Theor Appl Genet (1993) 85:761–769

THEORETICAL AND APPLIED GENETICS

Organellar segregation, rearrangement and recombination in protoplast fusion-derived *Brassica oleracea* calli

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Received January 21, 1992; Accepted May 7, 1992
Communicated by D. R. Pring

Summary. Cauliflower protoplasts were fused to determine the effect of protoplast source and pretreatment on organellar segregation in fusion products. Mitochondrial and chloroplast type were determined for over 250 calli from eight fusions between iodoacetate-treated or γ-irradiated leaf or hypocotyl protoplasts with fertile or Ogura cytoplasms. Organelles in fusion-derived calli were identified with five mitochondrial probes and one chloroplast probe. Mitochondrial and chloroplast segregation were independent but biased. Most calli had *B. oleracea* chloroplasts, but more calli had Ogura mitochondria than *B. oleracea* ones. Neither protoplast source nor pretreatment alone affected organelle segregation. However, iodoacetate treatment of hypocotyl protoplasts reduced their mitochondrial contribution to the fusion products although it did not affect chloroplast segregation. Over half of the calli had mitochondrial genomes distinct from those of either fusion partner; many of these contained the complete mitochondrial genome of one partner along with some mitochondrial DNA from the other. Out of 258 calli, 83 showed evidence of mitochondrial recombination, most commonly by formation of a novel 11-kb *Pst*I fragment near the *atp*9 region.

Key words: Organelle segregation – Mitochondrial recombination – *Brassica oleracea* – Ogura cytoplasmic male sterility

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Introduction

Several generalizations can be made regarding the fate of organelles following plant protoplast fusion (reviewed in Pelletier 1986). Chloroplasts usually sort out, so that plants regenerating from fusion products frequently (but not always) contain chloroplasts from only one fusion partner. Mitochondria similarly sort out and may also recombine, giving rise to novel mitochondrial genomes unlike those of either fusion partner. Chloroplast recombination is quite rare in higher plants.

These properties have been applied to various goals: to improve the Ogura male-sterile cytoplasm by organelle reassortment (e.g., Pelletier et al. 1983), to transfer *Nicotiana*, potato, *Citrus*, and *Brassica* cytoplasms intact from one nuclear background to another in a single step (e.g., Galun et al. 1987; Vardi et al. 1987; Yarrow et al. 1990), and to identify mitochondrial DNA (mtDNA) regions associated with cytoplasmic male sterility (CMS) in *Petunia* and *Brassica* (Rothenberg et al. 1985; Bonhomme et al. 1991).

Despite the importance of protoplast fusion methods in the manipulation of organellar traits, little is known about the role of protoplast pretreatment in organelle segregation. Irradiation and the metabolic inhibitors iodoacetate (IA) and Rhodamine 6-G are commonly used as pretreatments to prevent the division of unfused protoplasts. Irradiation also restricts a fusion partner's nuclear DNA contribution to the fusion product, but does not appear to affect organellar genomes as severely as the nucleus (Sidorov et al. 1981). Rhodamine 6-G, which is known to affect mitochondrial transmission in animal somatic hybrids, may have a similar effect in protoplast fusion products

(Böttcher et al. 1989). The effect of protoplast source (tissue used for protoplast isolation) is also incompletely understood, although Sundberg et al. (1991) reported that chloroplast segregation in *B. oleracea–B. napus* somatic hybrids was unaffected by the choice of hypocotyl or leaf-derived protoplasts for fusion.

The question of independent segregation has been difficult to address because the number of plants recovered from protoplast fusions have generally been rather small (for example, Kao et al. (1992) reported nine plants from two fusions). In an analysis of 42 calli derived from fused *B. napus* protoplasts with normal and Ogura cytoplasms, Morgan and Maliga (1987) reported that chloroplast segregation was complete, and biased towards *Brassica* chloroplasts. Mitochondrial segregation was incomplete and showed no such bias, implying that chloroplast and mitochondrial segregation were independent.

In this paper, we describe organelle segregation in over 250 calli from eight fusions of isogenic Ogura CMS and fertile cauliflower lines. Leaf or hypocotyl protoplasts of one partner were γ-irradiated before each fusion; those of the other partner were treated with iodoacetate. The organellar types of fusion-derived calli are compared to determine the effect of protoplast source and pretreatment on organelle segregation. We also describe the independent segregation of mitochondria and chloroplasts and mitochondrial rearrangements in the fusion products.

Materials and methods

Plant materials

Protoplasts were isolated from leaf and hypocotyl explants of cauliflower lines NY 7642A and NY 3317. NY 7642A is a male-sterile cauliflower inbred with the Ogura cytoplasm from *Raphanus sativus* (Dickson 1985). NY 3317 has the *B. oleracea* cytoplasm. It was selected from a protoplast regenerant of the maintainer line for NY 7642A (Jourdan et al. 1990), and so is nearly isogenic to NY 7642A.

