Protoplast Isolation, Fusion, and Culture

The upper two or three fully expanded leaves of *in vitro* cultured haploid plants were used as the source of protoplasts. Protoplasts of the three *Nicotiana* species were isolated separately as described by Huang and Chen (1988). They were washed once with W5 solution (Medgyesy *et al.*, 1980) and resuspended in this solution at a density of 5×10^5/ml. In one experiment protoplast suspensions of *N. plumbaginifolia, N. sylvestris*, and *N. otophora* were mixed in a 1:2:2 ratio, and in another experiment protoplast suspensions of *N. plumbaginifolia* and *N. otophora* were mixed in equal volumes. The protoplast mixtures were treated with polyethylene glycol (PEG) and cultured as described by Lee and Chen (1990). No selection against parental cells was applied during culture.

Cytological Techniques

The somatic chromosomes were prepared from root-tip cells of *in vitro* cultured plants. Excised roots were treated with 0.002 M 8-hydroxyquinoline at 18 to 20°C for 2.5 h, fixed in ethanol-acetic acid (3:1) overnight, stained by the Feulgen method for 1 h, and treated with 1% pectinase for 1 h. Root tips were squashed in 45% acetic acid.

For meiotic studies, flower buds were fixed in Carnoy's solution (6 parts ethanol: 3 parts chloroform: 1 part acetic acid) overnight, and anthers were squashed in propiono-carmine.

For determination of pollen fertility, anthers containing mature pollen grains were squashed in propiono-carmine. Only those grains that were plump and took up the stain were counted as fertile.

Results

Karyotypes of Parental Species

Haploid plants of *N. plumbaginifolia* have 5 large, 3 medium, and 2 small chromosomes. All of these were telocentric or acrocentric and one small chromosome possesses a satellite (Fig. 1A). The 12 chromosomes of haploid *N. sylvestris* are rather uniform in length and are metacentric or submetacentric. Two of these have a satellite and a third one often shows a secondary constriction which does not seem to be a nucleolar organizing region (Fig. 1B). The haploid complement of *N. otophora* consists of 5 large acrocentric and 7 small metacentric or submetacentric chromosomes. Two of the large acrocentric chromosomes possess a satellite (Fig. 1C). The satellited chromosomes of *N. otophora* are distinctly larger than those of *N. sylvestris*. Thus, karyotypes of the three *Nicotiana* species differ markedly, and these differences could be used as the basis to determine genome constitutions of plants regenerated from protoplast fusions.

Genome Constitutions of Regenerated Plants

Plant regeneration occurred in 70 calli in protoplast fusions involving three *Nicotiana* species. The time of shoot emergence following PEG treatment and genome constitutions of regenerated plants are shown in Table 1. Only 13 (18.6%) calli differentiated into parental plants and all of these were *N. plumbaginifolia*. Of the remaining calli, 24 (34.3%) gave rise

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Fig. 1. Somatic metaphase chromosomes of haploid plants of parental species and somatic hybrids. (A) Haploid *N. plumbaginifolia* showing 10 chromosomes. (B) Haploid *N. sylvestris* showing 12 chromosomes. The chromosome with a secondary constriction is indicated by an arrow. (C) Haploid *N. otophora* showing 12 chromosomes. (D) Somatic hybrid PS, $2n = 22$. (E) Somatic hybrid PSS, $2n = 34$. (F) Somatic hybrid SO, $2n = 24$. (G) Somatic hybrid SSO, $2n = 36$. (H) Somatic hybrid PSO, $2n = 34$. (I) Somatic hybrid PSO, $2n = 34$. The translocated chromosome with 2 satellites is indicated by an arrow. (J) Somatic hybrid PPO, $2n = 32$. (K) Somatic hybrid PPO, $2n = 30$. The large submetacentric chromosome derived from fusion of 2 telocentric or acrocentric chromosomes is indicated by an arrow. X 1300.

