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Production and analysis of asymmetric hybrid plants between monocotyledon (*Oryza sativa* L.) and dicotyledon (*Daucus carota* L.)

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Abstract Asymmetric hybrid plants were obtained from fused protoplasts of a monocotyledon (*Oryza sativa* L.) and a dicotyledon (*Daucus carota* L.). X-ray-irradiated protoplasts isolated from a cytoplasmic male-sterile (cms) carrot suspension culture were fused with iodoacetoamide-treated protoplasts isolated from a 5-methyltryptophan (5MT)-resistant rice suspension culture by electrofusion. The complementary recovered cells divided and formed colonies, which were then cultivated on regeneration medium supplemented with 25 mg/l 5MT to eliminate any escaped carrot cells. Somatic hybrids were regenerated from 5 of the 5MT-resistant colonies. The morphologies of most of the regenerated plants closely resembled that of the parental carrot plants. A cytological analysis of callus cultures induced from these plants indicated that most of the cells possessed 20–22 chromosomes and were resistant to 5MT. An isozyme analysis revealed that several regenerated plants had the peroxidase isozyme patterns of both parents. A Southern hybridization analysis with non-radioactively labelled DNA fragments of the *rgp1* gene showed that regenerated plants had hybridizing bands from both rice and carrot. Chloroplast (cp) and mitochondrial (mt) DNAs were also analyzed by Southern hybridization by using several probes. CpDNA patterns of the regenerated plants were indistinguishable from those of the carrot parent. However 1 of the regenerated plants had a novel band pattern of mtDNA that was not detected in either of the parents, indicating a possible recombination of mitochondrial genomes.

Key words Asymmetric hybrid plants · Monocotyledon · *Oryza sativa* L. · Dicotyledon *Daucus carota* L.

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Introduction

The production of somatic hybrid plants by protoplast fusion provides a useful approach for achieving the combination of genetic material. However, most of the hybrids between remote species, such as interfamilial hybrids, that have been described up-to-date have generally been unstable and have not formed any plants (Wetter and Kao 1980; Chien et al. 1982; Niizeki et al. 1985; Sala et al. 1985).

Several asymmetric hybrids between remote species have been obtained using selection systems that combine radioactive treatment and various selective markers (Dudits et al. 1980; Gupta et al. 1982, 1984; Imamura et al. 1987; Bates et al. 1987; Gleba et al. 1988; Perl et al. 1991; Hinnisdaels et al. 1991; Wolters et al. 1991; Feher et al. 1992). Interfamilial hybrid plants have also been produced by the same procedures (Dudits et al. 1987; Kisaka and Kameya 1994).

However, information concerning the genetic constitution of such fused products is still poor. In order to investigate nuclear and cytoplasmic traits of hybrids between remote species, we attempted to produce somatic hybrid plants between a 5-methyltryptophan (5MT)-resistant *Oryza sativa* and a cytoplasmic male-sterile *Daucus carota*. Regenerated plants were analyzed at the biochemical, molecular and cytological levels.

Materials and methods

Cell suspension materials and selection of 5MT-resistant cells

The callus of a cytoplasmic male-sterile (cms) *D. carota* was induced from surface-sterilized leaf segments cultured on Medium A [MS medium (Murashige and Skoog 1962) plus 1 mg/l 2,4-dichlorophenoxyacetic acid (2,4-D), 0.1 mg/l kinetin and 0.8% agar] under continuous fluorescent light (4 W/m^2) at 25 °C. The induced calli were then transferred to liquid Medium B (Medium A minus agar) and sub-cultured every 2 weeks. The suspension cultures of *O. sativa* (var 'Murasakidaikoku') were induced from anthers. 5MT-resistant calli were selected by a method described previously by Lee

and Kameya (1989). The selected calli were transferred to AA liquid medium (Toriyama and Hinata 1985) supplemented with 25 mg/l 5MT and sub-cultured every 2 weeks.