Protoplast isolation and pretreatments

Protoplasts were isolated from hypocotyls of 5- to 7-day-old etiolated seedlings (Jourdan et al. 1989a) and from young expanded plantlet leaves (Jourdan et al. 1990). Hypocotyl protoplasts were treated with fluorescein diacetate (FDA) for the last 20–30 min of incubation (1 µl of a 5 mg/ml acetone stock per ml enzyme solution). To pretreat protoplasts with iodoacetate (IA), an appropriate amount of a freshly prepared, filter-sterilized stock of 25 m*M* IA made up in W5 solution (Menczel and Wolfe 1984) was added to the protoplasts in the enzyme solution for a final concentration of 5 m*M*. Protoplasts were incubated with IA for 20–30 min before processing. A [^{60}Co] source was used to γ-irradiate protoplasts for 180 min at 112 rad/min during enzyme incubation; total dosage was 20.2 krad.

Protoplast fusion

Protoplast fusion and culture were as described in Walters and Earle (1990) and Walters et al. (1992). Six types of fusions were carried out; four types were leaf-hypocotyl and two were leaf-leaf fusions (Table 1). Hypocotyl-hypocotyl fusions were not under-

Table 1. Organellar type in calli from eight fusions between Ogura CMS cauliflower NY 7642A and near-isogenic maintainer NY 3317

	Fusion								Total
	1	2	3	3′	4	5	6	6′	
Fusion partners[a]									
NY 3317	L/IA	L/γ	H/γ	H/γ	L/IA	H/IA	L/γ	L/γ	
NY 7642A	L/γ	L/IA	L/IA	L/IA	H/γ	L/γ	H/IA	H/IA	
Chloroplasts									
ole	24	29	13	17	37	31	19	45	215
rph	1	13	1	0	0	3	0	0	18
Both	2	9	8	0	1	4	3	1	28
Total	27	51	22	17	38	38	22	46	261
Mitochondria									
ole	1	1	1	0	1	1	3	0	8
ogu	13	17	2	8	10	31	2	0	83
Both	3	4	4	1	3	0	0	4	19
Non-parental	9	19	14	8	36	3	17	42	148
N1[b]	0	5	6	3	15	0	14	38	81
N2	8	13	4	5	14	3	2	3	52
N3	1	1	0	0	3	0	0	1	6
N4	0	0	4	0	4	0	1	0	9
Total	26	41	21	17	50	35	22	46	258

[a] NY 3317 has *ole* chloroplasts and mitochondria; NY 7642A has *rph* chloroplasts and *ogu* mitochondria. Protoplasts were derived from leaves (L) or hypocotyls (H) and treated with 5 m*M* iodoacetate (IA) or 20.2 krad γ-irradiation (γ)

[b] Non-parental mtDNA types: N1, all *ole* fragments + some others (usually *ogu*); N2, all *ogu* fragments + some others (usually *ole*); N3, all *ole* and *ogu* fragments + novel fragment(s); N4, some *ole* fragments + some *ogu* fragments

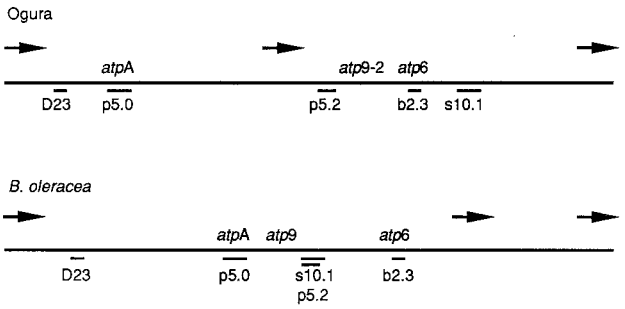

Fig. 1. Regions of Ogura and *B. oleracea* mitochondrial genomes hybridizing with the five probes used in this study, based on the maps published in Makaroff and Palmer (1988) and Palmer and Herborn (1988). Areas of strong hybridization are shown; some probes also hybridized weakly with other regions. Both maps are linearized at the *Bgl*I site internal to one of the recombination repeats, which are indicated by *arrows*

taken due to the large numbers of seeds required and the increased chance of contamination in these fusions.

DNA isolation and hybridization

Total DNA was isolated from calli weighing 50–200 mg using a scaled-down version of the Dellaporta et al. (1983) procedure. Volumes of all solutions were reduced 20 times; after the initial grinding and incubation steps, the samples were processed in microfuge tubes.

Samples were digested overnight with an excess of enzyme (*Pst*I or *Bgl*I), electrophoresed in 0.8% agarose, and blotted to Zetabind nylon membranes (AMF Cuno) with a dry blot procedure (Palmer 1986). Nick translations of probes with [^{32}P] dCTP and filter hybridizations were carried out according to standard procedures (Maniatis et al. 1982).