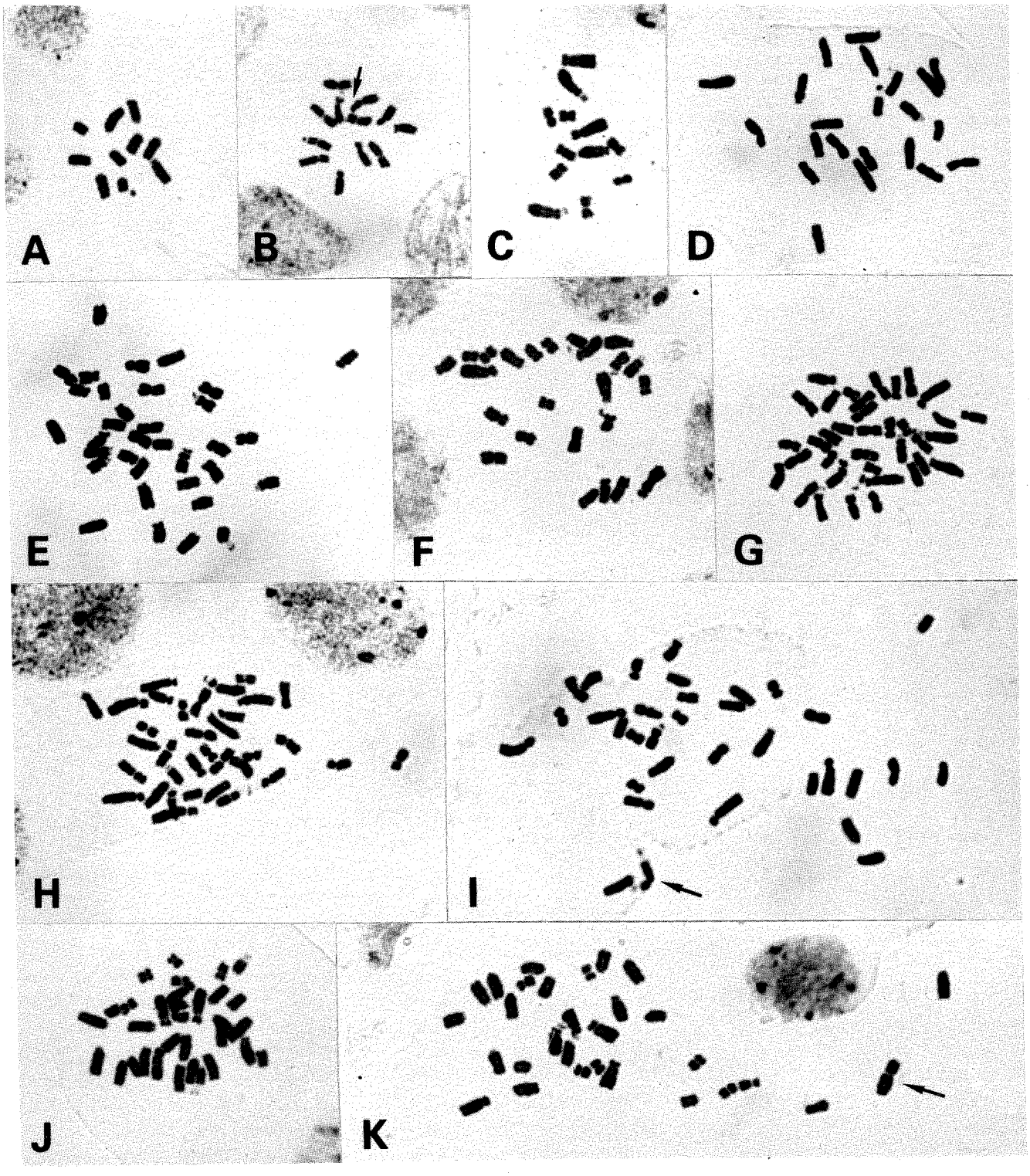
A
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Table 1. *Times of shoot emergence (days from beginning of culture) and genome constitutions of regenerated plants in fusions involving protoplasts of haploid* ***N. plumbaginifolia, N. sylvestris,*** *and* ***N. otophora***

Genome constitution	No. of calli				
	46-55	56-65	66-75	Total	%
P	0	1	1	2	2.9
PP	0	4	5	9	12.9
PPP	0	0	1	1	1.4
PPPP	0	1	0	1	1.4
PS	4	8	3	15	21.4
PPS	1	1	0	2	2.9
PSS	1	1	0	2	2.9
PPSS	2	1	0	3	4.3
PS/PPSS	0	1	0	1	1.4
PPS/PPSS	0	1	0	1	1.4
SO	8	3	1	12	17.1
SSO	3	0	0	3	4.3
SOO	2	0	0	2	2.9
SSOO	4	3	0	7	10.0
SSSO	1	0	0	1	1.4
SO/SSOO	4	0	0	4	5.7
SSO/SSOO	0	1	0	1	1.4
PSO	1	1	0	2	2.9
PPPSO	0	1	0	1	1.4
Total	31	28	11	70	100.0

