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Intergeneric symmetric and asymmetric somatic hybridization in *Festuca* and *Lolium*

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Summary

Intergeneric symmetric and asymmetric somatic hybrids have been obtained by fusion of metabolically inactivated protoplasts from embryogenic suspension cultures of tall fescue (*Festuca arundinacea* Schreb.) and unirradiated or 10–500 Gy-irradiated protoplasts from non-morphogenic cell suspensions of Italian ryegrass (*Lolium multiflorum* Lam.). Genotypically and phenotypically different somatic hybrid *Festulolium* mature flowering plants were regenerated.

Species-specific sequences from *F. arundinacea* and *L. multiflorum* being dispersed and evenly-represented in the corresponding genomes were isolated and used for the molecular characterization of the nuclear make-up of the intergeneric, somatic *Festulolium* plants recovered. The irradiation of Italian ryegrass protoplasts with $\leq$ 250 Gy X-rays prior to fusogenic treatment favoured the unidirectional elimination of most or part of the donor chromosomes. Irradiation of *L. multiflorum* protoplasts with 500 Gy produced highly asymmetric (over 80% donor genome elimination) nuclear hybrids and clones showing a complete loss of donor chromosomes.

The RFLP analysis of the organellar composition in symmetric and asymmetric tall fescue (+) Italian ryegrass regenerants confirmed their somatic hybrid character and revealed a bias towards recipient-type organelles when extensive donor nuclear genome elimination had occurred.

Approaches aimed at improving persistence of ryegrasses based on asymmetric somatic hybridization with largely sexually-incompatible grass species (*F. rubra* and *Alopecurus pratensis*), and at transferring the cytoplasmic male sterility trait by intra- and inter-specific hybridization in *L. multiflorum* and *L. perenne*, have been undertaken.

Abbreviations: cpDNA – chloroplast DNA, CMS – cytoplasmic male sterility, 2,4-D – 2,4-dichlorophenoxy-acetic acid, IOA – iodoacetamide, mtDNA – mitochondrial DNA, RFLP – restriction fragment length polymorphism

Introduction

Several species in the genera *Festuca* and *Lolium* are commonly grown cool-season perennial forage and turf grasses of the temperate region. Tall fescue (*F. arundinacea* Schreb.) has a wide range of distribution since it shows good persistence and tolerance to various environmental stresses. Concerning quality and palatability however, it compares unfavourably with the widely used ryegrasses: Italian ryegrass (*L. multiflorum* Lam.) and perennial ryegrass (*L. perenne* L.). For many years *Festulolium* hybrids have been produced by conventional crossing in order to combine the agronomically-desirable traits from fescues

and ryegrasses (Crowder, 1953). However, sexual hybrids between *L. multiflorum* and *F. arundinacea* have been readily obtained only unidirectionally (Italian ryegrass × tall fescue), while the reciprocal cross has been shown to be extremely difficult (Eizenga & Buckner, 1986). In addition, chromosomal instabilities and the poor female fertility observed in the early amphiploid *Festulolium* plants derived from these octaploid sexual hybrids have so far severly limited the release of valuable materials from these crosses.

Somatic hybridization by protoplast fusion shows promising solutions to these and other problems faced in conventional breeding programmes in ryegrasses and fescues, such as: 1) the transfer of genes from wild relatives to cultivated plants, by-passing existing sexual crossing barriers; 2) the rapid directed combination of partial genomes from different genetic sexually-(in)compatible origins that could serve as bridges to transfer specific traits, and 3) the transfer of cytoplasmically-encoded characters without requiring many backcrosses.

Here we summarize the present state of our research on: 1) the establishment of experimental protocols for the reproducible recovery of mature symmetric and asymmetric somatic *Festulolium* plants; 2) the isolation and characterization of species-specific, repetitive nuclear DNA sequences for the analysis of the genomic composition of sexual and somatic hybrids in the *Festuca-Lolium* complex; and 3) the characterization of the nuclear and organellar constitution of symmetric and asymmetric somatic *Festulolium* hybrids.

Materials and methods

Plant material, establishment of suspension cultures and isolation of protoplasts

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

Protoplasts were isolated from embryogenic cell suspensions of tall fescue and from cell lines of Italian ryegrass as previously reported (Spangenberg et al., 1994a). Italian ryegrass cells were X-ray-irradiated at doses of 10, 25, 50, 100, 250 and 500 Gy with an X-ray apparatus (Müller MG150, Type 70526/41; 80 kV, 17 mA, 1900 $Rmin^{-1}$, 0.2 mm Al-filter; Müller GmbH, Hamburg, FRG). Control unirradiated cell suspensions of Italian ryegrass were also used. Tall fescue protoplasts were resuspended for metabolic inactivation in WF solution (0.6 M mannitol, 10 mM $CaCl_2$, pH 5.8) containing 10 mM 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 with a commercial electrofusion setup (Elektro-Zellfusion CFA 400, Krüss, Hamburg, FRG) using the following conditions: ac-field (1 MHz, 80 V/cm for 30 s) followed by dc-pulses (0,75 kV/cm, 1–2 pulses, 30μs each) (Spangenberg et al., 1994a). Five to ten minutes after electrofusion, protoplasts were collected by centrifugation (80 g for 7 min), resuspended in 2.0 ml of double-concentrated AA medium (Müller & Grafe, 1978) supplemented with 2 mg/l 2,4-D and 0.6 M glucose, and plated on agarose solidified protoplast culture medium (Wang et al., 1992).

