Theor Appl Genet (1992) 84:763–770

THEORETICAL AND APPLIED GENETICS

Asymmetric protoplast fusion aimed at intraspecific transfer of cytoplasmic male sterility (CMS) in *Lolium perenne* L.

J. Creemers-Molenaar [1] *, R. D. Hall [2], and F. A. Krens

[1] Barenbrug Holland BV, Stationsstraat 40, NL-6678 AC Oosterhout, The Netherlands
[2] Department of Cell Biology, Centre for Plant Breeding and Reproduction Research (CPRO-DLO), P.O. Box 16, NL-6700 AA Wageningen, The Netherlands

Received October 1, 1991; Accepted December 19, 1991
Communicated by R. Hagemann

Summary. Techniques have been developed for the production of cybrids in *Lolium perenne* (perennial ryegrass). Gamma-irradiated protoplasts of a cytoplasmically male-sterile breeding line of perennial ryegrass (B200) were fused with iodoacetamide-treated protoplasts of a fertile breeding line (Jon 401). After fusion 25 putative cybrid calli were characterized to determine mitochondrion type and composition of the nuclear genome. Analysis of phosphoglucoisomerase isozyme profiles and determination of the ploidy level by flow cytometry indicated that all of the calli tested essentially contained the nuclear DNA of the fertile line. However, the presence of parts of the nuclear DNA from the sterile line could not be excluded. Southern blotting of total DNA isolated from the parental lines and putative cybrids combined with hybridizations using the mitochondrial probes *cox1* and *atp6* revealed that the mitochondria of the calli originated from the fertile line (5 calli), the sterile line (5 calli) or from both parental lines (15 calli). The hybridization patterns of the mtDNA from the cybrid calli showed extensive quantitative and qualitative variation, suggesting that fusion-induced inter- or intramolecular mitochondrial recombination had taken place.

Key words: *Lolium perenne* – Cytoplasmic male sterility – Asymmetric protoplast fusion – Mitochondrial DNA – Recombination

Introduction

For the genetic improvement of a given cultivar, agronomically important traits present in other cultivars or related species can be incorporated by sexual crossing. However, if sexual crossing barriers exist between plants, the transfer of desirable genes from wild relatives to cultivated plants by conventional breeding is prevented. By means of protoplast fusion the nuclear and cytoplasmic genomes of plants from different genetic origins can be rapidly combined in one step. Somatic hybrid plants have been regenerated from fusions between different species, genera and tribes (Glimelius et al. 1991). Depending on the taxonomic distance such hybrid plants often show reduced fertility and seed set; however, when used in combination with suitable selection pressure such plants might serve as bridges for the transfer of specific traits to crop plants (Glimelius 1991).

Several agronomically important traits, e.g. cytoplasmic male sterility (CMS), herbicide resistance, nectar production and resistance to fungal toxins, have been shown to be encoded by organellar DNA (Kumar and Cocking 1987). In the majority of higher plants, cytoplasmic organelles are maternally inherited, and thus the introduction of such cytoplasmically encoded traits into a cultivar via breeding requires many backcrosses. By asymmetric protoplast fusion the cytoplasms of two cells can be combined with the nucleus of only one of the parental lines. After regeneration, plants with the desired nucleus/cytoplasm combination can be identified. This technique requires that prior to fusion the nucleus of the cytoplasm donor line is eliminated, either by high-speed centrifugation or by irradiation (gamma- or X-rays), and that the recipient protoplasts are inactivated by, for example, iodoacetate or iodoacetamide. Thus, after fusion, the unfused or autofused enucleate and inactivated protoplasts die, whereas by a process of metabolic complementation, viable cybrids can be obtained and can divide in culture (Sidorov et al. 1981). Cybrid plants have been obtained in several dicotyledonous species such as *Nico-*

* *Present address*: Department of Cell Biology, Centre for Plant Breeding and Reproduction Research (CPRO-DLO), P.O. Box 16, NL-6700 AA Wageningen, The Netherlands
Correspondence to: J. Creemers-Molenaar

tiana and *Petunia*, and cybrid plants with improved breeding value have been produced in *Brassica* and *Solanum* species (Kumar and Cocking 1987).

Recent progress in the culture and regeneration of protoplasts from monocotyledonous species has stimulated research to apply protoplast fusion techniques to the cereals and grasses. Somatic hybridization of *Oryza* has been attempted with wild *Oryza* species (Finch et al. 1990; Hayashi et al. 1988), with barnyard grass (Terada et al. 1987) and between anther-derived haploid cultivars of *O. sativa* (Toriyama and Hinata 1988). Fertile hybrid plants have been obtained from fusions of *O. sativa* (+) *O. eichingeri*, *O. sativa* (+) *O. officinalis* (Hayashi et al. 1988) and haploid (+) haploid fusions within *O. sativa* (Toriyama and Hinata 1988). Somatic hybrid callus lines have been recovered from fusions of *Panicum americanum* (+) *Panicum maximum* (Ozias-Akins et al. 1986), *Panicum americanum* (+) *Saccharum officinarum* (Tabaeizadeh et al. 1986) and *Triticum monococcum* (+) *Pennisetum americanum* (Vasil et al. 1988). With the aim of transferring CMS within *O. sativa*, cybrid plants have been obtained by asymmetric somatic hybridization (Yang et al. 1988, 1989; Akagi et al. 1989; Kyozuka et al. 1989).