Table 2. *Times of shoot emergence (days from beginning of culture) and genome constitutions of regenerated plants in fusions involving protoplasts of haploid* ***N. plumbaginifolia*** and ***N. otophora***

Genome constitution	No. of calli					
	36-45	46-55	56-65	66-75	Total	%
P	0	1	0	0	1	1.2
PP	0	22	15	1	38	44.7
PPP	0	11	10	2	23	27.0
PPPP	0	8	1	4	13	15.3
PPPPP	0	1	0	1	2	2.3
PPPPPP	0	1	0	0	1	1.2
PP/PPP	0	0	1	0	1	1.2
OO	0	0	1	0	1	1.2
PPO	2	2	0	0	4	4.7
PPPPOO	1	0	0	0	1	1.2
Total	3	46	28	8	85	100.0

to plants containing genomes of *N. plumbaginifolia* and *N. sylvestris* (Figs. 1D, 1E), 30 (42.8%) to plants containing genomes of *N. sylvestris* and *N. otophora* (Figs. 1F, 1G), and 3 (4.3%) to plants having genomes of three species (Figs. 1H, 1I). No hybrids between *N. plumbaginifolia* and *N. otophora* were observed. Comparisons of the time of shoot emergence from the calli showed that hybrid shoots emerged earlier than shoots of *N. plumbaginifolia*. A few calli, such as PS/PPSS and PPS/PPSS, produced plants of two different genome constitutions.

The results from protoplast fusions between *N. plumbaginifolia* and *N. otophora* are shown in Table 2. Among the 85 calli in which plant regeneration occurred, 5 (5.9%) were somatic hybrids with PPO (Figs. 1J, 1K) and PPPPOO genome constitutions, a great majority of the rest being *N. plumbaginifolia*. Table 2 also indicated that hybrid shoots emerged earlier than those of parental species.

Aneuploidy and chromosome structural changes were common in somatic hybrids but relatively infrequent in *N. plumbaginifolia* plants regenerated from protoplasts. The most frequent structural changes were deficiencies and translocations (Fig. 1I). An unusual chromosome change observed in hybrids PPO and PSO was fusion of the centromeres of two telocentric or acrocentric chromosomes of the P genome (Fig. 1K). The maximum number of fused chromosomes in a cell was two, and the morphology of these chromosomes differed, indicating that several different chromosomes in the P genome could participate in fusion.

Meiotic Chromosome Associations

Chromosome associations at diakinesis and/or metaphase I in parental species and somatic hybrids with normal karyotypes are summarized in Table 3. Apart from the formation of low frequencies of univalents, meiosis in diploid plants of the parental species was generally normal. Among the three *Nicotiana* species, *N. otophora* had the highest frequency of univalents and the lowest frequency of fertile pollen. The univalents were formed probably as a result of failure of chiasma formation or of precocious chiasma terminalization in the small bivalents.

There was essentially no chromosome pairing in haploid plants of the parental species (Figs. 2A-2C). The univalents were either scattered in the cells or as-

sociated in groups with each group consisting of 2 to several univalents. The univalents in each group were often connected by chromatin strands but no chiasmata were evident (Fig. 2A). Pollen fertilities of the haploid plants were low.

The two diploid somatic hybrids, PS and SO, also showed predominantly univalent formation (Figs. 2D, 2E) and low pollen fertility. However, they differed in that pairing appeared to be better in PS than in SO. Groupings of univalents were also observed in the diploid hybrids (Figs. 2D, 2E).

All triploid hybrids except PSS showed a similar pattern of chromosome pairing. In these hybrids, the homologous chromosomes of the duplicated genome paired as bivalents whereas the chromosomes of the single genome formed univalents (Figs. 2F, 2G). Hybrid PSS differed from the others in that it had slightly better chromosome pairing but extremely low pollen fertility.

Tetraploid hybrids PPSS and SSOO formed predominantly bivalents during meiosis (Fig. 2H). Despite its diploid-like meiotic behavior, hybrid PPSS showed low pollen fertility.

