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A. Kanno · H. Kanzaki · T. Kameya

Detailed analyses of chloroplast and mitochondrial DNAs from the hybrid plant generated by asymmetric protoplast fusion between radish and cabbage

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Abstract In a previous report, intergeneric somatic hybrids between red cabbage (*Brassica oleracea* L. var. *capitata*) and radish (*Raphanus sativus* L. cv. Shougoin) were produced by protoplast fusion. Plant morphology, chromosome number, isozyme patterns, and *Sma*I cleavage pattern of chloroplast DNA indicated that the hybrid plants have the red cabbage nucleus and the radish chloroplasts. In this report, we analyzed the organization of chloroplast and mitochondrial DNAs from this hybrid using Southern hybridization. The restriction patterns of almost all regions of the chloroplast DNA from the hybrid were similar to that of radish, except for one region near the *rps16* gene, which encodes the chloroplast ribosomal protein S16. In contrast to chloroplast DNA, the restriction pattern of mitochondrial DNA from the hybrid was quite different from that of the parents.

Abbreviations *CMS* cytoplasmic male-sterility · *ctDNA* chloroplast DNA · *mtDNA* mitochondrial DNA

Introduction

The production of somatic hybrid plants by protoplast fusion provides a means of increasing genetic variability and overcoming sexual cross-incompatibility for plant breeding (Glimelius et al. 1991).

Evidence for genetic recombination and rearrangement of organelle DNA has been obtained in some somatic hybrid plants. For chloroplast DNA (ctDNA), there is not much evidence; however, a somatic hybrid between *Nicotiana tabacum* and *N. plumbaginifolia* was shown to be a product of interspecific ctDNA recombination (Medgyesy et al. 1985). Sequencing of the junction fragment showed homologous recombination between the two parental chloroplast genomes (Fejes et al. 1990). Additional evidence for recombination of ctDNA was obtained in somatic hybrids between *N. tabacum* and *Solanum tuberosum* (Thanh and Medgyesy 1989), *N. tabacum* and *N. debneyi* (Sproule et al. 1991), and within *Solanum* species (Sidirov et al. 1987). On the other hand, there are many reports of the recombination of mitochondrial DNA (mtDNA) in somatic hybrid plants. This was first suggested by Belliard et al. (1979) with two varieties of *N. tabacum* with different cytoplasms. mtDNAs from somatic hybrids have been characterized in *Nicotiana* (Nagy et al. 1981; 1983; Galun et al. 1982; Aviv et al. 1984), *Petunia* (Boeshore et al. 1983, 1985; Rothenberg et al. 1985; Rothenberg and Hanson 1987; Clark et al. 1986), *Solanum* (Kemble et al. 1986), *Daucus* (Matthews and Widholm 1985), Brassicaceae (Chetrit et al. 1985; Vedel et al. 1986; Landgren and Glimelius 1994), and Poaceae (Ozias-Atkins et al. 1987). Furthermore, evidence for the presence of rearranged or recombinant mtDNA in interfamilial somatic hybrids was obtained from hybrids between *N. tabacum* and *Daucus carota* (Smith et al. 1989) and between *Oryza sativa* and *Daucus carota* (Kisaka et al. 1994).

In our previous report (Kameya et al. 1989), intergeneric hybrid plants were obtained through protoplast fusion between red cabbage (*Brassica oleracea* L. var. *capitata*) and radish (*Raphanus sativus* L. cv. Shougoin). The plant morphology, chromosome number, isozyme patterns and the *Sma*I cleavage pattern of ctDNA indicated that the hybrid plants have the red cabbage nucleus and radish chloroplasts. These hybrids developed to the flowering stage and formed male-sterile flowers, which interestingly showed cytoplasmic inheritance. To verify the mechanism of the male sterility, we have analyzed in detail the organization of ctDNA and mtDNA from the intergeneric somatic hybrid plants between radish and red cabbage using Southern hybridization.

