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Identification of parental and recombined chromosomes in hybrid derivatives of *Lolium multiflorum* × *Festuca pratensis* by genomic in situ hybridization

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Abstract Genomic in situ hybridization (GISH) was used to identify *Festuca* chromatin in mitotic chromosomes of *Lolium multiflorum* (*Lm*)×*Festuca pratensis* (*Fp*) hybrids and hybrid derivatives. In two inverse autoallotriploids *LmLmFp* and *LmFpFp*, *in situ* hybridization was able to discriminate between the *Lolium* and *Festuca* chromosomes. In a third triploid hybrid produced by crossing an amphiploid of *L. multiflorum*×*F. pratensis* (2n=4x=28) with *L. multiflorum* (2n=2x=14), the technique identified chromosomes with interspecific recombination. Also, in an introgressed line of *L. multiflorum* which was homozygous for the recessive *sid* (senescence induced degradation) allele from *F. pratensis*, a pair of chromosome segments carrying the *sid* gene could be identified, indicating the suitability of GISH in showing the presence and location of introgressed genes. By screening backcross progeny for the presence of critical alien segments and the absence of other segments the reconstitution of the genome of the recipient species can be accelerated.

Key words *Lolium* · *Festuca* · Genomic in situ hybridization · Gene introgression

Introduction

The *Lolium/Festuca* complex of grass species includes an array of forms with distinct complementary agronomic characters (Thomas and Humphreys 1991). The possibility of combining these characters in a single genotype has been the incentive to undertake extensive studies of synthetic amphiploids containing the complete sets of chromosomes of two species within the complex (Lewis 1980). However, the production of commercial varieties of grasses based on synthetic amphiploids has been limited by irregularities in meiotic behaviour leading to genetic instability in later generations (Thomas and Humphreys 1991); the only successful example is the tetraploid hybrid *L. multiflorum*×*L. perenne* (Breese and Lewis 1984). Nevertheless, the extent of variation within the complex continues to merit attention when considering the production of more versatile cultivars of forage grasses to meet the future needs of grassland farming.

More recently, greater emphasis has been placed on the possible introgression of *Festuca* genes into diploid *Lolium* species (2n=14) through the development of appropriate crossing procedures. Pentaploid hybrids between autotetraploid *Lolium* and the hexaploid species *F. gigantea* (2n=6x=42) and *F. arundinacea* (2n=6x=42) have been shown to produce some lines with sufficient pollen fertility to be used as the male parent in backcrosses to diploid *Lolium* (Morgan et al. 1988; Humphreys 1989). Backcrossing the resulting BC_1 hybrids to diploid *Lolium* yielded more than 80% diploid progeny. The crossing scheme used accelerates the recovery of the diploid chromosome number because of the selective advantage of gametes with the haploid *Lolium* complement and the least number of *Festuca* chromosomes. However, Humphreys (1989) unequivocally demonstrated that a small proportion of the diploid BC_2 progeny includes *Festuca* genetic markers as a result of interspecific recombination. This crossing scheme provides a rapid method for the reconstitution of the recipient *Lolium* genotype and the controlled introgression of *Festuca* genes into *Lolium*. In an extensive introgression programme a range of morphological forms that fall outside the variability of the recipient *Lolium* parent has been isolated. These plants probably represent the products of interspecific recombination, and some have valuable agronomic characters such as drought resistance (Humphreys and Thomas 1993).

The development of appropriate techniques of in situ hybridization provides the means to identify segments of alien chromosomes introduced into the recipient species. The effective use of fluorescence in situ hybridization using total genomic DNA (genomic in situ hybridization,

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GISH) to identify segments of introgressed alien chromosomes in wheat has been demonstrated by Schwarzacher et al. (1992). In this report we describe the results of using this technique to discriminate between chromosomes in hybrids and derivatives in the *Lolium-Festuca* complex and assess its usefulness in detecting introgressed segments of alien chromosomes.

Materials and methods

Plant material

Triploid hybrids between *L. multiflorum* (*Lm*) and *F. pratensis* (*Fp*) were produced by crossing a synthetic autotetraploid cytotype (2n=4x=28) of one species with the diploid (2n=2x=14) of the other, giving the two inverse autoallotriploid types (2n=3x=21) *LmLmFp* and *LmFpFp*. A further triploid hybrid was produced by crossing diploid *L. multiflorum* with an established amphiploid line produced initially by doubling the chromosome number of the F_1 diploid hybrid *L. multiflorum* × *F. pratensis*. The amphiploid line had undergone a number of generations of seed multiplication as the cultivar 'Elmet' before being used to form the triploid. This triploid will be referred to as *Lm*(*LmFp Elmet*) to differentiate it from the other triploid hybrids.

