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Asymmetric somatic hybridization between tall fescue (*Festuca arundinacea* Schreb.) and irradiated Italian ryegrass (*Lolium multiflorum* Lam.) protoplasts

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Abstract Intergeneric asymmetric somatic hybrids have been obtained by the fusion of metabolically inactivated protoplasts from embryogenic suspension cultures of *Festuca arundinacea* (recipient) and protoplasts from a non-morphogenic cell suspension of *Lolium multiflorum* (donor) irradiated with 10, 25, 50, 100, 250 and 500 Gy of X-rays. Regenerating calli led to the recovery of genotypically and phenotypically different asymmetric somatic hybrid *Festulolium* plants. The genome composition of the asymmetric somatic hybrid clones was characterized by quantitative dot-blot hybridizations using dispersed repetitive DNA sequences specific to tall fescue and Italian ryegrass. Data from dot-blot hybridizations using two cloned Italian ryegrass-specific sequences as probes showed that irradiation favoured a unidirectional elimination of most or part of the donor chromosomes in asymmetric somatic hybrid clones obtained from fusion experiments using donor protoplasts irradiated at doses ≤ 250 Gy. Irradiation of cells of the donor parent with 500 Gy prior to protoplast fusion produced highly asymmetric nuclear hybrids with over 80% elimination of the donor genome as well as clones showing a complete loss of donor chromosomes. Further information on the degree of asymmetry in regenerated hybrid plants was obtained from chromosomal analysis including in situ hybridizations with *L. multiflorum*-specific repetitive sequences. A Southern blot hybridization analysis using one chloroplast and six mitochondrial-specific probes revealed preferentially recipient-type organelles in asymmetric somatic hybrid clones obtained from fusion experiments with donor protoplasts irradiated with doses higher than 100 Gy. It is concluded that the irradiation of donor cells before fusion at different doses can be used for producing both nuclear hybrids with limited donor DNA elimination or highly asymmetric nuclear hybrid plants in an intergeneric graminaceous combination. For a wide range of radiation doses tested (25–250 Gy), the degree of the species-specific genome elimination from the irradiated partner seems not to be dose dependent. A bias towards recipient-type organelles was apparent when extensive donor nuclear genome elimination occurred.

Key words Asymmetric somatic hybrids · forage grasses: fescues and ryegrasses · Plastome-genome interaction · *Festuca arundinacea* · *Lolium multiflorum*

Abbreviations cpDNA Chloroplast DNA · 2, 4-D 2,4-dichlorophenoxyacetic acid · FDA fluorescein diacetate · IOA iodoacetamide · mtDNA mitochondrial DNA · RFLP restriction fragment length polymorphism

Introduction

Protoplast fusion enables the generation of hybrid combinations between widely related plant species that cannot be sexually intercrossed. However, plants recovered from such fusion combinations are often sterile and morphologically abnormal and may show uncontrolled genomic instabilities, thus hampering the use of somatic cell fusion for increasing nuclear genetic variability in plants. Asymmetric somatic hybridization, based on the induction of unilateral chromosome elimination using lethal doses of X- or gamma-rays, has been developed as a means to create morphologically normal wide hybrids with improved fertility that also contain the whole genome of the non-irradiated species (recipient) but only a fraction of the genome from the irradiated fusion partner (donor) (Gleba and Sytnik 1984). Asymmetric

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somatic hybrids have been produced between many different dicot species in both intra- and intergeneric combinations (Gupta et al. 1984; Dudits et al. 1987; Bates et al. 1987; Imamura et al. 1987; Gleba et al. 1988; Famelaer et al. 1989; Yamashita et al. 1989; Wijbrandi et al. 1990a, b; Wolters et al. 1991), and the transferred donor genome has varied from a few traits (Gupta et al. 1984; Dudits et al. 1987) to many chromosomes (Imamura et al. 1987; Gleba et al. 1988; Yamashita et al. 1989; Wijbrandi et al. 1990b; Melzer and O'Connell 1990). However, information on donor genome elimination and the nuclear make-up of asymmetric somatic hybrids is mainly only available for a few species' combinations in Solanaceae and Brassiceae (Piastuch and Bates 1990; Parokonny et al. 1992; Wijbrandi et al. 1990a, b; Yamashita et al. 1989; Itoh et al. 1991), whereas analogous information is completely lacking for important graminaceous monocots.

Interspecific and intergeneric somatic hybridizations have been performed in some symmetric fusion combinations in Gramineae (Ozias-Atkins et al. 1986; Tabaeizadeh et al. 1986; Terada et al. 1987; Vasil et al. 1988; Hayashi et al. 1988), but viable somatic hybrid plants were only recovered for few fusion combinations between *Oryza* species (Hayashi et al. 1988). Similarly, the construction of cybrid plants in monocots is so far restricted to rice (Yang et al. 1988, 1989; Akagi et al. 1989; Kyozuka et al. 1989), with the main aim of transferring cytoplasmic male sterility. Recently, flowering somatic hybrid plants derived from graminaceous monocot species belonging to different genera were described for the first time – *Festuca arundinacea* (+) *Lolium multiflorum* (Takamizo et al. 1991) – but as yet no report on the recovery of asymmetric somatic hybrid/cybrid plants is available for any forage grass species. Cybrid calli only have been generated for one intraspecific combination in *L. perenne* (Creemers-Molenaar et al. 1992).

In the present article we report on the recovery of mature asymmetric somatic hybrid plants after fusion of metabolically inactivated tall fescue (*F. arundinacea* Schreb.) and X-ray irradiated (at different doses) protoplasts of Italian ryegrass (*L. multiflorum* Lam.). The effects of irradiation on the genomic constitution of the asymmetric somatic hybrids, as evaluated by quantitative dot-blot hybridizations using previously cloned repetitive dispersed species-specific sequences (Perez-Vicente et al. 1992) and by complementary in situ hybridizations, were analysed. A corresponding characterization of the organellar composition of the *Festulolium* asymmetric somatic hybrid clones obtained provided some insight on plastome-genome interactions for this intergeneric combination.

Materials and methods

Plant material, establishment of callus and suspension cultures

Hexaploid (2n = 42) tall fescue (*Festuca arundinacea* Schreb.) cv 'Fawn' and diploid (2n = 14) Italian ryegrass (*Lolium multiflorum* Lam.) cv 'Gorka Norodova' were used for the establishment of callus and suspension cultures as described earlier (Takamizo et al. 1990).