Protoplast isolation, fusion, selection and culture of somatic hybrids

The protoplasts of *D. carota* were isolated from 1-month-old suspension cultures by incubation in an enzyme solution [1.6% (w/v) Cellulase Onozuka R10 (Yakult Biochemicals), 0.3% (w/v) Macerozyme R10 (Yakult Biochemicals), 8% (w/v) mannitol and 0.1% (w/v) $CaCl_2 \cdot 2H_2O$ (pH 5.5)] for 3 h at 25 °C. The protoplasts of *O. sativa* were isolated from 25-month-old suspension cultures by incubation under the same conditions as those described for *D. carota*.

The protoplasts were filtered through a 50-μm mesh nylon net and washed twice with a washing solution [8% (w/v) mannitol and 0.1% ((w/v) $CaCl_2 \cdot 2H_2O$] with centrifugation at 80 g for 5 min. The protoplasts were purified by floating them in a 25% (w/v) sucrose solution followed by centrifugation at 80 g for 5 min. They were brought to a density of 5×10^5/ml in the washing solution. The protoplasts of *D. carota* were irradiated with 50 krad of X-rays, and the protoplasts of *O. sativa* were treated with a filter-sterilized iodoacetoamide (IOA) solution [10 m*M* IOA and 8% (w/v) mannitol (pH 5.5)] for 20 min at 4 °C before cells fusion. After the treatments, these protoplasts were washed once in the washing solution. The two kinds of protoplast populations were mixed in equal proportions to give a total population density of 5×10^5/ml and then submitted to electrofusion.

Aliquots containing the fused protoplasts were first diluted with an equal volume of gelrite solution [0.3% (w/v) gelrite, 3% (w/v) sucrose, 5% (w/v) glucose] and then dropped into a protoplast culture medium (Medium C, Table 1) in plastic petri dishes. During the first month of culture, the petri dishes were incubated in the dark at 25 °C. The microcalli were subsequently cultured in Medium D (Table 1) and transferred to continuous light (4 W/m^2) at 25 °C. After 2 weeks of culture, the microcalli were transferred to solid medium (Medium *E*, Table 1). When the visible colonies were about 1–2 mm in diameter, they were transferred to shooting medium (Medium F, Table 1). Regenerated shoots were transferred to a rooting medium (Medium G, Table 1).

5MT sensitivity of regenerated plants

The callus cultures were induced from leaves of regenerated plants on Medium A and then transferred to liquid Medium B. Subsequently, the established cell suspension cultures were transferred to Medium B supplemented with various concentrations of 5MT. Their growth was measured by the settled-cell volume technique after 2 weeks.

Cytological analysis

The number of chromosomes was determined by treating actively growing suspension cultures, established from each of the regenerated plants with 2 m*M* 8-quinolinol for 3 h and then fixing them with methanol-glacial acid (3:1, v/v) for 1 h. The cells were squashed and stained with 0.8% Giemsa, and the chromosome number was determined in about 200 metaphases per regenerated plant.

Analysis of DNA

Total DNAs were prepared from callus induced from *D. carota*, *O. sativa* and regenerated hybrid plants by the method described by Honda and Hirai (1990). Southern hybridizations were carried out using a non-radioactive DNA labelling and detection kit (Boehringer Mannheim, FRG). The cloned fragments of the *rgp1* gene (Sano and Youssefian 1991) were a gift from Dr. H. Sano, Akita Prefectural College of Agriculture, Japan. The clone for apocytochrome b (*cob*) and subunit 6 of F_1–F_0 ATPase (*atp6*) of rice mitochondrial (mt) DNA fragments and *Bam*HI-3 and *Bam*HI-8 of rice chloroplast (cp) DNA fragments were a gift from Dr. A. Hirai, The University of Tokyo, Japan. These clones were used as probes for Southern hybridizations.