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A. Kanno · T. Kameya (✉)
Institute of Genetic Ecology, Tohoku University, Sendai, 980-77, Japan

H. Kanzaki
Iwate Biotechnology Institute, Kitakami, Iwate, 024, Japan

Materials and methods

Plant material

Intergeneric hybrid plants were obtained through protoplast fusion between red cabbage (*B. oleracea* L. var. *capitata*) and radish (*Raphanus sativus* L. cv. Shougoin) as described by Kameya et al. (1989). Only two plantlets were regenerated and developed to the flowering stage. The morphology of these hybrid plants was very similar to that of red cabbage; however, these hybrid plants were male sterile. Since the isozyme patterns and the restriction patterns of ctDNA of the two hybrids were identical, we used only one hybrid line in the following experiments.

The crosses undertaken and the morphology of the plants are schematized in Fig. 1. The hybrids were maintained by backcrossing with *B. oleracea* pollen. The offspring (BC_1) from this cross, which were also male sterile, were crossed with *B. alboglabra* pollen twice. These offspring, which were also male sterile, and the parents of the hybrid, radish and red cabbage, were used for isolation of total DNA.

Isolation of total DNA and Southern hybridization

Total DNA was extracted from 1 g of mature green leaves using the method described by Honda and Hirai (1990). Total DNA was digested with restriction enzymes in accordance with the manufacturer's recommendations (Takara Shuzo Co., Kyoto, Japan). The DNA fragments were separated by electrophoresis on a 0.7% agarose gel and transferred to nylon membranes (MAGNA nylon 66, MSI). Southern hybridization analysis was carried out using a non-radioactive DNA labeling and detection kit (Boehringer Mannheim, Germany).

We used the *Bam*HI-1, -3, -8 and *Pst*I-7 fragments from rice ctDNA and the mitochondrial genes for the α-subunit of the F_1-ATPase (*atpA*), 26S rRNA, 18S rRNA and the subunit of cytochrome c oxidase (*coxII*) from pea as probes. The chloroplast and mitochondrial clones were kindly provided by Prof. A. Hirai (University of Tokyo, Japan) and Prof. K. Nakamura (Nagoya University, Japan), respectively.

Results

Genetic analysis of the intergeneric hybrid plants

As shown in Fig. 1a, the hybrids between red cabbage and radish (Kameya et al. 1989) were maintained by backcrossing with *B. oleracea* pollen, and the offspring (BC_1) were crossed with *B. alboglabra* pollen twice. The morphology of the offspring of the somatic hybrid is quite similar to that of cabbage, one of the parents of the hybrid (Fig. 1b). Because all of these offspring were male sterile and did not show segregation, this trait was maternally inherited stably.

ctDNA analysis of the offspring of the somatic hybrid

We compared the *Sma*I restriction pattern of the ctDNA of radish and red cabbage in the previous report (Kameya et al. 1989). We could detect only one distinguishing band in the cabbage ctDNA and the intergeneric hybrid plants did not have this band. This suggested that this region of the chloroplast DNA of the hybrid was derived from radish.

However, this result is not sufficient to show that the entire hybrid ctDNA was derived from radish. Therefore, to analyze more completely the ctDNA from the somatic hybrid, we used three restriction enzymes, and four ctDNA probes: *Bam*HI-1, -3, -8 and *Pst*I-7 fragments from rice ctDNA (Hirai et al. 1985). The restriction patterns of the ctDNA from radish and red cabbage were quite similar, but, some differences were found (Fig. 2). These results show that almost all regions of the ctDNA of the somatic hybrid offspring are of the radish type. Using the *Bam*HI-3 probe, however, a novel 2.1-kb band was found in the offspring of the somatic hybrid. Since the band from the hybrid was larger than that of radish and cabbage, this suggested that the change was caused by DNA recombination and/or rearrangement. The *Bam*HI-3 DNA fragment of rice ctDNA contains *trnK*, *trnQ*, and *trnS*, genes which encode tRNAs, *rps16*, which encodes a ribosome protein, and the *psbK* operon (Hiratsuka et al. 1989) (Fig. 3a). For further analyses, we carried out Southern hybridization with four shorter DNA fragments as probes (A–D, Fig. 3). The 2.1-kb unique band was detected by three probes, A, B, and C, but not by probe D. This indicates that the recombination and/or rearrangement site was near probe B, which covers the *rps16* gene that encodes the chloroplast ribosomal protein S16.