Isolation of protoplasts

Protoplasts were isolated from embryogenic cell suspensions of tall fescue (*F. arundinacea* cv 'Fawn') and from one cell line of Italian ryegrass (*L. multiflorum* cv 'Gorka Norodova'). Four days after subculture approximately 2–4 ml settled volume of tall fescue and Italian ryegrass cells were resuspended in 12–14 ml enzyme solution EF consisting of 3% (w/v) cellulase Onozuka RS, 1% (w/v) Macerozyme R10 (both from Yakult Inc, Tokyo, Japan) and 0.1% (w/v) Pectolyase Y-23 (Seishin Pharmaceutical Co Tokyo, Japan) in washing solution WF (0.6 *M* mannitol, 10 m*M* $CaCl_2$, pH 5.8). Italian ryegrass cells in the enzyme solution were X-ray irradiated. As a control, unirradiated cell suspensions of Italian ryegrass were also used. Irradiation at doses of 10, 25, 50, 100, 250 and 500 Gy was performed with an X-ray apparatus (Müller MG150, Type 70526/41; 80 kV, 17 mA, 1900 R min^{-1}, 0.2 mm Al-filter; Müller GmbH, Hamburg, FRG). After subsequent incubation for 4 h on a rotary shaker at 40 rpm, 25 °C, in the dark, all of the protoplast isolation mixtures were passed sequentially through 280-, 100- and 50-μm mesh size metallic sieves. Protoplasts were washed twice in WF by centrifugation (80 *g* for 7 min). Italian ryegrass protoplasts were resuspended in 0.6 *M* mannitol, and tall fescue protoplasts were resuspended for metabolic inactivation in WF solution containing 10 m*M* iodoacetamide (IOA) and incubated at 4 °C for 15 min. After IOA treatment, tall fescue protoplasts were washed twice by centrifugation in 0.6 *M* mannitol and used in fusion experiments.

Fusion of protoplasts

Fusion experiments were performed in the genotype combination *F. arundinacea* cv 'Fawn' (+) *L. multiflorum* cv 'Gorka Norodova' using a commercial electrofusion setup (Elektro-Zellfusion CFA 400, Krüss, Hamburg, FRG). Equal numbers of protoplasts from both species were suspended with a final density of 10^6/ml in 1.5 ml 0.6 *M* mannitol and transferred into plastic spectrophotometer-cuvettes used as electrofusion chambers. The electrofusion conditions used were: a.c.-field (1 MHz, 80 V/cm for 30 s) followed by d.c.-pulses (0.75 kV/cm, 1–2 pulses, 30 μs each), at room temperature. Between 5 and 10 min after electrofusion, protoplasts were collected by centrifugation (80 *g* for 7 min) and resuspended in 2.0 ml of double-concentrated AA medium (Müller and Grafe 1978) supplemented with 2 mg/l 2, 4-D and 0.6 *M* glucose and plated in agarose-solidified protoplast culture medium (Wang et al. 1992).

Culture of protoplasts after fusion

Protoplasts were cultured using the agarose bead-type culture method (Shillito et al. 1983) with nurse cells (Kyozuka et al. 1987) as previously described (Wang et al. 1993). About 400 mg (fresh weight) of suspension-cultured cells from tall fescue cell lines were added to the AA medium around the agarose beads and used as nurse cells during the first week in culture. Cultures were kept on a rotary shaker at 60 rpm in the dark at 25 °C.

Proliferation and plant regeneration from putative fusants-derived colonies

After about 1 month in culture, the agarose beads containing visible colonies or individual colonies growing on the agarose beads were transferred onto MS medium (Murashige and Skoog 1962) supplemented with 1 mg/l 2, 4-D, 500 mg/l casein hydrolysate, 90 m*M* sucrose, 0.8% (w/v) agarose (Type I, Sigma). About 4 weeks later proliferating calli were placed on MSK medium consisting of MS basal medium supplemented with 90 m*M* sucrose and 0.2 mg/l ki-

netin. The regenerated shoots with or without roots were then transferred to hormone-free MS medium for stimulating rooting. All cultures were kept under fluorescent light conditions (40 $\mu E\,m^{-2}\,s^{-1}$) with a 16/8 h (light/dark) photoperiod at 25 °C. Rooted plants were then potted in soil and grown until maturity under greenhouse-conditions (natural daylength; 23 °C/18 °C). Pollen viability was assessed by fluorescein diacetate (FDA) staining.

DNA isolation and gel electrophoresis

Total genomic cellular DNA was isolated from freeze-dried regenerating calli and leaf material from shoot cultures and greenhouse-grown symmetric and asymmetric somatic hybrids and their parental forms. The isolation and digestion of genomic DNA were performed according to Lichtenstein and Draper (1985). Restriction enzyme analysis, gel electrophoresis and DNA blotting were carried out following standard protocols (Sambrook et al. 1989).

Hybridization experiments

Southern blot hybridization experiments using digested (*Eco*RI or *Bam*HI) total genomic DNA from putative symmetric and asymmetric somatic hybrids and their parents were performed following standard protocols as described in Sambrook et al. (1989). Hybridization probes were [^{32}P]dATP labeled by random priming (Feinberg and Vogelstein 1983).

Repetitive and evenly dispersed species-specific sequences of *L. multiflorum* (pLMH2 and pLMB4) and *F. arundinacea* (pFAH1) (Perez-Vicente et al. 1992) were used in quantitative dot-blot analyses for the characterization of the nuclear composition of putative tall fescue (+) Italian ryegrass somatic hybrid-regenerating clones. The quantitative dot-blots that were performed in identical triplicates (for later hybridizing with the *L. multiflorum*- and *F. arundinacea*-specific sequences) and the corresponding calculations of genomic composition of somatic *Festulolium* clones were as described earlier (Perez-Vicente et al. 1992), and were based on estimates for a nuclear DNA amount of 4.31 pg per 2C nucleus in *L. multiflorum* (Hutchinson et al. 1979) and 2C-value of 6.7 pg for *F. arundinacea* (Bennett et al. 1982).

For the characterization of the organellar genomes of somatic hybrids, the following plant mitochondrial DNA (mtDNA) gene-specific probes were used: *cox1* (3.94-kb *Bam*HI-*Eco*RI fragment from pBN6601; Isaac et al. 1985a), *cox2* (2.4-kb *Eco*RI fragment from pZMEI; Fox and Leaver 1981), *atpA* (4.8-kb *Hin*dIII fragment from atpA-copyV; Isaac et al. 1985a)—all kindly provided by Prof. C. J. Leaver; *cox3* (1.1-kb *Eco*RI-*Pst*I fragment, W. Schuster, personal communication), kindly supplied by Prof. A. Brennicke; *atp9* (2.2-kb *Xba*I fragment; Dewey et al. 1985a), *atp6* (2.7-kb *Hin*dIII fragment; Dewey et al. 1985b) – generously made available by Prof. C. S. Levings. The chloroplast DNA (cpDNA) gene-specific clone from the spinach ribulose-1,5-biphoshate carboxylase large subunit (*rbcL*, *Bam*HI fragment from pWHsp403) was kindly provided by Prof. R. Herrmann.

