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Analysis of genetic stability of plants regenerated from suspension cultures and protoplasts of meadow fescue (*Festuca pratensis* Huds.)

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ABSTRACT. A cytological and molecular analysis was performed to assess the genetic uniformity and true-to-type character of plants regenerated from 20 week-old embryogenic suspension cultures of meadow fescue (*Festuca pratensis* Huds.), and compared to protoplast-derived plants obtained from the same cell suspension. Cytological variation was not observed in a representative sample of plants regenerated directly from the embryogenic suspensions and from protoplasts isolated therefrom. Similarly, no restriction fragment length polymorphisms (RFLPs) were detected in the mitochondrial, plastid and nuclear genomes in the plants analyzed. Randomly amplified polymorphic DNA markers (RAPDs) have been used to characterise molecularly a set of mature meadow fescue plants regenerated from these *in vitro* cultures. RAPD markers using 18 different short oligonucleotide primers of arbitrary nucleotide sequence in combination with polymerase chain reaction (PCR) allowed the detection of pre-existing polymorphisms in the donor genotypes, but failed to reveal newly generated variation in the protoplast-derived plants compared to their equivalent suspension-culture regenerated materials.
The genetic stability of meadow fescue plants regenerated from suspension cultures and protoplasts isolated therefrom and its implications on gene transfer technology for this species are discussed.

Abbreviations: PCR: polymerase chain reaction; RAPD: random amplified polymorphic DNA; RFLP: restriction fragment length polymorphism.

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Key words: *Festuca pratensis* - suspension cultures - protoplasts - plant regeneration - somaclonal variation - genetic fidelity

INTRODUCTION

Meadow fescue (*Festuca pratensis* Huds.) is a high-yielding grass with good digestibility and is one of the most important components of permanent grasslands and leys in Northern latitudes (Hulten 1971; Aastveit and Aastveit 1989). Regeneration of mature, greenhouse-grown plants from protoplasts and suspension cultures of meadow fescue has been recently achieved (Wang et al., see accompanying paper), thus opening up possibilities for genetic transformation of this species.
The presence of genetic variation, induced during tissue culture, in plants regenerated from *in vitro* cultures is a matter deserving special consideration in the context of genetic transformation. The recovery of forage grass plants which are phenotypically and cytologically similar to the donor plants has been reported from embryogenic tissue cultures of *Pennisetum americanum* (Vasil and Vasil 1981; Swedlund and Vasil 1985) and *P. purpureum* (Haydu and Vasil 1981; Chandler and Vasil 1984). Recently, evidence for the genetic uniformity of plants derived from embryogenic callus cultures of *P. purpureum*, based on an analysis using biochemical (isozyme) and RFLP markers, has been provided (Shenoy and Vasil 1992).
On the other hand, regenerants originating from cell suspensions of *Festuca arundinacea* (Humphreys and Dalton 1991) and of one pentaploid *Festulolium* hybrid (Humphreys and Dalton 1992) have been shown to be unstable at one locus and to show cytological changes.

However, no similar information is available for plants regenerated from protoplasts isolated from embryogenic cultures of any forage grass using molecular markers known to allow the detection of genomic variability in different cultivars and genotypes of the corresponding species.
Here we describe the determination of the genetic stability of plants regenerated from suspension cultures and protoplasts of *Festuca pratensis* screened by RFLP and RAPD markers.

MATERIALS AND METHODS

Plant material

Plants were regenerated from 20 week-old suspension cultures established from a single genotype of *Festuca pratensis* cv. Barmondo. Plants regenerated from protoplasts isolated from the same suspension cultures of meadow fescue cv. Barmondo are described in Wang et al. (accompanying paper).

DNA isolation, gel electrophoresis and hybridization experiments

Total genomic cellular DNA was isolated from freeze-dried cell suspensions and leaf material from sterile shoot cultures and greenhouse-grown plants. Isolation and digestion of genomic DNA was performed according to Lichtenstein and Draper (1985). Restriction enzyme analysis, gel electrophoresis, DNA blotting and Southern hybridizations were carried out following standard protocols (Sambrook et al. 1989). Hybridization probes were [^{32}P]dATP-labeled by random priming (Feinberg and Vogelstein 1983). The following plant mtDNA gene-specific probes were used: *cox1* (3.94 kb BamHI-EcoRI fragment from pBN6601); *cox2* (2.4 kb EcoRI fragment from pZMEl); and *atpA* (4.8 kb HindIII fragment from atpA-copyV), all kindly provided by Prof. C.J. Leaver. A chloroplast DNA gene-specific clone from spinach ribulose-1,5-biphosphate carboxylase large subunit (*rbcL*, BamHI fragment from pWHsp403) was kindly provided by Prof. R. Herrmann. A nuclear repetitive DNA sequence (pFAH1) cloned from *F. arundinacea*, which is present in the genome of *F. pratensis* (Pérez-Vicente et al. 1992), was also used as hybridization probe. Corresponding plasmid inserts, PCR amplified fragments excised from agarose gels and partially digested total genomic DNA were used for labeling.

