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Limited chloroplast gene transfer via recombination overcomes plastome-genome incompatibility between *Nicotiana tabacum* and *Solanum tuberosum*

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Abstract

Green cybrids with a new nucleus-chloroplast combination cannot be selected after protoplast fusion in the intersubfamilial *Nicotiana-Solanum* combination. As an approach to overcome the supposed plastome-genome incompatibility, a partial plastome transfer by genetic recombination has been considered. After fusions of protoplasts of a light-sensitive *Nicotiana tabacum* (tobacco) plastome mutant and lethally irradiated protoplasts of wild-type *Solanum tuberosum* (potato), a single green colony was recovered among 2.5×10^4 colonies. The regenerated plants had tobacco-like (although abnormal) morphology, but were normally green, and sensitive to tentoxin, demonstrating chloroplast markers of the potato parent. Restriction enzyme analysis of the chloroplast DNA (cpDNA) revealed recombinant, nonparental patterns. A comparison with physical maps of the parental cpDNA demonstrated the presence of a considerable part of the potato plastome flanked by tobacco-specific regions. This "potacco" plastome proved to be stable in backcross and backfusion experiments, and normally functional in the presence solely of *N. tabacum* nucleus.

Introduction

One of the most stimulating areas of research into cell organization and phylogenesis is the development and mechanism of the delicately concerted interplay between nuclear and organelle genomes [3, 30]. The challenge is to explore its genetic background concealed by a network of pleiotropic nucleus-organelle-environment interactions. One of the approaches that may be adopted is the analysis of plants possessing functional chloroplasts with a plastome derived entirely or partially from a very different species. Both traditional and somatic cell genetics offer methods for the creation of such cybrids, but there are still major limitations to their application. In genera with biparental organelle inheritance (most thoroughly studied in *Oenothera*) even interspecific crosses may result in various chlorophyll deficiencies, interpreted as different forms of a genome-plastome incompatibility [19, 43]. Protoplast fusion techniques have opened the way to chloroplast transfer between species from distinct genera or tribes [14, 18, 42], but the failure to produce functional *Nicotiana-Solanum* cybrids [42] also suggests a taxonomical barrier to the transfer of entire chloroplast genomes in more remote species

combinations. In the unicellular-uniplastidic green alga *Chlamydomonas*, where sexual mating naturally involves the fusion of the parental chloroplasts, chloroplast DNA recombination has been used extensively for mapping different plastid mutations genetically [33, 12] and physically [21, 22]. In higher plants an obvious barrier to the exchange of plastid genes by sexual crosses is the maternal (cytoplasmic) inheritance of organelles in most species [43, 36, 5]. Furthermore, interorganellar chloroplast DNA recombination appears to be a rare phenomenon, because no spontaneous appearance of genetic recombination of plastids was detectable, even if the plastid markers used allowed visual identification of subgroups of cells with recombinant plastids, either after protoplast fusion [10], or in sexual crosses of species with biparental organelle inheritance [4]. A stringent selection for the recombination of different parental plastid markers in protoplast fusion derived cell cultures did result in the isolation of a single *N. tabacum-N. plumbaginifolia* somatic hybrid plant possessing stable recombinant chloroplasts [24].

Biochemical analysis of this plastome demonstrated numerous homologous recombinations all around the genome [24, 9]. This result raised the prospect that disturbances in the cooperation between the nuclear and plastid genomes in remote species combinations might be overcome by a partial plastome transfer via genetic recombination. This approach, whereby a particular mosaic of the parental plastid genes in the recombinant plastome might maintain cooperativity to one parental nucleus, while allowing substantial plastid gene transfer from the other parent, has been attempted between *N. tabacum* and *S. tuberosum*, species from different subfamilies [6] of the family Solanaceae.