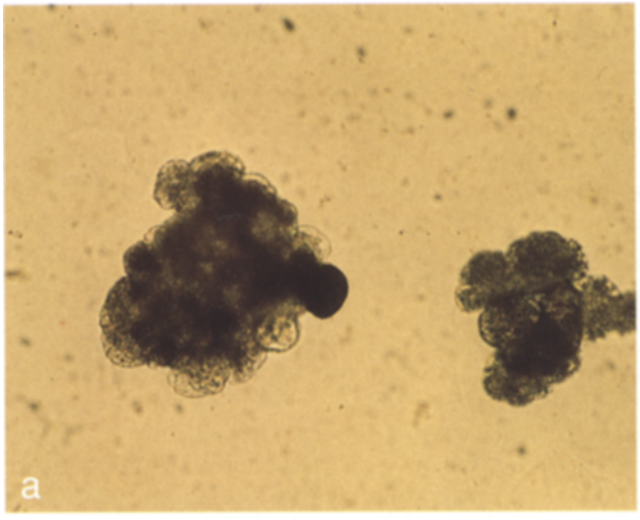
Chromosome preparation and in situ hybridization

Chromosome preparations were made from shoot meristems isolated and pre-treated following de Lautour and Cooper (1971) with modifications. Chromosome counts were based on an inspection of at least three independent metaphases. In situ hybridization with digoxigenin-labeled probes from *L. multiflorum*-specific repetitive sequences pLMH2 and pLMB4 (Perez-Vicente et al. 1992) and detection was done according to the manufactor's instructions (Boehringer Mannheim, FRG) with modifications (Perez-Vicente et al. 1992). Both Italian ryegrass-specific sequences have been shown, by in situ hybridization analysis, to be represented in all *L. multiflorum* chromosomes (Perez-Vicente et al. 1992).

Results

Regeneration of asymmetric somatic hybrid plants between *F. arundinacea* and *L. multiflorum*

Asymmetric somatic hybrids were obtained by the "donor-recipient" method using IOA-inactivated protoplasts of tall fescue (recipient) and X-ray irradiated (at doses ranging from 10 to 500 Gy) non-morphogenic protoplasts of Italian ryegrass (donor) (Fig. 1). Unfused metabolically inactivated protoplasts of tall fescue failed to divide. Dose response experiments measuring the effect of X-ray irradiation on Italian ryegrass protoplasts revealed that doses $\geq$100 Gy fully abolished colony formation. Thus, fusion experiments were carried out using donor protoplasts that had been treated with sublethal and lethal irradiation doses of X-rays. In addition, unirradiated protoplasts of *L. multiflorum* were used for control symmetric fusions. After 1 month in bead-type culture, numerous colonies developed for the different fusion combinations performed using unirradiated Italian ryegrass protoplasts or protoplasts irradiated at sublethal doses (10, 25 and 50 Gy). For these cases, a large proportion of the colonies observed was not expected to be able to regenerate green plantlets since they would be derived from unfused dividing but non-morphogenic Italian ryegrass protoplasts. Overall fusion frequencies (number of growing visible colonies/number of plated protoplasts after fusion) in the range of $1–5 \times 10^{-5}$ were obtained from fusion experiments involving lethal (100, 250 and 500 Gy) irradiation doses of donor Italian ryegrass protoplasts (Fig. 1A, B). No colony formation was observed in control experiments using mixed unfused populations of metabolically inactivated tall fescue protoplasts and donor Italian ryegrass protoplasts irradiated at lethal doses. These asymmetric fusions in the cultivar combination *F. arundinacea* cv 'Fawn' (+) *L. multiflorum* cv 'Gorka Norodova' led to the recovery of more than 50 regenerating asymmetric somatic hybrid callus clones (Fig. 1C) for the cases in which donor protoplasts were treated with X-ray irradiation doses of 10, 25, 50, 100, 250 and 500 Gy. From 32 "donor-recipient" electrofusion experiments, 25 in vitro well-rooted plantlets (Fig. 1D) could be transferred to soil and further grown under greenhouse conditions. Of these 13 were derived from 500-Gy fusions, while 2 or 3 mature plants were established from each of the other X-ray doses. Soil-grown asymmetric somatic hybrid plants varied phenotypically with respect to leaf shape and plant growth habit (Fig. 1E). Nine of them were vernalized in order to assess their fertility, and one asymmetric somatic *Festulolium* plant has already flowered and shown inflorescences with protruding anthers (Fig. 1F). Anthers of this first flowering plant contained 30–40% FDA stainable pollen (Fig. 1G).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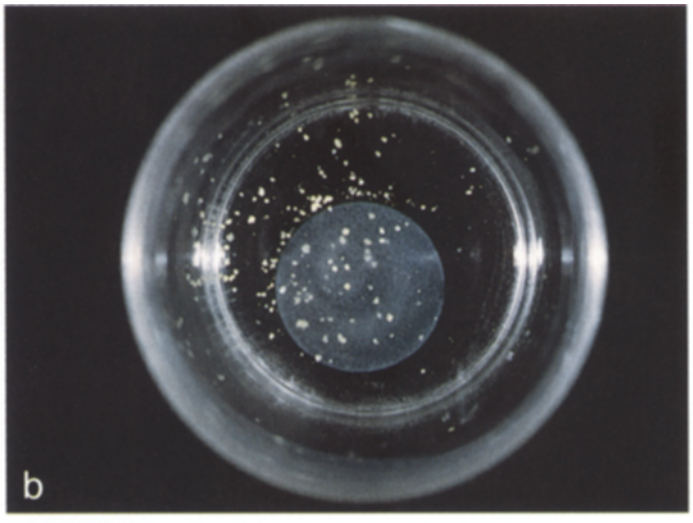
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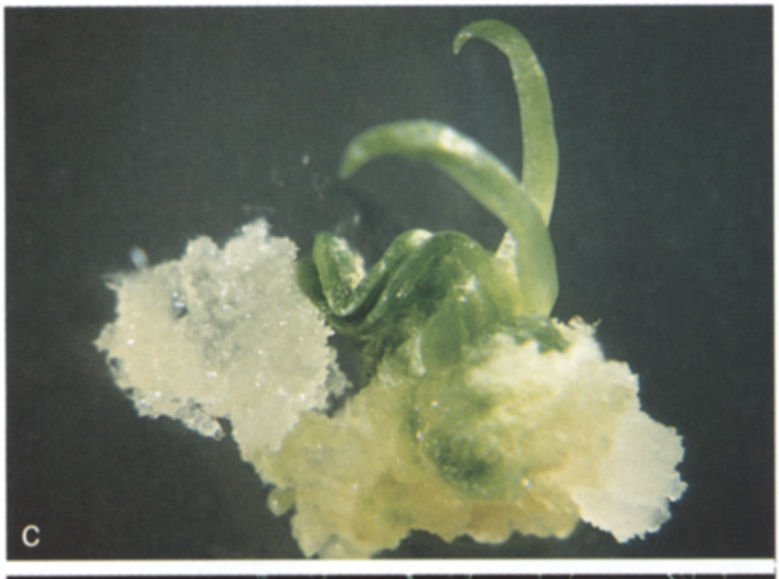
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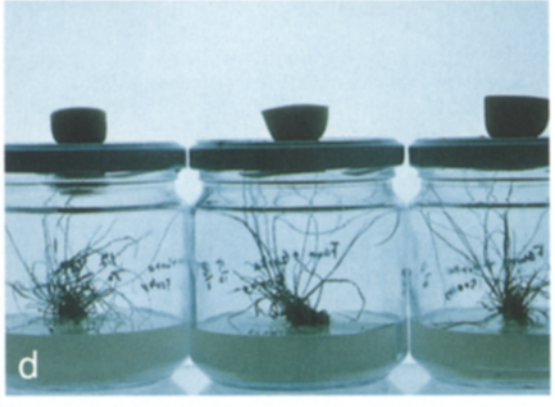
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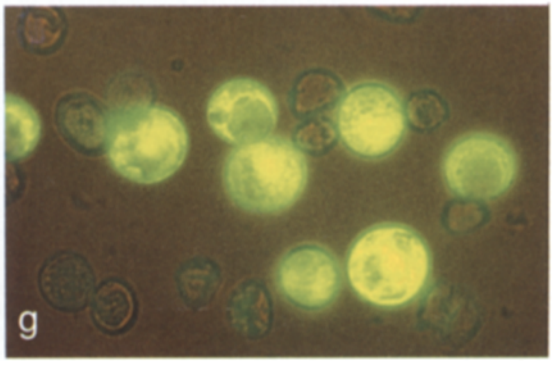
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Fig. 1A–G Production of asymmetric somatic hybrids between iodoacetamide-inactivated tall fescue protoplasts and X-ray-irradiated Italian ryegrass protoplasts. **A** Putative asymmetric somatic hybrid colonies 2 weeks after protoplast fusion growing in bead-type culture, **B** bead-type culture showing proliferating asymmetric somatic hybrid clones 4 weeks after protoplast fusion, **C** regenerating clone from **B** 2 months after protoplast fusion, **D** rooted asymmetric somatic hybrid regenerants 3 months after protoplast fusion, **E** greenhouse-grown asymmetric somatic hybrid plants, **F** inflorescence from greenhouse-grown asymmetric somatic hybrid plant with protruding anthers, **G** fluorescein diacetate-stained pollen from inflorescence from **F**