In perennial ryegrass (*Lolium perenne* L.) a stable CMS-type is available that originated from a sexual crossing between an F_4 hybrid of *L. perenne* × *L. multiflorum* with *Festuca pratensis* (Wit 1974) and that has been used in a breeding program aimed at the production of F_1-hybrid seed. Transfer of this CMS type to other valuable breeding lines by asymmetric protoplast fusion might greatly enhance the perspectives of this breeding program. Recent progress in the regeneration of plants from protoplasts of *L. perenne* (Creemers-Molenaar et al. 1989; Creemers-Molenaar 1991) allow the application of protoplast fusion techniques in this species. In the study presented here we investigated the possibility of transferring cytoplasmic male sterility from a sterile to a fertile perennial ryegrass breeding line by donor/recipient protoplast fusion.

Materials and methods

Plant material

The cytoplasm donor line used in this study was the CMS inbred line B200, and the recipient line was a selected, fully fertile breeding line, Jon 401. Both lines were provided by Barenburg Research, Wolfheze, The Netherlands. Suspension cultures were initiated either from immature inflorescence-derived callus (B200) or directly from mature embryos (Jon 401), as described previously (Creemers-Molenaar et al. 1989). A 3-year-old suspension culture (Lp9A, derived from B200) and a 10-month-old suspension culture (Lp25, derived from Jon 401) were used in the fusion experiments. At the time of experimentation, both cultures had lost the potential for plant regeneration.

Protoplast isolation and treatments

Protoplasts were isolated by overnight incubation in CPW enzyme solution (Creemers-Molenaar et al. 1989) and cultured in conditioned RY-2 medium (Creemers-Molenaar et al. 1992). Prior to fusion, protoplasts from suspension culture Lp9A were washed twice, resuspended in CPW13M medium and then gamma-irradiated at doses ranging from 0 to 30 krad. The intensity of the ^{60}Co radiation was 150 krad/h. During transport and irradiation the protoplasts were kept on ice. After irradiation the protoplasts were centrifuged and resuspended in CPW13M medium.

Protoplasts from suspension Lp25 were treated with different concentrations of iodoacetate or iodoacetamide. The protoplasts were washed only once after overnight enzyme incubation and resuspended in CPW13M medium at a density of 5×10^5/ml. Freshly prepared stock solutions (0.1 *M*) of iodoacetate or iodoacetamide in CPW13M medium were added to the protoplast suspensions to give the final concentrations described in the Results section. After incubation for 10 min at 4 °C in the dark, the protoplasts were centrifuged for 5 min, washed 3 times and, finally, resuspended in CPW13M medium.

PEG-mediated protoplast fusion

Protoplast fusion was performed using polyethylene glycol (PEG) as the fusing agent, according to the method of Gilmour et al. (1989) with minor modifications. Mass fusions were carried out in 10-ml polystyrene tubes (Greiner) with 1.0×10^6 protoplasts from each parent in a final volume of 0.3 ml CPW13M medium. The PEG solution (0.4 ml, 30%, PEG6000 from Serva) was added dropwise, without mixing, and the tubes were incubated undisturbed for 30 min at room temperature. Aggregated protoplasts were induced to fuse by the addition of 0.8 ml high pH/Ca^{2+} solution (Keller and Melchers 1973). After incubation for 10 min the protoplasts were washed 3 times in CPWC13M medium (CPW13M supplemented with 0.74% $CaCl_2 \cdot 2H_2O$) and cultured in conditioned RY-2 medium. Control treatments consisted of: (1) separate culture of gamma-irradiated and iodoacetamide/iodoacetate-treated protoplasts; (2) separate culture of autofused, treated protoplasts; and (3) cocultivation of autofused, treated protoplasts.

Southern blotting and hybridization

Total DNA from fusion-derived calli and from protoplast-derived calli from the parental lines was essentially extracted as described by Mettler (1987). Callus tissue (0.3 g) was homogenized in liquid nitrogen with 400 µl extraction buffer using a pestle and mortar. The aqueous phase of the phenol extraction was treated with 0.1 volume (96% v/v) ethanol to reduce contamination by polysaccharides. Precipitated DNA was redissolved in 60 µl TE (10 m*M* TRIS, 1 m*M* EDTA, pH 8.0). DNA was digested with *Eco*RI at a 2 times higher concentration than was recommended by the manufacturer and in the presence of spermidine (final concentration 2.0 m*M*). The incubation was for 2–3 h at 37 °C. Southern blotting and DNA hybridizations were performed according to the protocol of Kreike et al. (1990). Heterologous probes coding for the mitochondrial genes *atp6* (atpase subunit 6, Dewey et al. 1985) and *cox1* (cytochrome c oxidase subunit 1, Isaac et al. 1985) were labeled with biotin as recommended by the manufacturer (Boehringer Mannheim) and used for hybridization. The light-emitting product AMPPD (Tropix, Bedford, Md.) was used as substrate for the enzyme alkaline phosphatase, which enabled the detection of DNA-DNA hybrids by luminography (Kreike et al. 1990).