Chloroplast type was identified by probing *Bgl*I digests with *Petunia* chloroplast fragment s8 (provided by Maureen Hanson, Cornell University); mitochondrial type was identified with five probes hybridizing to regions throughout the Ogura and *B. oleracea* mitochondrial genomes (Fig. 1). *Pst*I digests were probed with four clones (s10.1, p5.2, p5.0, and b2.3) provided by Jeffrey Palmer, University of Michigan and Chris Makaroff, Miami University. Probe s10.1 hybridizes to the ORF 87 92/*trn*W P region, p5.2 hybridizes to a region near but not including the *atp*9 gene, p5.0 hybridizes to a region including *atp*A, and b2.3 hybridizes to the *atp*6 region. *Bgl*II digests were probed with a fifth mitochondrial probe, D23 (also from Chris Makaroff). D23 hybridizes to a region of Ogura mtDNA near one of the recombination repeats, within the 8.6-kb *Bgl*II fragment and including the *Pst*I site between the 13- and 5.2-kb *Pst*I fragments. This region is correlated with the CMS phenotype (Bonhomme et al. 1991; Poleman-Stephenson 1991; Walters et al. 1992).

Results

The IA and γ-irradiation pretreatments prevented the division of unfused protoplasts; unfused fusion partner protoplasts did not form colonies when cultured. Calli were recovered from all six types of fusions (Table 1). In each fusion, 0.4–2.7 $\times$ 10^5 protoplasts were plated, producing 52–429 calli for transfer to solidified

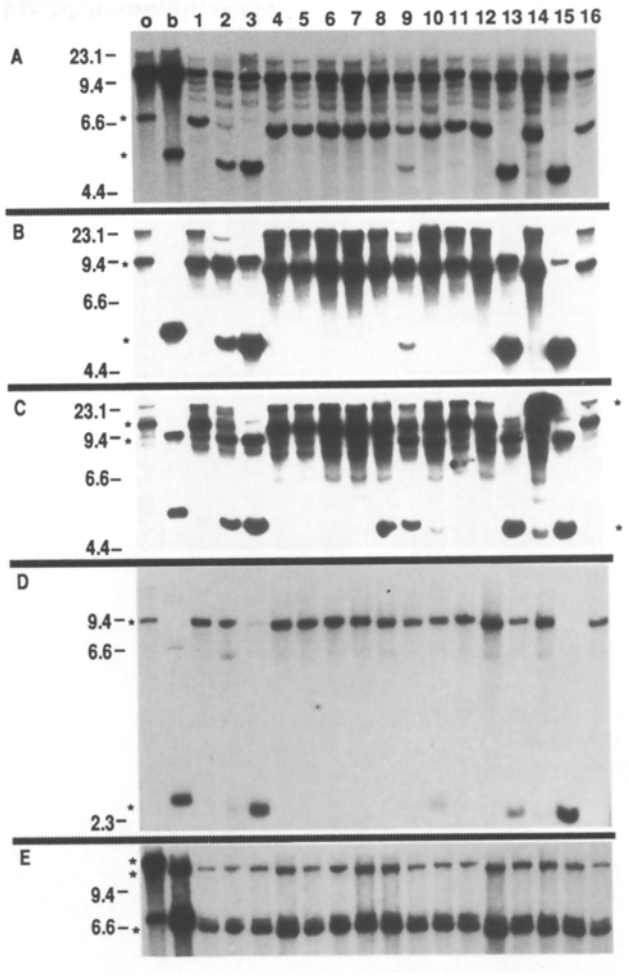

Fig. 2. Hybridization of organellar probes to digests of total DNA from fusion partners and fusion-derived calli. *Asterisks* indicate fragments that hybridize strongly for one partner only. *o* fusion partner NY 7642A (*ogu* cytoplasm), *b* fusion partner NY 3317 (*ole* cytoplasm), *1–16* individual calli from fusion 3′. **A** s10.1 mitochondrial probe, *Pst*I digest; **B** s5.2 mitochondrial probe, *Pst*I digest. There is a novel 11-kb band (slightly larger than the *ogu*-specific band) in *lanes 13* and *15*; the same band is present but less distinct in *lane 3*. **C** p5.0 (*asterisks* on *left*) and b2.3 (*asterisks* on *right*) mitochondrial probes, *Pst*I digest; **D** D23 mitochondrial probe, *Bgl*I digest; **E** s8 chloroplast probe, *Bgl*I digest

medium. These calli represented 0.11–0.24% of the protoplasts plated, considerably less than the plating efficiency of unfused protoplasts in this culture system (typically 3–5%, Walters and Earle 1990). The reduced plating efficiency presumably reflected the inability of treated, unfused protoplasts to divide. Over 50 calli grew large enough for analysis in four fusion experiments. In the other four experiments, 17–46 calli were used.