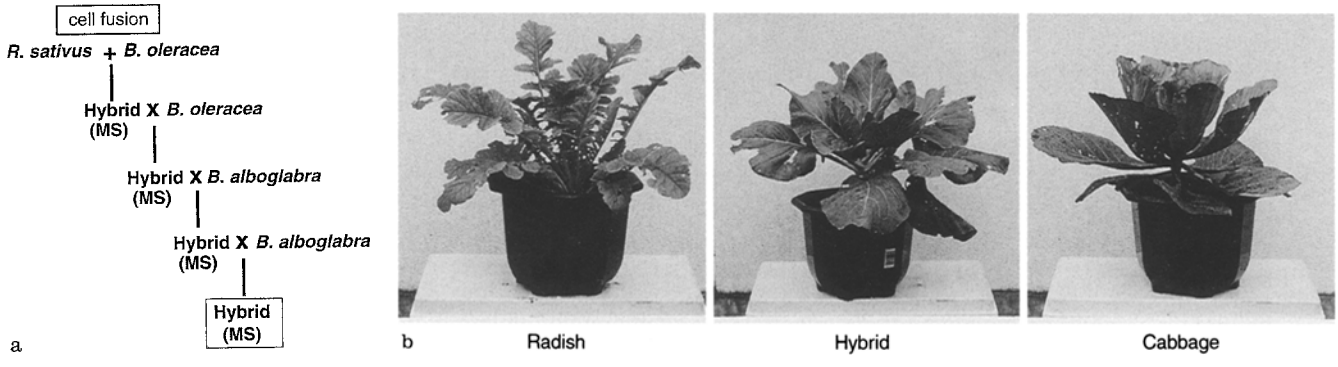

Fig. 1a Schematic representation of the steps involved in producing the offspring of the somatic hybrid generated by protoplast fusion between radish and cabbage. **b** Morphology of the offspring of the somatic hybrid, and the parents of the protoplast fusion – radish (*Raphanus sativus* L. cv. Shougoin) and cabbage (*Brassica oleracea* L. var. *capitata*)