Discussion

Preferential Recovery of Somatic Hybrids

The preferential recovery of somatic hybrids in the absence of artificial selection may be attributed to several causes. Since the protoplast culture method used was appropriate for all three *Nicotiana* species (Huang and Chen, 1988; Chen, unpublished results), the recovery of no or few *N. sylvestris* and *N. otophora* plants suggests that haploid protoplasts of these two species may be more sensitive to PEG compared with those of *N. plumbaginifolia*. There are two explanations for the early appearance of hybrid shoots and late appearance of the shoots of *N. plumbaginifolia*. First, hybrid cells may grow and differentiate faster as a result of hybrid vigor (Smith *et al.*, 1976; Schieder, 1978, 1980; O'Connell and Hanson, 1987). Alternatively, slow plant regeneration may be the inherent property of the protoplasts of *N. plumbaginifolia*. All the hybrids between *N. plumbaginifolia* and *N. otophora* had a genome constitution of either PPO or PPPPOO. This suggests that the hybrids are viable only when the ratio of P to O is 2:1.

Table 3. *Chromosome associations at diakinesis and/or metaphase I in parental species and somatic hybrids*

Genome constitution	$2n$	No. of PMCs	Chromosome association I mean	I range	II mean	II range	III mean	III range	Pollen fertility (%)
P	10	53	9.96	9–10	0.02	0–1	0	0	5.2
S	12	61	11.97	11–12	0.02	0–1	0	0	5.4
O	12	54	11.85	11–12	0.07	0–1	0	0	6.2
PP	20	60	0.07	0–2	9.97	9–10	0	0	92.8
SS	24	52	0.50	0–4	11.75	10–12	0	0	96.6
OO	24	52	1.08	0–6	11.46	9–12	0	0	45.7–88.3
PS	22	60	18.30	14–22	1.80	0–4	0.03	0–1	8.0
SO	24	62	22.77	16–24	0.61	0–4	0	0	4.3
PPS	32	58	11.38	6–12	10.31	10–13	0	0	11.5
PSS	34	54	8.25	3–12	12.81	11–15	0.04	0–1	0.8
SSO	36	53	11.43	8–14	12.26	11–14	0	0	36.9
SOO	36	53	11.89	8–14	12.06	10–14	0	0	25.5–44.6
PPO	32	58	11.48	6–14	10.26	9–13	0	0	15.9
PPSS	44	50	0.52	0–6	21.74	19–22	0	0	10.4
SSOO	48	49	0.53	0–4	23.73	22–24	0	0	85.3