Culture of protoplasts after fusion and plant regeneration from putative fusants-derived colonies

Protoplasts were cultured using the agarose bead-type culture method (Shillito et al., 1983) with nurse cells (Kyozuka et al., 1987) as previously described by Wang et al. (1993a). After about one month in culture, colonies were transferred onto MS medium (Murashige & Skoog, 1962) supplemented with 1 mg/l 2,4-D, 500 mg/l casein hydrolysate, 90 mM sucrose solidified with 0.8% (w/v) agarose. About four weeks later proliferating calli were placed on MSK medium consisting of MS basal medium supplemented with 90 mM sucrose and 0.2 mg/l kinetin. The regenerated shoots were then transferred to hormone-free MS medium for rooting. All cultures were kept under fluorescent light conditions (40 μmol m^{-2} s^{-1}) with a 16 h photoperiod at 25° C. Rooted plants were then potted in soil and grown until maturity under greenhouse conditions (natural daylength; 23° C/18° C).

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 asym-

metric somatic hybrids and their parental lines. Isolation and digestion of genomic DNA was performed according to Lichtenstein & 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 (EcoRI or BamHI), 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 & Vogelstein, 1983).

Repetitive and evenly-dispersed species-specific sequences of *L. multiflorum* (LMH2 and LMB4) and *F. arundinacea* (FAH1) (Perez-Vicente et al., 1992) were used for the characterization by quantitative dot blot analysis of the nuclear composition of putative tall fescue (+) Italian ryegrass, somatic hybrid regenerating clones (Spangenberg et al., 1994a).

For the characterization of the organellar genomes of somatic hybrids, different plant mtDNA gene-specific probes (*cox1, cox2, cox3, atpA, atp6* and *atp9*) and one cpDNA gene-specific clone (*rbcL*) were used.

Isolation of species-specific repetitive DNA sequences

The construction of DNA probes and the isolation of repetitive clones specific to *L. multiflorum, F. arundinacea, F. rubra* and *Alopecurus pratensis* was basically performed according to Saul & Potrykus (1984). Colonies containing inserts of plant DNA were picked at random and used for colony hybridization screenings. Two replicas of the total bank of clones were produced on Biodyne (Pall, Glen Cove, USA) filters and hybridized with probes created by random priming of total DNA. Clones preliminarily identified as species-specific were further tested as described earlier (Pérez-Vicente et al., 1992). Inserts from species-specific, repetitive DNA clones were sequenced by the dideoxynucleotide chain termination procedure of Sanger et al. (1980) using the T7 sequencing kit (Pharmacia, Sweden).

Chromosome preparation and in situ *hybridization*

Chromosome preparations were made from shoot meristems isolated and pre-treated following with modifications de Lautour & Cooper (1971). *In situ* hybridization with digoxigenin-labeled probes and detection was done according to the manufactor's instructions (Boehringer Mannheim, FRG) with modifications (Perez-Vicente et al., 1992).

Results

Protoplast fusion, culture of fusion products and regeneration of symmetric and asymmetric somatic hybrid Festulolium *plants*

Protoplasts of tall fescue (*F. arundinacea*) and Italian ryegrass (*L. multiflorum*) were readily-obtained from embryogenic (Fig. 1A) and non-morphogenic (Fig. 1B) cell suspensions, respectively. The protoplast yield varied in the range 0.5–2 $\times$ 10^6/g fresh weight cells depending on the suspension culture used.

The selection scheme used for the enrichment of protoplast fusion products to generate symmetric and asymmetric somatic hybrids, was based on metabolically-inactivated, totipotent protoplasts of tall fescue and (unirradiated or X-ray irradiated) non-morphogenic protoplasts of Italian ryegrass, respectively. Dose response experiments on the inactivation of tall fescue protoplasts with IOA in the range of 2–20 mM, showed that 10 mM IOA treatment for 15 min completely inhibited colony formation. Dose response experiments using X-ray irradiation of Italian ryegrass protoplasts in the range of 50–500 Gy, revealed that doses of 100 Gy and higher, fully prevented colony formation.

No regeneration of green plantlets occurred in the following control experiments: 1) unirradiated protoplasts isolated from non-morphogenic suspension cultures of Italian ryegrass; 2) X-ray-irradiated protoplasts from suspensions as in 1); 3) IOA-inactivated protoplasts of tall fescue isolated from morphogenic suspensions; and 4) mixtures of unfused protoplasts from 1) with 3) or from 2) with 3).

