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Chloroplast segregation in somatic hybrids of *Nicotiana plumbaginifolia* and *N. sylvestris* having different ratios of parental nuclear genomes

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Summary. Fusion of mesophyll protoplasts of haploid *Nicotiana plumbaginifolia* (P) and *N. sylvestris* (S) resulted in the production of somatic hybrid plants of various ploidy levels. Analysis of the restriction fragment patterns of chloroplast DNA from 118 plants belonging to genome constitutions PS, PPS, PSS, and PPSS revealed that two had a pattern corresponding to a mixture of parental DNA while all the others had the pattern of either *N. plumbaginifolia* or *N. sylvestris*. In the latter case, the ratio of the two parental types fits 1:1 in all the four genome constitutions studied. Since the protoplasts used in the fusion experiment were physiologically similar and the hybrid cells were not deliberately selected, these results suggest that chloroplast segregation in the somatic hybrids is independent of the chloroplast input of the fusion partners and the nuclear background of the fusion products.

Introduction

The analysis of chloroplasts in somatic hybrids of higher plants has revealed that following protoplast fusion there is rapid segregation of parental chloroplasts. In some cases segregation of chloroplasts seems to be random (Chen et al. 1977; Belliard et al. 1978; Scowcroft and Larkin 1981; Sundberg et al. 1987), while in other cases it is biased (Kumar et al. 1982; Sundberg et al. 1991; Sundberg and Glimelius 1991; Li and Sink 1992). The mechanism involved in chloroplast segregation is not fully understood.

Recently, two studies have suggested that the proportion of parental nuclear genomes in the fusion products influences chloroplast segregation. Sundberg and Glimelius (1991) compared the restriction fragment patterns of chloroplast DNA (cpDNA) from plants recovered from protoplast fusions between tetraploid *Brassica napus* and diploid related species and found that chloroplast segregation was biased towards the type of the tetraploid species. Bonnema et al. (1992) studied the cpDNA of plants recovered from fusions of mesophyll protoplasts of *Lycopersicon esculentum* with suspension cell protoplasts of *L. pennellii* that had been irradiated with different doses of ^{60}Co. They found that chloroplast segregation was random in symmetric hybrids but biased in asymmetric hybrids, the degree of bias being correlated with the extent of nuclear asymmetry. However, in these studies involvement of other factors affecting chloroplast segregation has not been excluded.

We have shown that the somatic hybrids of *Nicotiana plumbaginifolia* (PP) and *N. sylvestris* (SS) are more vigorous and grow faster than both parents during culture (Lee and Chen 1992). Thus, when leaf protoplasts isolated from haploid plants of these two species were fused, large numbers of somatic hybrids differing in the ratio of parental nuclear genomes (such as PS, PPS, PSS, and PPSS) could be recovered without the application of selection (Lin and Chen, 1990). These somatic hybrids are particularly useful for investigation of the effect of parental nuclear genome on chloroplast segregation. We therefore reestablished the somatic hybrids and analyzed the restriction fragment patterns of cpDNA of these hybrids. The results of these studies are presented in this paper.

Materials and Methods

Plant material. Haploid plantlets of *Nicotiana plumbaginifolia* Viviani and *N. sylvestris* Spegazzini & Comes 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. Protoplasts were isolated from leaves of in vitro cultured haploid plantlets as described by Huang and Chen (1988). They were washed once with W5 solution (Medgyesy et al. 1980) and resuspended in