Total DNA was isolated from calli derived from each fusion experiment, as well as from 6 calli each from unfused NY 7642A leaf and NY 3317 hypocotyl

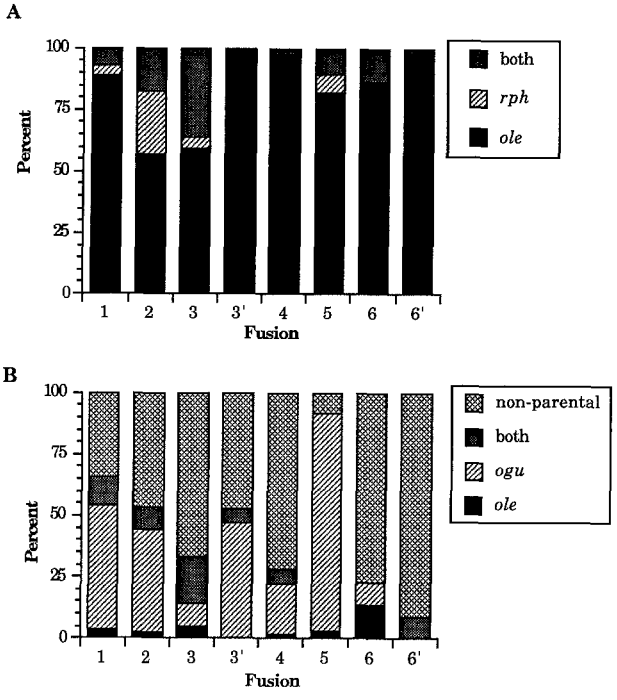

Fig. 3A, B. Percent calli with **A** *ole*, *rph*, or both chloroplast types, and with **B** *ole*, *ogu*, both or non-parental mtDNA types, based on Table 1

protoplasts. Each sample was digested with restriction enzymes, blotted, and hybridized with the organellar probes (Fig. 2). No alterations were detected in the chloroplast or mitochondrial DNA of calli from unfused protoplasts. Fusion-derived calli contained *B. oleracea* (*ole*), *Raphanus* (*rph*) or both chloroplast genomes, and *B. oleracea*, Ogura (*ogu*), both, or non-parental mtDNA types.

Chloroplast segregation

Calli with *ole* chloroplasts greatly outnumbered those with *rph* chloroplasts in all of the fusions (Table 1, Fig. 3A). Calli with *rph* chloroplasts were recovered only when leaf protoplasts of NY 7642A were used for fusion (fusions 1, 2, 3, and 5). The presence of *rph* chloroplasts in the calli was not assured by the use of NY 7642A leaf protoplasts however (note fusion 3′).

Two types of fusion experiment (fusions 3 and 6) were carried out twice. In one instance (experiments 6 and 6′) the chloroplast types were quite similar, but in other case (experiments 3 and 3′) they were different ($\chi^2 = 9.04$, $P < 0.05$). Of the calli in fusion 3, 59.1% contained *ole*, 4.5% had *rph* and 36.4% had both chloroplast types; all of the calli from fusion 3′ had *ole* chloroplasts (Fig. 3A). Although the proportions of chloroplast types differed in fusions 3 and 3′, *ole* chloroplasts were favored in both fusions.

The proportion of calli containing both chloroplast types varied from 0% to 36%. This variation was unrelated to protoplast type or pretreatment; fusions 3 and 3′ were identical in type, but represented the extremes in proportion of calli with both chloroplasts. If the smaller or lighter calli had completed fewer divisions and were therefore more likely to retain both chloroplast types, then isolation of DNA from such calli in some fusions would account for these differences. To investigate this, the average callus age and mass at the time of DNA isolation (80.7–120.7 days and 61.6–166.2 mg, respectively) were plotted against the percentage of calli with both chloroplasts from each fusion. Neither callus age nor callus mass accounted for the differences in chloroplast segregation among fusions; best-fitting regression lines accounted for only 1.6% and 2%, respectively, of the variation in calli with both chloroplasts (data not shown). Similarly, callus age and mass within fusions were unrelated to the presence of both chloroplast types. Calli from fusion 2 (which had the most calli with both types of chloroplasts) were divided into two classes according to age and into three classes according to mass, but the proportion of calli with mixed chloroplasts was not substantially different in any of these classes (data not shown).

Mitochondrial segregation

Calli were assigned an mtDNA type according to results with five mitochondrial probes (Table 1). These probes hybridize to regions dispersed throughout the *ogu* and *ole* mitochondrial genomes, but only hybridize to a portion of the mtDNA. Although they probably provide a good representation of the mtDNA type in the fusion products, they do not absolutely determine mitochondrial genome type. Some calli had only *ogu* or *ole* fragments for all five probes. Others had both *ole*- and *ogu*-specific fragments for all of the probes, suggesting the retention of both mitochondrial genomes. The remaining calli had non-parental mtDNA types; most of these had all the specific fragments of one fusion partner and some of the other partner's.