Fusion experiments performed using unirradiated (symmetric fusions) or 10–500 Gy X-ray-irradiated (asymmetric fusions) Italian ryegrass protoplasts (Fig. 1C) led to colony formation within 3–4 weeks in bead type culture (Fig. 1DE). After a further 4–6 weeks on proliferation and regeneration medium, more than 60

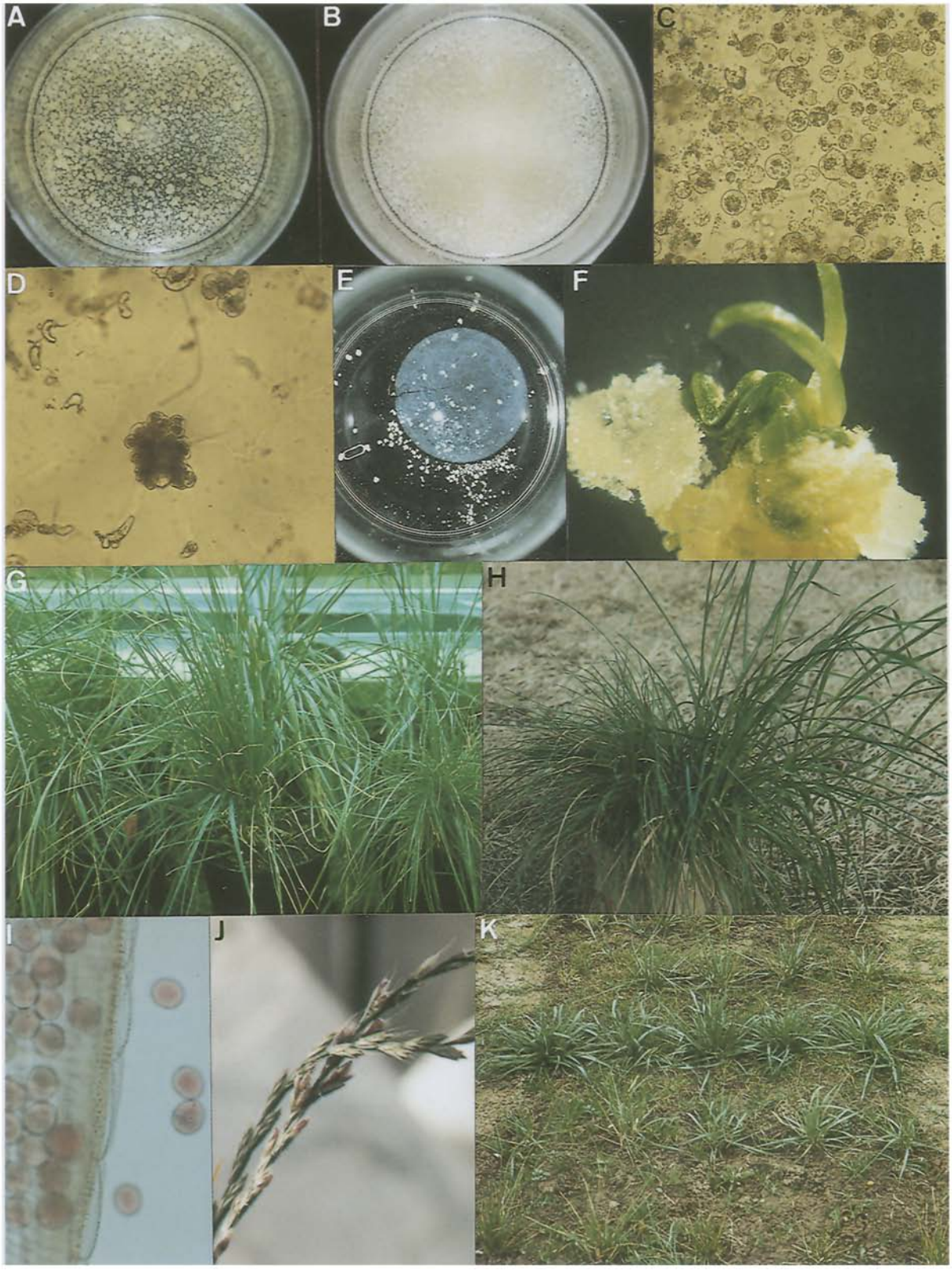
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Fig. 1. Recovery of symmetric and asymmetric somatic hybrid plants between *Festuca arundinacea* Schreb. and *Lolium multiflorum* Lam. A) Embryogenic cell suspension of *F. arundinacea* used for isolation of totipotent protoplasts; B) Non-morphogenic cell suspension of *L. multiflorum* used for isolation of protoplasts; C) Protoplast mixture after fusion of IOA-inactivated protoplasts of *F. arundinacea* and non-morphogenic protoplasts of *L. multiflorum* isolated from A) and B), respectively; D) Multiple divisions of putative fusant from asymmetric fusion between IOA-inactivated tall fescue protoplasts and 250 Gy X-ray-irradiated Italian ryegrass protoplasts two weeks after fusion; E) Putative somatic hybrid colonies obtained from fusion experiments between tall fescue protoplasts and 500 Gy-irradiated Italian ryegrass protoplasts after 4 weeks in bead type culture; F) Regenerating clone from E) two months after protoplast fusion; G) Greenhouse-growing, mature somatic hybrid *Festulolium* plants obtained from symmetric and asymmetric protoplast fusions; H) Morphology of clonally-unstable somatic hybrid plant (right side of the plant is morphologically similar to Italian ryegrass); I) Acetocarmine-stained pollen of somatic hybrid *Festulolium* plant shown on H); J) Seeds developing on symmetric somatic hybrid *Festulolium* plant after pollination with Italian ryegrass; K) Somatic hybrid *Festutolium* plants growing in the field.