Materials and methods

Plant material

Wild-type *Solanum tuberosum* cv. Cardia was maintained as shoot cultures on RM salts [28] with 2% sucrose and 0.7% Bacto-Agar (RM medium) supplemented with choline dichloride (CCC) (0.3 mg/ml), in light (1500 lx, 16-h day, 26 °C). A light-sensitive plastid mutant (LS1) of *Nicotiana tabacum* cv. Turkish Samsun [13] was maintained as shoot cultures on RM medium supplemented with thiamine-HCl (1 mg/ml) and 1-naphthaleneacetic acid (0.1 mg/ml), under low light (400 lx, 16-h day).

Protoplast fusion and cell culture

Protoplasts were isolated from leaves [26], a procedure facilitated in the case of potato by a substantial increase in leaf area of aseptically cultured plants on CCC-containing medium. Those of the appropriate partner were irradiated with a dose of 200 J/kg (0.066 J/kg·s dose rate) of ^{60}Co gamma rays [27]. Protoplasts were fused as described previously [26], with the exception of the time of polyethylene glycol treatment (1–2 min). Cultures were grown in liquid medium for five weeks, after which green colonies were isolated in solid medium, and plants were regenerated, as described previously [25, 42].

Chloroplast DNA analysis

Chloroplasts were isolated from leaves of aseptically grown plants in a high ionic strength homogenization buffer as described [2]. CpDNA was purified by phenol-chloroform deproteinization [23], digested with restriction enzymes according to the instructions of the supplier (Boehringer Mannheim GmbH), separated by agarose (0.3% and 1.6%) gel electrophoresis (1.75 V/cm, 18 h), and stained with ethidium bromide.

Results

Production of normal tobacco plants with genetically recombinant chloroplasts

Selection of green colonies following fusions of protoplasts of a light-sensitive *N. tabacum* plastome mutant (LS1) and lethally irradiated protoplasts of wild-type *S. tuberosum* led to the recovery of one cell line among 2.5×10^4 bleached colonies. This green

callus showed apparently no regeneration capability. In a small-scale experiment, therefore, irradiated protoplasts of this callus were again fused with those of the LS1 mutant. The single green cell line obtained produced green, tobacco-like shoots, but with an abnormal morphology. Considering that the production of a plant with a completely normal morphology is essential to exclude a possible partial nuclear hybridity, irradiated protoplasts of this fusion derived plant were again backfused to the LS1 mutant. Of the 72 green colonies which appeared among 3.0 $\times$ 10^4 bleached ones, several produced shoots identical to those of wild type *N. tabacum*. In the meantime the original green callus, after numerous subcultures on regenerating medium, also produced tobacco-like shoots. Plants from each of the three sequential fusion experiments remained green after transfer to the greenhouse, but the first line had very distorted leaves, the second one had a slightly abnormal morphology, and only the third one chosen for further investigation was completely normal. F_1 progeny of the three lines proved to be sensitive to (bleached by) tentoxin (20 mg/l), as is characteristic of *S. tuberosum*, in contrast to *N. tabacum*, which is naturally resistant to this phytotoxin [8]. This marker of the chloroplasts, together with their normal color, suggested the presence of recombinant chloroplasts in the plants. Backcross ((R $\times$ Nt) $\times$ Nt) progenies of each of the three regenerates were normal green either on RM medium or in soil (500 seedlings examined in each cross).

Restriction enzyme analysis of the recombinant "potacco" plastome

Restriction enzyme analysis of cpDNA of the plants with each of *Bam* HI, *Pvu* II, *Xho* I (Fig. 1), *Kpn* I, *Pst* I, *Sal* I, *Sma* I (not shown) revealed recombinant, nonparental fragmentation patterns, containing some parent-specific fragments from both tobacco and potato, in addition to nonparental and common fragments. A comparison of the three regenerates and their F_1 progenies (six plants tested) demonstrated identical patterns with *Bam* HI, *Pst* I, *Pvu* II, *Sal* I, *Sma* I, and *Xho* I. No difference was detected between wild-type *N. tabacum*