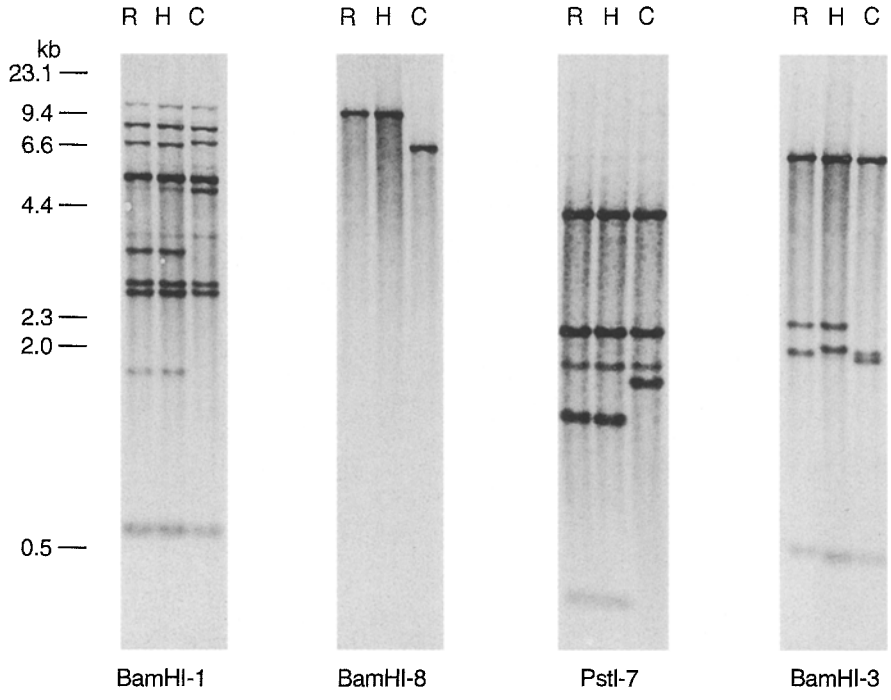

Fig. 2 Southern hybridization of total DNA from radish (*R*), the offspring of the somatic hybrid (*H*), and red cabbage (*C*). Total DNA was digested with *Eco*RI and hybridized with *Bam*H1-1, -3, -8, and *Pst*I-7 DNA fragments of rice chloroplast DNA (ctDNA) as probes

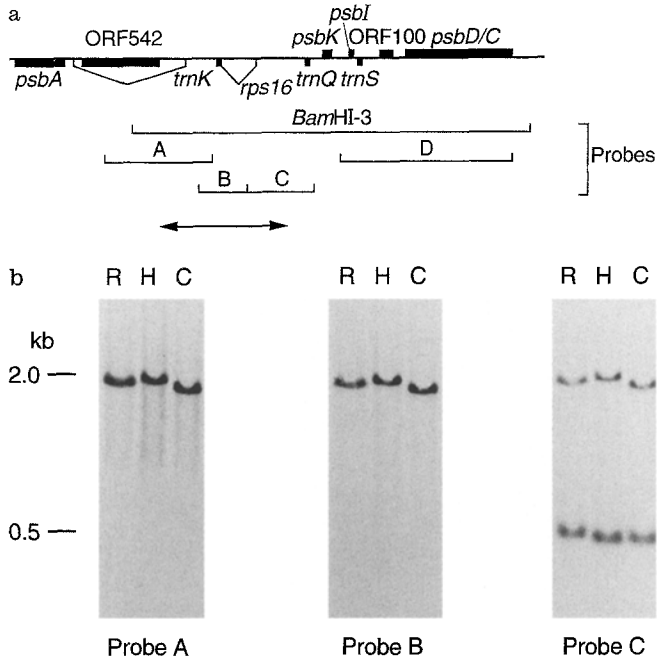

Fig. 3a Genetic map near *Bam*HI-3 DNA fragment of rice ctDNA. the location of the recombination and/or rearrangement site is indicated by the *arrow*. **b** Southern hybridization of total DNA from radish (*R*), the offspring of the somatic hybrid (*H*), and red cabbage (*C*). Total DNA was digested with *Eco*RI and hybridized with probes A, B, and C

mtDNA analysis of the offspring of the somatic hybrid

To identify the restriction pattern of the mtDNA from the offspring of the somatic hybrid, Southern hybridization was carried out using mitochondrial genes as probes (Fig. 4). We used four probes: *atpA*, 26S and 18S rRNA, and *coxII* genes. Some novel DNA fragments were detected in the offspring of the hybrid by probes *atpA* and 26S and 18S rRNA genes. The pattern of the offspring of the hybrid using the *atpA* probe was considerably different from that of the parents. To determine the sites of mtDNA recombinations and/or rearrangements, further Southern hybridizations were carried out using three mitochondrial probes, *atpA* and 26S and 18S rRNA genes, and three restriction enzymes, *Bam*HI, *Eco*RI and *Hin*dIII. However, we could not determine the sites of recombination and/or rearrangement (data not shown).

In contrast, the hybridization pattern of the offspring of the somatic hybrid obtained with the *coxII* probe was of the radish type. Other experiments showed that cabbage has two copies of the *coxII* gene and one of them gave a pattern identical to that of radish (data not shown). Thus, we cannot identify the origin of the *coxII* gene of the somatic hybrid.