Isozyme analysis

Protoplast-derived calli from the parental lines and calli obtained from fusions between these lines were analyzed for isozyme patterns 10 days after the eighth subculture (9 months after the fusion experiment). Samples were prepared by homogenizing 0.3 g callus with 20 µl extraction buffer in microfuge tubes by means of a power-driven grinding pestle. The extraction buffer was 0.1 *M* TRIS-HCl pH 7.1, supplemented with 3% (w/v) sucrose, 1% (w/v) dithiothreitol (DTT) and 0.04% (w/v) amido black. The homogenates were centrifuged for 5 min at 11,000 *g* after which 1 µl of the supernatant was used for electrophoresis. For the electrophoresis, the native-PAGE PhastSystem from Pharmacia was used following the procedure developed by Van Dreven and Booy (personal communication, CPRO, Wageningen). The gel separation media were PhastGel homogeneous 7.5 medium and PhastGel native buffer strips. The gels were run for 60 min and stained for phosphoglucoisomerase (PGI) and acid phosphatase (ACP) directly after the end of the run. For PGI, the gels were incubated for 30 min at 37 °C in the dark in 20 ml enzyme-staining solution (Hayward and McAdam 1977). For ACP, the gels were incubated for 2–3 h at room temperature under indirect light conditions in 20 ml enzyme-staining solution (Östergaard et al. 1985). After staining, the gels were washed with tap water, incubated in 2.5% (v/v) glycerine for 3 min and dried at 37 °C.

Ploidy level

The ploidy levels of the calli were determined by flow cytometry 7 months after protoplast fusion. Nuclear suspensions were prepared by chopping 0.25–0.5 g callus with a sharp razor blade in 2 ml isolation buffer, followed by filtration through a 35-µm mesh nylon filter. The nuclear isolation buffer was prepared as described by Saxena and King (1989) and modified by using 0.2 *M* sucrose, 10 m*M* spermine tetrahydrochloride and 0.25 µg/l 4,6-diaminodino-2-phenylindole (DAPI) (HA Verhoeven, CPRO, Wageningen). A nuclear suspension prepared from leaves of *Nicotiana plumbaginifolia* plantlets was used as an internal standard for all the samples. Nuclear suspensions prepared from leaves of greenhouse-grown diploid and tetraploid *L. perenne* varieties were used as controls. The samples were analyzed on a Partec PAS-II flow cytometer as described by Verhoeven et al. (1990). The coefficients of variation (CV) were determined as $CV(\%) = (FWHM/2.355 \times MEAN) \times 100$. In this formula FWHM = full width at half the maximum peak length, and MEAN = peak distance.

Results

Inactivation treatments and PEG fusion

The effects of gamma-irradiation on the plating efficiency of protoplasts from the cytoplasm donor line Lp9A were investigated, and the results are shown in Fig. 1. After irradiation of the protoplasts at 2.5 krad there was a 20-fold decrease in plating efficiency. After irradiation of the protoplasts at 5 krad, microcolonies of five to ten cells were formed. However, these colonies did not proliferate. No microcolonies were formed after irradiation of the protoplasts at 15 or 30 krad. In further experiments protoplasts of line Lp9A were irradiated at 15 krad prior to fusion.

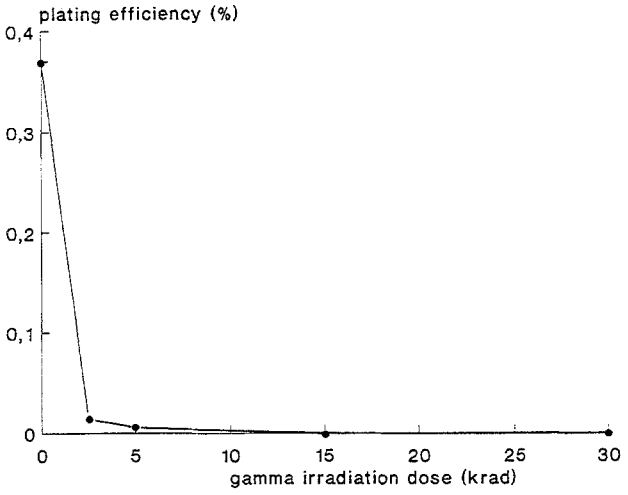

Fig. 1. The effect of gamma-irradiation on the plating efficiencies of protoplasts isolated from suspension cells of *L. perenne* line Lp9A. The intensity of the ^{60}Co irradiation was 150 krad/h

For inactivation of cells from the recipient line Lp25, freshly isolated protoplasts were initially treated with iodoacetate at 1, 2 and 4 m*M*. However, after 1 day of culture only ruptured protoplasts were observed in all treatments, and when iodoacetate-treated protoplasts were fused or cocultivated with gamma-irradiated protoplasts from line Lp9A, no microcolonies were formed (results not shown). After the treatment of protoplasts from line Lp25 with 1 m*M* iodoacetamide, microcolonies were still formed but at a reduced frequency (0.2%) (see Table 1); at higher concentrations, no microcolonies were formed. However, when iodoacetamide-treated protoplasts of line LP25 were subsequently cocultivated with gamma-irradiated protoplasts from line Lp9A, microcolonies were still formed from cells treated with as high as 4 m*M* iodoacetamide. Nevertheless, further proliferation to calli was only observed when ≤ 2 m*M* iodoacetamide had been used.