RAPD analysis

Eighteen commercial 10-mer primers (kit U) from Operon Technologies, Alameda, California, USA were used for PCR amplification. Amplification reactions were performed in 15 µl volumes containing: 1x reaction buffer (100µg/ml gelatine, 10 mM Tris-HCl, pH 8.5, 50 mM KCl, 2 mM $MgCl_2$), 100 µM dATP, dGTP, dTTP, dCTP, 0.2 µM primer, 0.6 units Taq polymerase (Boehringer Mannheim,5 U/µl) and 5-10 ng of genomic DNA. The PCR conditions were essentially as described by Williams et al. (1990). The following conditions were provided by a Perkin Elmer Cetus thermocycler: 20 cycles using denaturation temperature (94°C; 1 min), annealing temperature (36°C; 1 min) and extension temperature (72°C; 2 min), followed by further 20 cycles using denaturation temperature (94°C; 1 min), annealing temperature (36°C; 1 min) and extension temperature (72°C; 3 min). PCR amplification products were analyzed by electrophoresis in 1.5% agarose/ethidium bromide gels.

Chromosome counts

Chromosome counts were based on evaluation of at least three well spread metaphase plates prepared from meristems following procedures described earlier (Pérez-Vicente et al. 1992).

RESULTS AND DISCUSSION

A total number of 82 *in vitro* plantlets were obtained from 20 week-old embryogenic suspension cultures established from a single genotype of meadow fescue cv. Barmondo. More than 40 independent plantlets were regenerated from protoplasts isolated from the same 20 week-old suspension cultures. On average 10% of these *in vitro* regenerants were albino; some of the regenerating cultures were chimeric (green/albino) (Fig. 1A). A representative sample of these plants regenerated from embryogenic suspension cultures and protoplasts was grown under greenhouse conditions. They did not show any gross morphological variation when evaluated at maturity. Chromosome counts performed for nine independent plants regenerated from cell suspensions and seven independent plants from protoplasts isolated from the same suspension cultures revealed in all cases the expected number 2n = 14 (Fig. 1B).
Some variant cells are present in embryogenic tissue and cell cultures of *Gramineae*, but plants regenerated from such cultures, through somatic embryogenesis, have been shown to be in most cases phenotypically and cytologically uniform (Swedlund and Vasil 1985). The regeneration of albino plantlets on the one hand, and of phenotypically uniform mature green plants from suspension and protoplast cultures in meadow fescue on the other, supports this view.

Analysis of genetic uniformity of plants screened by RFLP markers

In order to determine if the plants regenerated from protoplasts show newly induced genetic variation compared to plants regenerated directly