Since Sakai and Imamura (1992; 1993) reported that cybrid progeny have different mtDNA patterns caused by the mitochondrial subgenome, we analyzed the mtDNA restriction pattern from individual plants of the offspring of the somatic hybrids. Total DNA was prepared from six independent plants and Southern hybridizations were carried out using mtDNA probes (Fig. 5). These results showed that there were no differences in the restriction patterns of mtDNA among the individual offspring.

Discussion

Chromosome elimination occurs during the regeneration of fused cells, (Shepard et al. 1983; Babiychuk et al. 1992). In addition, two different types of chloroplast may be

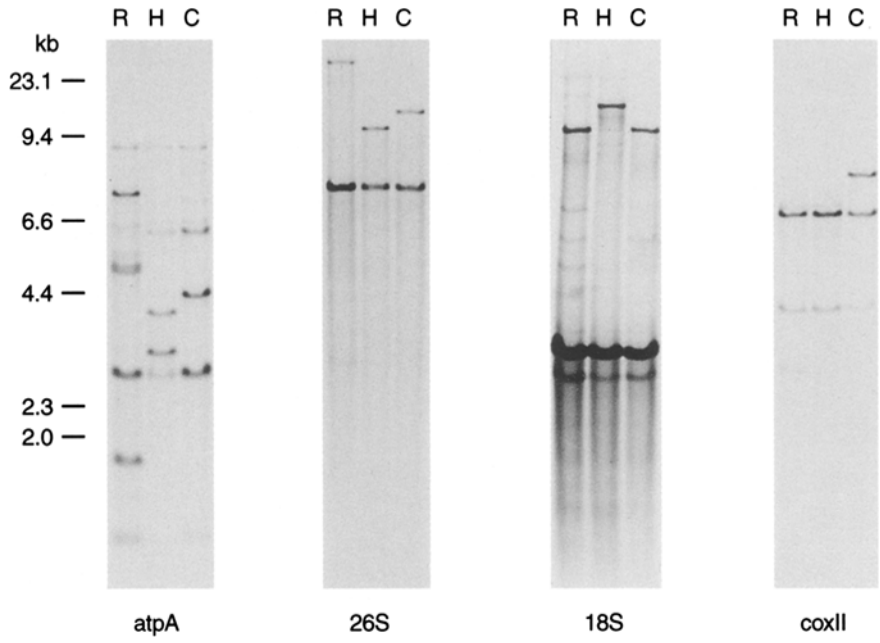

Fig. 4 Southern hybridization of total DNA from radish (*R*), the offspring of the somatic hybrid (*H*), and red cabbage (*C*). Total DNA was digested with *Bam*HI and hybridized with *atpA*, 26 S and 18S rRNA, and *coxII* genes from pea mitochondrial DNA as probes

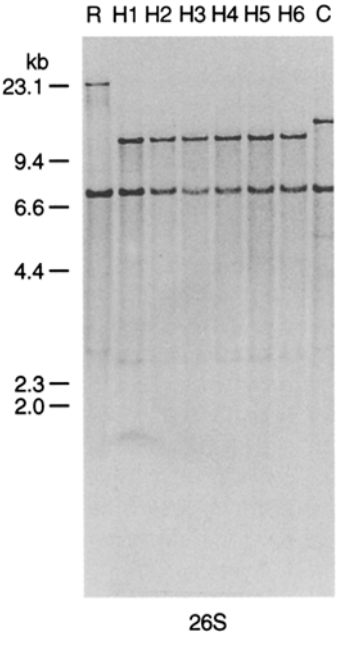

Fig. 5 Southern hybridization of total DNA from radish (*R*), six independent offspring of the somatic hybrid (*H1–H6*), and red cabbage (*C*). The probe was the mitochondrial 26S rRNA gene and the restriction enzyme was *Bam*HI

