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H. Kisaka · M. Kisaka · A. Kanno · T. Kameya

Production and analysis of plants that are somatic hybrids of barley (*Hordeum vulgare* L.) and carrot (*Daucus carota* L.)

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Abstract In order to obtain plants that were somatic hybrids of barley (*Hordeum vulgare* L.) and carrot (*Daucus carota* L.), we fused protoplasts that had been isolated from 6-month-old suspension cultures of carrot cells with protoplasts isolated from barley mesophyll by electrofusion. After culture for 1 month at 25°C , the cells were cultured for 5 weeks at 4°C , and were then returned to 25°C for culture on a shoot-inducing medium. Three plants (nos. 1, 2 and 3) were regenerated from the cells. The morphology 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 had about 24 chromosomes, fewer than the sum of the numbers of parent chromosomes which was 32. Southern hybridization analysis with fragments of the *rgp1* gene used as probe showed that the regenerated plants contained both barley and carrot genomic DNA. Chloroplast (ct) and mitochondrial (mt) DNAs were also analyzed with several probes. The ctDNA of the regenerated plants yielded hybridization bands specific for both barley and carrot when one fragment of rice ctDNA was used as probe. Furthermore, the regenerated plants yielded a barley specific band and a novel band with another fragment of rice ct DNA as a probe. One of the regenerated plants (no. 1) yielded a novel pattern of hybridized bands of mt DNA (with an *atp6* probe) that was not detected with either of the parents. These results indicated that the regenerated plants were somatic hybrids of barley and carrot and that recombination of both the chloroplast genomes and the mitochondrial genomes might have occurred.

Key words *Hordeum vulgare* L. · *Daucus carota* L. · Interfamilial somatic hybrid · Low temperature · Recombination

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

Introduction

The goal of plant breeding is the construction of new genotypes by the introduction and the manipulation of genetic variations. The production of somatic hybrid plants by protoplast fusion is a useful method for combining genetic materials. Protoplast fusion can sometimes lead to the production of new genetic variants as a consequence of the recombination of nuclear and cytoplasmic genomes. Many intra- and inter-specific, and several intergeneric, somatic hybrid plants have been reported (Melchers et al. 1978; Aviv et al. 1980; Gupta et al. 1982, 1984; Menczel et al. 1983; Negrutiu et al. 1986; O'Connell and Hanson 1986; Pental et al. 1986; Toriyama et al. 1987; Gleba et al. 1988; Kameya et al. 1989; Toki et al. 1990; Perl et al. 1991; Kostenyuk et al. 1991; Babiychuk et al. 1992). Recently, asymmetric hybrids between remote species, for example interfamilial hybrid plants, have been obtained by exploiting various systems for the selection of hybrids (Somers et al. 1986; Dudits et al. 1987; Kisaka and Kameya 1994; Kisaka et al. 1994).

Barley (*Hordeum vulgare* L.) is a crop plant that tolerates low temperatures and salinity. To examine the possibility that these characteristics of barley might be transferable to other crops by protoplast fusion, we attempted to produce plants that were somatic hybrids of barley and carrot (*Daucus carota* L.), utilizing the low-temperature tolerance of barley for the selection of hybrids. In the present report, we describe the production and analysis of somatic hybrids of barley and carrot.

Material and methods

Cultivation of plants and cells

Seeds of *H. vulgare* (var. 'Hashirihadaka') were surface-sterilized in 70% (v/v) ethanol for 30 s and then in a 2% (v/v) solution of sodium hypochlorite for 15 min. They were washed twice in sterile distilled water, placed on medium A (Table 1), and cultured under continuous fluorescent light (4 W/m^2) at 25°C. After 1 week, the young leaves were used for the isolation of protoplasts. Callus was induced

Table 1 Composition of culture media. 2,4-D=2,4-Dichlorophenoxyacetic acid; NAA=naphthaleneacetic acid; BAP=N^6-benzylaminopurine; MS=Murashige and Shoog's (1962) formulation

Component	Medium A	Medium B	Medium C	Medium D	Medium E
Mineral salts	MS	MS	MS	MS	MS
Vitamins	MS	MS	MS	MS	MS
Sucrose (w/v)	3.0%	3.0%	3.0%	3.0%	3.0%
Glucose (w/v)			5.0%		
2,4-D		1.0 mg/l	1.0 mg/l		
Kinetin		0.1 mg/l	0.5 mg/l		
NAA				0.1 mg/l	
BAP				1.0 mg/l	
Agar (w/v)	0.8%	0.8%		1.0%	0.8%

from surface-sterilized leaf segments of a cytoplasmic male-sterile (cms) strain of *D. carota* (Kanzaki et al. 1991) cultured on Medium B (Table 1) under continuous fluorescent light (4 W/m^2) at 25°C. The resulting calli were transferred to a liquid medium (medium B minus agar) and sub-cultured at 2-week intervals.