Enzyme analysis

The callus extracts of regenerated plants and their parents were subjected to polyacrylamide gel electrophoresis for peroxidase by a method described previously (Yamamoto and Momotani 1971). Anthranilate synthase activity was measured in cell extracts from the callus of some regenerated plants and their parents according to the method of Ranch et al. (1983).

Results

Effect of X-irradiation and iodoacetamide treatment on colony formation

The protoplasts of *D. carota* were X-irradiated at various dosages and then cultured for about 2 weeks. The number of dividing cells were then counted. As shown in Fig. 1a, exposure to more than 50 krad of X-rays usually inhibited cell division. The protoplasts of *O. sativa* were treated with 10 m*M* IOA for different periods and subsequently cultured for about 2 weeks. As shown in Fig. 1b, no protoplast division was observed when the treatments time exceeded 15 min. On the basis of these results, pre-fusion treatments of protoplasts were performed using 50 krad of X-rays for *D. carota* and 10 m*M* IOA for 20 min for *O. sativa*. No colony formation was observed in cultures of X-irradiated protoplasts, IOA-treated protoplasts and their mixed protoplasts without the fusion treatment.

Table 1 Composition of culture media[a] (*2, 4-D* 2, 4-dichlorophenoxyacetic acid, *NAA*, α-naphthaleneacetic acid, *BAP* N^6-benzylaminopurine, *MS* Murashige and Skoog (1962) formulation

Addenda	Medium C	Medium D	Medium E	Medium F	Medium G
Mineral salts MS	MS	MS	MS	MS	MS
Vitamins	MS	MS	MS	MS	MS
Sucrose (w/v)	3%	3%	3%	3%	3%
Glucose (w/v)	5%				
2, 4-D	1.0 mg/l	1.0 mg/l	1.0 mg/l		
Kinetin	0.5 mg/l	0.5 mg/l	0.5 mg/l		
NAA				1.0 mg/l	
BAP				0.1 mg/l	
5MT				25 mg/l	
Agar (w/v)			0.8%	0.8%	0.8%

[a] pH was adjusted to 5.8

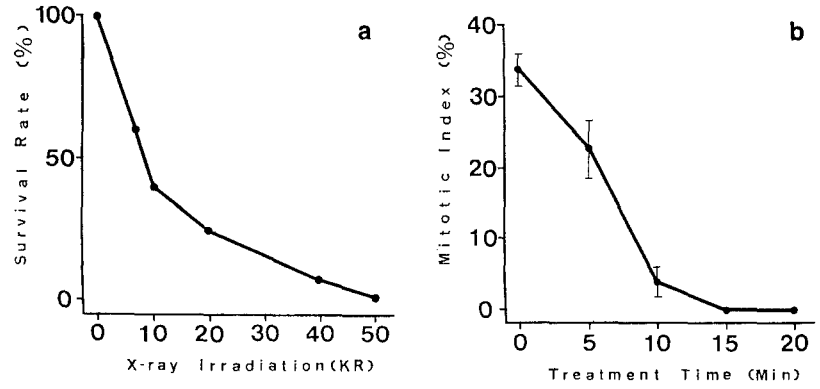


Fig. 1a, b Establishment of selective conditions to obtain somatic hybrids. **a** Effect of X-irradiation dosage on cell division of *D. carota* protoplasts. **b** Effect of iodoacetamide on cell division of *O. sativa* protoplasts. These results were measured after 2 weeks of culture

Protoplast fusion and culture

Following the pre-fusion treatments, protoplast populations of *D. carota* and *O. sativa* were hybridized by electrofusion using the selection design schematized in Fig. 3. After 2 months of culture, some colonies were observed on solid Medium E. As there was a possibility that some colonies were formed through the homoplasmic fusion of X-irradiated protoplasts, these colonies were transferred to shooting Medium F supplemented with 25 mg/l 5MT (Fig. 2b). *D. carota* callus growth was inhibited in the presence of 5MT (Fig. 2a). Five of the colonies regenerated green shoots on the same medium (Fig. 2c). The shoots regenerated from these colonies were then transferred to rooting Medium G (Fig. 2d). The regenerated plants closely resembled *D. carota* in morphology (Fig. 2f), but a few regenerants having narrow leaves without notches were also observed (Fig. 2e).