Analysis of the nuclear composition of asymmetric somatic hybrids

The amount of *L. multiflorum* DNA in the *F. arundinacea* (+) *L. multiflorum* asymmetric somatic hybrids was estimated by dot-blot hybridization analysis. Genomic DNA isolated from 5 asymmetric somatic hybrid regenerating clones (Fig. 1C, D) derived from each of the fusion experiments using Italian donor protoplasts X-ray irradiated with 10, 25, 50, 100 and 250 Gy, and from the parental forms *F. arundinacea* and *L. multiflorum* in concentration series and mixes representing different ratios of genome equivalents, were applied to identical triplicate dot-blot filters. The filters were hybridized with two cloned Italian ryegrass-specific dispersed repetitive DNA probes (pLMB4 and pLMH2) (Fig. 2A, B) and with one tall fescue-specific repetitive DNA sequence (pFAH1) as control (data not shown). The concentration series of parental DNAs enabled us to make a calibration plot of the radioactivity per dot in relation to the amount of DNA from one species and for mixes representing different ratios of genome equivalents (Fig. 2C).

The suitability of these cloned *L. multiflorum*- and *F. arundinacea*-specific dispersed repetitive DNA sequences as probes for the characterization of the nuclear composition of *Festulolium* hybrids has been previously demonstrated (Perez-Vicente et al. 1992). By means of the calibration plots for all three species-specific probes the amount of Italian ryegrass and tall fescue DNA per dot could be estimated and the fraction of the nucler DNA of the asymmetric somatic hybrids derived from Italian ryegrass could be determined (Fig. 3). All of the analysed regenerating calli derived from 10- to 250-Gy fusion products contained Italian ryegrass nuclear DNA, however a large variation in the contribution of *L. multiflorum* DNA to their genomes was evident (Figs. 2A, B and 3). Differences in the estimates of genome equivalents of *L. multiflorum* in the asymmetric somatic hybrids corresponding to the different X-ray radiation doses were revealed for the two independent Italian ryegrass-specific repetitive sequences used as hybridization probes (Fig. 3). However, for both of the probes used, evidence was obtained in the same direc-

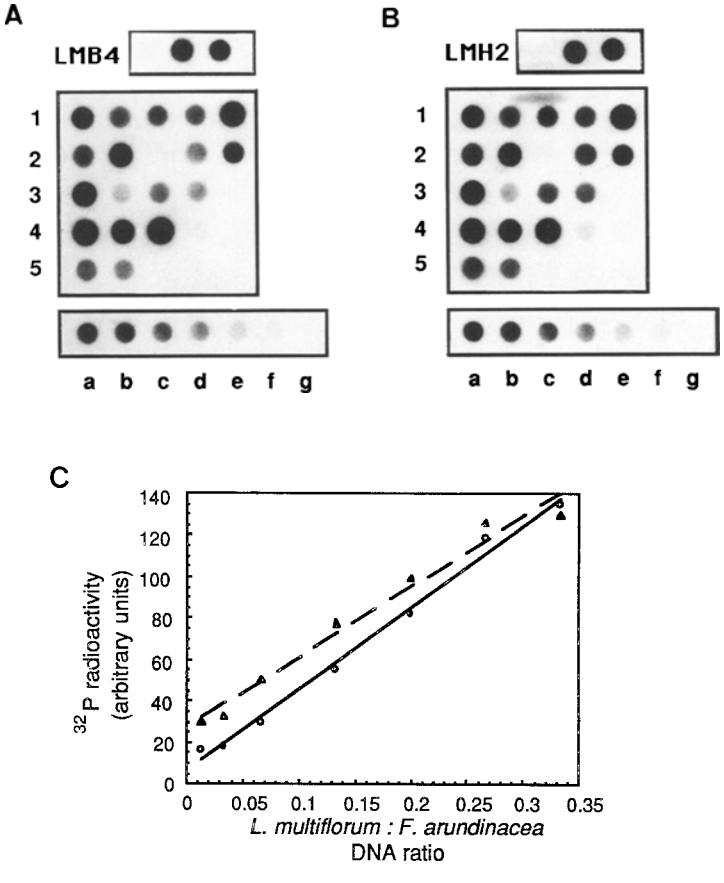

Fig. 2A–C Dot-blot analysis of *F. arundinacea* (+) *L. multiflorum* asymmetric somatic hybrids. Quantitative dot-blot analysis using *L. multiflorum*-specific sequences pLMB4 (**A**) and pLMH2 (**B**) of asymmetric somatic hybrid clones (independent regenerating calli and plants) obtained from independent fusion products between recipient protoplasts of *F. arundinacea* and donor protoplasts of *L. multiflorum* irradiated at: *1* 10 Gy, *2* 25 Gy, *3* 50 Gy, *4* 100 Gy, and *5* 250 Gy. *Uppermost row* of dots represents (from *left* to *right*): 7.5 µg DNA of *F. arundinacea*, mix of 2.5 µg DNA of *L. multiflorum* and 7.5 µg of *F. arundinacea*, 2.5 µg of *L multiflorum*. Calibration plots (**C**) of the radioactivity per dot for decreasing amounts of *L. multiflorum* DNA: *a* 2.5 µg, *b* 2.0 µg, *c* 1.5 µg, *d* 1.0 µg, *e* 0.5 µg, *f* 0.25 µg, *g* 0.1 µg in a constant (7.5 µg) background of *F. arundinacea* DNA (*lowest row*). *Open circles* refer to data obtained for hybridization probe LMB4, *triangles* for probe LMH2

tion, namely on asymmetric somatic hybrid clones showing either limited donor genome elimination and thus being almost symmetric or being highly asymmetric and retaining even less than 5% of the *L. multiflorum* genome (Figs. 2 and 3).