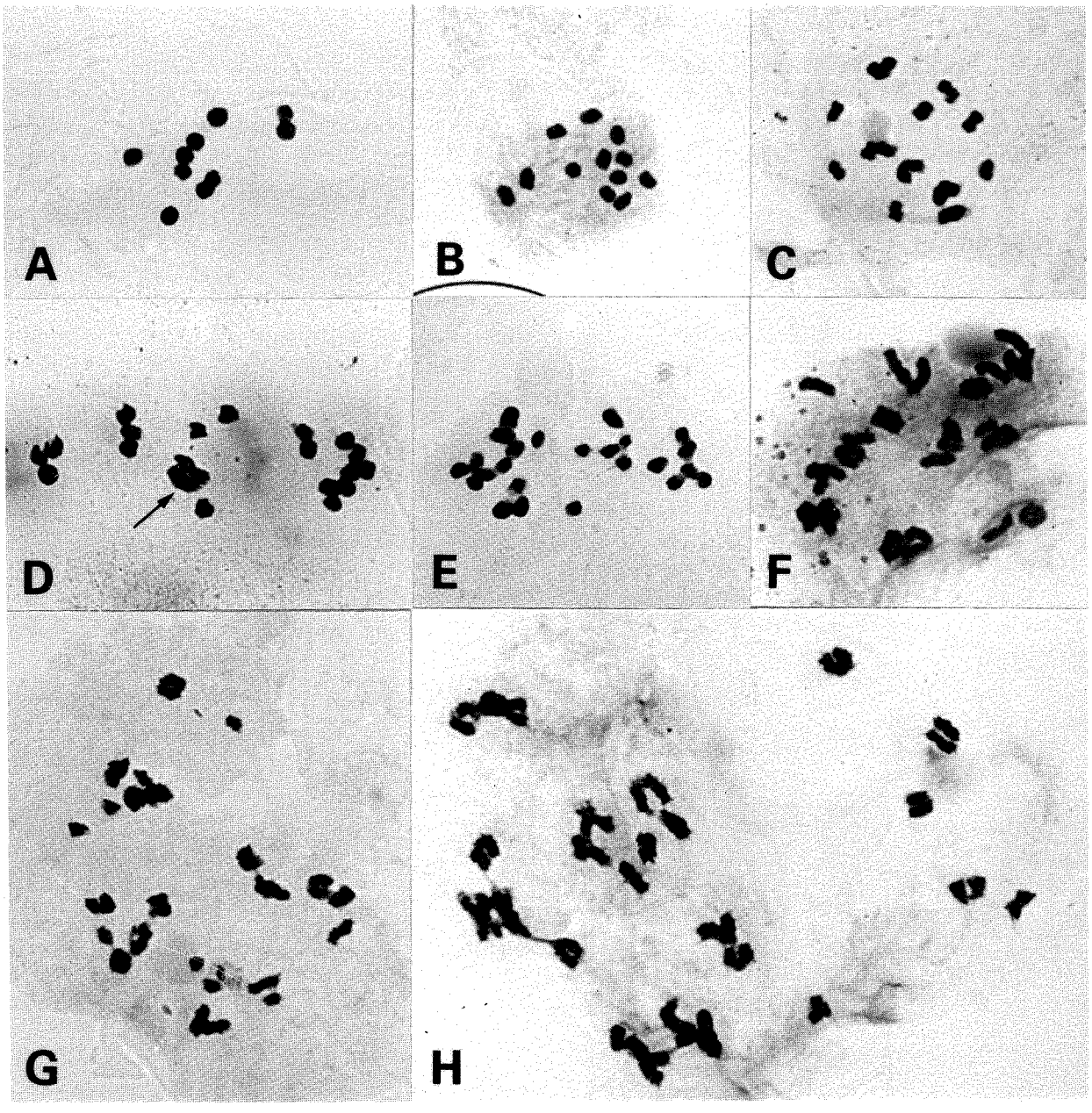

Fig. 2. Chromosome associations at diakinesis or MI in haploid plants of parental species and somatic hybrids. (A) Haploid *N. plumbaginifolia* showing 10 I. (B) Haploid *N. sylvestris* showing 12 I. (C) Haploid *N. otophora* showing 12 I. (D) Somatic hybrid PS showing 2 II + 18 I. The bivalents are indicated by an arrow. (E) Somatic hybrid SO showing 24 I. (F) Somatic hybrid PSS showing 12 II + 10 I. (G) Somatic hybrid SSO showing 12 II + 12 I. (H) Somatic hybrid SSOO showing 24 II. X 1100.

Origins of Chromosome Variation in Somatic Hybrids

Although the protoplasts were isolated from haploid plants, large numbers of somatic hybrids recovered from the fusion experiments were polyploids. Triple protoplast fusion is the most likely explanation for origin of the triploid calli, although other possibilities cannot be excluded. Because quadruple protoplast fusion is rare (Lee and Chen, 1990), the occurrence of high frequencies of tetraploid calli, such as PPSS, and chimeric calli consisting of diploid and tetraploid cells, such as

PS and PPSS, is attributed to spontaneous chromosome doubling of diploid hybrid cells during culture. Likewise, hexaploid callus PPPPOO may originate from a triploid hybrid cell through the same mechanism. "Chimeric" calli PPS/PPSS and SSO/SSOO could be the result of joint development of two different hybrid cells which happen to be adjacent in the agarose plate. The origins of calli SSSO and PPPSO are difficult to explain.

We have found that aneuploidy and chromosome structural changes are more common in somatic hybrids than in *N. plumbaginifolia* plants regenerated from protoplasts. This suggests that hybridity is a cause for the occurrence of these abnormalities. The centric fusions observed in hybrids PPO and PSO are very similar to those reported in mice (Chen and Ruddle, 1971; Lin *et al.*, 1974) in that the chromosomes (*N. plumbaginifolia*) participating in fusion are telocentric or acrocentric and possess constitutive heterochromatin near the centromeres (Chen, unpublished results). The fact that they occur only when the O genome is present suggests that the large heterochromatin blocks of *N. otophora* (Burns, 1966; Wang and Chen, 1988) may be responsible.