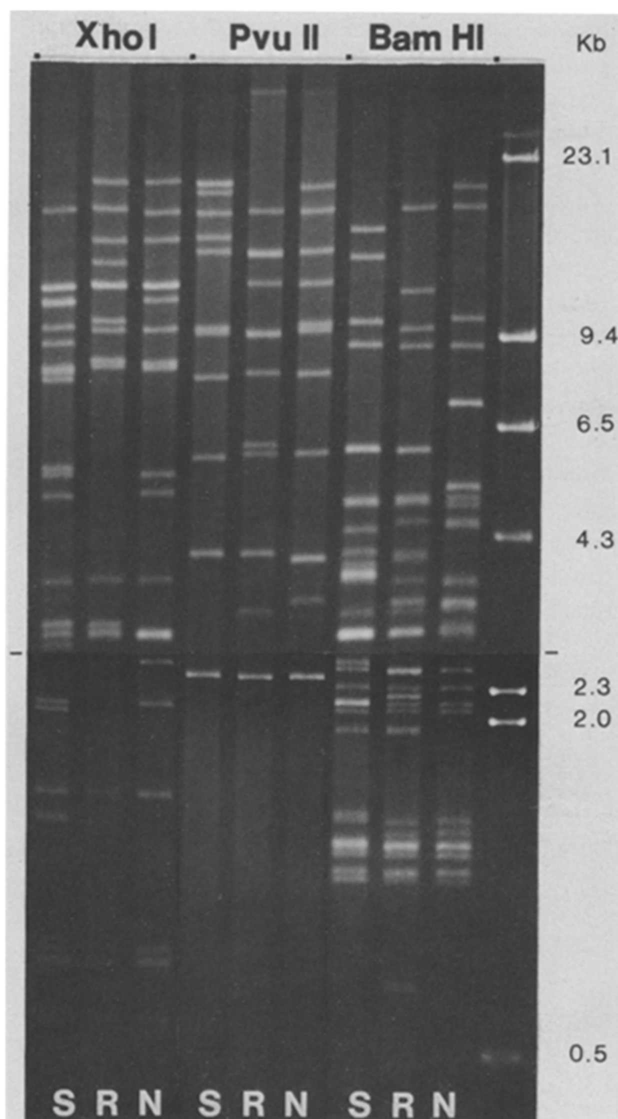

Fig. 1. Restriction endonuclease fragment patterns of chloroplast DNA from *Solanum tuberosum* (S), a tobacco plant with recombinant chloroplasts (R), and *Nicotiana tabacum* (N). On the right a lambda *Hin*d III digest is also shown. Fragments larger and smaller than 2.5 kb were separated in 0.3% and 1.6% agarose gels, respectively.

and the LS1 mutant with *Bam* HI, *Pvu* II, *Sal* I, and *Xho* I (data not shown). Scoring of individual parent-specific bands in the recombinant cpDNA restriction patterns indicated that only one of the alternative polymorphic parental DNA regions was present, if the region remained parental. A preliminary identification of the position of one or other

of the parental genome parts on the recombinant plastome was feasible due to the availability of physical maps of both *N. tabacum* [37, 11, 34, 16, 41] and *S. tuberosum* [15] chloroplast DNA. The physical map of *Lycopersicon esculentum* cpDNA [31, 32] was also of use because the number and size of tomato cpDNA fragments generated by certain restriction endonucleases are identical with or similar to those of *S. tuberosum* [35]. A comparison of the recombinant cpDNA fragmentation patterns, generated by different enzymes, with the parental patterns and physical maps, demonstrated the presence of numerous potato-specific restriction sites in the inverted repeat region, flanked by tobacco-specific regions (Fig. 2). Further smaller recombination sites in the large single-copy region might also be concluded from a comparison of the proper restriction fragment polymorphisms (Fig. 1).