Protoplast fusions were carried out between gamma-irradiated protoplasts from Lp9A and iodoacetamide-treated protoplasts from Lp25 at concentrations between 1 and 7 m*M* iodoacetamide. Although no differential staining of the donor and the recipient protoplasts had been applied, due to slow rounding up, the fusion frequency could be estimated by microscopic examination immediately after PEG fusion. Irrespective of the iodoacetamide concentration used, the frequency of hetero + homofusion events was 5%. At low concentrations of iodoacetamide (1–2 m*M*), the plating efficiencies and the number of calli that proliferated after fusion were similar to those obtained in cocultivation (Table 1). However, at higher concentrations (4–7 m*M*) calli were only formed in the fusion dishes and not in the cocultivation dishes. At these higher iodoacetamide concentrations, the plating efficiencies, as well as the number of calli that

Table 1. The effect of iodoacetamide treatment of protoplasts from the recipient line Lp25 on the PE of protoplasts after PEG-mediated fusion with gamma-irradiated protoplasts of the donor line Lp9A in perennial ryegrass

Concentration of IA (m*M*)	Culture[a]	Cocultivation[b]		Fusion[d]	
	pe	pe	Calli[c]	pe	Calli[c]
0	0.5	/	/	/	/
1	0.2	0.07	9	0.1	11
2	0	0.05	7	0.04	8
4	0	0.02	0	0.01	2
5	0	0	0	< 0.01	9
6	0	0	0	< 0.01	7
7	0	0	0	< 0.01	11

IA, Iodoacetamide; pe, plating efficiency
pe was expressed as the percentage of protoplasts that formed microcolonies after 4 weeks of culture and was determined from 2–4 replicate plates
[a] Protoplasts of Lp25, cultured after iodoacetamide-treatment
[b] Iodoacetamide-treated protoplasts of Lp25 and gamma-irradiated protoplasts of Lp9A, mixed in a ratio of 1:1 after autofusion
[c] The number of microcolonies per 3.5×10^5 protoplasts that proliferated further to form calli
[d] Fusion of iodoacetamide-treated protoplasts of Lp25 with gamma-irradiated protoplasts of Lp9A
/ = Not applicable

proliferated after fusion, were low (<0.01% and 2–11 calli/3.5×10^5 protoplasts, respectively). While the results given in Table 1 represent the data from one experiment, this experiment was repeated twice, and in both cases similar results were obtained. A number of the calli that were obtained from one of these experiments were subcultured for 5–9 months for further characterization.

Characterization of fusion-derived calli

To determine the mtDNA composition of fusion-derived calli, Southern blots were hybridized with *cox1* and *atp6* probes. DNA from 25 fusion-derived callus lines and the parental callus lines were examined. The hybridization patterns of 5 fusion-derived calli and calli from the parental lines are shown in Fig. 2. The *cox1* probe hybridized strongly to fragments of 7.1, 5.6, 4.6, 4.4, 2.4 and 1.3 kb, and weakly to a 3.7-kb and a 3.5-kb fragment, all present in the donor line Lp9A (Fig. 2a). The same probe hybridized strongly to only one fragment of 3.4 kb present in the recipient line Lp25. In callus line 5-1, hybridization with the *cox1* probe revealed four fragments (5.6, 4.6, 3.7 and 1.3 kb), corresponding to those of Lp9A and 1 new fragment of 2.7 kb. However, the relative intensity of the 3.7-kb fragment in callus line 5-1 was substantially increased compared with that of Lp9A. In callus line 5-8, the 7.1-kb and the 2.4-kb fragments of Lp9A were also present, while compared with callus line 5-1 the stoichiometry of several other signals

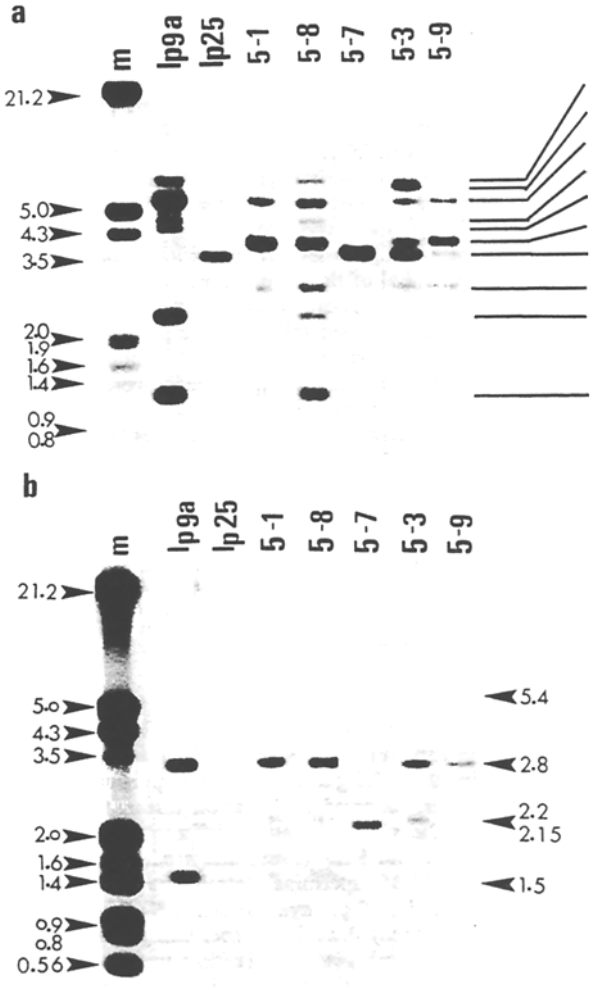

Fig. 2 a, b. Southern blot hybridization of *Eco*R1-digested total DNA of protoplast-derived calli from *L. perenne* with the mitochondrial probes *cox1* (**a**) and *atp6* (**b**). A non-radioactive labeling method was used for the probes, and DNA-DNA hybrids were visualized by luminography. *m Eco*R1 + *Hin*d111-digested lambda DNA size markers, *Lp9A* CMS parental line, *lp25* fertile recipient line, *5-1*, *5-8*, *5-7*, *5-3* and *5-9* fusion-derived calli