Averages of the data obtained with the two Italian ryegrass-specific sequences pLMH2 and pLMB4 revealed no or very limited donor genome elimination for asymmetric somatic clones regenerated from donor protoplasts irradiated with 10 Gy (Figs. 2A, B and 3).

No donor genome elimination was detectable for 4 regenerating clones obtained from control symmetric-type fusions involving unirradiated Italian ryegrass protoplasts and using regeneration capacity as the selection criterion. Estimates from dot-blots hybridized with the tall fescue-specific repetitive sequence pFAH1 as probe indicated the presence of approximately a complete chromosome set of the recipient *F. arundinacea* in all asymmetric and symmetric somatic hybrids analyzed (data not shown). The variation detected could be due to changes in the ploidy level of recipient and donor protoplasts and/or multiple fusion events. Thus, the irradiation of donor cells prior to fusion led to an unidirectional species-specific elimination of Italian ryegrass chromosomes in asymmetric somatic hybrids for this intergeneric fusion combination. Interestingly, some of the asymmetric somatic hybrid clones recovered after irradiation with doses as different as 25 and 250 Gy retained comparable amounts of donor nuclear DNA, whereas the degree of asymmetry in independent clones within each dose varied even more (Fig. 3A, B). Therefore, no strict correlation between the level of the species-specific *Lolium* genome elimination and the radiation dose used was apparent for the 25- to 250-Gy range of doses tested. Although the number of independent clones analysed for the individual radiation doses was small, it is speculated that with increasing doses relatively more plants showing an extensive donor nuclear genome elimination are present within each category (Fig. 3).

In an analogous manner, a set of 24 regenerating asymmetric somatic hybrid clones obtained from donor protoplasts irradiated with 500 Gy was analysed by quantitative dot-blots (data not shown). An extensive (on average > 85%) and similar Italian ryegrass nuclear genome elimination in all of these 500-Gy asymmetric somatic hybrid regenerating clones was revealed for both of the Italian ryegrass-specific probes tested (Fig. 3A, B). In 4 out to 24 regenerating 500-Gy asymmetric somatic hybrid clones analysed no *L. multiflorum* DNA above background levels was detected when pLMB4 was used as the hybridization probe, thus suggesting that putative cybrid clones completely lacking Italian ryegrass chromosomes might have been recovered.

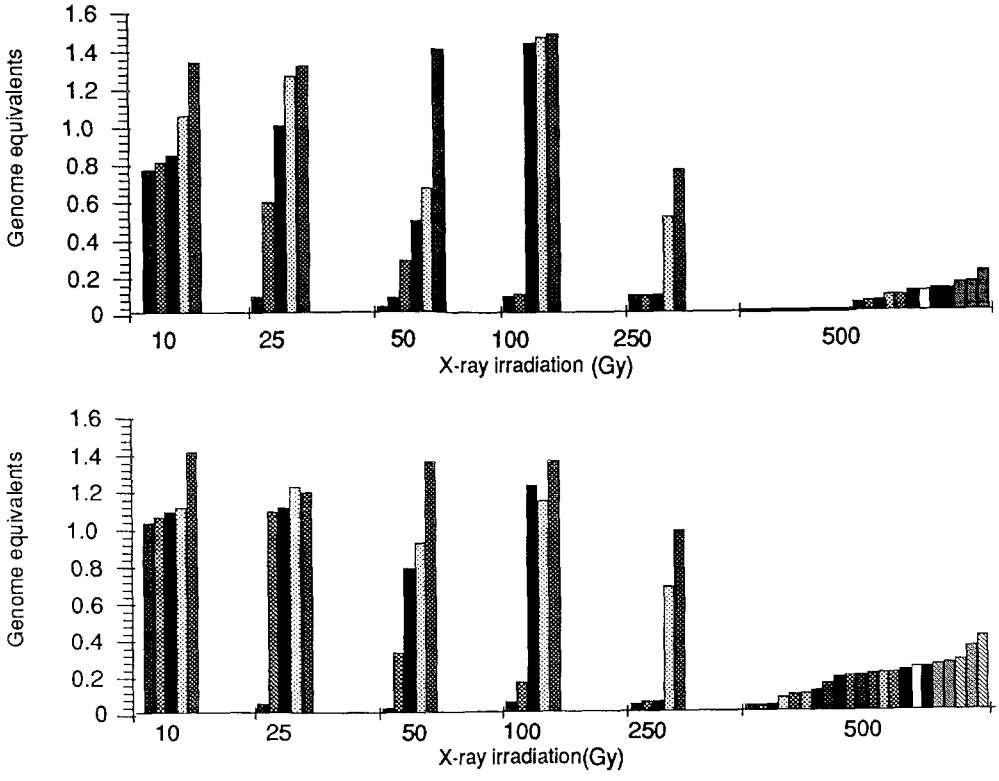

Fig. 3 Genome composition of asymmetric *F. arundinacea* (+) *L. multiflorum* somatic hybrids. Quantitative dot-blots of asymmetric somatic *Festulolium* clones (*bars* correspond to independent regenerating calli and plants) obtained from different doses (10–500 Gy) of X-ray irradiation of donor protoplasts were hybridized with the *L. multiflorum*-specific repetitive sequences LMB4 (*upper graph*) and LMH2 (*lower graph*). The genomic composition of independent clones is shown as genome equivalents of *L. multiflorum* per genome equivalent of *F. arundinacea*

To provide further information on the chromosomal composition of the regenerants obtained and the relative genomic contribution of both parents to them, both chromosome counts and a preliminary analysis by in situ hybridization were performed on metaphase chromosomes using the Italian ryegrass-specific sequences pLMB4 and pLMH2 for representative asymmetric somatic hybrid plants.

Plants derived from the 500-Gy-irradiated protoplasts showed chromosome counts lower than (e.g. $2n = 37 \pm 1$ for plant 500.2) or close to (e.g. $2n = 39 \pm 1$ for plant 500.3) the corresponding count for the recipient tall fescue ($2n = 42$). No detectable hybridization signals were found on chromosomes of these plants when probed in situ with either of the Italian ryegrass-specific sequences pLMB4 or pLMH2, thus supporting the hypothesis of complete donor genome elimination. In contrast, one 500-Gy plant that belonged to the category of asymmetric somatic hybrid clones containing $\geq 20\%$ of the *L. multiflorum* genome in the corresponding quntitative dot-blot analysis using pLMH2 as the hybridization probe (Fig. 3) showed 49 ± 1 chromosomes, clearly higher than the recipient. A representative plant from the 10-Gy asymmetric somatic hybrid clones showed a chromosome count close to the expected additive number ($2n = 56$) for a symmetric clone and hybridization signals on 10 chromosomes when probed in situ with the sequence pLMB4 (data not shown). Thus, these results further support the evidence obtained from quantitative dot-blot hybridizations for the presence of clones showing either limited donor DNA elimination or high asymmetry (Figs. 2, 3).