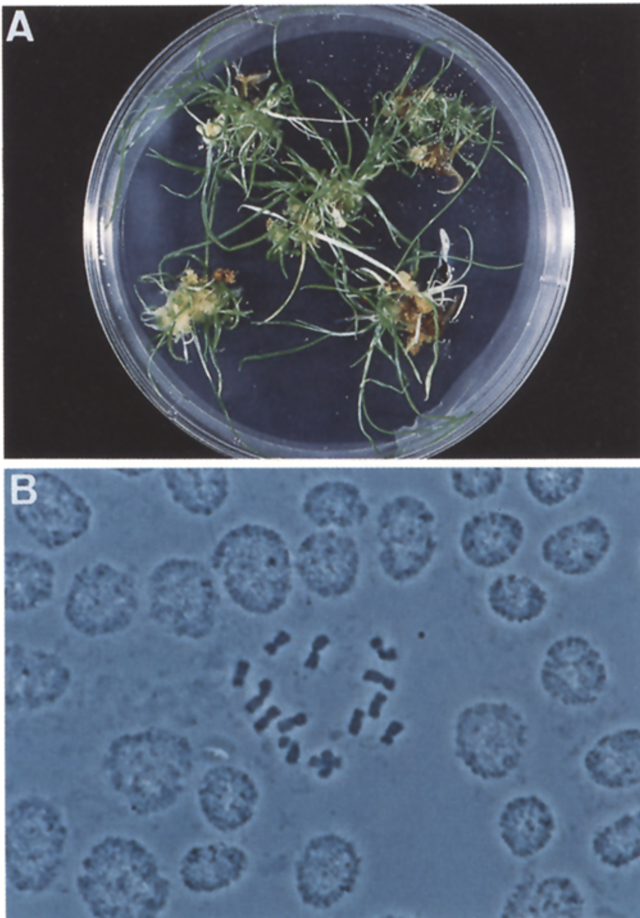


Figure 1: Morphological and cytological evaluation of plants regenerated from cell suspensions and protoplasts of *Festuca pratensis*.
A) *In vitro* regenerating green and albino plantlets from suspension cultures of meadow fescue cv. Barmondo; B) Metaphase in a meristem cell prepared from one representative plant regenerated from protoplasts isolated from the same cell suspension used for A).

from the suspensions used for protoplast isolation, a screen by RFLP markers was performed. Analysis involving three mitochondrial gene specific probes (Fig. 2A, B), one chloroplast gene specific probe (Fig. 2C) and one nuclear repetitive DNA sequence isolated from *F. arundinacea* (data not shown) did not reveal any variation in either sets of plants. The same hybridization probes, however, failed to detect polymorphisms among different genotypes within one cultivar and even among different cultivars of *F. pratensis*, e.g. for *cox2* (Fig. 2A), although they did reveal RFLPs in different *Festuca* species, e.g. between *F. pratensis* and *F. rubra* for *cox2* (Fig. 2A) or between *F. pratensis*, *F. arundinacea* and *F. rubra* for *atpA* (Fig. 2B).
A similar analysis of the genetic fidelity of a population of napier grass plants regenerated from embryogenic callus cultures also failed to reveal variation among them (Shenoy and Vasil 1992). These results supported the view that there is a direct selection *in vitro* towards genetically normal cells in the formation of somatic embryos, and that plants obtained from such somatic embryos are largely stable. However, that analysis did not indicate whether the biochemical and molecular markers used were at all suitable for detecting (e.g. pre-existing) genetic variability in *P. purpureum*.
Genetic variation has been observed in an analysis of plants regenerating from cell suspensions of *F. arundinacea* (Humphreys and Dalton 1991) and from a pentaploid *Lolium multiflorum* x *F. arundinacea* hybrid (Humphreys and Dalton 1992).

Analysis of genetic uniformity of plants screened by RAPD markers

The recent development of randomly amplified polymorphic DNA markers for genetic mapping and fingerprinting (Williams et al. 1990), provides a possible method for assessing the genetic uniformity of plants regenerated from suspension and protoplast cultures. Since the procedure depends on the differential PCR amplification of DNA sequences using arbitrary oligonucleotide primers, polymorphisms resulting from for example, deletions or insertions in the amplified regions or base changes altering primer binding sites etc., will be revealed (Baird et al. 1992). These are mutational events which have been proposed to be involved in creating heritable variation in plant tissue cultures (Evans 1989).
An analysis based on RAPD markers using 18 different primers allowed discrimination between different *Festuca* species, and even different genotypes and cell suspensions within one particular cultivar of meadow fescue (Fig. 3A, lanes 1 and Fig. 3B, lanes 1 and 3). Different patterns of PCR amplification products were obtained for five independent suspension cultures initiated from different genotypes of the cv. "Barmondo" in the case of 4 primers out of 18 tested (Fig. 3B, lanes 3). This suggests a utility of RAPD technology for revealing pre-existing genetic variation among single-seed derived suspension cultures obtained from a highly heterozygous out-crossing species. However, while analyzing independent plants regenerated from a single-genotype-derived cell suspension, no variation among the regenerants was revealed (Fig. 3A, B, lanes 2). The same holds true for the