Isolation and fusion of protoplasts and the selection and culture of hybrid microcalli

Protoplasts of *H. vulgare* were isolated from young leaves by incubation in an enzyme solution [1.6% (w/v) Cellulase Onozuka R10 (Yakult Biochemicals, Tokyo, Japan), 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 *D. carota* were isolated from cells in 6-month-old suspension cultures by the incubation of cells under the same conditions as those described for *H. vulgare*. The protoplasts were filtered through a nylon net (50-μm mesh) and washed twice with 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) solution of sucrose with subsequent centrifugation at 80× g for 5 min, and then they were washed once with the washing solution. Equal numbers of the two kinds of protoplast were combined to give a final density of 5 10^3/ml and were subjected to electrofusion using a BTX Electro Cell Manipulator 200.

The fused protoplasts were washed once with washing solution and diluted with an equal volume of a solution of Gelrite [0.3% (w/v) Gelrite (Sigma), 3% (w/v) sucrose and 5% (w/v) glucose]. The suspension of protoplasts was added to the protoplast culture medium (medium C, Table 1) in plastic Petri dishes. Non-fused protoplasts isolated from *H. vulgare* and *D. carota* were cultured, as controls, under the same conditions as those described for fused protoplasts. During the first month of culture, the Petri dishes were incubated in darkness at 25°C. The resultant microcalli were subsequently cultured in medium D (Table 1) and incubated in darkness at 4°C . After 5 weeks at 4°C, the petri dishes were transferred to continuous light (4 W/m^2) at 25°C for 1 month. The resultant visible colonies of about 1–2 mm in diameter were transferred to fresh shooting medium (medium D, Table 1). Regenerated shoots were transferred to root-inducing medium (medium E, Table 1).

Cytological analysis

Chromosome numbers were determined by treating actively growing cells in suspension culture (from the regenerated plants and from *D. carota*) and root tips (*H. vulgare*) with 0.03% (w/v) 8-quinolinol for 3 h at 25°C, with subsequent fixation in a mixture of ethanol and glacial acid (1:3, v/v) for 16 h at 25°C. The cells were then treated with 1 N HCl for 7 min at 60°C and stained with Schiff's reagent for 1 h at 25°C. Chromosomes were counted in about ten metaphases per regenerated plant.

Analysis of DNA

Total DNA was prepared from callus (from regenerated plants and from *D. carota*) and from the intact plant (*H. vulgare*) by the method described by Honda and Hirai (1990). Southern hybridization was performed with a non-radioactive DNA labelling and detection kit (Boehringer Mannheim, Town, Germany). The cloned fragment of the *rgp1* gene (Sano and Youssefian 1991) was a gift from Dr. H. Sano, (NAIST, Nara, Japan). The clones corresponding to subunit 6 of F_1-F_0 ATPase (*atp6*) and cytochrome b (*cob*) of rice mitochondrial DNA together with fragments of rice ctDNA were a gift from Dr. A. Hirai (University of Tokyo, Japan). Plasmids containing mitochondrial genes for 26*s* and 18*s* ribosomal RNA, and for subunit 9 of F_1-F_0 ATPase (*atp9*) were provided by Dr. K. Nakamura (Nagoya University, Nagoya, Japan). The cloned genes were used as probes for Southern hybridization.

Results

Effects of low-temperature treatment

When cells from 6-month-old suspension cultures of cells isolated from *D. carota* were plated on medium D (Table 1) and incubated at 4°C for various periods and the calli then