mixed in a fused hybrid cell between two different species. However, the parental chloroplasts segregate during callus development and, in most cases, the regenerated hybrid plants have only one chloroplast type (Akada and Hirai 1986). Clear evidence for the recombination of ctDNA in higher plants is very limited; however, a somatic hybrid of *N. tabacum* and *N. plumbaginifolia* was shown to be a product of homologous recombination of ctDNA (Medgyesy et al. 1985; Fejes et al. 1990). As shown in Fig. 2, the restriction pattern of the ctDNA from the somatic hybrid was quite similar to that from radish, except for one region. This suggests that the novel fragment of ctDNA from the hybrid has been constructed by recombination between radish and cabbage ctDNA. As shown in Fig. 3, a novel DNA fragment was detected by probes A, B, and C in the hybrid, indicating that the rearrangement and/or recombination site is near the *rps16* gene. For a more precise analysis, further investigations are necessary to determine the sequence of the novel fragment.

In contrast to ctDNA recombination and/or rearrangement of mtDNAs of somatic hybrids has been reported in many combinations between species among the Solanaceae, Umbelliferae, Brassicaceae and Poaceae (Nagy et al. 1983; Aviv et al. 1984; Boeshore et al. 1985; Matthews and Widholm 1985; Kemble et al. 1986; Ozias-Atkins et al. 1987; Rothenberg and Hanson 1987; Landgren and Glimelius 1994). As shown in Fig. 4, novel DNA fragments were detected in the mtDNA of the somatic hybrid by three of four probes. This indicates that rearrangements and/or recombinations may be occurring in many regions of the mtDNA of the somatic hybrid.

Sakai and Imamura (1992; 1993) have shown that cybrid progeny have different mtDNA patterns caused by changes in the amounts of mitochondrial subgenomes. However, the offspring of the somatic hybrid in the present study have the same mtDNA pattern, as shown in Fig. 5, indicating that the mtDNAs of the somatic hybrid have been inherited stably.

Genetic analysis of the intergeneric somatic hybrids shows that the male-sterile trait of the hybrids did not segregate but was inherited maternally. The generation of cytoplasmic male sterility (CMS) by protoplast fusion has been reported for *N. tabacum* and *N. africana* (Kumashiro et al. 1988) and with tomato and *Solanum* protoplasts (Melchers et al. 1992). However, the mechanism of generation of cytoplasmic male-sterile plants by protoplast fusion is not known. Although the cause of CMS has not been clarified, reorganization of mtDNAs is closely correlated with the CMS character, and it is thought that reorganiza-

tion of mtDNAs results in the construction of chimeric genes, which inhibit the expression of normal mitochondrial genes (Newton 1988; Levings 1990; Hanson 1991). Therefore, the CMS trait of the somatic hybrid may have been caused by the reorganization of the parental mtDNA following construction of chimeric genes, or by inhibition of the transcription of normal mitochondrial genes. To determine alterations in the mitochondrial gene products, we are now carrying out Northern hybridization using mitochondrial genes as probes. In addition, since we do not exclude the possibility that the male sterility was caused by nuclear-mitochondrial incompatibility, we still need to investigate whether such incompatibility is present or not.

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References

Akada S, Hirai A (1986) Studies on the mode of separation of chloroplast genomes in parasexual hybrid calli. III. Random separation of two types of chloroplast genomes in a hybrid callus. Jpn J Genet 61:437–445

Aviv D, Arzee-Gonen P, Bleichman S, Galun E (1984) Novel alloplasmic *Nicotiana* plants by donor-recipient protoplast fusion: cybrids having *N. tabacum* or *N. sylvestris* nuclear genomes and either or both plastomes and chondriomes from alien species. Mol Gen Genet 196:244–253

Babiychuk E, Kushnir S, Gleba YY (1992) Spontaneous extensive chromosome elimination in somatic hybrids between somatically congruent species *Nicotiana tabacum* L. and *Atropa belladonna* L. Theor Appl Genet 84:87–91

Belliard G, Vedel F, Pelletier G (1979) Mitochondrial recombination in cytoplasmic hybrids of *Nicotiana tabacum* by protoplast fusion. Nature 281:401–403