Most fusions produced more calli with *ogu* than with *ole* mitochondria. The proportion of calli with both mitochondrial types varied from 0% to 19%, which was slightly lower than the proportion of calli with mixed chloroplasts. Fusions producing the most calli with both chloroplasts did not always have the most calli with both mtDNA types, but individual calli with both chloroplast types were more likely to have both mitochondrial types (see section on independent segregation below).

The proportions of calli with the various mtDNA types were different in repeated fusions (fusions 3 and

3′; and 6 and 6′). This difference was statistically significant ($\chi^2 = 10.68$, $P < 0.05$) for fusions 6 and 6′, but not for fusions 3 and 3′. Taken together with the chloroplast data, these results show that there is considerable variation in organelle sorting-out, even in fusions of the same type conducted in the same laboratory. Slight or moderate differences between the eight fusions described here are probably due to this variation and cannot be attributed to specific pretreatment or cell type effects.

As in the chloroplast analysis, callus mass and age were not associated with the proportion of calli with both mitochondrial types. Similarly, there was no association between these factors and the number of calli with non-parental mtDNA types, either between or within fusions (data not shown).

Non-parental mtDNA types

Over half of the calli evaluated (148/258, or 57%) had non-parental mtDNA types that were distinct from *ole* and/or *ogu* mitochondrial genomes. Although the fusions varied considerably in the proportion of calli with non-parental mtDNA types (from 11% to 91%), these differences were not related to cell type or to pretreatment. Calli with non-parental mtDNA types were further divided into four categories (Table 1). N1: These had the *ole*-specific fragments for all of the probes and also had others (usually *ogu*-specific) for some probes. N2: Conversely to N1 calli, these had *ogu*-specific fragments for all probes and others, usually *ole*-specific, for some probes. N3: These calli had all of the *ogu* and *ole*-specific fragments as well as one or more novel bands. N4: These calli contained *ole*-specific fragments for some probes and *ogu*-specific fragments for others.

Almost 90% of the calli with non-parental mtDNA types were type N1 or N2; these appeared to have the complete mitochondrial genome from one fusion partner and a partial genome from the other. Fewer calli with non-parental mtDNA had both complete mitochondrial genomes (N3: 4%) or had neither complete parental mitochondrial genome but only some regions from each type (N4: 6%).

About half of the calli with non-parental mtDNA types had novel fragments for at least one mitochondrial probe (83/148, or 56%). By far the most common of these (72/83) was an 11-kb band observed by hybridizing *Pst*I digests with the p5.2 probe (Fig. 2B). This novel band was found in association with the *ole*-specific fragment and with both parental bands, but rarely with only the *ogu*-specific fragment, and never alone (Table 2). This 11-kb fragment was not found in any of the 12 calli regenerated from unfused protoplasts, and mtDNA of fusion-derived calli with this band was usually 'non-parental' in some other way as well, strongly sugggesting that the band is induced by fusion-related mitochondrial interactions.

Iodoacetate treatment of hypocotyl protoplasts consistently reduced their mitochondrial contribution to the fusion products. In fusion 5 (hypocotyl protoplasts of *ole* partner treated with IA) 31 calli had *ogu* mitochondria, and only 1 had *ole* ones. Most of the calli in fusions 6 and 6′ (*ogu* hypocotyl protoplasts treated with IA) had N1 mtDNA, suggesting partial loss of the *ogu* mitochondrial genome (Table 1).

Analyses of individual mitochondrial probes

Table 3 summarizes the hybridization patterns for each of the mitochondrial probes in 241 calli from the eight fusions. Numbers of calli with *ole*, *ogu* or both bands were relatively similar for probes s10.1, p5.2, b2.3, and p5.0, but were quite different for D23. With this probe very few colonies had only *ole*-specific fragments, and many had both *ole* and *ogu* bands. One interpretation is that the Ogura region hybridizing with D23 is better retained than the rest of the mitochondrial genome. Interestingly, the region hybridizing to the p5.0 probe is close to the D23 region

Table 2. Association of the novel 11-kb *Pst*I fragment with the *ole*-specific fragment in hybridization with mitochondrial probe p5.2

Other fragments	Total calli	Calli with 11-kb band
ole	52	32
ogu	127	3
Both	78	37
Novel	1	0
Total	258	72

Table 3. Numbers of calli with *ole*- and/or *ogu*-specific fragments for five mitochondrial probes, and coincidence of fragments

	Probe				
	s10.1	p5.2	b2.3	p5.0	D23
Fragment type					
ole	88	52	81	85	12
ogu	44	70	64	66	106
Both	109	119	96	90	123
Average coincidence[a]	2.13	2.18	2.28	2.29	1.79