Characterization of the regenerated plants

Callus cultures induced from leaf segments removed from five regenerated plants and their parents were analyzed at the cytological and molecular levels. Cell suspension cultures were obtained from the induced calli and tested for 5MT resistance. All suspension cultures induced from the regenerated plants were resistant to 25 mg/l 5MT (Fig. 2g), but the observed resistance was intermediate between that of the two parents (Table 2, Fig. 4). The measurements of anthranilate synthase activity showed that the activity in regenerated plants activity no. 1 and 2 was about 3 times higher than that found in *D. carota* and similar to that observed in *O. sativa*. (Table 3).

Cytological analysis showed that the chromosome number of the regenerated plants ranged from 20 to 22, which is significantly lower than the additive chromosome number of the parents (Table 2).

The peroxidase isozyme was compared by polyacrylamide gel electrophoresis using callus cultures induced from regenerated plants and their parents. The isozyme band patterns of the regenerated plants resembled those of *D. carota*, but two bands of *O. sativa* had been added to these band patterns (Fig. 5).

Genomic DNA was analyzed by Southern hybridization using non-radioactively labelled cloned fragments of the *rgp1* gene. The regenerated plants had two hybridizing bands that corresponded to the bands from both parents as shown in Fig. 6. One band corresponding to a 18-kbp segment of *O. sativa* DNA was clearly present in extracts of all of the regenerated plants, which demonstrates their hybrid nature. MtDNA of the regenerated plants was analyzed by Southern hybridization. As shown in Fig. 7a and b, 1 of the regenerated plants (lane 2) had a *D. carota* band but also a unique band not present in either of the parents. CpDNA analysis revealed that regenerated plants had hybridization patterns identical to those of *D. carota* (Fig. 8a, b).

Discussion

We attempted to produce asymmetric hybrid plants between *D. carota* and 5MT-resistant *O. sativa* using the selection design schematized in Fig. 3. The morphology of the plants obtained by cell fusion closely resembled that of *D. carota*. Resistance of 5MT has been shown to be a useful marker when selecting for somatic hybrids (Kameya et al. 1981; Horn et al. 1983; Toriyama et al. 1986; Lee and Kameya 1989). In our study, all of the callus cultures induced from the regenerated plants were resistant to 5MT, which shows a clear relationship with anthranilate synthase activity as observed in selected 5MT-resistant lines (Widholm et al. 1977).

The hybrid plants possessed 20–22 chromosomes, fewer than the additive chromosome number of the parents. The results of the isozyme and chromosome analyses indicated that the regenerated plants had many *D. carota* and only a few *O. sativa* chromosomes. It is interesting to mention that although we treated *D. carota* protoplasts with X-ray irradiation, the regenerated plants had many *D. carota* chromosomes. Genomic DNA analysis using the *rgp1* gene as a probe revealed that regenerated plants had genomic DNA