was clearly different. The hybridization patterns of callus line 5-7 and the recipient line Lp25 were identical except for a faint extra band at the 4.6-kb fragment position. For callus line 5-3 the *cox1* probe hybridized to all of the fragments mentioned earlier and, in addition, a new hybridizing fragment of 6.2 kb could be identified. The hybridization pattern of callus line 5-9 showed fragments corresponding to the Lp9A-specific 5.6-kb fragment, the amplified 3.7-kb and the novel 2.7-kb fragments as well as a faint signal at the position of the Lp25-specific 3.4-kb fragment. The hybridization patterns of these 5 fusion-derived calli with *cox1*, as shown in Fig. 2a, represent but a small selection from a broad spectrum of qualitative and quantitative variation in mtDNA that was observed among the 25 calli examined. In total, 16

Table 2. Summary of the characterization of fusion-derived calli giving the proposed parental origin of the nucleus and of the mitochondria

Callus[a] line	Ploidy (2n = ..x)	MtDNA[b]						Parental origin	
		cox1			*atp6*			Nucleus[c]	Mitochondria
		S	F	R	S	F	R		
Lp9A	2	7	0	0	3	0	0	S	S
Lp25	4	0	1	0	0	1	0	F	F
5-1	4	4	0	1	2	0	1	F	S
5-2	3.4	0	1	0	1	0	1	F	SF
5-3	4.1	7	1	2	2	0	1	F	SF
5-4	4.9	0	1	0	0	1	0	F	F
5-5	2.8+5.7	0	1	0	0	1	0	F	F
5-6	6.5	0	1	0	0	1	0	F	F
5-7	2.9+5.1	0	1	0	0	1	0	F	F
5-8	3.1+5.1	6	0	1	2	0	1	F	S
5-9	4.3	2	1	1	1	0	1	F	SF
6-1	4.6	1	1	0	1	1	0	F	SF
6-2	3.8	6	0	1	2	0	1	F	S
6-3	4.7	4	1	2	3	0	1	F	SF
6-4	4.0	3	1	2	2	1	0	F	SF
6-5	4.9	4	1	1	1	0	1	F	SF
6-6	4.3	7	1	1	2	0	1	F	SF
7-1	4.4	1	1	1	1	0	1	F	SF
7-2	3.6	7	0	1	2	0	1	F	S
7-3	5.1	3	1	0	1	1	0	F	SF
7-4	4.4	4	1	0	1	1	0	F	SF
7-5	4.2	0	1	0	0	1	0	F	F
7-6	7.8	2	1	1	1	1	0	F	SF
7-7	4.2	7	1	1	2	1	0	F	SF
7-8	4.2	6	1	1	1	1	0	F	SF
7-9	4.4+6.9	3	1	1	2	0	1	F	SF
7-10	4.0	7	0	1	2	0	1	F	S

[a] Lp9A, sterile donor line; Lp25, fertile recipient line; lines 5- (1–9), 6- (1–6) and 7- (1–10) are fusion-derived calli; iodoacetamide treatments of the recipient line were 5, 6, and 7 m*M*, respectively

[b] *cox1*: S, 1–7 hybridizing fragments of the sterile line; F, hybridization to the unique Lp25 fragment; R, novel hybridizing fragments of 6.2 and/or 2.7 kb. *atp6*: S, hybridization to 1–3 fragments of the sterile line; F, hybridization to the 2.15 kb fragment of Lp25; R, novel hybridizing fragment of 2.2 kb

[c] Based on ploidy and the presence of Lp25-specific GPI isozyme bands in all of the fusion-derived calli

different hybridization patterns could be discriminated when *cox1* was used as a probe. The amplified 3.7-kb and the novel 2.7-kb fragments were observed in 17 and 16, respectively, of the 25 calli that were examined. The novel 6.2-kb fragment was detected in DNA from 3 calli. While 5 calli showed mtDNA hybridization patterns identical to Lp25, none of the calli showed banding patterns identical to Lp9A or to a summation of both parents (Table 2). Treatment of Lp9A with PEG (autofusion) did not alter the DNA hybridization pattern of the calli subsequently obtained (results not shown).

Figure 2b shows the hybridization patterns of the same calli as in Fig. 2a when *atp6* was used as the probe. For Lp9A this probe hybridized strongly to fragments of 2.8 kb and 1.5 kb and weakly to a 5.4-kb fragment. For Lp25, *atp6* hybridized to only a single 2.15-kb fragment. The hybridization pattern of callus line 5-7 was identical to that of Lp25. DNA from calli 5-1, 5-8 and 5-3 all showed hybridization bands corresponding to the Lp9A-specific fragments of 2.8 kb and 1.5 kb, while this probe only hybridized weakly to the 2.8-kb fragment from DNA of callus 5-9. Neither the calli represented in Fig. 2b nor the other fusion-derived calli examined contained the Lp9A-specific 5.4-kb fragment. DNA from all of the fusion-derived calli shown in Fig. 2 b, as well as all other such calli that have been examined, had an *atp6*-homologous fragment of either 2.15 or 2.2 kb (results not shown). The 2.2-kb fragment was considered to be a novel fragment. The number of Lp9A- and Lp25-specific fragments, as well as novel fragments that hybridized to *cox1* and *atp6* of the calli examined, are summarized in Table 2.