Boeshore ML, Lifshitz I, Hanson MR, Izhar S (1983) Novel composition of mitochondrial genomes in *Petunia* somatic hybrids derived from cytoplasmic male sterile and fertile plants. Mol Gen Genet 190:459–467

Boeshore ML, Hanson MR, Izhar S (1985) A variant mitochondrial DNA arrangement specific to *Petunia* stable sterile somatic hybrids. Plant Mol Biol 4:125–132

Chetrit P, Mathieu C, Vedel F, Pelletier G, Primard C (1985) Mitochondrial DNA polymorphism induced by protoplast fusion in Cruciferae. Theor Appl Genet 69:361–366

Clark E, Schnabelrauch L, Hanson MR, Sink KC (1986) Differential fate of plastid and mitochondrial genomes in *Petunia* somatic hybrids. Theor Appl Genet 72:748–755

Fejes E, Engler D, Maliga P (1990) Extensive homologous chloroplast DNA recombination in the pt14 *Nicotiana* somatic hybrid. Theor Appl Genet 79:28–32

Galun E, Arzee-Gonen P, Fluhr R, Edelman M, Aviv D (1982) Cytoplasmic hybridization in *Nicotiana*: Mitochondrial DNA analysis in progenies resulting from fusion between protoplasts having different organelle constitutions. Mol Gen Genet 186:50–56

Glimelius K, Fahlesson J, Landgren M, Sjodin C, Sundberg E (1991) Gene transfer via somatic hybridization in plants. Trends Biotechnol 9: 24–30

Hanson MR (1991) Plant mitochondrial mutations and male sterility. Annu Rev Genet 25:461–486

Hirai A, Ishibashi T, Morikami A, Iwatsuki N, Shinozaki K, Sugiura M (1985) Rice chloroplast DNA: a physical map and the location of the genes for the large subunit of ribulose 1,5-bisphosphate carboxylase and 32KD photosystem II reaction center protein. Theor Appl Genet 70:117–122

Hiratsuka J, Shimada H, Whittier R, Ishibashi T, Sakamoto M, Mori M, Kondo C, Honji Y, Sun CR, Meng BY, Li YQ, Kanno A, Nishizawa Y, Hirai A, Shinozaki K, Sugiura M (1989) The complete sequence of the rice (*Oryza sativa*) chloroplast genome: intermolecular recombination between distinct tRNA genes accounts for a major plastid DNA inversion during the evolution of the cereals. Mol Gen Genet 217:185–194

Honda H, Hirai A (1990) A simple and efficient method for identification of hybrids using nonradioactive rDNA as probe. Jpn J Breed 40:339–348

Kameya T, Kanzaki H, Toki S, Abe T (1989) Transfer of radish (*Raphanus sativus* L.) chloroplasts into cabbage (*Brassica oleracea* L.) by protoplast fusion. Jpn J Genet 64:27–34

Kemble RJ, Barsby TL, Wong RSC, Shepard JF (1986) Mitochondrial DNA rearrangements in somatic hybrids of *Solanum tuberosum* and *Solanum brevidens*. Theor Appl Genet 72:787–793

Kisaka H, Lee H, Kisaka M, Kanno A, Kang K, Kameya T (1994) Production and analysis of asymmetric hybrid plants between monocotyledon (*Oryza sativa* L.) and dicotyledon (*Daucus carota* L.). Theor Appl Genet 84:365–371

Kumashiro T, Asahi T, Komari T (1988) A new source of cytoplasmic male sterile tobacco obtained by fusion between *Nicotiana tabacum* and X-irradiated *N. africana* protoplasts. Plant Sci 55:247–254

Landgren M, Glimelius K (1994) A high frequency of intergenomic mitochondrial recombination and an overall biased segregation of *B. campestris* or recombined *B. campestris* mitochondria were found in somatic hybrids made within Brassicaceae. Theor Appl Genet 87:854–862