Analysis of the organellar constitution of asymmetric somatic hybrids

The organellar composition of intergeneric *F. arundinacea* (+) *L. multiflorum* asymmetric somatic hybrids was analysed by the generation of species-specific patterns obtained after hybridization of DNA digests with mtDNA- and cpDNA-specific heterologous gene probes.

Six mitochondrial gene-specific probes (*cox1*, *cox 2*, *cox 3*, *atpA*, *atp6* and *atp 9*) from heterologous (maize and *Oenothera*) systems were used in a Southern blot analysis of digested total DNA from asymmetric somatic hybrid regenerating clones (Fig. 1C–E) that arose from fusions with donor protoplasts irradiated at different X-ray doses. The results were compared to those from corresponding parental forms. Representative results for *Bam*HI digests hybridized with the *atpA* probe (Fig. 4) and for DNA samples digested with *Eco*RI that were used in hybridizations with the *cox1* probe (Fig. 5) are shown. Four symmetric-type regenerants from fusions with unirradiated (0 Gy) Italian ryegrass protoplasts were included for comparison. Independent asymmetric somatic hybrids (10–500 Gy) showed either parental-like, additive (including both the tall fescue and the Italian ryegrass diagnostic bands) and novel patterns (involving the absence of parental-like bands and the presence of non-parental bands) when total DNA digests were blotted and hybridized with different mitochondrial gene-specific probes (Table 1). For Southern hybridizations in which *Bam*HI-digested DNA samples and *atpA* as probes were used, additive-type patterns were found for all 10-Gy- and 25-Gy-asymmetric somatic hybrids (Fig. 4; 10 Gy and 25 Gy). The Italian ryegrass parental-like pattern was present in one 100-Gy clone (Fig. 4; 100 Gy, lane 3), tall fescue parental-like patterns were revealed for most 250-Gy and 500-Gy clones (Fig. 4, 250 Gy, lanes 2–6; 500 Gy, lanes 3–9) and novel patterns were also detected (Fig. 4; 50 Gy, lane 1; 100 Gy, lane 7). Equivalent types of patterns were obtained for *Eco*RI digests hybridized with the *cox 1* probe (Fig. 5). For most of the hybridization probes tested, it was apparent that additive-like patterns predominated in asymmetric somatic hybrids derived from radiation doses in the range of 10 Gy to 50 Gy (Table 1), for asymmetric somatic hybrids corresponding to X-ray doses ≥ 100 Gy, mainly tall fescue parental-like patterns were observed (Table 1). The same held true for 500-Gy asymmetric somatic hybrids (Figs. 4 and 5), where only 6 clones showed either novel or additive patterns and the remaining 19 500-Gy clones analyzed revealed recipient-like RFLPs (data not shown).

In an analogous manner, the chloroplast type of regenerating tall fescue (+) Italian ryegrass symmetric and asymmetric somatic hybrid clones was determined using the spinach *rbcL* gene as hybridization probe. Tall fescue chloroplasts were revealed in 4 individual symmetric somatic hybrids. The analysis of a set of 10- to 500-Gy clones obtained from asymmetric protoplast fusions revealed the presence of Italian ryegrass chloroplasts in one case (Table 1, clone 50.1); for doses of ≤ 250 Gy mixes of both chloroplast types were still present in the regenerating calli analysed in a few cases (Table 1). However, for 250-Gy and 500-Gy clones tall fescue chloroplasts were preferentially detected. Although the final assessment of possibly biased organellar transmission in *F. arundinacea* (+) *L. multiflorum* asymmetric somatic hybrids would require the analysis of larger numbers of independent regenerated plants, a preferential transmission towards recipient-type organelles occurring concomitantly with donor genome elimination was apparent (Figs. 4, 5; Table 1).

Discussion

Asymmetric somatic hybridization was performed for one intergeneric combination in Gramineae in which X-ray-irradiated protoplasts of *L. multiflorum* were used as the donor and metabolically inactivated protoplasts of *F. arundinacea* were used as the recipient. A series of asymmetric somatic hybrid clones were obtained, and