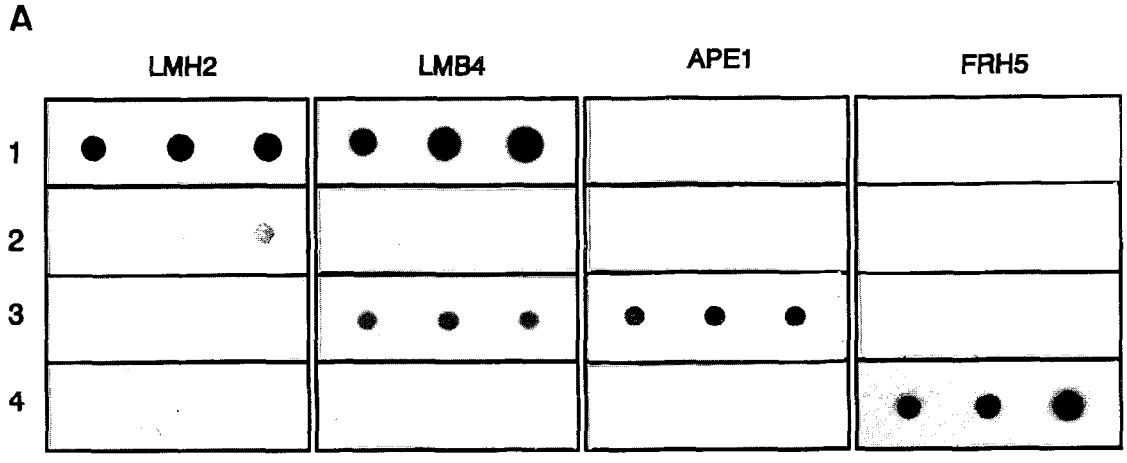
calli differentiating green shoots were obtained from over 40 symmetric and asymmetric fusion experiments involving different X-ray irradiation doses of donor protoplasts (Fig. 1F). These regenerating calli could thus be preliminarily identified as putative symmetric and asymmetric somatic hybrid clones. Green shoots grew vigorously, rooted after transfer to MS hormone-free medium and 31 plantlets were successfully transferred to soil and grown until maturity under greenhouse conditions (Fig. 1G). Of these, 6 plants were obtained from symmetric fusions, 13 were derived from 500-Gy fusions, and 2 or 3 mature plants were recovered from each of the other X-ray doses (Spangenberg et al., 1994; Takamizo & Spangenberg, 1994). Plant habit and leaf morphology varied among these independent symmetric and asymmetric tall fescue (+) Italian ryegrass somatic hybrid plants (Fig. 1G). Some showed an intermediate character when compared with the parental plants, others were closer to tall fescue (Fig. 1G). In other cases chimaeric clones revealing plant sectors with leaf morphology and plant habit similar to Italian ryegrass were observed (Fig. 1H). When brought to flower they produced inflorescences with protruding anthers which contained stainable pollen (Fig. 1I). Crosses of these clonal unstable plants with Italian ryegrass pollen led to seed formation (Fig. 1J). Representative intergeneric somatic hybrid *Festulolium* plants obtained from symmetric and asymmetric fusion combinations are now growing under field conditions (Fig. 1K).

Isolation and characterization of species-specific repetitive DNA sequences for analysing hybrid nuclear genomes in the Festuca-Lolium *complex*

For the analysis of hybrid nuclear genomes, cloned DNA sequences need to be: 1) highly species-specific; 2) repetitive; 3) dispersed in the corresponding genome; and 4) evenly distributed in all or most chromosomes. We have isolated repetitive nuclear DNA sequences which fulfil these requirements from *L. multiflorum* and *F. arundinacea* (Pérez-Vicente et al., 1992). In addition, repetitive nuclear DNA sequences specific for red fescue (*F. rubra*) and meadow foxtail (*A. pratensis*) were isolated and characterized at the molecular and cytological level, to be used as tools for the analysis of further intergeneric somatic hybrids to be generated.

In order to isolate repetitive species-specific DNA sequences, ca. 250 recombinant plasmid clones, each containing random sequences from *L. multiflorum, F. arundinacea, F. rubra* and *A. pratensis*, were screened using labelled total DNA from two species as hybridization probes. As expected for multicopy sequences, between 5 and 10% of the clones showed strong hybridization signals when probed with DNA from the same species. DNA inserts from one *F. arundinacea* clone, six *F. rubra* clones, eight *L. multiflorum* clones and one *A. pratensis* clone showing differential hybridization were isolated and their species-specificity was tested by dot blot hybridization analysis to total DNA from different grass species within and outside the *Festuca-Lolium* complex: *L. multiflorum, L. perenne, F. arundinacea, F. pratensis, F. rubra* and *A. pratensis*. Representative results from this analysis are shown in Fig. 2A. The Italian ryegrass sequence LMH2 was only present in *L. multiflorum* and *L. perenne*, the sequence LMB4 from *L. multiflorum* hybridized in addition to total DNA from *A. pratensis* (Fig. 2A). The meadow foxtail sequence APE1 was only present in *A. pratensis*. Similarly, the clone FRH5 from red fescue hybridized exclusively to DNA from *F. rubra* (Fig. 2A), while the sequence FAH1 cloned from *F. arundinacea* cross-hybridized to *F. pratensis* DNA but was absent in the genome of the other species considered.