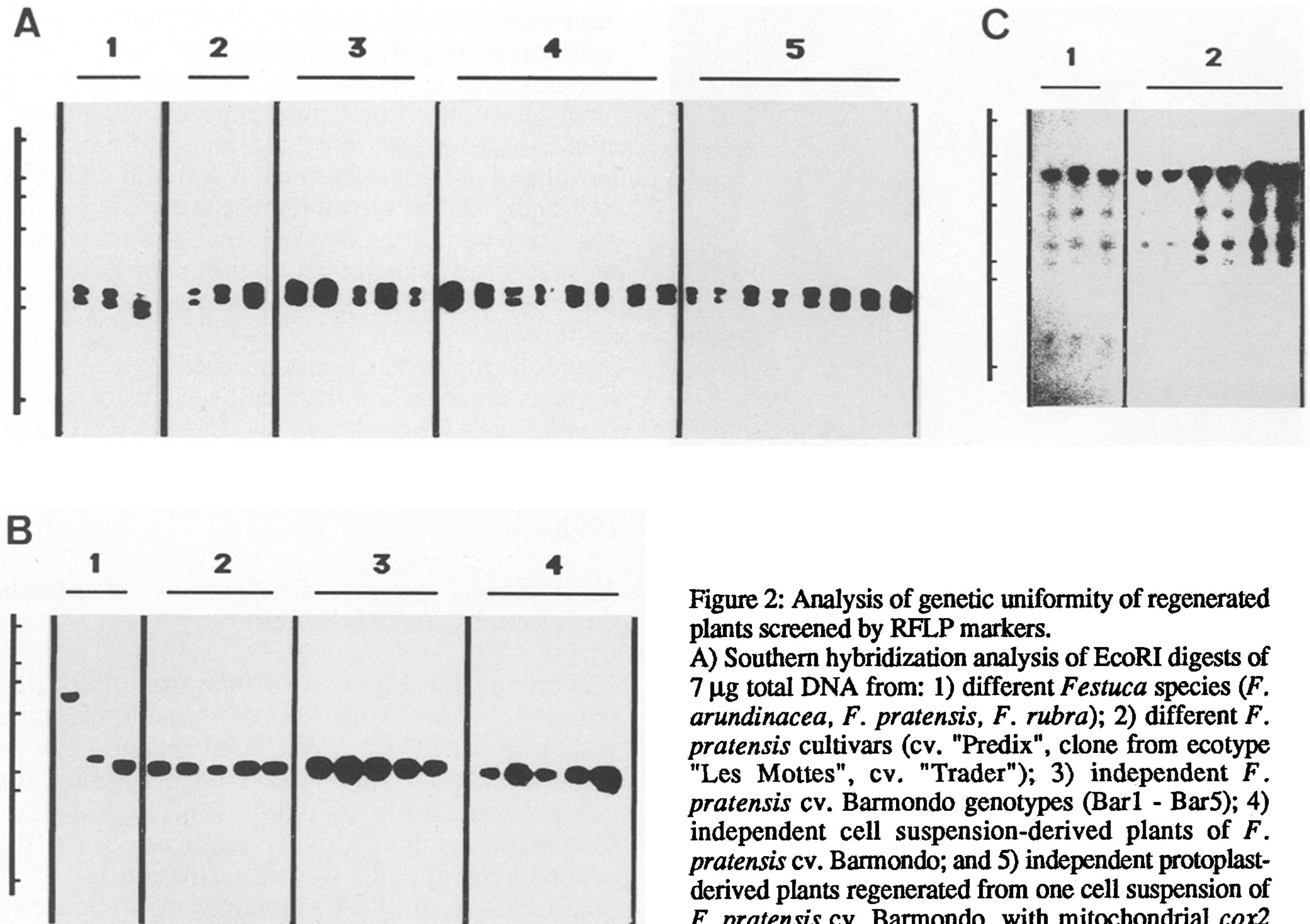
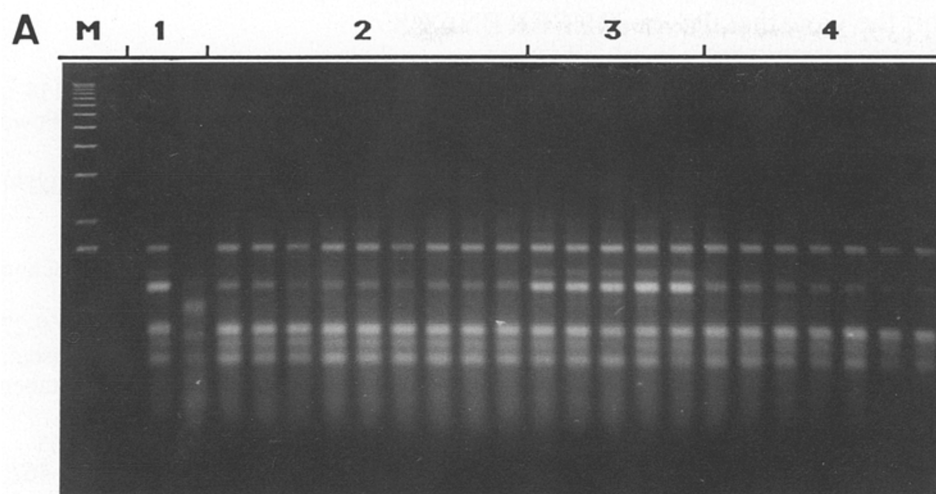