Discussion

Functional *Nicotiana-Solanum* cybrids with a new nucleus-chloroplast combination could not be selected [42], suggesting an incompatibility of the entire nuclear and plastid genomes in this intersubfamilial combination. *Nicotiana-Solanum* somatic hybrids, however, have a normal green color [38]. During the selection of *N. tabacum* cell lines with recombinant chloroplasts, based on their greening ability, therefore, elimination of the *S. tuberosum* chromosomes was of crucial importance. Ionizing irradiation is effective in the elimination of the irradiated nuclei in protoplast fusion experiments [45, 27], but at least some of the fusion derived plants could be partial hybrids [27]. In the present experiment, regeneration problems and abnormal morphologies in the selected lines, therefore, necessitated repeated backfusions to *N. tabacum* until a completely normal plants was obtained. The lack of appearance of

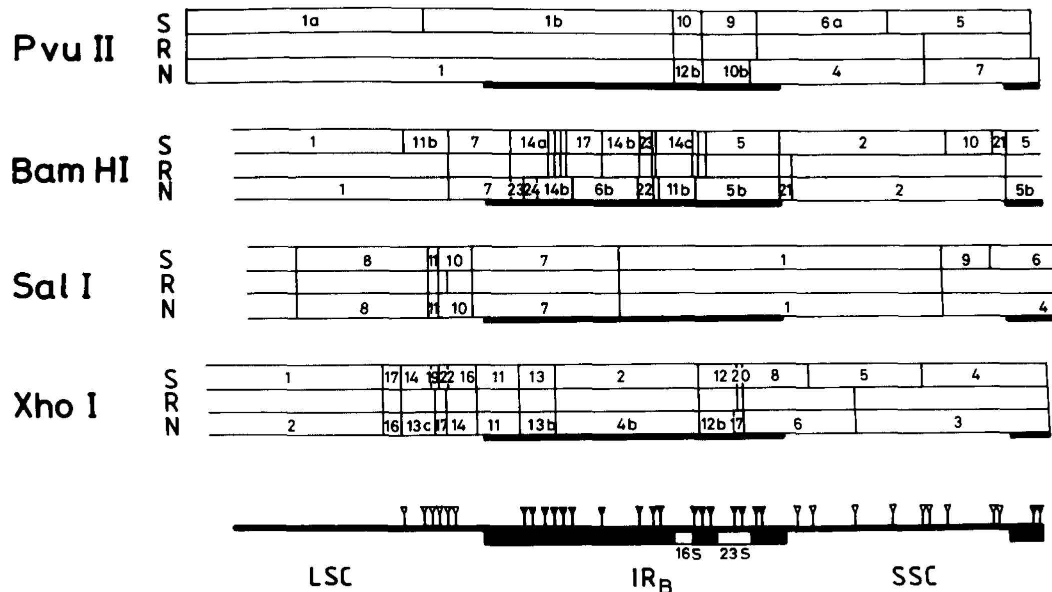

Fig. 2. Comparative restriction endonuclease cleavage site maps of a part of the chloroplast DNA from *Solanum tuberosum* (S), the chloroplast recombinant (R), and *Nicotiana tabacum* (N). The recombinant map was constructed from a comparison of the parental and recombinant restriction fragment patterns with physical maps of tobacco [37, 16, 41], potato [15], and tomato [31, 32, 35] cpDNA. Numbering of the fragments follows the original publications. The inverted repeat regions are marked by thick lines. A summation of the parent-specific restriction sites from tobacco (open triangles) and potato (solid triangles), present in the recombinant plastome, is marked on a simplified map, showing (from left to right) a part of the large single-copy region (LSC), the left inverted repeat (IR_B) with the 16S and 23S rRNA genes, and the full small single-copy region (SSC) [40, 41]. The distribution of the parent-specific restriction sites was also supported by the use of *Kpn* I, *Pst* I, and *Sma* I fragmentation patterns (data not shown).