To increase knowledge of the organization, degree of dispersion and chromosomal localization of selected, highly species-specific, repetitive DNA sequences,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LMH2
LMB4
APE1
FRH5
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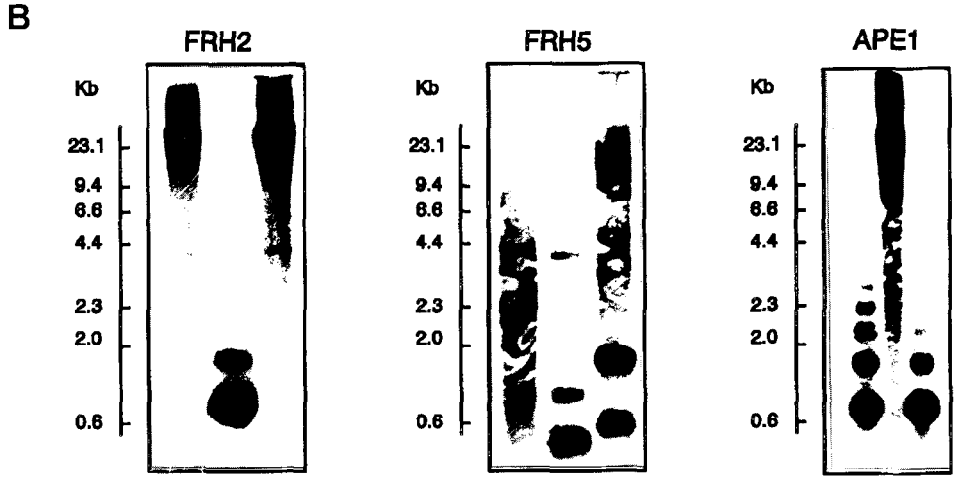
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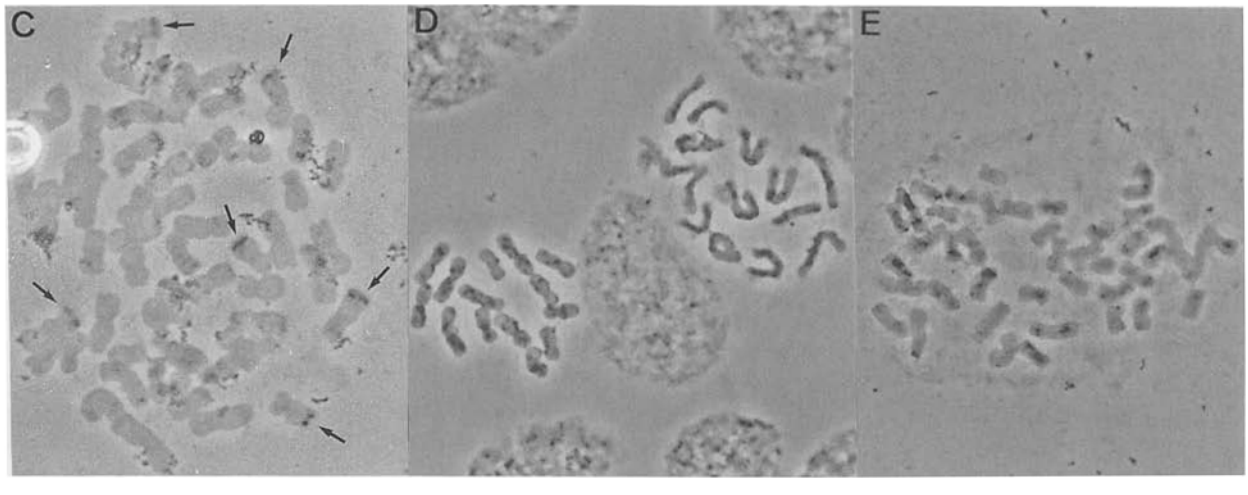
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Fig. 2. Isolation and characterization of species-specific repetitive DNA sequences in *Festuca, Lolium* and *Alopecurus*. A) Test for species-specificity of repetitive DNA sequences isolated from *L. multiflorum* (LMH2 and LMB4), *A. pratensis* (APE1) and *F. rubra* (FRH5). Labelled probes were hybridized to dot blots containing (from left to right) 0.5, 1 and 2 μg of (1) *L. multiflorum*, (2) *L. perenne*, (3) *A. pratensis*, and (4) *F. rubra* DNA; B) Southern blot hybridization patterns from *F. rubra* and *A. pratensis* DNA were probed with repetitive DNA sequences FRH2, FRH5 and APE1, respectively. In all cases, 7 μg of total DNA digested with BamHI, HindIII or EcoRI (lanes from left to right) were loaded; C–E) *In situ* hybridization analysis of repetitive DNA sequences from: C) *F. rubra* (FRH2), D) *L. multiflorum* (LMH2), and E) *F. arundinacea* (FAH1) hybridized to metaphase chromosomes of red fescue, Italian ryegrass and tall fescue, respectively. Arrows indicate hybridization signals on red fescue chromosomes (C).