[a] A ratio of observed: expected coincidence was calculated for each of the ten possible probe pairs. This was calculated as the number of calli with fragments from the same partner for both probes divided by the number predicted based on the frequency of each type. For each probe the average ratio of coincidence with the other four probes is shown

Table 4. Mitochondrial composition of calli with the complete mitochondrial genome from one partner and some mtDNA from the other

Mitochondrial type	Total calli	Calli with both *ole*- and *ogu*-specific fragments in region				
		s10.1	p5.2	b2.3	p5.0	D23
N1 (complete *ole*+ partial *ogu*)	81	4	38	9	7	77
N2 (complete *ogu*+ partial *ole*)	52	18	12	35	41	10

in Ogura (Fig. 1), but it is not similarly retained. The use of D23 or probes hybridizing to the same region might therefore be especially important in distinguishing individuals with exclusively *ole* mitochondrial genomes from those that also have some *ogu* mtDNA. We compared our analysis with and without D23; without this probe, the mtDNA type of 13 calli would have been incorrectly identified as *ole*. The total number of calli with *ole* mitochondria would have been 21, many more than the 8 indicated in Table 1.

All ten pairwise combinations of the five probes were not independent at a highly significant level (241 calli analyzed, $\chi^2 > 151$, $P < 0.001$ for every pair), strongly suggesting linkage on the mitochondrial genome. The D23 probe showed the weakest linkage to other probes (lowest average coincidence in Table 3); many calli had mtDNA from both partners that hybridized with this probe.

Different regions of the *ogu* and *ole* mitochondrial genomes were retained in calli with N1 and N2 mtDNA (Table 4). A high proportion of the calli with N1 mtDNA (which had the complete *ole* mitochondrial genome) had the *ogu* D23 and p5.2 regions, but many calli with N2 mtDNA (with the complete *ogu* mitochondrial genome) had the *ole* b2.3 and p5.0 regions. These calli also revealed an association between the b2.3 and p5.0 regions. Of the 7 calli with N1 mtDNA having the *ogu* b2.3 or p5.0 region, 5 had both regions. There was a similar linkage between these regions in the N2 calli (data not shown). The 9 calli with type N4 mtDNA (which had some mtDNA regions from one fusion partner and some from the other) tended to have *ole* mtDNA in the s10.1, p5.2 and p5.0 regions, together with the *ogu* D23 region.

Independent segregation

Results from the eight fusions were pooled to investigate the independence of organelle segregation. It was assumed that protoplast type or pretreatment did not affect the independence of organelle segregation. Segregation of mitochondrial type (*ole*, *ogu*, both or non-parental) and chloroplast type (*ole*, *rph* or both) was not independent (256 calli analyzed, $\chi^2 = 13.80$, $P < 0.05$). Much of the deviation from the expected values resulted from many calli with both chloroplast types and with mtDNA from both partners; perhaps organelle segregation in general was delayed in these calli. When calli with chloroplasts or mitochondria from both partners were omitted from the analysis, organellar segregation in the remaining calli was independent (203 calli; $\chi^2 = 0.65$, $P > 0.05$).

Plant regeneration

Four plants were regenerated from 3 calli produced in fusion 2; 1 plant was also recovered from a callus from fusion 4. All 5 plants had *ole* chloroplasts and *ogu* mitochondria [they are described in more detail as plants from fusions DF2 and DF3 in Walters et al. (1992)]. The uniform organellar composition of these plants was unexpected since only 11/41 and 8/38 calli from fusions 2 and 4, respectively, had this organelle combination. When we included calli with both chloroplasts and those with "both", N1 or N3, mtDNA types only 25/41 and 19/38 calli from these fusions had the potential to regenerate plants with *B. oleracea* chloroplasts and Ogura mitochondria.

Discussion

There was a strong bias towards the *ole* chloroplasts in all of these fusions, regardless of protoplast type or pretreatment. Leaf protoplasts may be slightly more effective at transmitting chloroplasts, since the only fusions in which calli with *rph* chloroplasts were recovered were those fusions in which they came through leaf protoplasts.

Others have also noted biased chloroplast segregation following fusion. Normal *Brassica* chloroplasts are more common in fusion products than atrazine-resistant chloroplasts when plants are regenerated in the absence of triazines (Yarrow et al. 1986; Thomzik and Hain 1988). In fusions between *B. napus* lines with normal and Ogura cytoplasms, Morgan and Maliga (1987) documented a bias towards *B. napus* chloroplasts. Atrazine-resistant *Brassica* chloroplasts were favored over *Raphanus* ones (Poleman-Stephanson 1991). It appears that there is an order of preferred chloroplast type in *Brassica: Brassica* > Atr^r *Brassica* > *Raphanus*. *B. napus* chloroplasts were favored in fusions with *Eruca sativa*, *R. sativus*, *B. nigra*, and *B. juncea*, but chloroplast segregation was unbiased in *B. oleracea–B. campestris* somatic hybrids (Lundgren and Glimelius 1990; Sundberg and Glimelius 1991). *B. napus* chloroplasts were similarly favored in

B. napus–B. oleracea fusions (Sundberg et al. 1991). Biased chloroplast segregation has also been reported in *Nicotiana* (Thanh et al. 1988; Böttcher et al. 1989; Pental et al. 1989).