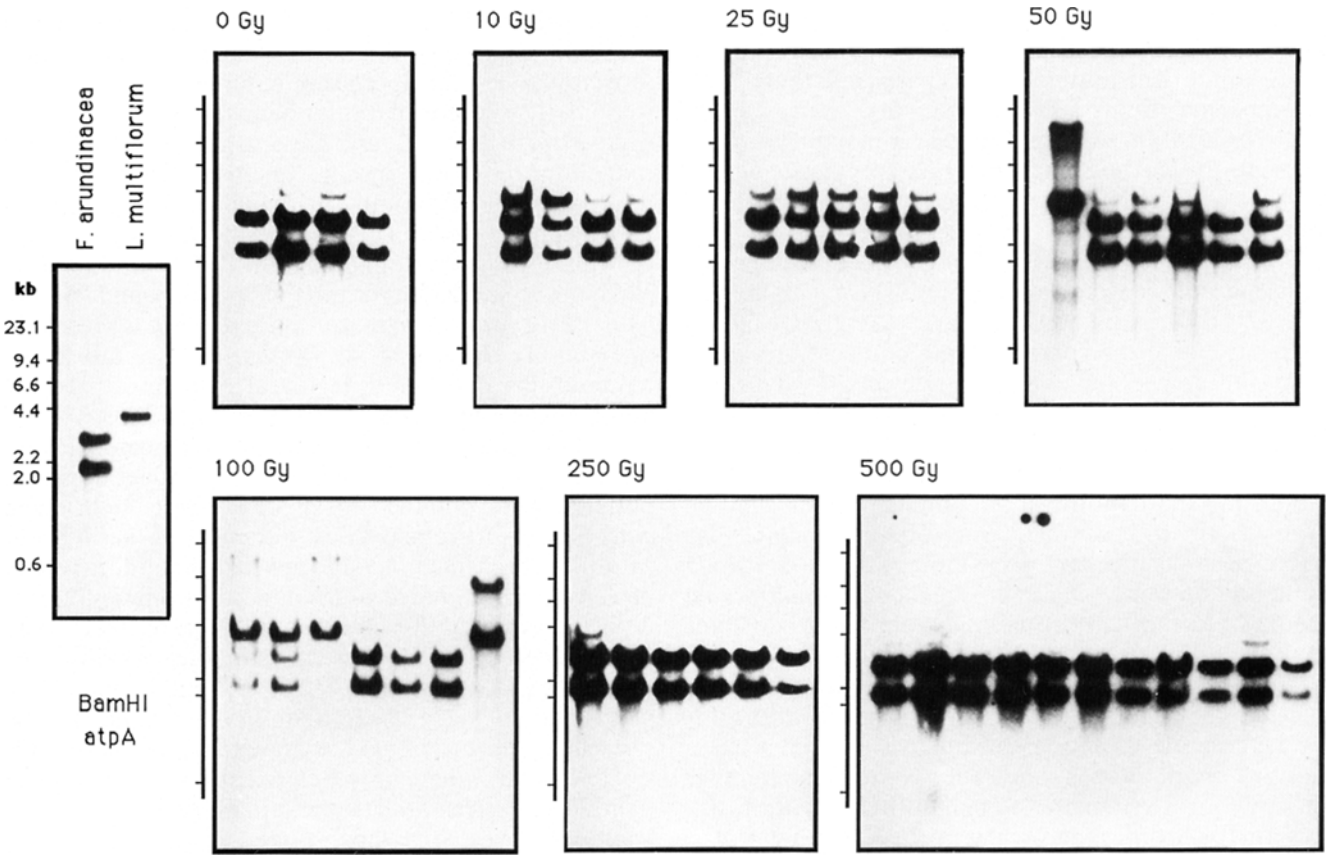

Fig. 4 Restriction fragment length polymorphism (RFLP) analysis of the organellar composition of asymmetric somatic hybrid clones (independent regenerating calli and plants) between IOA-inactivated *F. arundinacea* protoplasts and control (0 Gy) or X-ray-irradiated (10 Gy, 25 Gy, 50 Gy, 100 Gy, 250 Gy, 500 Gy) *L. multiflorum* protoplasts, with the *atpA* mitochondrial gene-specific probe. DNA samples were digested with *Bam*HI. Corresponding hybridization patterns are shown for the parental forms (*F. arundinacea* and *L. multiflorum*)

plants were regenerated for each radiation dose category.

The nuclear constitution of a set of regenerating calli and plants was estimated by dot-blot hybridizations using tall fescue and Italian ryegrass-specific repetitive dispersed sequences. These sequences have been previously shown to be suitable for the characterization of symmetric somatic hybrid plants between tall fescue and Italian ryegrass (Takamizo et al. 1991) and for the analysis of the nuclear composition of *Festulolium* plants obtained from wide crosses (Perez-Vicente et al. 1992). Based on the experiments described here, it was concluded that X-ray irradiation favoured an unidirectional elimination of most or few of the donor *L. multiflorum* chromosomes. X-ray irradiation of donor protoplasts prior to fusion and "gamma-fusion" have been shown to be two reliable methods for inducing species-specific chromosome elimination from the irradiated partner and for the production of asymmetric somatic hybrids in interspecific *Brassica* (Yamashita et al. 1989) and intergeneric *Nicotiana-Atropa* (Gleba et al. 1988) combinations.

In our study, further information on the nuclear genomic make-up of representative asymmetric somatic hybrid plants was obtained from chromosome counts and in situ hybridizations using Italian ryegrass-specific repetitive sequences. In few cases where a comparison could be made, correlative evidence was found between the number of excess chromosomes, presence or absence of in situ hybridization signals to metaphase chromosomes and the estimates from corresponding dot-blot values for individual asymmetric somatic hybrids. This confirmed the validity of the genome composition estimates obtained for all of the regenerating clones on the basis of dot-blot hybridizations. Similar results were obtained from chromosomal analysis of *Nicotiana tabacum* (+) *N. plumbaginifolia* asymmetric somatic hybrids by dot-blot and in situ hybridizations in which two main categories of regenerants were revealed: one containing only 1%–5% *N. plumbaginifolia* DNA, the other 15%–25% of the donor genome (Piastuch and Bates 1990). In addition, for these and other interspecific *Nicotina* asymmetric somatic hybrids, the occurrence of intergenomic translocations have been demonstrated (Piastuch and Bates 1990; Parokonny et al. 1992). Detailed in situ hybridization studies, however, would be needed to assess if intergenomic translocations have taken place in our case. Furthermore, our data suggest that the degree of elimination of donor chromosomes from X-ray-irradiated Italian ryegrass protoplasts was not dose dependent for asymmetric somatic hybrids in