Sequence	Size (bp)	Copy Number*	Characteristics**
LMH2	173	1.3×10^4	dispersed, (D, E).
LMB4	300	2.0×10^3	dispersed, (D, F).
FAH1	102	2.0×10^4	clustered, (A, B).
FRH5	78	1.1×10^5	tandemly arranged, (C).
APE1	385	7.0×10^3	tandemly arranged, (F).

* Estimated values per haploid genome
**Sequences cross-hybridizing to the corresponding probes are present in: (A) *F. arundinacea*, (B) *F. pratensis*, (C) *F. rubra*, (D) *L. multiflorum*, (E) *L. perenne*, (F) *A. pratensis*.

LMH2

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AGACTTTGTG CAATGTCAGA AGTGTTAAGA ATGATTATGT CACCTCTGAA   50
TGTATGAATT TTTTATTATG CACTAACCCT CTAATGAGTT TGCTTGAAGT   100
TTGGTGTGGA GGAAGTTTTC AAGGGTCAAG AGAAGAGGAT GATACAATAT   150
GATCAAGAAG AGTGAAAGGT CTA
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FAH1

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AGCTTGGCTA GAGCTGCTTG CCTCCTGACC TTTTCCGGTT CCGGCCTTGG   50
GAGCAGAGGG GGAGGCACTC ATCCGGTCGA TCTCGGCTTC AAGCCCGTCG   100
GA
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FRH5

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AGCTTTAAAA ATTCATCGTT AAACGCTTGG TTAGTAGAGC CAAAGATAAT   50
ATCATCAACA TATAGTTGGC ATATAAAA
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APE1

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AATTCCCTCG GTATGTTACA TTCTTTCATC AGCTTATTCC CCGTTTCTTT   50
TCTAGTTTGA GCCTAACACA CTATTGTAGC TATCCATGGG TTTGGCTCTT   100
AAACCCTGAT GGCCAAATGG ATCCACGGCA CCATTGAATT TCAAACTTTA   150
AGTGCCAAAA CTCCTGTTTT TCATCTCGTA GGCCCTTTTA GAAATGCTAA   200
ACGTCCTTTT TTGGGGCCTA GATTTCTAGG AACGCTTTTT CGGGATTTGT   250
GTGCATGAAT TTCAAATCAT ACGAACTTAT AAAATAGAAG TCATTCCATC   300
GTTATTCCCC TAGAGCCTTC CAGCAGTTCC TAAACCGCCT CAGTCGGAGC   350
ACGTTATCAT TCGTCTTGTT AGTTCCGAGT GAATT
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Fig. 3. Characteristics and nucleotide sequences of repetitive DNA clones from *L. multiflorum, F. arundinacea, F. rubra* and *A. pratensis*. Size of the cloned repetitive DNA sequences was estimated by comparing the electrophoretic mobility of the corresponding inserts to size standards. Estimated number of copies per haploid genome was determined by comparison of the extent of hybridization of the indicated sequence to defined amounts of genomic DNA and of the corresponding DNA sequence. *L. multiflorum*-specific, repetitive clones LMH2, LMB4 and *F. arundinacea*-specific, repetitive sequence FAH1 (Pérez-Vicente et al, 1992; Theor. Appl. Genet. 84: 145–154), *F. rubra*-specific, repetitive clone FRH5, and *A. pratensis*-specific, repetitive sequence APE1.

Southern blot hybridizations and *in situ* hybridizations to metaphase chromosomes were performed. This analysis revealed: 1) a complex multiple band, ladder-pattern indicating a tandem arrangement and hybridization to 6 and 2 chromosomes for the red fescue sequences FRH2 and FRH5, respectively (Fig.

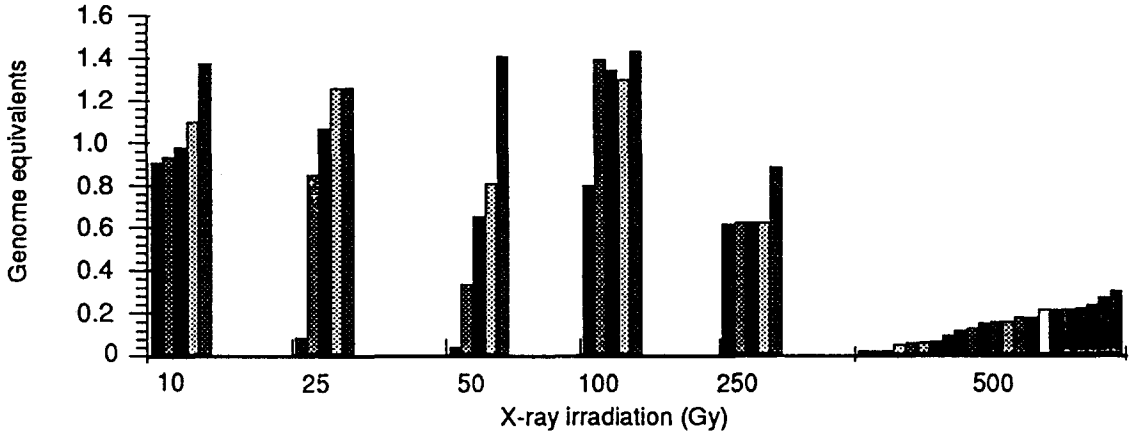

Fig. 4. Genome composition of asymmetric *F. arundinacea* (+) *L. multiflorum* somatic hybrids: Quantitative dot blots of asymmetric somatic *Festulolium* clones (bars correspond to independent regenerating calli and plants) obtained from different doses (10–500 Gy) of X-ray irradiation of donor protoplasts were hybridized with the *L. multiflorum*-specific, repetitive sequences LMH2 and LMB4. Genomic composition of independent clones based on average of data obtained from hybridization with both probes is shown as genome equivalents of *L. multiflorum* per genome equivalent of *F. arundinacea*.