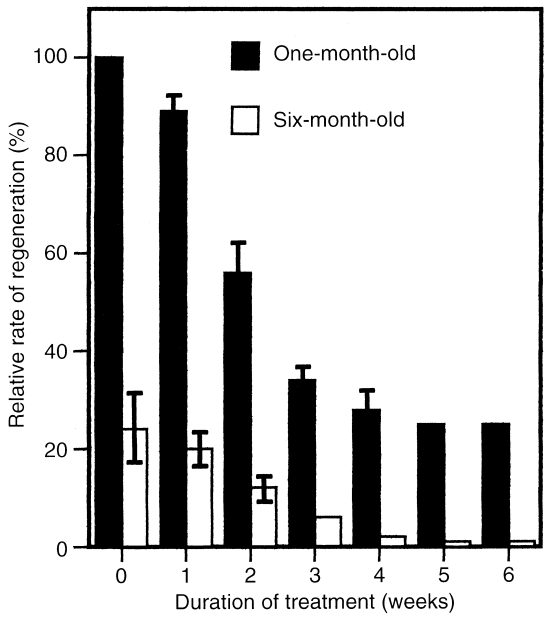


Fig. 1 Effects of low-temperature treatment on cell regeneration. One-month-old and 6-month-old calli of *D. carota* were cultured at 4°C for various periods, and were then transferred to regeneration medium and cultured at 25°C for 6 weeks. The regeneration rate in control cells (1-month-old) without low-temperature treatment was taken as 100%

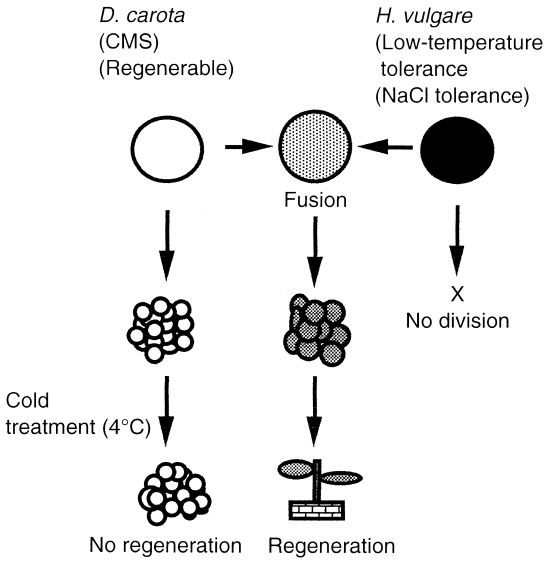


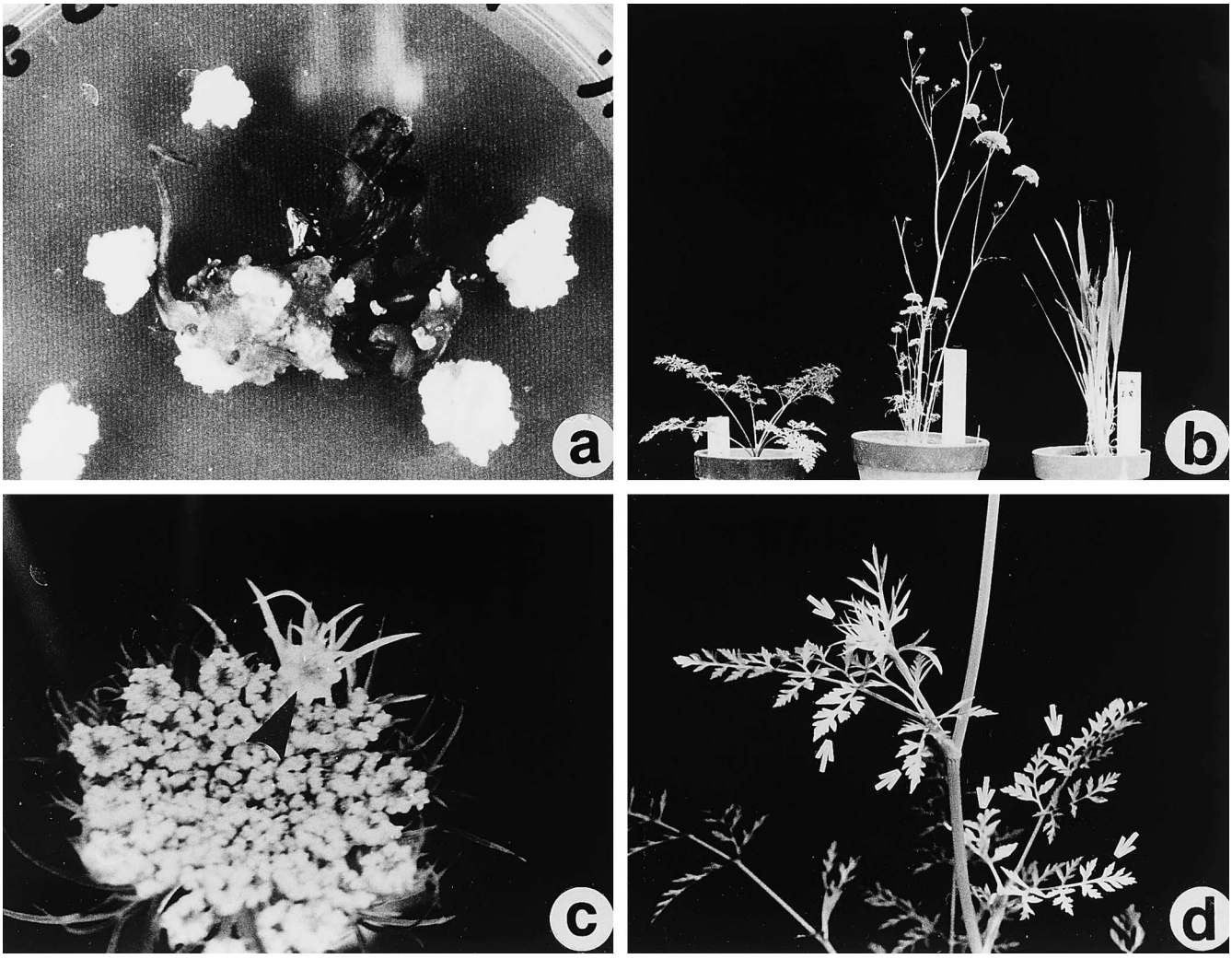
Fig. 2 Scheme for selection of hybrids between *H. vulgare* and *D. carota*