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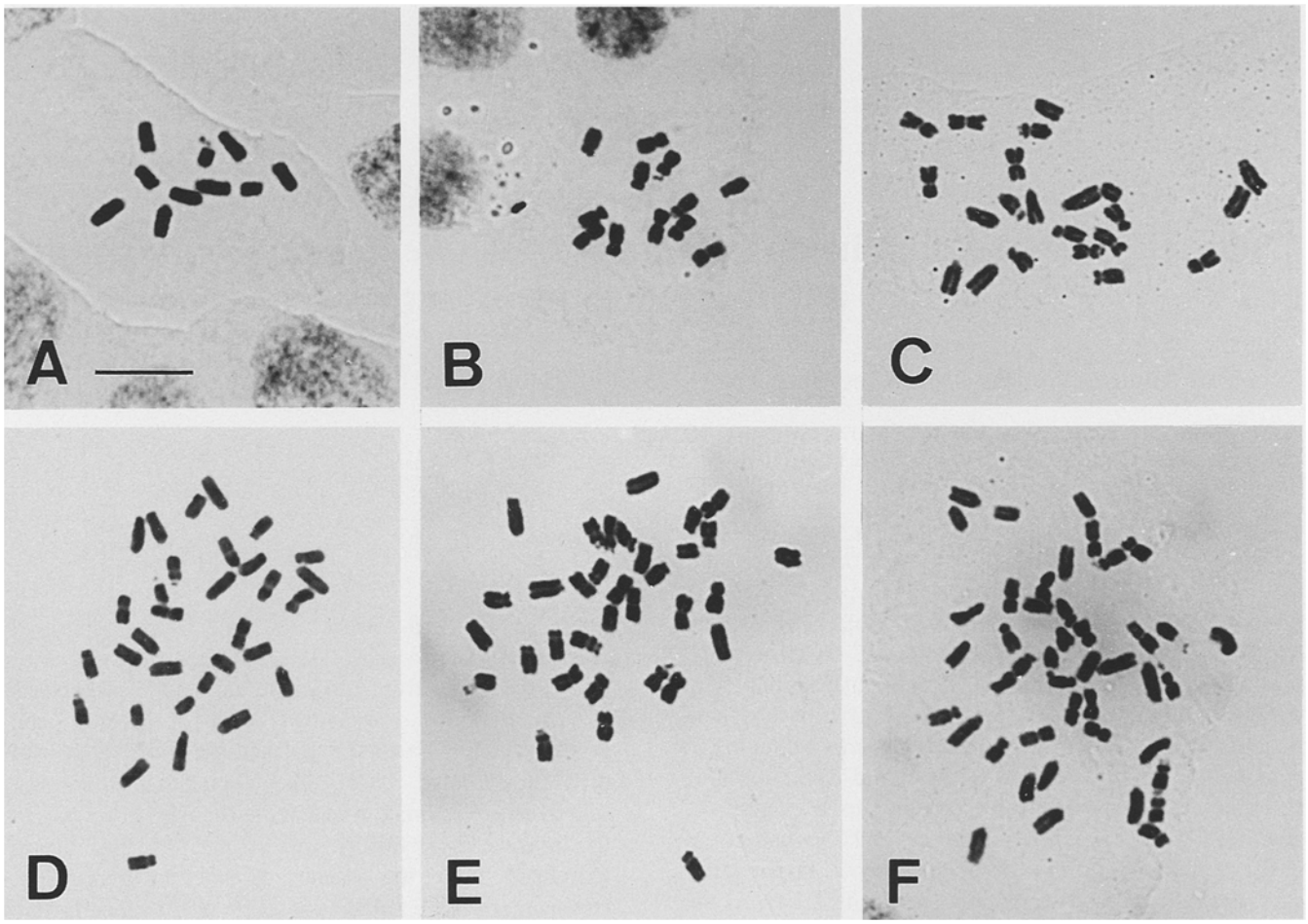

Fig. 1. Somatic metaphase chromosomes of parental species and somatic hybrids. (A) Haploid *Nicotiana plumbaginifolia* (P) showing 10 chromosomes. (B) Haploid *N. sylvestris* (S) showing 12 chromosomes. (C) Somatic hybrid PS, $2n = 22$. (D) Somatic hybrid PPS, $2n = 32$. (E) Somatic hybrid PSS, $2n = 34$. (F) Somatic hybrid PPSS, $2n = 44$. Bar = 10 μm.

the same solution at a density of 2 x 10^5/ml. Protoplast suspensions of the two *Nicotiana* species were mixed at equal volumes. The protoplast mixture was treated with polyethylene glycol (PEG) and cultured as described by Lee and Chen (1990). No selection against parental cells was applied during culture.

Chromosome preparation. Somatic chromosomes were prepared from root-tip cells of in vitro grown plantlets. 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, hydrolyzed with 1 N HCl at 60°C for 7 min, stained in leuco basic fuchsin for 1 h, and treated with 1% pectinase for 1 h. Root tips were squashed in 45% acetic acid.

Restriction endonuclease analysis of cpDNA. When in vitro cultured plantlets reached the stage of 5-6 leaves, they were transplanted to pots for further growth. Three weeks later, cpDNA was isolated from leaves of potted plants (To et al. 1992) and digested with appropriate restriction enzymes under the conditions as recommended by the manufacturer (Boehringer Mannheim). The restriction fragments were separated on 0.8% agarose gel and stained with ethidium bromide.

Results and Discussion

Karyotypes of parental species. Karyotypes of the two parental species have been described previously (Goodspeed 1954; Lin and Chen 1990; Lee and Chen 1992). Haploid *N. plumbaginifolia* has 5 large, 3 medium, and 2 small chromosomes; all of them are telocentric or acrocentric, and one small chromosome possesses a satellite (Fig. 1A). The 12 chromosomes of haploid *N. sylvestris* are rather uniform in size and are metacentric or submetacentric; two chromosomes have a satellite (Fig. 1B). Thus, karyotypes of the two parental species differ markedly, and these differences could be used as a basis to determine genome constitutions of plants regenerated from protoplast fusion.