2BC); 2) a similar banding pattern and hybridization only to 24 meadow foxtail chromosomes for the *A. pratensis*-specific sequence APE1 (Fig. 2B); 3) a partially-dispersed arrangement of the *L. multiflorum* sequences LMH2 and LMB4 and hybridization to all Italian ryegrass chromosomes (Fig. 2D); and 4) a similar banding pattern with some clustering and hybridization only to some tall fescue chromosomes for the *F. arundinacea*-specific sequence FAH1 (Fig. 2E).

Insert size and copy number of the highly species-specific sequences were estimated to range from 80–400 bp and from 2,000 to 100,000 copies per haploid genome, respectively (Fig. 3). Representative species-specific, repetitive DNA sequences were subjected to a sequence analysis: the Italian ryegrass clone LMH2 (173 bp) (Pérez-Vicente et al., 1992), the tall fescue clone FAH1 (102 bp) (Pérez-Vicente et al., 1992), the red fescue clone FRH5 and the meadow foxtail clone APE1 (Fig. 3).

Analysis of nuclear and organellar composition of symmetric and asymmetric somatic Festulolium *hybrids*

The nuclear composition of symmetric and asymmetric intergeneric *F. arundinacea* (+) *L. multiflorum* somatic hybrid plants, was characterized by chromosome counts and quantitative dot blot hybridizations using interdispersed repetitive DNA probes specific for Italian ryegrass (LMH2 and LMB4) and tall fescue (FAH1) (Spangenberg et al., 1994a; Takamizo & Spangenberg, 1994). Chromosome counts performed in representative somatic *Festulolium* plants recovered from symmetric and asymmetric protoplast fusions were 18 and 20 (slightly higher than the count 2n = 14 for Italian ryegrass), 37 and 39 (slightly lower or close to the count 2n = 42 for tall fescue), 49 (clearly higher than the count for the recipient tall fescue), 53 (close to the expected additive count of 2n = 56 for a symmetric hybrid) and one plant showing 90 chromosomes (Spangenberg et al., 1994a; Takamizo & Spangenberg, 1994). The suitability of the 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 (Pérez-Vicente et al., 1992). This analysis revealed the presence of Italian ryegrass repetitive DNA sequences in the nuclear genome of all primary regenerants from symmetric fusions and showed hybridization signals estimated to about one genome equivalent of *L. multiflorum* per haploid genome of *F. arundinacea*. Similarly, the analysis of the asymmetric somatic hybrid clones derived from fusion experiments using Italian ryegrass donor protoplasts irradiated with 10, 25, 50, 100 and 250 Gy and 500 Gy of X-rays, was performed. A concentration series of parental DNAs allowed a calibration plot of the radioactivity per dot in rela-

tion to the amount of DNA from one species and for mixtures representing different ratios of genome equivalents to be made. With the calibration plots for all three species-specific probes used, the amount of Italian ryegrass and tall fescue DNA per dot could be estimated, and the fraction of the nuclear DNA of the asymmetric somatic hybrids derived from Italian ryegrass could be determined (Spangenberg et al., 1994a). All analysed regenerating clones derived from 10–250 Gy-fusion products contained Italian ryegrass nuclear DNA but showed a large variation in the contribution of *L. multiflorum* DNA to their genomes (Fig. 4). These asymmetric somatic hybrid clones showed either no or limited donor genome elimination (being thus almost symmetric) or retained even < 5% of the *L. multiflorum* genome (being thus highly asymmetric). Some 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. 4). The analysed asymmetric somatic hybrid clones obtained from donor protoplasts irradiated with 500 Gy, revealed an extensive (> 85%) and similar Italian ryegrass nuclear genome elimination for both Italian ryegrass-specific probes tested. For some of these 500 Gy-asymmetric somatic hybrid clones analysed, no *L. multiflorum* DNA above background was detectable. Estimates from dot blots hybridized with the tall fescue-specific repetitive sequence FAH1 as probe, indicated the presence of approximately a complete chromosome set of the recipient *F. arundinacea* in all asymmetric and symmetric somatic hybrids analysed. The results obtained indicated: 1) the true nuclear hybrid nature of all green plants regenerated from symmetric fusions, 2) the tightness of the IOA metabolic inactivation of tall fescue protoplasts, 3) the value of species-specific repetitive DNA probes for the identification of somatic hybrids in intergeneric fusion combinations, 4) the suitability of these sequences for the analysis of donor genome elimination in asymmetric somatic hybrids, 5) irradiation of donor cells prior to fusion leading to the unidirectional species-specific elimination of Italian ryegrass chromosomes in asymmetric somatic hybrids, 6) no strict correlation between the level of the species-specific *Lolium* genome elimination and the radiation dose used, being apparent for a wide (25–250 Gy) range of X-ray doses tested.