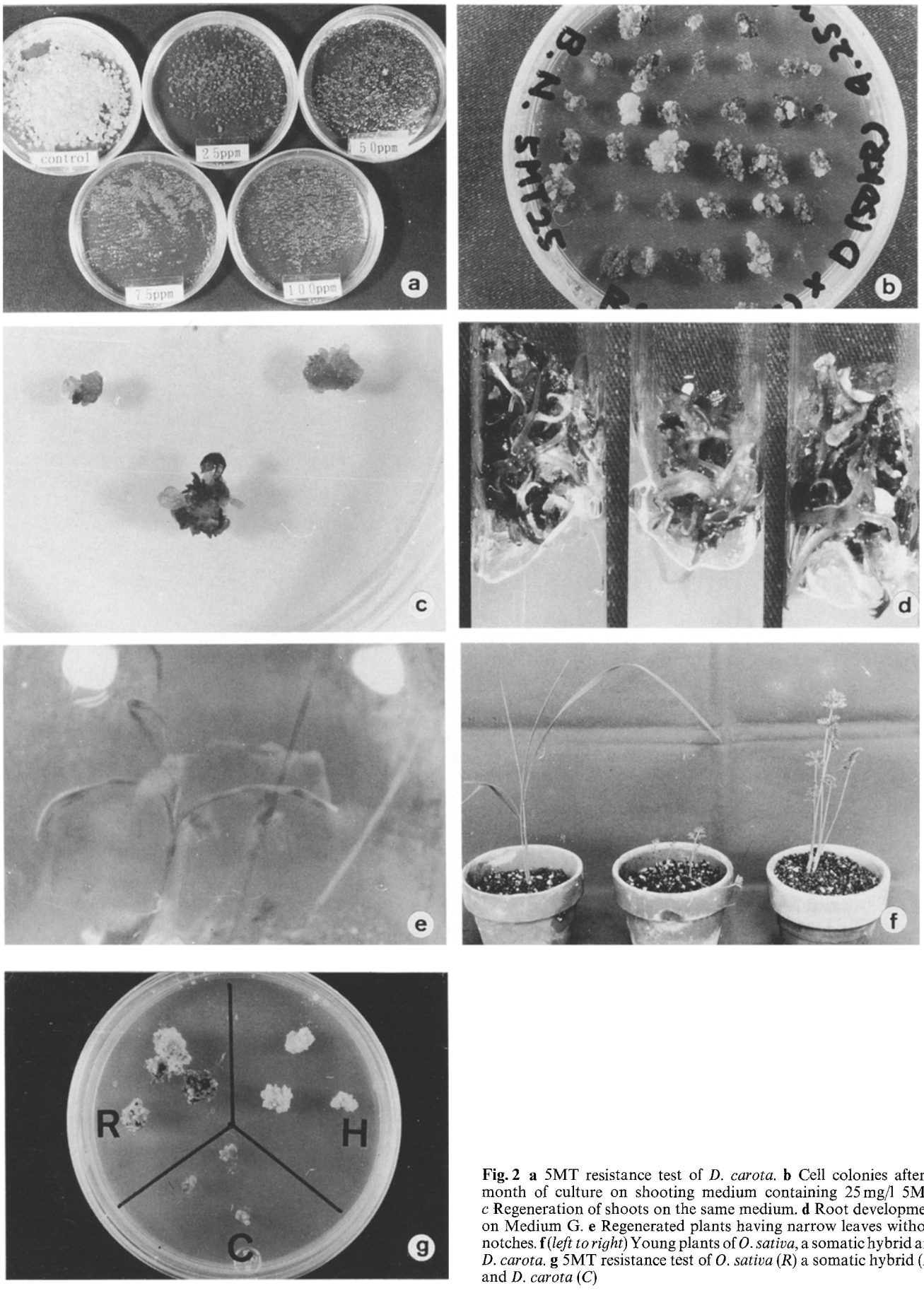


Fig. 2 **a** 5MT resistance test of *D. carota*. **b** Cell colonies after 1 month of culture on shooting medium containing 25 mg/l 5MT. *c* Regeneration of shoots on the same medium. **d** Root development on Medium G. **e** Regenerated plants having narrow leaves without notches. **f** (*left to right*) Young plants of *O. sativa*, a somatic hybrid and *D. carota*. **g** 5MT resistance test of *O. sativa* (*R*) a somatic hybrid (*H*) and *D. carota* (*C*)

Table 2 Expression of resistance marker, Chromosome number, isozyme pattern and cytoplasmic genotype in regenerated plants