Genome constitutions of regenerated plants. More than 3,000 calli were recovered from protoplast fusion between haploid *N. plumbaginifolia* and *N. sylvestris*, but only 259 fast regenerating calli were selected for further studies. From each of these calli 2 to 6 shoots were

transferred to hormone-free medium for root formation. Genome constitutions of plants regenerated from the 259 calli are presented in Table 1. Among these calli, one gave rise to *N. plumbaginifolia* plants, 256 to hybrid plants of varying ploidy levels such as PS, PPS, PSS, PPSS, etc. (Fig. 1C-F), and two (PP/PSS and SS/PPSS) to both hybrid and parental plants. These results confirm our previous study (Lee and Chen 1992) that hybrid cells of these two species grow and develop faster than both parents. In addition to changes in ploidy level, loss of 1 or 2 chromosomes and of chromosome segments was observed in a small fraction of the somatic hybrids.

Table 1. Genome constitutions of calli derived from fusion of mesophyll protoplasts of haploid *Nicotiana plumbaginifolia* (P) and *N. sylvestris* (S)

Genome constitution	No. of calli	% of calli
PP	1	0.39
PS	132	50.97
PPS	11	4.25
PSS	7	2.70
PPSS	93	35.91
PSSS	1	0.39
PPSSS	2	0.77
PPPSS	2	0.77
PPPSSS	2	0.77
PPPPSS	1	0.39
PS/PPSS	5	1.93
SS/PPSS	1	0.39
PP/PSS	1	0.39
Total	259	100.00

Chloroplasts in somatic hybrids. Chloroplasts in the somatic hybrids PS, PPS, PSS, and PPSS were determined by the restriction fragment patterns of cpDNA digested with *Hind*III. Differentiation of parental patterns was due to the presence of an extra *Hind*III site in the cpDNA of *N. plumbaginifolia* (Yang et al. 1992). As a result, the 5.9 kb band in the *N. sylvestris* pattern was replaced by two bands of 5.4 kb and 0.5 kb in the pattern of *N. plumbaginifolia* (Fig. 2).

A total of 118 somatic hybrid plants regenerated from 32 calli were analyzed as to their cpDNA. Plants derived from one piece of callus had same or different restriction fragment patterns. Of the 118 plants, two had a pattern corresponding to a mixture of parental cpDNA, while all the others showed the pattern of either *N. plumbaginifolia* or *N. sylvestris* (Fig. 2). In the latter case, the ratio of the two patterns fits 1:1 in all the four classes of somatic hybrids (PS, PPS, PSS, and PPSS) studied (Table 2). These results were confirmd by analysis of the restriction fragment patterns of cpDNA digested with *Sma*I (data not shown).

Although the technique used in this study may not be sensitive enough to detect a minior fraction of

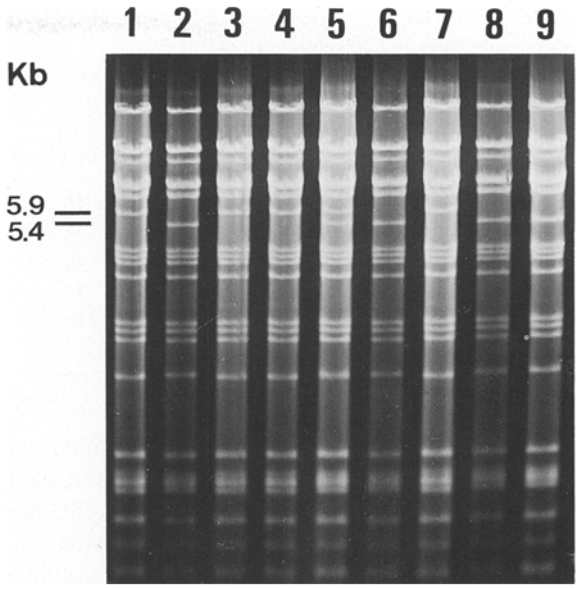

Fig.2. Restriction fragment patterns of *Hind*III digests of cpDNA isolated from *Nicotiana sylvestris* (lane 1), *N. plumbaginifolia* (lane 2), and their somatic hybrids (lanes 3-9). The sample in lane 5 represents a pattern corresponding to a mixture of parental cpDNA.