Levings CS III (1990) The Texas cytoplasm of maize: cytoplasmic male sterility and disease susceptibility. Science 250: 942–947

Matthews BF, Widholm JM (1985) Organelle DNA compositions and isoenzyme expression in an interspecific somatic hybrid of *Daucus*. Mol Gen Genet 198:371–376

Medgyesy P, Fejes E, Maliga P (1985) Interspecific chloroplast recombination in a *Nicotiana* somatic hybrid. Proc Natl Acad Sci USA 82:6960–6964

Melchers G, Mohri Y, Watanabe K, Wakabayashi S, Harada K (1992) One-step generation of cytoplasmic male sterility by fusion of mitochondrial-inactivated tomato protoplasts with nuclear-inactivated *Solanum* protoplasts. Proc Natl Acad Sci USA 89:6832–6836

Nagy F, Torok I, Maliga P (1981) Extensive rearrangements in the mitochondrial DNA in somatic hybrids of *Nicotiana tabacum* and *Nicotiana knightiana*. Mol Gen Genet 183:437–439

Nagy F, Lazar G, Menczel L, Maliga P (1983) A heteroplasmic state induced by protoplast fusion is a necessary condition for detecting rearrangements in *Nicotiana* mitochondrial DNA. Theor Appl Genet 66:203–207

Newton KJ (1988) Plant mitochondrial genomes: organization, expression, and variation. Annu Rev Plant Physiol Plant Mol Biol 39: 503–532

Ozias-Atkins P, Pring DR, Vasil IK (1987) Rearrangements in the mitochondrial genome of somatic hybrid cell lines of *Pennisetum americanum* (L.) K. Schum. + *Panicum maximum* Jacq. Theor Appl Genet 74:15–20

Rothenberg M, Hanson MR (1987) Recombination between parental mitochondrial DNA following protoplast fusion can occur in a region which normally does not undergo intragenomic recombination in parental plants. Curr Genet 12:235–240

Rothenberg M, Boeshore ML, Hanson MR, Izhar S (1985) Intergenomic recombination of mitochondrial genomes in a somatic hybrid plant. Curr Genet 9:615–618

Sakai T, Imamura J (1992) Alteration of mitochondrial genomes containing *atpA* genes in the sexual progeny of cybrids between *Raphanus sativus* cms line and *Brassica napus* cv. Westar. Theor Appl Genet 84:923–929

Sakai T, Imamura J (1993) Evidence for a mitochondrial sub-genome containing radish *atpA* in a *Brassica napus* cybrid. Plant Sci 90:95–103

Shepard JF, Bindney K, Barsby T, Kemble R (1983) Gene transfer in plants through interspecific protoplast fusion. Science 219:683–688

Sidirov VA, Zubko MK, Kuchko AA, Komarnitsky IK, Gleba YY (1987) Somatic hybridization in potato: use of γ-irradiated protoplasts of *Solanum pinnatisectum* in genetic reconstruction. Theor Appl Genet 74:364–368

Smith MA, Pay A, Dudits D (1989) Analysis of chloroplast and mitochondrial DNAs in asymmetric somatic hybrids between tobacco and carrot. Theor Appl Genet 77:641–644

Sproule A, Donaldson P, Dijak M, Bevis E, Pandeya R, Keller WA, Gleddie S (1991) Fertile somatic hybrids between transgenic *Nicotiana tabacum* and transgenic *N. debneyi* selected by dual-antibiotic resistance. Theor Appl Genet 82:450–456

Thanh ND, Medgyesy P (1989) Limited chloroplast gene transfer via recombination overcomes plastome-genome incompatibility between *Nicotiana tabacum* and *Solanum tuberosum*. Plant Mol Biol 12:87–93

Vedel F, Chetrit P, Mathieu C, Pelletier G, Primard C (1986) Several different mitochondrial DNA regions are involved in intergenomic recombination in *Brassica napus* cybrid plants. Curr Genet 11:17–24