Fig. 3 **a** Regeneration of shoots from selected callus. **b** (left to right) Plants of *D. carota*, a regenerant, and *H. vulgare*. **c** and **d**. The green and white leaves (**c**) and flowers (**d**). The *arrow* indicates a white flower

transferred to 25°C, the number of the regenerated calli decreased with increasing duration of the low-temperature treatment (Fig. 1). On the basis of this result, the low-temperature treatment for the selection of hybrid calli consisted of incubation at 4°C for 5 weeks after incubation for 1 month at 25°C of the fused cells.

Protoplast fusion and culture of fused cells

Protoplasts of *D. carota*, isolated from cells in suspension culture, and those of *H. vulgare*, isolated from young leaves, were fused by electrofusion, and the fused cells were then cultured according to the scheme outlined for the selection of hybrids in Fig. 2. After culture for 1 month at 25°C, the fused cells were transferred to medium D (Table 1) and incubated at low temperature (4°C) for 5 weeks in darkness. The resultant calli were transferred to continuous light (4 W/m^2) at 25°C. When visible colonies had developed to about 1–2 mm in diameter, about 2700 colonies were transferred to fresh medium D (Table 1). Three shoots were regenerated (Fig. 3a) and these were transferred to medium E (Table 1). The three



regenerated plants were potted in soil and designated no.1, no. 2 and no. 3.

The protoplasts of *H. vulgare* that had been isolated from young leaves failed to divide. The protoplasts of *D. carota* that had been isolated from 6-month-old suspension cultures proliferated and formed colonies. However, about 1400 colonies that had been incubated at 4°C for 5 weeks failed to regenerate any shoots. Furthermore, no plants were obtained from protoplasts of either *H. vulgare* or *D. carota* were cultured under the same conditions but without fusion treatment.

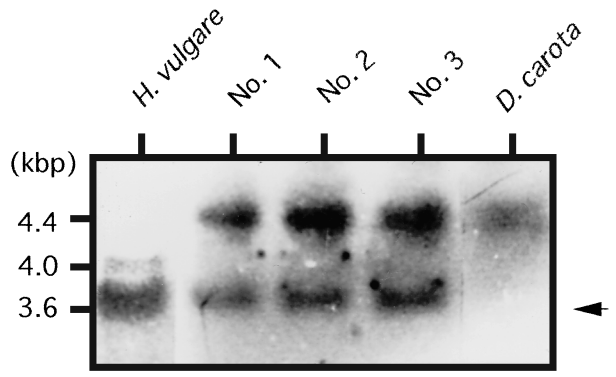


Fig. 4 Results of Southern-hybridization analysis of genomic DNA. Total DNA was digested with *Hin*dIII, and a fragment of the *rgp1* gene was used as the probe. The *arrow* indicates a band specific for *H. vulgare*

Table 2 Chromosome numbers of the parental and the somatic hybrid plants

Cells	Chromosome number	Standard error[a]
D. carota	18.4	1.2
H. vulgare	14.0	–
Somatic hybrids		
No. 1	24.4	1.4
No. 2	24.9	2.6
No. 3	24.2	1.8

[a] n≥10

Analysis and characterization of the three regenerated plants

The somatic hybrid plants closely resembled *D. carota* in morphology (Fig. 3b). Hybrid no. 1 had variegated green and white leaves and flowers, which developed without vernalization (Fig. 3c, d). The morphology of the roots of the somatic hybrids was similar to that of roots of *D. carota*. The flowers exhibited male sterility as did those of the parent strain of *D. carota*.