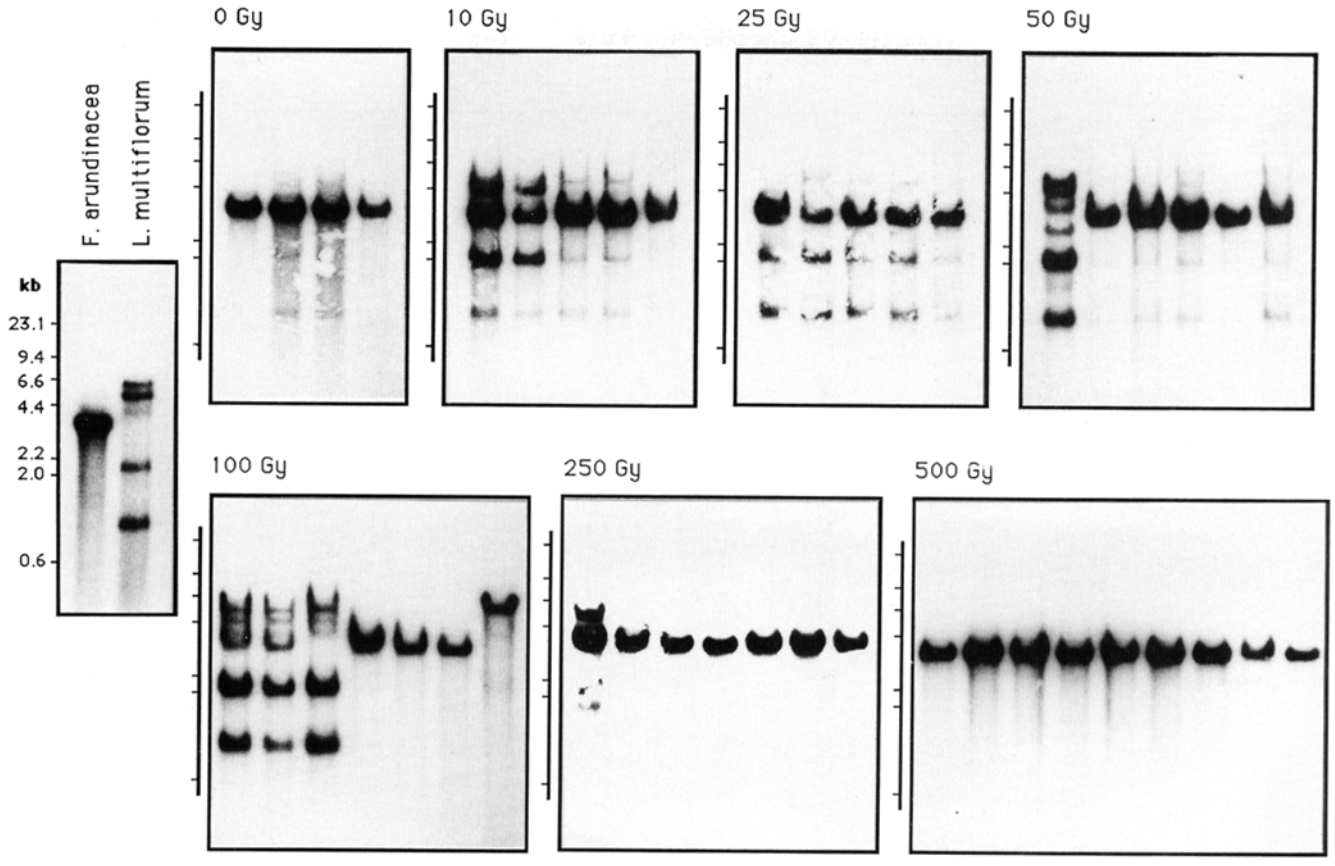

Fig. 5 RFLP analysis of the organellar composition of asymmetric somatic hybrid clones (independent regenerating calli and plants) obtained from fusions between IOA-inactivated *F. arundinacea* protoplasts and control (0 Gy) or X-ray-irradiated (10 Gy, 25 Gy, 50 Gy, 100 Gy, 250 Gy, 500 Gy) *L. multiflorum* protoplasts, with the *cox1* mitochondrial gene-specific probe. DNA samples were disgeted with *Eco*RI. Corresponding hybridization patterns are shown for the parental forms (*F. arundinacea* and *L. multiflorum*)

the range of 25–250 Gy and that a larger variability of the asymmetry level is detectable in independent clones within each of these dose categories. Analogous results were obtained in a thorough evaluation of intertribal asymmetric somatic hybrids between *N. plumbaginifolia* and *Atropa belladonna* obtained by "gamma-fusion" (Gleba et al. 1988). However, these results are in contrast to those obtained for asymmetric somatic hybrids between *Lycopersicon esculentum* and *L. pennelli*, where the amount of nuclear DNA transferred from the irradiated fusion partner was found to be inversely proportional to the radiation dose (Melzer and O'Connell 1992).

In our case, only a very limited elimination of *L. multiflorum* DNA was observed in some regenerating asymmetric somatic hybrid clones obtained from donor protoplasts irradiated with 10 Gy to 250 Gy. Similarly, the irradiation of protoplasts prior to fusion has been shown to eliminate the donor genome to only a limited extent in interspecific asymmetric somatic hybrids between *Lycopersicon esculentum* and *L. peruvianum* (Wijbrandi et al. 1990a, b) and in intergeneric clones between *L. esculentum* and *Solanum tuberosum* (Wolters et al. 1991). When Italian ryegrass protoplasts were irradiated with 500-Gy of X-rays, an extensive donor genome elimination was found in all cases. However, individual clones with an equivalent extensive donor genome elimination were also found for X-ray doses of 25–250 Gy.

In few cases, the complete absence of *L. multiflorum* DNA was demonstrated in 500-Gy plants. Recently, asymmetric protoplast fusion aimed at the intraspecific transfer of cytoplasmic male sterility has been reported for *Lolium perenne* (Creemers-Molenaar et al. 1992). In this case, cybrid calli showing extensive quantitative and qualitative variation in hybridization patterns based on two mitochondrial probes were described. However, the analysis of the nuclear genome performed for these putative cybrid perennial ryegrass calli did not allow the investigators to exclude the presence of donor DNA (Creemers-Molenaar et al. 1992). In our case, mature plants derived from "donor-recipient" protoplast fusion and lacking donor chromosomes were obtained for an intergeneric combination in Gramineae. Most of the asymmetric somatic hybrid clones from 500-Gy-irradiated donor protoplasts revealed an organellar composition closer to that of tall fescue (recipient) when subjected to an RFLP analysis involving six different mitochondrial and one chloroplast probe. Interestingly, earlier we reported a similar observation for symmetric somatic *Festulolium* plants obtained from a different genotype combination between *F. arundinacea* and *L. multiflorum* (Takamizo et al. 1991). However, compa-

Table 1 Summary of organellar RFLP patterns of asymmetric somatic hybrid clones (independent regenerating calli and plants) obtained from fusions between IOA-inactivated *F. arundinacea* protoplasts and X-ray-irradiated (10 Gy, 25 Gy, 50 Gy, 100 Gy and 250 Gy) *L. multiflorum* protoplasts with mitochondrial (*cox1*, *cox2*, *cox3*, *atpA*, *atp6*, and *atp9*) and chloroplast (*rbcL*) gene-specific probes. Patterns for DNA digests with *Eco*RI (E) and *Bam*HI (B) are given (*T* Tall fescue pattern, *I* Italian ryegrass pattern, *TI* additive pattern, *n*0 new pattern (completely new pattern), *n*1 novel pattern (additive but missing some parental bands); *n*2 novel pattern (additive with additional non-parental bands). Nomenclature of clones indicates X-ray dose used for irradiation of donor protoplasts followed by consecutive numbers for the independent clones

Asymmetric hybrids	Hybridization probe/Restriction enzyme combination						
	cox1/E	*cox2*/B	*cox3*/E	*atp*A/B	*atp6*/E	*atp9*/B	*rbcL*/E
10.1	TI	TI	TI	TI	TI	TI	TI
10.2	TI	TI	TI	TI	TI	TI	TI
10.3	TI	TI	TI	TI	TI	TI	T
10.4	TI	TI	TI	TI	TI	TI	T
10.5	T	n1	n0	TI	TI	T	T
25.1	TI	TI	TI	TI	TI	TI	T1
25.2	TI	TI	TI	TI	TI	TI	TI
25.3	TI	TI	T	TI	TI	TI	TI
25.4	TI	TI	TI	TI	TI	TI	TI
25.6	TI	n2	TI	TI	TI	TI	TI
50.1	n0	I	n2	n0	n0	I	I
50.2	TI	TI	T	TI	TI	TI	T
50.3	TI	TI	TI	TI	TI	TI	T
50.4	TI	TI	TI	TI	TI	TI	T
50.5	T	T	T	T	TI	T	T
50.6	TI	TI	TI	TI	TI	TI	T
100.1	TI	I	TI	TI	TI	TI	TI
100.2	TI	TI	TI	TI	TI	TI	TI
100.3	I	I	TI	I	I	I	I
100.4	T	T	T	T	T	T	T
100.5	T	T	T	T	T	T	T
100.6	T	T	T	T	T	T	T
100.7	n0	n0	T	n0	n0	n0	I
250.1	n0	n1	TI	TI	TI	TI	TI
250.2	TI	TI	TI	TI	TI	TI	T
250.3	T	T	T	T	T	T	T
250.4	T	T	T	T	T	T	T
250.5	T	T	T	T	T	T	T
250.6	T	T	T	T	T	T	T
250.7	T	T	T	T	T	T	T
250.8	T	T	T	T	T	T	T

rable regenerating clones from symmetric-type fusions incuded in the present study as controls and obtained for exactly the same genotype combination as the 500-Gy asymmetric somatic hybrids did not reveal the same phenomenon. Thus, nuclear-organellar interactions for this intergeneric combination could explain a probable bias towards recipient (tall fescue) organelles in highly asymmetric somatic clones when an extensive donor (Italian ryegrass) nuclear genome elimination was induced by irradiation. A thorough characterization of the organellar composition for a larger number of highly asymmetric somatic hybrid plants would be required to substantiate this speculation.

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