"contaminating" DNA, the rarity (2 out of 118) of plants with a mixture of parental DNA suggests rapid chloroplast segregation following protoplast fusion. The protoplasts of the two parents were physiologically similar as they were isolated from leaves of plantlets that were cultured under identical conditions and were the same age, and the hybrid cells were not deliberately selected. In addition, the numbers of somatic hybrid plants were sufficiently large for statistical analysis. For these reasons, the fit of the two parental chloroplast types to a 1:1 ratio in somatic hybrids PS, PPS, PSS, and PPSS is interpreted to indicate that the nuclear background has no significant effect on chloroplast segregation. This interpretation is consistent with Goodspeed's (1954) taxonomic treatment of *Nicotiana* in which the two parental species were placed in the same section (*Alatae*), and with the finding of Cséplö et al. (1984) that the chloroplasts of *N. sylvestris* can be successfully introduced into the nuclear background of *N. plumbaginifolia* via somatic cybridization.

Sundberg and Glimelius (1991) reported that in the somatic hybrids between tetraploid *Brassica napus* (AACC) and diploid *B. oleracea* (CC), *B. nigra* (BB), *Raphanus sativus* (RR), or *Eruca sativa* (EE), chloroplast segregation was biased favoring the type of *B. napus*. They interpreted that the biased segregation, besides being an effect of genetic divergence, could also be the result of an unequal input of organelles of the two fusion partners. In the present study, tetraploid PPSS could originate from quadruple protoplast fusion or from

Table 2. Chloroplast segregation in somatic hybrids derived from fusion of mesophyll protoplasts of haploid *Nicotiana plumbaginifolia* and *N. sylvestris*

Nuclear genome	No. of calli	No. of plants	Chloroplast genome			Chi- square analysis (1:1)	
			plum	syl	mixed	χ^2	P
PS	10	29	10	17	2	1.81	0.2-0.1
PPS	6	25	14	11	0	1.20	0.3-0.2
PSS	7	30	12	18	0	0.36	0.7-0.5
PPSS	9	34	21	13	0	1.88	0.2-0.1
Pooled	32	118	57	59	2	0.03	0.9-0.7
Homogeneity (3 df)						5.22	0.2-0.1

fusion of two protoplasts followed by chromosome doubling. Triple protoplast fusions are the most likely explanation for the origin of somatic hybrids PPS and PSS, although other possibilities such as fusion of a normal protoplast and an endoreduplicated protoplast (Chen et al. 1988) cannot be excluded. Assuming that mesophyll protoplasts of the two parental species contain approximately equal numbers of chloroplasts, the ratio of *N. plumbaginifolia* to *N. sylvestris* chloroplasts in the initial fusion products of PS (PPSS), PPS, and PSS would be 1:1, 2:1, and 1:2, respectively. However, as our results indicate, the distribution of chloroplasts in regenerated plants does not conform to this expectation, suggesting lack of effect of the initial chloroplast input on chloroplast segregation.

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References

Belliard G, Pelletier G, Vedel F, Quetier F (1978) Mol Gen Genet 165: 231-237

Bonnema AB, Melzer JM, Murray LW, O'Connell MA (1992) Theor Appl Genet 84: 435-442

Chen CC, Huang CR, To KY (1985) Bot Bull Acad Sin 26: 147-153

Chen CC, Lee FM, Kao YY (1988) Genome 30: 615-620

Chen K, Wildman SG, Smith HH (1977) Proc Natl Acad Sci USA 74: 5109-5112

Cséplö A, Nagy F, Maliga P (1984) Mol Gen Genet 198: 7-11

Goodspeed TH (1954) The genus *Nicotiana*. Chronica Botanica, Waltham, Massachusetts

Huang HC, Chen CC (1988) J Hered 79: 28-32

Kumar A, Cocking EC, Bovenberg WA, Kool AJ (1982) Theor Appl Genet 62: 377-383

Lee FM, Chen CC (1990) J Hered 81: 313-317

Lee FM, Chen CC (1992) Taiwania 37:11-18

Li Y, Sink KC (1992) Curr Genet 22: 167-171

Lin RF, Chen CC (1990) Bot Bull Acad Sin 31: 179-187

Medgyesy P, Menczel L, Maliga P (1980) Mol Gen Genet 179: 693-698

Negrutiu I, Mousseau J (1980) Z Pflanzenphysiol 100: 373-376

Scowcroft WR, Larkin PJ (1981) Theor Appl Genet 60: 179-184

Sundberg E, Glimelius K (1991) Theor Appl Genet 83: 81-88

Sundberg E, Lagercrantz U, Glimelius K (1991) Plant Sci 78: 89-98

Sundberg E, Landgren M, Glimelius K (1987) Theor Appl Genet 75: 96-104

To KY, Lai YK, Feng TY, Chen CC (1992) Genome 35: 220-224

Yang DI, Feng TY, Chen CC, Lai YK (1992) Theor Appl Genet 83: 515-527