Callus cultures induced from leaf segments of the regenerated plants and their parents were analyzed at the cytological and molecular levels. Cytological analysis revealed that the chromosome number of the regenerated plants was about 24 (Table 2), significantly lower than the sum of the chromosome numbers (32) of the parents. Genomic DNA was analyzed by Southern hybridization with a non-radioactively labelled DNA fragment of the *rgp1* gene. The regenerated plants generated both a band specific for *D. carota* (4.4 kbp) and a band specific for *H. vulgare* (3.6 kbp) (Fig. 4). Chloroplast (ct) and mitochondrial (mt) DNAs were also analyzed by Southern hybridization with fragments of ctDNA and mtDNA (Table 3). The results of the analysis of ctDNA with a non-radioactively labelled *Bam*HI-8 fragment of rice ctDNA as a probe indicated that the regenerated plants yielded both bands specific for *D. carota* (4.2 kbp and 2.2 kbp) and a band specific for *H. vulgare* (9.0 kbp) (Fig. 5a). The regenerated plants also yielded a band specific for *H. vulgare* (4.4 kbp) and a unique band (8.6 kbp) when the *Bam*HI-3 fragment of rice ctDNA was used as the probe (Fig. 5b). In the analysis of mtDNA, one of the regenerated plants (no. 1) yielded a novel band (9.0 kbp) that was not detected in the

Table 3 Analysis of ctDNA and mtDNA in regenerated plants

Cell line	Fragments of mtDNA				
	atp6	*atp9*	*cob*	18*s* rRNA	26*s* rRNA
No. 1	D.C.+U[a]	D.C.[b]	D.C.	D.C.	D.C.
No. 2	D.C.	D.C.	D.C.	D.C.	D.C.
No. 3	D.C.	D.C.	D.C.	D.C.	D.C.
Cell line	Fragments of mtDNA				
	*Bam*HI-3	*Bam*HI-8	*Bam*HI-10	*Bam*HI-13	*Bam*HI-16
No. 1	H.V.+U[c]	D.C.+H.V.[d]	D.C.	D.C.	D.C.
No. 2	H.V.+U	D.C.+H.V.	D.C.	D.C.	D.C.
No. 3	H.V.+U	D.C.+H.V.	D.C.	D.C.	D.C.

[a] D.C.+U, *D. carota*-type plus a unique band
[b] D.C., *D. carota*-type
[c] H.V.+U, *H. vulgare*-type plus a unique band
[d] D.C.+H.V., *D. carota* bands plus *H. vulgare* band