Origins and Relationships of Parental Species

Based on the occurrence of bivalents and secondary associations of univalents in haploid plants of the species with 12 pairs of chromosomes, Kostoff (1942) suggested that the basic number in *Nicotiana* was six. Goodspeed (1954) reached the same conclusion as he observed 4 to 8 bivalents in interspecific hybrids between distantly related species of the genus. In the present study, although bivalents were present in haploid plants of the parental species and in diploid somatic hybrids, the frequencies were too low (see Table 3) to be evidence for chromosome homology within the haploid complements. Furthermore, we found that the association of univalents in groups were not chromosome-specific. Because all three *Nicotiana* species possess heterochromatin (Merritt, 1974), we believe that secondary associations may be the result of chance fusion or stickiness of the heterochromatin of nonhomologous chromosomes (Riley and Chapman, 1957; Collins and Sadasivaiah, 1972). Thus, in contrast to the observations of Kostoff (1942) and Goodspeed (1954), the cytological data obtained in this study suggest that the *Nicotiana* species with 12 pairs and aneuploid numbers of chromosomes are true diploids. This hypothesis is consistent with the results of extensive genetic analyses of two biochemical mutants, valine uptake deficiency (Bourgin *et al.*, 1985; Marion-Poll *et al.*, 1988) and the nia type of nitrate reductase deficiency (Müller, 1983; Gabard *et al.*, 1987). These studies showed that the mutant phenotypes were recessive and were transmitted to the progeny as monogenic Mendelian traits in *N. plumbaginifolia* ($2n=20$) but as digenic traits in *N. tabacum* ($2n=48$).

The preponderance of univalents in the diploid hybrid PS and the rarity of multivalents in polyploid hybrids PPS, PSS, and PPSS suggest that there is little homology between genomes P and S. Likewise, there is no homology between S and O. Interspecific hybrids between *N. sylvestris* and *N. otophora* were also obtained from sexual crosses and chromosome associations in the hybrids were studied (Goodspeed, 1954; Takenaka, 1956). The frequencies of bivalents in the sexual hybrids appear to be higher than that observed in this study. This discrepancy is likely caused by the utilization of different criteria for classification of chromosome associations. The only somatic hybrid between *N. plumbaginifolia* and *N. otophora* raised to the flowering stage was a triploid with PPO genome constitution. Analysis of chromosome associations in this hybrid suggests that there is no homology between the genomes of these two species.

Potential Applications of Somatic Hybrids

In addition to providing information concerning genome relationships, the somatic hybrids obtained in this study have many other potential applications. Because the somatic hybrids were isolated in the absence of artificial selection, they should be ideal materials for investigations on the randomness of organelle segregation. Moreover, the availability of interspecific somatic hybrids at various ploidy levels, such as PS, PPS, PSS, and PPSS, offers a unique opportunity to study the influence of nuclear genomes on segregation of cytoplasmic organelles. It would also be interesting to investigate the chloroplasts and mitochondria in the somatic hybrids derived from fusion of protoplasts of three different species.

The triploid somatic hybrids, such as PPS, PSS, SSO, SOO and PPO, are useful materials for cytogenetic studies. For example, from crosses of each triploid hybrid with its respective diploid parent a

series of chromosome addition lines could be established and used for gene mapping.

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菸草屬體細胞雜種細胞學之研究

林榮芳　陳其昌

國立臺灣大學植物學系

將單倍體*Nicotiana plumbaginifolia* (P)、*N. sylvestris* (S) 及 *N. otophora* (O) 的葉肉原生質體混合液以 polyethylene glycol 處理，培養之後得到各種染色體組 (genome) 組成的體細胞雜種。一些體細胞雜種在染色體數目及構造上發生了變異。在染色體組組成爲 PPO 及 PSO 的體細胞雜種中，常見到兩條末端中節的 *N. plumbaginifolia* 染色體在中節處融合，變成了一條中位或次中位中節的染色體。三種菸草的單倍體植物在減數分裂時均形成單價體，顯示染色體組內沒有同源性。分析體細胞雜種減數分裂時染色體配對的行爲，發現三種菸草的染色體組間也沒有同源性。文中並討論了這些體細胞雜種在遺傳學研究上的用途。