One hypothesis regarding biased chloroplast segregation is that the bias reflects a nuclear-plastid incompatibility, and is therefore to be expected when the genetic distance between fusion partners is large. In the Lundgren and Glimelius (1990) and Sundberg and Glimelius (1991) reports, chloroplast segregation was biased in the widest fusions, but not in a fusion between the more closely related *B. oleracea* and *B. campestris*. Our own results are consistent with the nuclear-plastid incompatibility hypothesis: there was a clear bias against *R. sativus* chloroplasts in fusion products with *B. oleracea* nuclear DNA. However, genetic distance cannot be the sole factor affecting chloroplast segregation: Pental et al. (1989) effectively eliminated *N. tabacum* chloroplasts in fusions with *N. rustica* protoplasts by using microspore protoplasts of the *N. tabacum* partner.

There was a surprising bias towards Ogura mitochondria in these fusions. This contrasts with previous work in this laboratory (Jourdan et al. 1989a, b) in which no fusion-derived plants were recovered with the complete Ogura mitochondrial genome. Previous fusions used the same source of Ogura cytoplasm (cauliflower NY 7642A) but differed in the second fusion partner (*B. napus* or *B. campestris*) and in the fusion and culture protocols. We do not know which of these factors is responsible for the differences.

Treating hypocotyl protoplasts with iodoacetate apparently reduced their mtDNA contribution to the fusion products. Iodoacetate, a sulfhydryl reagent, is not a specific mitochondrial inhibitor (Wright 1978). Hypocotyl cells divide and grow more rapidly than leaf cells (Glimelius 1984); perhaps the enhanced mitochondrial activity in hypocotyl protoplasts makes those mitochondria more susceptible to iodoacetate. Whatever the mechanism, mitochondrial elimination with iodoacetate could improve control over the outcome of a fusion. For example, one might be able to reliably introduce CMS mitochondria into an otherwise desirable fertile genotype by irradiating the CMS partner to eliminate the nucleus and then fusing it with iodoacetate-treated hypocotyl protoplasts of the fertile line. Rhodamine 6-G has been used for mitochondrial elimination in mammalian cell fusions (Ziegler and Davidson 1981), but its action in plant protoplast fusions is less straightforward (Galun et al. 1988). Böttcher et al. (1989) reported that many *Nicotiana* fusion products involving a rhodamine-treated partner had more extensively recombined mitochondria, but there were few instances of complete mitochondrial elimination. Jourdan et al. (1989a) found that rhodamine 6-G was ineffective for mitochondrial elimination in *B. oleracea–B. campestris* fusions.

Differences in callus mass or age did not account for the differences in numbers of calli with mixed chloroplasts within or among fusions, nor did they account for difference in numbers of calli with mixed or non-parental mtDNA types. Apparently, organelle sorting-out did not progress much within the range of age (81–122 days after fusion) and mass (62–166 mg) analyzed. Obviously, some sorting-out took place before the calli were analyzed, and the organellar type of regenerated plants suggests further sorting-out or selection later in the regeneration process; perhaps there are distinct periods of sorting-out during culture. It may be that sorting-out is mainly limited to those times when a fusion product passes through a bottleneck of low cell number, making mixed organellar populations in those cells susceptible to genetic drift. These bottlenecks occur when the fusion product is just formed, and again when a few cells in a callus form a shoot primordium during the earliest stage of regeneration. Morgan and Maliga (1987) found that some fusion-derived plants had mtDNA different from that in the calli that gave rise to them, but they also reported a general agreement between the organellar types of calli and plants.

The Ogura mitochondrial genome is reportedly unstable in callus culture (Morgan and Maliga 1987). We found no evidence of mtDNA alterations in calli from unfused protoplasts, nor did Jourdan et al. (1990) find any alterations in the mtDNA of numerous protoplast-derived Ogura cauliflower plants. This lack of rearrangements may be related to the brief (2–3 month) period of callus culture both in our study and in that of Jourdan et al. (1990). Mitochondrial rearrangements in *B. campestris* have been observed to be associated with time in culture (Shirzadegan et al. 1991).