any kind of chlorophyll deficiencies in regenerates derived from the backfusions, and progenies obtained from the backcrosses, strongly indicated that the abnormality of the first and second regenerates was due to their polyploid-aneuploid chromosome numbers, induced by the cell culture itself [1, 20]. Using the amphidiploid, and therefore relatively unstable *N. tabacum* [39], similar problems have also been reported in other protoplast fusion experiments [42, 7]. In the LS1 plastid mutant no revertants have been found in our and other laboratories [13]. Green color and tentoxin sensitivity of the normal tobacco regenerate was, therefore, a strong indication of the recombinant nature of its chloroplasts. In contrast to the case of the intrageneric *Nicotiana* chloroplast recombinant [24], each of the enzymes used revealed a restriction fragmentation pattern markedly different from those of the parents, which was to be anticipated considering the much wider taxonomical distance [44]. The absence of any variation in the patterns of the vegetative, somatic, or sexual derivatives of the originally selected cell line demonstrates the stability of the recombinant plastome, and the termination of the intraorganellar DNA and intracellular organelle segregation, at the end of the selection period. A preliminary view of the recombinant DNA suggests a homologous recombination between the parental DNAs. It seems that the major step in the formation of the recombinant plastome was an exchange of (at least most of) the inverted repeat region of tobacco with that of potato, which is not surprising considering its high intramolecular recombination activity [29, 17].

To clarify the mechanism of the recombination, to identify all of the recombination sites, and to explore the plastome for possible smaller deletions, insertions, or inversions, fine mapping and sequencing of numerous cloned fragments will be necessary. With respect to the main approach in this work, however, the motivation for a detailed biochemical analysis is frustrated by the great number of genes present from both species, which prevents the localization of the plastid markers (chlorophyll deficiency and tentoxin resistance) used in the selection, and the identification of the genes essential in the cooperation with the nucleus. In this "potacco" plastome it is likely that a particular combination of tobacco genes, crucial in the interaction with the nucleus, and potato genes, neutral from this viewpoint, but complementing the plastome deficiency, ensure functionality of the chloroplast genome in the presence of a tobacco nucleus. Results presented here demonstrate that chloroplast DNA recombination, brought to light by the power of cell culture selection, may be considered as an efficient tool to achieve chloroplast gene transfer between somatically/sexually incompatible species.

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References

1. Bayliss MW: Chromosomal variation in plant tissues in culture. Int Rev Cytol Suppl 11A: 113–144 (1980).
2. Bookjans G, Stumman BM, Henningsen KW: Preparation of chloroplast DNA from pea plastids isolated in a medium of high ionic strength. Anal Biochem 141: 244–247 (1984).
3. Bottomley W, Bohnert HJ: The biosynthesis of chloroplast proteins. In: Parthier B, Boulter D (eds) Encyclopedia of Plant Physiology, Vol. 14B. Nucleic Acids and Proteins in Plants II, pp. 531–596. Springer-Verlag, Berlin (1982).
4. Chiu W-L, Sears BB: Recombination between chloroplast DNAs does not occur in sexual crosses of *Oenothera*. Mol Gen Genet 198: 525–528 (1985).
5. Connett MB: Mechanisms of maternal inheritance of plastids and mitochondria: developmental and ultrastructural evidence. Plant Mol Biol Rep 4: 193–205 (1987).
6. D'Arcy WG: The classification of the Solanaceae. In: Hawkes JG, Lester RN, Skelding AG (eds) The Biology and Taxonomy of the Solanaceae, pp. 3–47. Academic Press, London (1979).
7. Dudits D, Maróy E, Praznovszky T, Oláh Z, Györgyey J, Cella R: Transfer of resistance traits from carrot into tobacco by asymmetric somatic hybridization: regeneration of fertile plants. Proc Natl Acad Sci USA 84: 8434–8438 (1988).
8. Durbin RD, Uchytil TF: A survey of plant insensitivity to tentoxin. Phytopathology 67: 602–603 (1977).
9. Fejes E, Engler D, Maliga P: Extensive homologous chloroplast DNA recombination in the pt14 *Nicotiana* somatic hybrid. Genetics (submitted).