To determine the parental origin of the nuclear DNA in the calli, GPI isozyme patterns of the parental callus

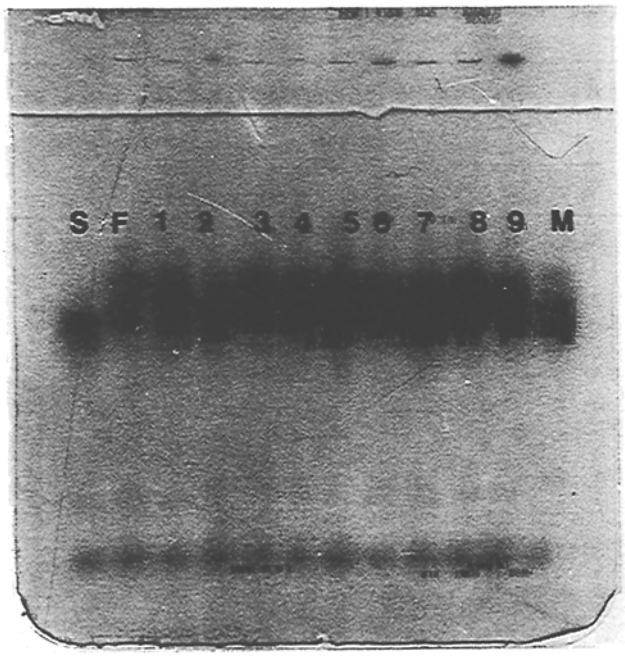

Fig. 3. Zymogram of phosphoglucoisomerase isozyme patterns of protoplast-derived calli from *L. perenne*: CMS parental line Lp9A (*S*), fertile parental line Lp25 (*F*), mix of both parental lines (*M*) and fusion-derived calli 5-7, 5-8, 5-9, 6-1, 6-2, 6-3, 6-4, 6-5, 6-6 (*1–9*)

lines and the fusion-derived calli were examined. The results are shown in Fig. 3. In line Lp9A only one strong band was present. Lp25 showed the same band but at a lower intensity and, in addition, two extra bands were present. The isozyme patterns of all 25 calli examined were identical to that of Lp25. In addition, ACP was analyzed, but the isozyme patterns obtained for this enzyme showed no clear differences between parental lines (results not shown).

To determine the ploidy levels of the calli, the DNA content of isolated nuclei was determined by flow cytometry. The results are summarized in column 2 of Table 2. The CV values of the DNA histograms of the internal *N. plumbaginifolia* standard and of all the callus lines examined varied between 8.8% and 14.6% and 6.2% and 14.8%, respectively. The ploidy of the callus from the donor line Lp9A was diploid and that of the recipient line Lp25 was tetraploid. Whereas none of the fusion-derived calli were diploid, 8 were tetraploid ($2n = 3.8-4.2\times$). Twelve of the calli predominantly contained nuclei with a higher DNA content than the tetraploid recipient line Lp25, and 1 callus contained a lower DNA content. Four calli were chimeric, containing both nuclei with a high DNA content ($2n = 5.1-6.9\times$) and a low DNA content ($2n = 2.8-4.4\times$).

Discussion

In this paper a method has been described that achieves asymmetric protoplast fusion in perennial ryegrass by the incorporation of gamma-irradiated donor protoplasts and iodoacetamide-inactivated recipient protoplasts. Isozyme patterns and ploidy levels of the calli indicated that all of the 25 fusion-derived calli examined contained the nuclear genome of the fertile recipient line Lp25. MtDNA analysis revealed that 15 calli contained a mixture of mtDNA sequences from both lines, 5 calli contained only mtDNA sequences of the sterile donor line and 5 calli contained only mtDNA sequences of the fertile recipient line. In addition, the hybridization patterns of Southern blots with *cox1* and *atp6* as probes revealed that distinct mtDNA rearrangements had taken place in the majority of the calli that contained DNA fragments complementary to the sterile line.

Although the nuclear DNA of all the fusion-derived calli originated from the fertile recipient line, as established through PGI isozyme analysis, it cannot be excluded, considering the, on average, high ploidy levels, that fractions of the nuclear genome of the sterile line were also present. Sidorov et al. (1981) found that after PEG fusion of gamma-irradiated protoplasts of *N. tabacum* with iodoacetate-treated protoplasts of *N. plumbaginifolia*, 60% of the regenerated plants contained some nuclear genetic material from the irradiated cells. The variable ploidy levels and the chimeric nature of several of the fusion-derived calli that were observed in this study may have been due to the presence of chromosome fragments of the donor line, which induce amplification and/or chromosome loss in the recipient genome. Recently, an efficient procedure for the isolation of cytoplasts from suspension-derived protoplasts of *Lolium perenne* has been developed (Van Ark et al. 1992). Fusion of the recipient protoplasts with cytoplasts instead of irradiated protoplasts may be a means to circumvent partial transfer of nuclear DNA from the donor line.