Figure 2: Analysis of genetic uniformity of regenerated plants screened by RFLP markers.
A) Southern hybridization analysis of EcoRI digests of 7 µg total DNA from: 1) different *Festuca* species (*F. arundinacea, F. pratensis, F. rubra*); 2) different *F. pratensis* cultivars (cv. "Predix", clone from ecotype "Les Mottes", cv. "Trader"); 3) independent *F. pratensis* cv. Barmondo genotypes (Bar1 - Bar5); 4) independent cell suspension-derived plants of *F. pratensis* cv. Barmondo; and 5) independent protoplast-derived plants regenerated from one cell suspension of *F. pratensis* cv. Barmondo, with mitochondrial *cox2* gene specific probe.
B) Southern hybridization analysis of EcoRI digests of 7 µg total DNA from: 1) different *Festuca* species (*F. rubra, F. arundinacea, F. pratensis*); 2) independent *F. pratensis* cv. Barmondo genotypes (Bar1 - Bar5); 3) independent cell suspension-derived plants of *F. pratensis* cv. Barmondo; and 4) independent protoplast-derived plants regenerated from one cell suspension of *F. pratensis* cv. Barmondo, with mitochondrial *atpA* gene specific probe.
C) Southern hybridization analysis of EcoRI digests of 7 µg total DNA from: 1) different *F. pratensis* cultivars (cv. "Predix", clone from ecotype "Les Mottes", cv. "Trader"); 2) independent protoplast-derived plants regenerated from one cell suspension of *F. pratensis* cv. Barmondo, with chloroplast *rbcL* gene specific probe. (HindIII digested λ-DNA was used as size marker)

analysis of independent protoplast-derived plants regenerated from the same suspension culture (Fig. 3A, B, lanes 4), irrespective of the primer used.To confirm the nature of the PCR amplification products and to estimate the copy number of the amplified target DNA sequences, labeled total DNA isolated from one suspension culture of meadow fescue cv. Barmondo was used to probe a Southern blot of amplification products from different PCR reactions, corresponding to independent plants regenerated from cell suspensions. A hybridization pattern equivalent to that from the ethidium bromide/agarose gel was obtained (Fig. 3C), and thus is indicative of the true nature of the PCR bands observed and the equivalent stoichiometry in the meadow fescue genome of sequences corresponding to the PCR amplification products. To further confirm the specificity of the PCR amplification, a representative and relatively minor common amplification product was excised from the agarose, labeled, and used to probe a Southern blot of digested total DNA from different cultivars, different genotypes, independent regenerants from cell suspensions and protoplasts of meadow fescue. Here again, identical hybridization patterns were obtained (Fig. 3D).