Clone number	Resistance to 5MT (25 mg/l)[a]	Chromosome number	Isozyme pattern[b]	Chloroplast DNA[b]	Mitochondrial DNA[b]	
					atp6	*cob*
No. 1	++	20.4 ± 0.8	D.C. + s0	D.C.	D.C. + N	D.C + N
No. 2	+++	22.2 ± 1.1	D.C. + s0	D.C.	D.C	D.C.
No. 3	++	20.3 ± 0.9	D.C. + s0	D.C.	D.C.	D.C.
No. 4	++	20.1 ± 0.7	D.C. + s0	D.C.	D.C.	D.C.
No. 5	++	20.1 ± 0.7	D.C. + s0	D.C.	D.C.	D.C.

[a] Resistance test: +++, 100 ~ 80% callus relative growth; ++, 80 ~ 50% callus relative growth; +, 50 ~ 30% callus relative growth in the presence of 25 mg/l 5MT

[b] D.C. + s0, *D. carota* bands plus some *O. sativa* bands; D.C., *D. carota* type; D.C. + N, *D. carota* type plus novel patterns

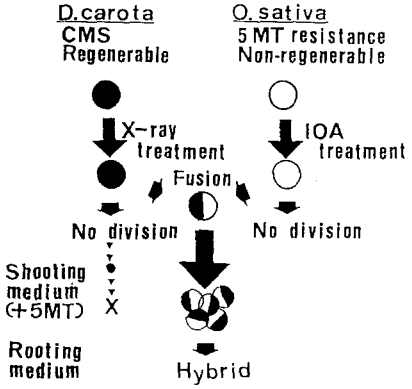
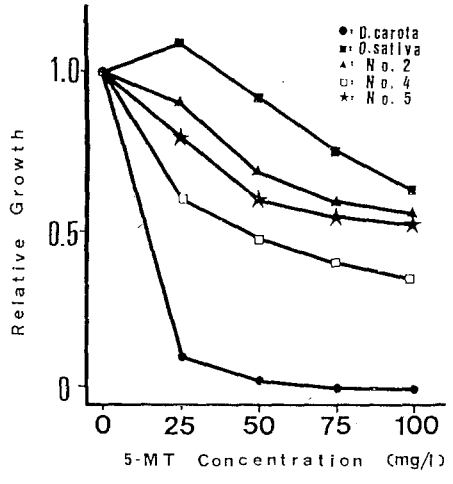


Fig. 3 Selection scheme for hybrid plants between *D. carota* and *O. sativa*

Table 3 Anthranilate synthase activity

Cell line	Anthranilate synthase activity (nM/30 min/g FW)	Relative values
D. carota	9.05	1.00
O. sativa	22.02	2.32
Hybrids		
No. 1	20.93	2.31
No. 2	35.13	3.88

Fig. 4 5MT resistance test of *D. carota*, *O. sativa* and somatic hybrids (no. 2, 4, 5)



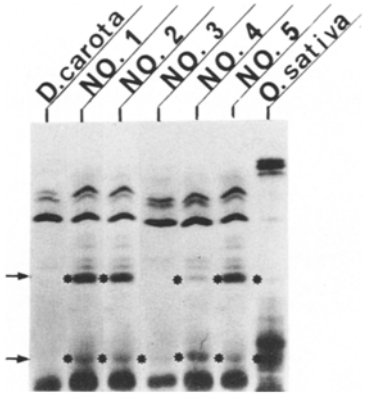


Fig. 5 Isozyme analysis of peroxidase. *D. carota* and *O. sativa* and somatic hybrids (no. 1–5). *Arrows* show bands of *O. sativa*