The inactivation treatment of the recipient line was a critical factor. Perennial ryegrass protoplasts showed an extreme sensitivity to iodoacetate and, therefore, this metabolic inhibitor was not suitable for inactivation purposes. These results contrast with those obtained for *Nicotiana* (Sidorov et al. 1981), *Solanum* (Kemble et al. 1986), *Panicum maximum* (Ozias-Akins 1986) and *Saccharum officinarum* (Tabazaeideh et al. 1986), for which iodoacetate has been applied successfully for the inactivation of protoplasts to obtain cybrids. On the other hand, in perennial ryegrass iodoacetamide prevented growth of the recipient protoplasts, but allowed the proliferation of heterokaryons after fusion with irradiated protoplasts. High concentrations of iodoacetamide (5–7 m*M*) were essential to prevent the division of autofused, iodoacetamide-treated protoplasts in the presence

of autofused, irradiated protoplasts. The irradiated protoplasts appeared to exert a feeder effect on the iodoacetamide-treated protoplasts.

The hybridization patterns of cybrid *Lolium* calli with *cox1* showed particular qualitative, and a range of quantitative, changes as compared to calli of the parental lines. However, as the DNA hybridization patterns obtained after autofusion were identical to those of protoplast-derived calli, these mtDNA rearrangements resulted from protoplast heterofusion and were not induced by tissue culture itself. This is in contrast with tissue culture-induced mtDNA rearrangements, independent of a fusion process, which have previously been observed in *Solanum* (Kemble and Shepard 1984), *Triticum* (Rode et al. 1987) *Beta* (Brears et al. 1989) and *Oryza* (Chowdhury et al. 1990).

The extent of variation between the mtDNA hybridization patterns of cybrid calli in perennial ryegrass and the occurrence of novel fragments suggest that intermolecular recombination has taken place (Boeshore et al. 1983). Intermolecular recombination has been demonstrated in somatic hybrids of *Petunia* (Rothenberg et al. 1985). If intermolecular recombination has taken place in *Lolium*, the appearance of the common non-parental 3.7-kb and 2.7-kb fragments in 17 and 16 of the cybrid calli, respectively, may indicate the presence of recombination "hot spots". The occurrence of hot spots of recombination has been suggested to explain identical mtDNA restriction patterns in somatic hybrid plants of rice. These patterns differed from the starting material in that several non-parental bands were present (Yang et al. 1988). Alternatively, the heteroplasmic state might have induced preferential amplification of specific mtDNA molecules that were already present at substoichiometric concentrations in the parental lines, as has been demonstrated in hybrids of pearl millet and Guinea grass (Ozias-Akins et al. 1988). However, more extensive mtDNA analysis will be necessary to determine which process caused the observed mtDNA variation in perennial ryegrass cybrid calli.

This is the first report of experiments aimed at obtaining the intraspecific transfer of cytoplasmic male sterility by asymmetric protoplast fusion in the economically important forage crop *Lolium perenne*. Cybrid calli were obtained that contained the recipient nuclear DNA at the expected ploidy level and only the mitochondria of the donor line. The high percentage of cybrid calli that was obtained in this study offers promising perspectives for the regeneration of cybrid plants when the described procedure is applied to regeneration-competent suspension lines.

Acknowledgements. The authors appreciate the skilful technical assistance of Y. van Oort. We thank G.J.A. Rouwendal (ATO, The Netherlands) for development and teaching of the mtDNA characterization procedures and Prof. Dr. G.J. Wullems (University of Nijmegen, The Netherlands) and Dr. C.M. Colijn-Hooymans (CPRO-DLO, The Netherlands) for stimulating discussions and critically reading the manuscript. The assistance of F. van Dreven (CPRO-DLO) with the isozyme determinations is highly appreciated. We would like to thank those authors listed for their kind gifts of DNA probes. This work has been part of a research cooperation between Barenbrug and CPRO-DLO.

References

Akagi H, Sakamoto M, Negishi T, Fujimura T (1989) Construction of rice cybrid plants. Mol Gen Genet 215:501–506

Van Ark HF, Hall RD Creemers-Molenaar J, Krens FA (1992) High yields of cytoplasts from protoplasts of *Lolium perenne* and *Beta vulgaris* using gradient centrifugation. (In press, Plant Cell Tissue and Organ Culture)

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

Brears T, Curtis GJ, Lonsdale DM (1989) A specific rearrangement of mitochondrial DNA induced by tissue culture. Theor Appl Genet 77:620–624

Chowdhury MKU, Schaeffer GW, Smith RL, Debonte LR, Matthews BF (1990) Mitochondrial DNA variation in long-term tissue cultured rice lines. Theor Appl Genet 80:81–87

Creemers-Molenaar J (1991) Regeneration from protoplasts of perennial ryegrass; progress and applications. In: den Nijs APM, Elgersma A (eds), Fodder crops breeding: achievements, novel strategies and biotechnology. Proc 16th meeting Fodder Crops Section Eucarpia. Wageningen, The Netherlands, pp 123–128

Creemers-Molenaar J, Van der Valk P, Loeffen JPM, Zaal MACM (1989) Plant regeneration from suspension cultures and protoplasts of *Lolium perenne* L. Plant Sci 63:167–176

Creemers-Molenaar J, van Eeuwijk FA, Krens FA (1992) Culture optimization of perennial ryegrass protoplasts. J Plant Physiol 139:303–308

Dewey RE, Levings CS III, Timothy DH (1985) Nucleotide sequences of atpase subunit 6 of maize mitochondria. Plant Physiol 79:914–919