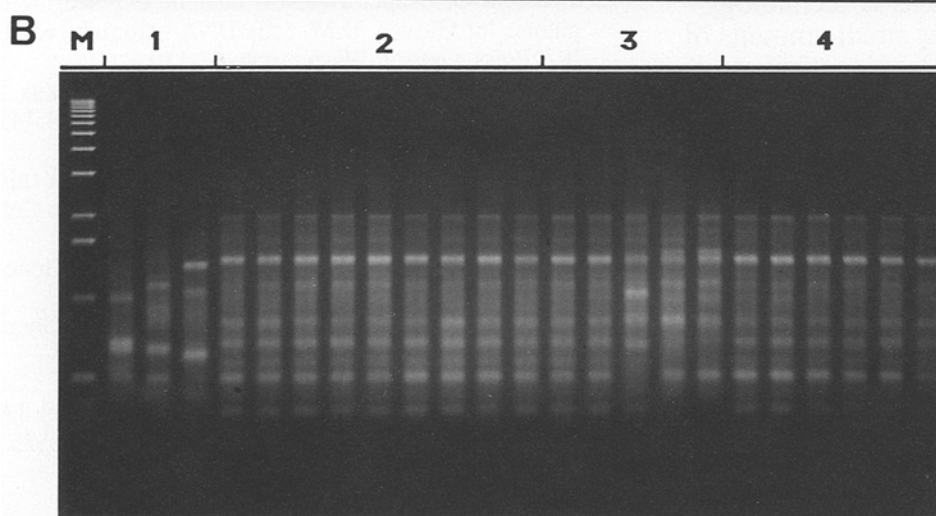
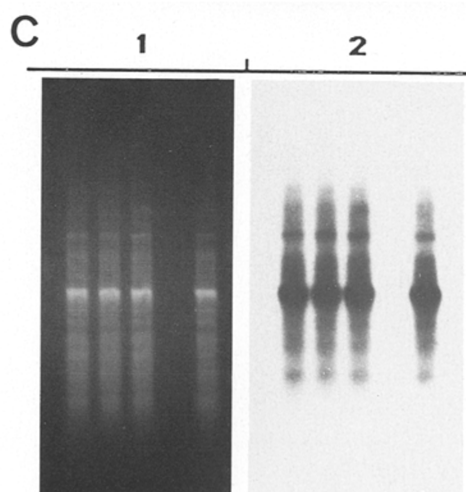
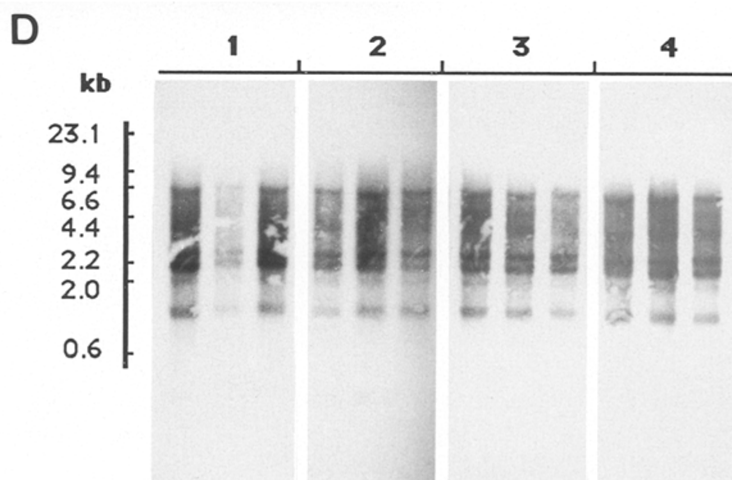


Figure 3: Analysis of genetic uniformity of regenerated plants screened by RAPD markers.

A) Screening by RAPDs generated with primer OPU-8: 1) different *Festuca* species (*F. pratensis, F. arundinacea*); 2) independent cell suspension-derived plants of a single genotype of *F. pratensis* cv. Barmondo; 3) independent *F. pratensis* cv. Barmondo genotypes (Bar1 - Bar5) and 4) independent protoplast-derived plants regenerated from one cell suspension of *F. pratensis* cv. Barmondo.

B) Screening by RAPDs generated with primer OPU-10: 1) different *Festuca* species (*F. rubra, F. pratensis, F. arundinacea*); and 2), 3), 4) as in A).





C) Southern hybridization analysis of RAPDs: 1) Amplification products of independent PCR reactions using primer OPU-3 for different cell suspension-derived plants of *F. pratensis* cv. Barmondo of a single genotype; 2) Southern blot from gel of 1) hybridized with labelled total DNA from a cell suspension of *F. pratensis* cv. Barmondo.

D) Southern hybridization analysis of EcoRI digests of 7 μg total DNA from: 1) different *F. pratensis* cultivars (cv. "Predix", clone from ecotype "Les Mottes", cv. "Trader"); 2) independent *F. pratensis* cv. Barmondo genotypes (Bar3 - Bar5); 3) independent cell suspension-derived plants of a single genotype of *F. pratensis* cv. Barmondo and 4) independent protoplast-derived plants regenerated from one cell suspension of *F. pratensis* cv. Barmondo, hybridized with a common PCR amplified fragment (ca. 1.8 kb) with primer OPU-3 excised from the gel.

In summary, our results support the view that the mature plants of *F. pratensis* regenerated from protoplasts isolated from embryogenic suspension cultures do not show newly generated variation - compared with the corresponding suspension-derived plants - at any of the sequences screened by DNA markers and are largely genetically stable and true-to-type.
Therefore, in the context of production of transgenic plants of meadow fescue, no significant difference regarding tissue culture induced genetic variation would seem to be expected between particle bombardment of embryogenic suspension cultures and direct gene transfer to protoplasts.
In addition, the potential of RAPD technology - which is technically easy, uses small amounts of DNA and does not require radioactivity - is illustrated in the context of the evaluation of somaclonal variation.

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