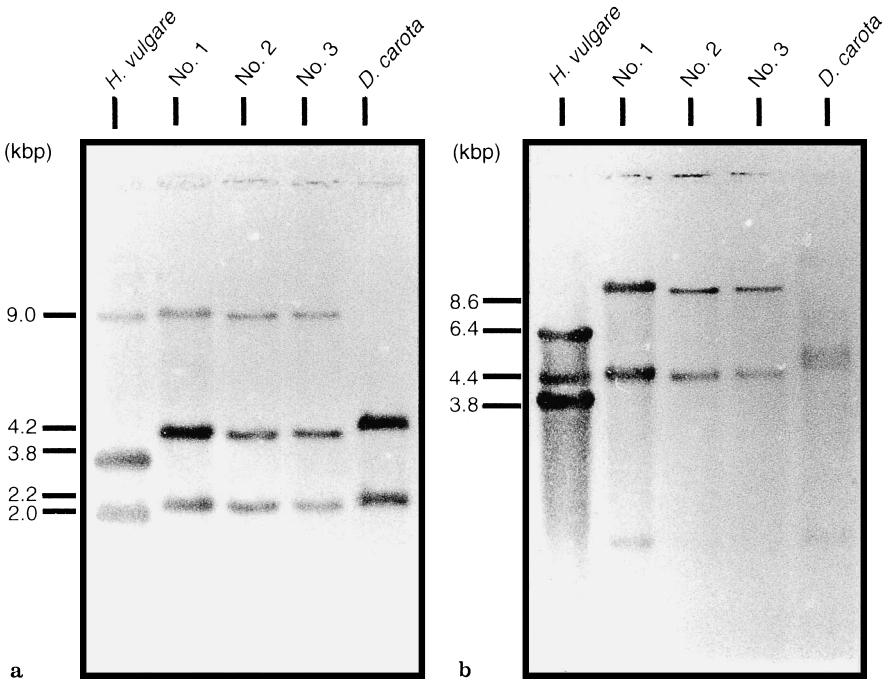


Fig. 5 a, **b** Results of Southern hybridization of ctDNA. Total cellular DNA was digested with *Hin*dIII, and the *Bam*HI-8 fragment (**a**) and the *Bam*HI-3 fragment (**b**) of rice ctDNA were used as probes

analysis of either parent when a fragment of *atp6* was used as the probe (Fig. 6). These results indicated that the regenerated plants were somatic hybrids of *H. vulgare* and *D. carota*.

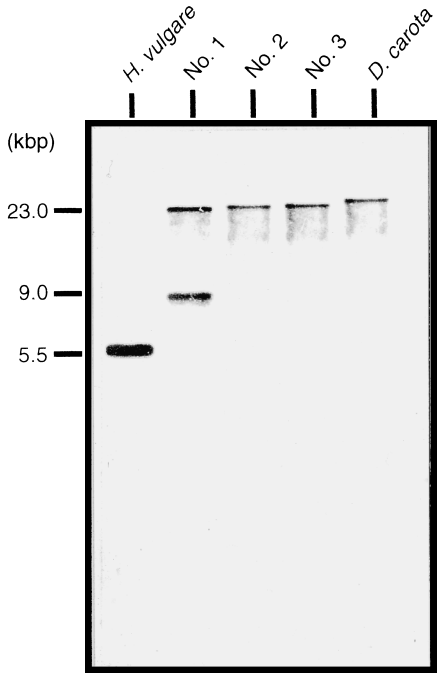


Fig. 6 Results of Southern hybridization of mtDNA. Total cellular DNA was digested with *Eco*RI, and a fragment of *atp6* was used as the probe

Discussion

To produce somatic hybrids between *H. vulgare* and *D. carota*, we incubated fused cells at a low temperature (4°C) for 5 weeks after fusion, and obtained three somatic hybrid plants. *Hordeum* species are very difficult to regenerate from protoplasts (Wang and Lorz, 1994). In the present study, *H. vulgare* protoplasts isolated from young leaves also failed to divide. Furthermore, *D. carota* protoplasts isolated from 6-month-old callus did not yield any plants because the callus had low plant-regenerative ability and this was further decreased with low-temperature treatment for a long time. As a result, low-temperature incubation was effective for the selection of hybrids.

Evidence for the recombination of chloroplast genomes from different genetic lines of higher plants has been reported (Medgyesy et al. 1985; Thanh and Medgyesy 1989). In our experiment, five fragments of ctDNA were used as probes for Southern hybridization to analyze the ctDNA of the regenerated plants. With *Bam*HI-8 and *Bam*HI-3 fragments of rice ct DNA as probes the regenerated plants yielded both the parental bands or else a band specific for *H. vulgare* and a novel band. Our results indicate that part of the ctDNA was transferred from *H. vulgare* to the regenerated plants. The recombination of mtDNA in interfamilial hybrids has been reported previously (Smith et al. 1989; Kisaka et al. 1994). In the present study, plant no. 1 yielded a novel band when *atp6* was used as a probe for mtDNA. This result is evidence of the possible recombination of the mitochondrial genomes of *D. carota* and *H. vulgare*. When several other fragments of ctDNA and mtDNA were used as probes, the regenerated plants yielded the same patterns of bands as *D. carota*. Further-

more, the regenerated plants yielded both parental bands with a DNA fragment of the nuclear *rgp1* gene as probe. Taken together, these results indicated that the three regenerated plants were somatic hybrids between *H. vulgare* and *D. carota*.

From the chromosome number and pattern of Southern hybridization of genomic DNA, ctDNA and mtDNA, it seems possible that these three somatic hybrid plants might have been be regenerated from the same fused cell because the chromosome number and the Southern hybridization patterns of genomic DNA and ctDNA were the same, respectively, for all three somatic hybrids. Thus, the recombination of the chloroplast and mitochondrial genomes of two remote species, from different families, seems to have occurred as a result of cell fusion.

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