10. Fluhr R, Aviv D, Galun E, Edelman M: Generation of heteroplastidic *Nicotiana* cybrids by protoplast fusion: analysis for plastid recombinant types. Theor Appl Genet 67: 491–497 (1984).
11. Fluhr R, Fromm H, Edelman M: Clone bank of *Nicotiana tabacum* chloroplast DNA: mapping of the alpha, beta and epsilon subunits of the ATPase coupling factor, the large subunit of ribulosebisphosphate carboxylase, and the 32-kD membrane protein. Gene 25: 271–280 (1983).
12. Gillham NW: Organelle Heredity. Raven Press, New York (1978).
13. Gleba YY: Nonchromosomal inheritance in higher plants as studied by somatic cell hybridization. In: Sharp W, Larsen PO, Paddock EF, Raghaven V (eds) Plant Cell and Tissue Culture – Principles and Applications. pp. 775–788. Ohio State University Press, Columbus, OH (1979).
14. Glimelius K, Bonnett HT: *Nicotiana* cybrids with *Petunia* chloroplasts. Theor Appl Genet 72: 794–798 (1986).
15. Heinhorst S, Gannon GC, Galun E, Kenschaft L, Weissbach A: Clone bank and physical and genetic map of potato chloroplast DNA. Theor Apl Genet 75: 244–251 (1988).
16. Hildebrand M, Jurgenson JE, Ramage RT, Bourque DP: Derivation of a physical map of chloroplast DNA from *Nicotiana tabacum* by two-dimensional gel and computer-aided restriction analysis. Plasmid 14: 64–79 (1985).
17. Howe CJ: Recombinogenic sequences in chloroplast DNA. In: Mantell SH, Chapman GP, Street PFS (eds) The Chondriome – Chloroplast and Mitochondrial Genomes, pp. 164–173. Longman House, London (1986).
18. Kushnir SG, Shlumukov LR, Pobregnyak NJ, Berger S, Gleba Y: Functional cybrid plants possessing a *Nicotiana* genome and an *Atropa* plastome. Mol Gen Genet 209: 159–163 (1987).
19. Kutzelnigg H, Stubbe W: Investigations on plastome mutants in *Oenothera*. I. General considerations. Subcell Biochem 3: 73–89 (1974).
20. Larkin PJ, Scowcroft WR: Somaclonal variation – a novel source of variability from cell cultures for plant improvement. Theor Appl Genet 60: 197–214 (1981).
21. Lemieux C, Turmel M, Seligy VL, Lee RW: Chloroplast DNA recombination in interspecific hybrids of *Chlamydomonas*: linkage between a nonmendelian locus for streptomycin resistance and restriction fragments coding for 16S rRNA. Proc Natl Acad Sci USA 81: 1164–1168 (1984).
22. Lemieux C, Lee RW: Nonreciprocal recombinatoin between alleles of the chloroplast 23S rRNA gene in interspecific *Chlamydomonas* crosses. Proc Natl Acad Sci USA 84: 4166–4170 (1987).
23. Maniatis T, Fritsch EF, Sambrook J: Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY (1982).
24. Medgyesy P, Fejes E, Maliga P: Interspecific chloroplast recombination in a *Nicotiana* somatic hybrid. Proc Natl Acad Sci USA 82: 6960–6964 (1985).
25. Medgyesy P, Golling R, Nagy F: A light sensitive recipient for the effective transfer of chloroplast and mitochondrial traits by protoplast fusion in *Nicotiana*. Theor Appl Genet 70: 590–594 (1985).
26. Menczel L, Nagy F, Kiss Z, Maliga P: Streptomycin resistant and sensitive somatic hybrids of *Nicotiana tabacum* + *Nicotiana knightiana*: correlation of resistance to *N. tabacum* plastids. Theor Appl Genet 59: 191–195 (1981).