Finch RP, Slamet IH, Cocking EC (1990) Production of heterokaryons by the fusion of mesophyll protoplasts of *Porteresia coarctata* and cell suspension-derived protoplasts of *Oryza sativa*: a new approach to somatic hybridization in rice. J Plant Physiol 136:592–598

Gilmour DM, Davey MR, Cocking EC (1989) Production of somatic hybrid tissues following chemical and electrical fusion of protoplasts from albino cell suspensions of *Medicago sativa* and *M. borealis*. Plant Cell Rep 8:29–32

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

Hayashi Y, Kyozuka J, Shimamoto K (1988) Hybrids of rice (*Oryza sativa* L.) and wild *Oryza* species obtained by cell fusion. Mol Gen Genet 214:6–10

Hayward MD, McAdam NJ (1977) Isozyme polymorphism as a measure of distinctiveness and stability in cultivars of *Lolium perenne*. Z Pflanzenzucht 81:228–234

Isaac P, Jones VP, Leaver CJ (1985) The maize cytochrome c oxidase subunit I gene: sequence, expression and rearrangement in cytoplasmic male sterile plants. EMBO J 4:1617–1623

Keller WA, Melchers G (1973) The effect of high pH and calcium on tobacco leaf protoplast fusion. Z Naturforsch 28C:737–741

Kemble RJ, Shepard JF (1984) Cytoplasmic DNA variation in a potato protoclonal population. Theor Appl Genet 69:211–216

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

Kreike CM, de Koning JRA, Krens FA (1990) Non-radioactive detection of single-copy DNA-DNA hybrids. Plant Mol Biol Rep 8:172–179

Kumar A, Cocking EC (1987) Protoplast fusion: a novel approach to organelle genetics in higher plants. Am J Bot 74:1289–1303

Kyozuka J, Kaneda T, Shimamoto K (1989) Production of cytoplasmic male sterile rice (*Oryza sativa* L.) by cell fusion. Bio/Technology 7:1171–1174

Mettler IJ (1987) A simple and rapid method for minipreparation of DNA from tissue cultured plant cells. Plant Mol Biol 5:346–349

Östergaard H, Nielsen G, Johansen H (1985) Genetic variation in cultivars of diploid ryegrass, *Lolium perenne* and *L. multiflorum*, at five enzyme systems. Theor Appl Genet 69:409–421

Ozias-Akins P, Ferl RJ, Vasil IK (1986) Somatic hybridization in the gramineae: *Pennisetum americanum* (L.) K. Schum. (Pearl millet) + *Panicum maximum* Jacq. (Guinea grass). Mol Gen Genet 203:365–370

Ozias-Akins P, Tabaeizadeh Z, Pring DR, Vasil IK (1988) Preferential amplification of mitochondrial DNA fragments in somatic hybrids of the Gramineae. Curr Genet 13:241–245

Rode A, Hartmann C, Falconet D, Lejeune B, Quetier F, Benslimane A, Henry Y, de Buyser J (1987) Extensive mitochondrial DNA variation in somatic tissue cultures initiated from wheat immature embryos. Curr Genet 12:369–376

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

Saxena PK, King J (1989) Isolation of nuclei and their transplantation into plant protoplasts. In: Bajaj YPS (ed) Biotechnology in agriculture and forestry 9: plant protoplasts and genetic engineering, II. Springer, Berlin Heidelberg New York, pp 328–342

Sidorov VA, Menczel L, Nagy F, Maliga P (1981) Chloroplast transfer in *Nicotiana* based on metabolic complementation between irradiated and iodoacetate-treated protoplasts. Planta 152:341–345

Tabaeizadeh Z, Ferl RJ, Vasil IK (1986) Somatic hybridization in the Gramineae: *Saccharum officinarum* L. (sugarcane) + *Pennisetum americanum* (L.) K. Schum. (Pearl millet). Proc Natl Acad Sci USA 83:5616–5619

Terada R, Kyozuka J, Nishibayashi S, Shimamoto K (1987) Plantlet regeneration from somatic hybrids of rice (*Oryza sativa* L.) and barnyard grass (*Echinochloa oryzicola* Vasing). Mol Gen Genet 210:39–43

Toriyama K, Hinata K (1988) Diploid somatic-hybrid plants regenerated from rice cultivars. Theor Appl Genet 76:665–668

Vasil V, Ferl RJ, Vasil IK (1988) Somatic hybridization in the Gramineae: *Triticum monococcum* L. (Einkorn) + *Pennisetum americanum* (L.) K. Schum. (pearl millet). J Plant Physiol 132:160–163

Verhoeven HA, Sree Ramulu K, Dijkhuis P (1990) A comparison of the effects of various spindle toxins on metaphase arrest and formation of micronuclei in cell-suspension cultures of *Nicotiana plumbaginifolia*. Planta 182:408–414

Wit F (1974) Cytoplasmic male sterility in ryegrasses (*Lolium* spp.) detected after intergeneric hybridization. Euphytica 23:31–38

Yang Z-Q, Shikanai T, Yamada Y (1988) Asymmetric hybridization between cytoplasmic male sterile (CMS) and fertile rice (*Oryza sativa* L.) protoplasts. Theor Appl Genet 76:801–808

Yang Z-Q, Shikanai T, Mori K, Yamada Y (1989) Plant regeneration from cytoplasmic hybrids of rice (*Oryza sativa* L.). Theor Appl Genet 77:305–310