Many fusion-derived calli had non-parental mtDNA types; most of these seemed to have one complete mitochondrial genome and part of the other (N1 and N2 mtDNA). N1 calli were likely to retain the p5.2 and D23 Ogura mtDNA regions, but calli with N2 mtDNA usually had the b2.3 and p5.0 regions from *B. oleracea*. Based upon their work with *Petunia* fusion products, Boeshore et al. (1983) proposed a model in which subgenomic mtDNA molecules assort separately during the culture and regeneration of fusion products. However, comparisons of Table 4 and Fig. 1 show that the linkage of mtDNA regions in calli with N1 and N2 mtDNA are not consistent with predictions based on the positions of major recombinations repeats (Palmer 1988). Our results could reflect selective amplification of pre-existing sublimons; Shirzadegan et al. (1991) found evidence that extensively rearranged sublimons were present in the *B. campestris* mt genome and amplified in culture.

The D23 region was the one most likely to be contributed from both fusion partners. D23 was also the most loosely associated with other probes. One explanation might be that D23 hybridizes to an Ogura mtDNA region that is particularly competent for autonomous replication. If so, that region must be other than the subgenomic circle defined by the Ogura 10-kb repeats (Makaroff and Palmer 1988) because other probes hybridizing to the same circle do not cosegregate with D23. Böttcher et al. (1989) also found that some regions of mtDNA were much more likely than others to be transmitted from both fusion partners to the fusion products.

The mtDNA region hybridizing with the p5.2 probe showed the greatest evidence of rearrangement, frequently showing a novel 11-kb fragment in *Pst*I digests of fusion-derived calli. Robertson et al. (1987) and Jourdan et al. (1989a) described an identical novel fragment in several *B. napus* plants produced by fusion between Ogura CMS *B. oleracea* and atrazine-resistant *B. campestris*. In our calli the band is almost always seen with the *B. oleracea* parental-specific fragment, and with or without the Ogura-specific fragment. The novel fragment could result from amplification of a sublimon, but we did not see this fragment in calli from unfused protoplasts, even at long exposures. *Pst*I digests of *B. oleracea* and *B. campestris* both show a 5.2-kb band when probed with p5.2; a simple explanation for the 11-kb fragment is an insertion of 5.5–6-kb of Ogura mtDNA within the 5.2-kb fragment. In their investigations of culture-induced mtDNA changes in *B. campestris*, Shrizadegan et al. (1991) identified the endpoints of inversions and other rearrangements to either side of the p5.2 fragment.

In spite of numerous fusion between plants with Ogura and *Brassica* cytoplasms reported in the literature, the recovery of plants with the combination of *Raphanus* chloroplasts from Ogura and *Brassica* mitochondria has not been reported (except for M. Temple, personal communication). This has led to speculation that the *Raphanus* chloroplasts and *Brassica* mitochondria are somehow incompatible (Pelletier 1986; Bonnett and Glimelius 1990). This combination was also absent in the present analysis of over 250 fusion-derived calli. However, our results clearly indicate that mitochondria and chloroplasts sort out independently. The absence of this particular organelle combination was a result of bias against the *Raphanus* chloroplasts and of a similar but independent bias against the *Brassica* mitochondria. Given the proportions of calli with *B. oleracea* mitochondria (8/258) and with *Raphanus* chloroplasts (18/261), only 1 in 467 fusion products would be expected to have this combination of organelles. The chance of recovering a fusion product with *B. oleracea* mitochondria and *Raphanus* chloroplasts might be maximized in fusions with one or both of these biases eliminated; for example, one could treat hypocotyl protoplasts of the Ogura partner to reduce its contribution of mtDNA.

The analysis of a large number of fusion-derived calli led us to several conclusions. Both chloroplast and mitochondrial segregation were biased in this particular fusion combination, and these biases were generally unaffected by protoplast type or pretreatment. However, mitochondrial segregation was affected by pretreatment of hypocotyl protoplasts with iodoacetate; further investigations are needed to determine the general utility of this technique for influencing the mitochondrial type of fusion products. Organelle segregation had progressed considerably by the time the calli were analyzed, but it was not in a state of rapid flux within the window of callus age and mass examined. On the basis of comparisons of mtDNA in calli and plants from calli in the same fusions, mitochondrial sorting-out in the calli appeared incomplete. There was evidence of mitochondrial recombination in about one-third of the calli, especially in a region near the *atp*9 gene. Finally, the independence of mitochondrial and chloroplast segregation suggests that any combination of organelles can be obtained provided that there is no overriding nuclear-organelle incompatibility and that a large enough population of fusion products is generated.

Acknowledgements. We thank Drs. J. Palmer, C. Makaroff and M. Hanson for probes. This work was supported by USDA grant no. 85-CRCR-1-1608 and by the Cornell NSF Plant Science Center, a unit in the USDA-DOE-NSF Plant Science Centers Program. The Plant Science Center is also a unit of the Cornell Biotechnology Program, which is sponsored by the New York State Science and Technology Foundation, a consortium of industries and the U.S. Army Research Office.

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