27. Menczel L, Galiba G, Nagy F, Maliga P: Effect of radiation dosage on efficiency of chloroplast transfer by protoplast fusion in *Nicotiana*. Genetics 100: 487–495 (1982).
28. Murashige T, Skoog F: A revised medium for rapid growth and bioassays with tobacco tissue cultures. Phys Plant 15: 473–497 (1962).
29. Palmer J: Comparative organization of chloroplast genomes. Annu Rev Genet 19: 325–354 (1985).
30. Parthier B: The cooperation of nuclear and plastid genomes in plastid biogenesis and differentiation. Biochem Physiol Pflanzen 177: 283–317 (1982).
31. Phillips AL: Restriction map and clone bank of tomato plastic DNA. Curr Genet 10: 147–152 (1985).
32. Phillips AL: Localisation of genes for chloroplast components in tomato plastid DNA. Curr Genet 10: 153–161 (1985).
33. Sager R: Genetic analysis of chloroplast DNA in *Chlamydomonas*. Adv Genet 19: 287–340 (1977).
34. Salts Y, Herrman RG, Peleg N, Lavi U, Izhar S, Frankel R, Beckmann JS: Physical mapping of plastid DNA variation among eleven *Nicotiana* species. Theor Appl Genet 69: 1–14 (1984).
35. Schiller B, Herrmann RG, Melchers G: Restriction endonuclease analysis of plastid DNA from tomato, potato and some of their somatic hybrids. Mol Gen Genet 186: 453–459 (1982).
36. Sears BB: Elimination of plastids during spermatogenesis and fertilization in the plant kingdom. Plasmid 4: 233–255 (1908).
37. Seyer P, Kowallik KV, Herrmann RG: A physical map of *Nicotiana tabacum* plastid DNA including the location of structural genes for ribosomal RNAs and the large subunit of ribulose bisphosphate carboxylase-oxygenase. Curr Genet 3: 189–204 (1981).
38. Skarzhynskaya MV, Cherep NN, Gleba Y: Potato and tobacco hybrid cell lines and plants obtained by cloning individual protoplast fusion products. Sov Cytol Genet 6: 42–48 (1982).
39. Smith HH: Recent cytogenetic studies in the genus *Nicotiana*. Adv Genet 14: 1–54 (1968).
40. Sugita M, Kato A, Shimada H, Sugiura M: Sequence analysis of the junctions between a large inverted repeat and single-copy regions in tobacco chloroplast DNA. Mol Gen Genet 194: 200–205 (1984).
41. Sugiura M, Shinozaki K, Zaita N, Kusuda M, Kumano M: Clone bank of the tobacco (*Nicotiana tabacum*) chloroplast genome as a set of overlapping restriction endonuclease fragments: mapping of eleven ribosomal protein genes. Plant Sci 44: 211–216 (1986).
42. Thanh ND, Páy A, Smith MA, Medgyesy P, Márton L: Intertribal chloroplast transfer by protoplast fusion between

Nicotiana tabacum and *Salpiglossis sinuata*. Mol Gen Genet 213: 186–190 (1988).

43. Tilney-Bassett RAE: The inheritance and genetic behaviour of plastids. In: Kirk JTO, Tilney-Bassett RAE (eds) The Plastids, pp. 251–524. Elsevier Biomedical Press, Amsterdam (1978).
44. Whitfeld PR, Bottomley W: Organization and structure of chloroplast genes. Annu Rev Plant Physiol 34: 279–310 (1983).
45. Zelcer A, Aviv D, Galun E: Interspecific transfer of cytoplasmic male sterility by fusion between protoplasts of normal *Nicotiana sylvestris* and X-ray irradiated protoplasts of male sterile *N. tabacum*. Z Pflanzenphysiol 90: 397–407 (1978).