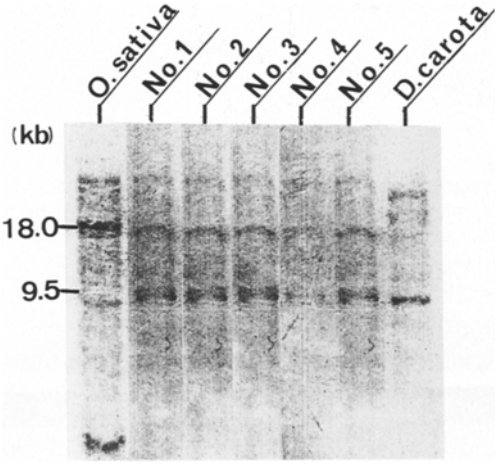


Fig. 6 Southern hybridization of genomic from *D. carota*, *O. sativa* and their somatic hybrids (no. 1–5). DNA was digested with *Bam*HI, and fragments of the *rgp1* gene were used as the probe

fragments from both *D. carota* and *O. sativa*. These results suggest that the regenerated plants were asymmetric hybrids between *D. carota* and *O. sativa*.

The recombination of mtDNA is interfamilial hybrids has been reported by Smith et al. (1989). We

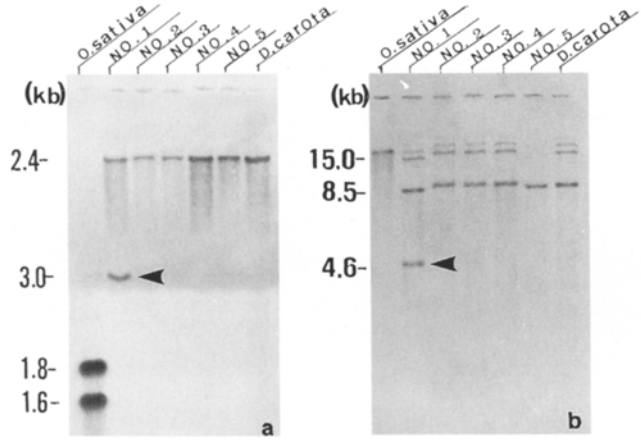


Fig. 7a, b Southern hybridization of mtDNA from *D. carota*, *O. sativa* and their somatic hybrids. MtDNAs were digested with *Eco*RI, and fragments of *atp6* (**a**) and *cob* (**b**) were used as probes

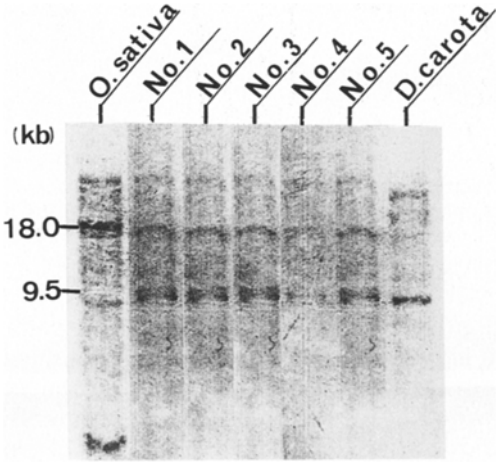


Fig. 8a, b Southern hybridization of cpDNA from *D. carota*, *O. sativa* and their hybrids. **a** CpDNAs were digested with *Bam*HI, and a *Bam*HI-7 fragment of rice cpDNA was used as the probe. **b** CpDNAs were digested with *Eco*RI, and a *Bam*HI-3 fragment of rice cpDNA was used as the probe

analyzed mtDNA of hybrid plants and their parents by Southern hybridization. Four cell lines contained only *D. carota* mtDNA fragments. However, one cell line had a novel band not present in either of the parents. This result indicated that recombinant mitocondrial genomes were generated in this hybrid.

Further, an analysis of cpDNA by Southern hybridization showed that all of the five hybrid plants had only *D. carota* cpDNA fragments.

These results allow us to conclude that we have successfully produced somatic hybrid plants between *D. carota* and *O. sativa* by cell fusion. As far as we know, this is the first report of somatic hybrid plants between a monocotyledon and a dicotyledon. We believe that these results provide useful data on hybridization between two remote species.

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