The organellar composition of intergeneric *F. arundinacea* (+) *L. multiflorum* symmetric and asymmetric somatic hybrid clones was analysed by the generation of species-specific patterns obtained after hybridization of total DNA digests with six mtDNA (*cox1*, *cox2*, *cox3*, *atpA*, *atp6* and *atp9*) and one cpDNA (*rbcL*)-specific heterologous gene probes. Parental-like additive and novel (involving the absence of parental-like bands and/or the presence of non-parental bands) patterns, were observed depending on the hybridization probe and the somatic *Festulolium* clone considered. A general overview of the results obtained in the RFLP analysis performed for a set of *Festulolium* clones regenerated from symmetric and asymmetric protoplast fusions has been provided (Spangenberg et al., 1994a; Takamizo & Spangenberg, 1994). While additive patterns are preferentially observed in the mtDNA RFLP analysis of symmetric and < 50 Gy asymmetric clones, together with an extensive nuclear genome elimination of the donor, tall fescue patterns become predominant for the > 100 Gy asymmetric *Festulolium* clones (Spangenberg et al., 1994a; Takamizo & Spangenberg, 1994).

Discussion

Symmetric and asymmetric somatic fusions were performed for one intergeneric combination in the Gramineae involving unirradiated or X-ray-irradiated protoplasts of *L. multiflorum* (donor) and metabolically inactivated protoplasts of *F. arundinacea* (recipient). A series of symmetric and asymmetric somatic hybrid clones were obtained and plants were regenerated for each radiation dose category (0–500 Gy). Regeneration of mature somatic hybrid plants in the Gramineae other than rice, in which successful somatic hybridization and cybridization have been reported (Terada et al., 1987; Hayashi et al., 1988; Akagi et al., 1989; Kyozuka et al., 1989; Yang et al., 1989), is thus feasible. For rice, somatic hybridization in the intergeneric combination *O. sativa* (+) *Echinochloa oryzicola* has been attempted but the recovery of mature green plants failed (Terada et al., 1987). The same holds true for all intergeneric somatic hybrids so far reported in the Gramineae, e.g. *Triticum monococcum* (+) *Pennisetum americanum* (Vasil et al., 1988). Thus, the greenhouse-grown, flowering *Festulolium* somatic hybrid plants described represent the first case of mature plant regeneration for intergeneric symmetric and asymmetric somatic hybridization in the Gramineae (Takamizo et al., 1991; Spangenberg et al., 1994a; Takamizo & Spangenberg, 1994).

Repetitive species-specific sequences were isolated from forage grass species: *F. arundinacea* and *L. multiflorum* (Pérez-Vicente et al. 1992) and from *F. rubra* and *A. pratensis*. They were characterized at the molecular and cytological level. Some were found to be dispersed and represented in all or most chromosomes of the corresponding species, proving suitable for the analysis of the nuclear composition of *Festulolium* plants obtained from wide crosses (Pérez-Vicente et al., 1992) and from somatic hybridization (Takamizo et al., 1991; Spangenberg et al., 1994a).

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* (e.g. Yamashita et al., 1989) and intergeneric *Nicotiana-Atropa* (e.g. Gleba et al., 1988) combinations. In the case of asymmetric somatic *Festulolium* we found correlative evidence between the number of excess chromosomes, presence or absence of *in situ* hybridization signals on metaphase chromosomes and the estimates from corresponding dot blot values for individual hybrids (Spangenberg et al., 1994a). Furthermore, out 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 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 reported for intertribal, asymmetric somatic hybrids between *N. plumbaginifolia* and *Atropa belladonna* obtained by 'gamma-fusion' (Gleba et al., 1988).

Approaches aimed at evaluating the potential contribution of somatic hybridization and cybridization supporting conventional breeding programmes in ryegrasses and fescues are now conceivable. Asymmetric protoplast fusion aimed at intraspecific transfer of cytoplasmic male sterility (CMS) has been described for *L. perenne*, but since non-morphogenic cell suspensions were used, only cybrid calli were recovered (Creemers-Molenaar et al., 1992). Recently, we have established an efficient plant regeneration system from protoplasts isolated from single genotype-derived embryogenic cell suspensions in *L. multiflorum, L. perenne* and *L.* × *boucheanum* (Wang et al., 1993b). These protoplast-to-mature plant regeneration systems opened up opportunities for the use of *Lolium* protoplasts as recipients in asymmetric somatic hybridization and cybridization experiments. Somatic cybridizations aimed at transferrring organellar-coded traits using morphogenic ryegrass protoplasts as recipients and X-ray-irradiated protoplasts from established non-morphogenic suspension cultures of CMS lines in *L. multiflorum* and *L. perenne*, are now in progress. In addition, a programme to generate fertile asymmetric somatic hybrids combining quality traits from perennial ryegrass and persistence/resistance traits of red fescue and meadow foxtail has been initiated. A reproducible protoplast-to-plant regeneration system is now available for *F. rubra* (Spangenberg et al., 1994b) and first regenerating asymmetric somatic hybrid clones in *F. rubra* (+) *L. perenne* have been recovered.

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