A line of *L. multiflorum* homozygous for a recessive allele for delayed senescence (*sid* - senescence induced degradation) transferred from *F. pratensis* was also studied to test whether the segment of chromosome carrying this specific locus could be identified. The homozygous line had been isolated from the progeny of interpollinated BC_1 plants from the following cross:

L. multiflorum (4x) YYYY × *F. pratensis* (2x) yy
↓
L. multiflorum (2x) YY × *LmLmFp* (3x) YYy
↓
BC_1 (2x) Yy and YY interpollinated

Preparation of cells

Roots were produced from detached vegetative tillers on an aerated culture tank (Morgan 1976). Excised root tips were placed in ice cold water for 16 h and then fixed for a minimum of 1 h and a maximum of 24 h in ethanol:acetic acid (3:1). After fixation, the root tips were washed in citric acid-sodium citrate buffer (pH 4.8) and treated with an enzyme solution of 2% cellulase, 20% pectinase for 15 min at 37 °C. Treated root tips were again rinsed in citrate buffer, transferred to 45% acetic acid for not more than 15 min, and squashed in 45% acetic acid on acid cleaned slides. Slide preparations were examined under a phase contrast microscope, and those with acceptable numbers of somatic metaphase cells were frozen in liquid nitrogen and the cover glass removed. Preparations were stored at –20°C until required.

Preparation of the probe and in situ hybridization

Total genomic DNA was extracted from young actively growing leaves of *F. pratensis* and *L. multiflorum* according to the method of Dellaporta et al. (1983). The genomic probes were prepared by mechanically shearing DNA of *F. pratensis* to approximately 5 kb by repeated passing through a hypodermic needle and then labelling it by nick translation with either biotin-14-dATP (Life Technologies), digoxigenin-11-dUTP (Boehringer Mannheim) or rhodamine-4-dUTP (Amersham). Blocking DNA was prepared by autoclaving the DNA of *L. multiflorum* for 2 min which fragmented it into approximately 200 bp.

The protocol followed for in situ hybridization and detection of the digoxigenin labelled probe was essentially that of Leitch et al. (1991). Forty microliters of hybridization mixture containing 100 ng of *Festuca* DNA probe and 4 μg of blocking *Lolium* DNA was applied to each slide. The method of detection of sites of hybridization of the biotin labelled probe is described by Leggett et al. (1993). Both the digoxigenin and biotin hybridization sites were visualized with the appropriate fluorescein conjugates. The rhodamine-4-dUTP-labelled probe requires no detection and can be examined directly.

All preparations were counterstained with DAPI, and the fluorescein preparations of the *LmLmFp* triploid were also stained with propidium iodide. The preparations were finally mounted in Vectashield antifade, examined using a Leitz Laborlux fluorescence microscope and photographed on Kodak Ektachrome 400HC slide film.

Results

Genomic in situ hybridization in triploid hybrids

The triploid hybrid *LmLmFp* combines the diploid genome of *L. multiflorum* and the haploid genome of *F. pratensis*. Somatic chromosomes of the *LmLmFp* hybrid were probed with DNA of *F. pratensis* labelled with biotin (Fig. 1a). The haploid complement of *F. pratensis* fluoresces yellow with fluorescein, while the *L. multiflorum* chromosomes show little sign of probe hybridization and appear red as a result of the propidium iodide counterstain.

Digoxigenin labelled *Festuca* DNA was hybridized onto the chromosomes of the inverse triploid hybrid *LmFpFp*. As expected the 14 chromosomes of *F. pratensis* fluoresce yellow, with the 7 *L. multiflorum* chromosomes not being fluorescent (Fig. 1b). The results from these inverse triploid hybrids clearly show the usefulness of *in situ* hybridization in discriminating between the chromosomes of the two species.

The triploid hybrid *Lm*(*LmFp Elmet*) theoretically has two genomes of *L. multiflorum* and a single genome of *F. pratensis*. However, as chromosome pairing in the amphiploid is not completely confined to homologous pairs (Lewis 1980), some interspecific recombination would be expected to occur. There was, therefore, a chance that only the genome from the diploid *L. multiflorum* parent would be intact because the two genomes derived from 'Elmet' could have changed through recombination during the generations of seed multiplication. For this hybrid, *F. pratensis* DNA labelled with rhodamine was used as the probe, and the *Festuca* chromatin is, therefore, coloured red. In some cells, the *Lolium* chromosomes were hardly discernible, and comparison with the same preparation stained with DAPI was required to locate these chromosomes (Fig. 1c, d). The cell shown in Fig. 1d illustrates that considerable interspecific recombination has occurred. Only 7 of the *Lolium* chromosomes do not show any hybridization, and these have been derived from the diploid *L. multiflorum* parent. The other seven *Lolium* chromosomes that have come from the amphiploid have segments that fluoresce and are clearly seen to be recombined chromosomes. The size of the *Festuca* segment is variable. There are also 7 brightly fluorescing *Festuca* chromosomes, and 6 of these have segments of chromosome that are nonfluorescent and represent *Festuca/Lolium* exchanges. The segments of *Lolium* onto *Festuca* chromosomes are also of variable length, and there are narrow interstitial segments

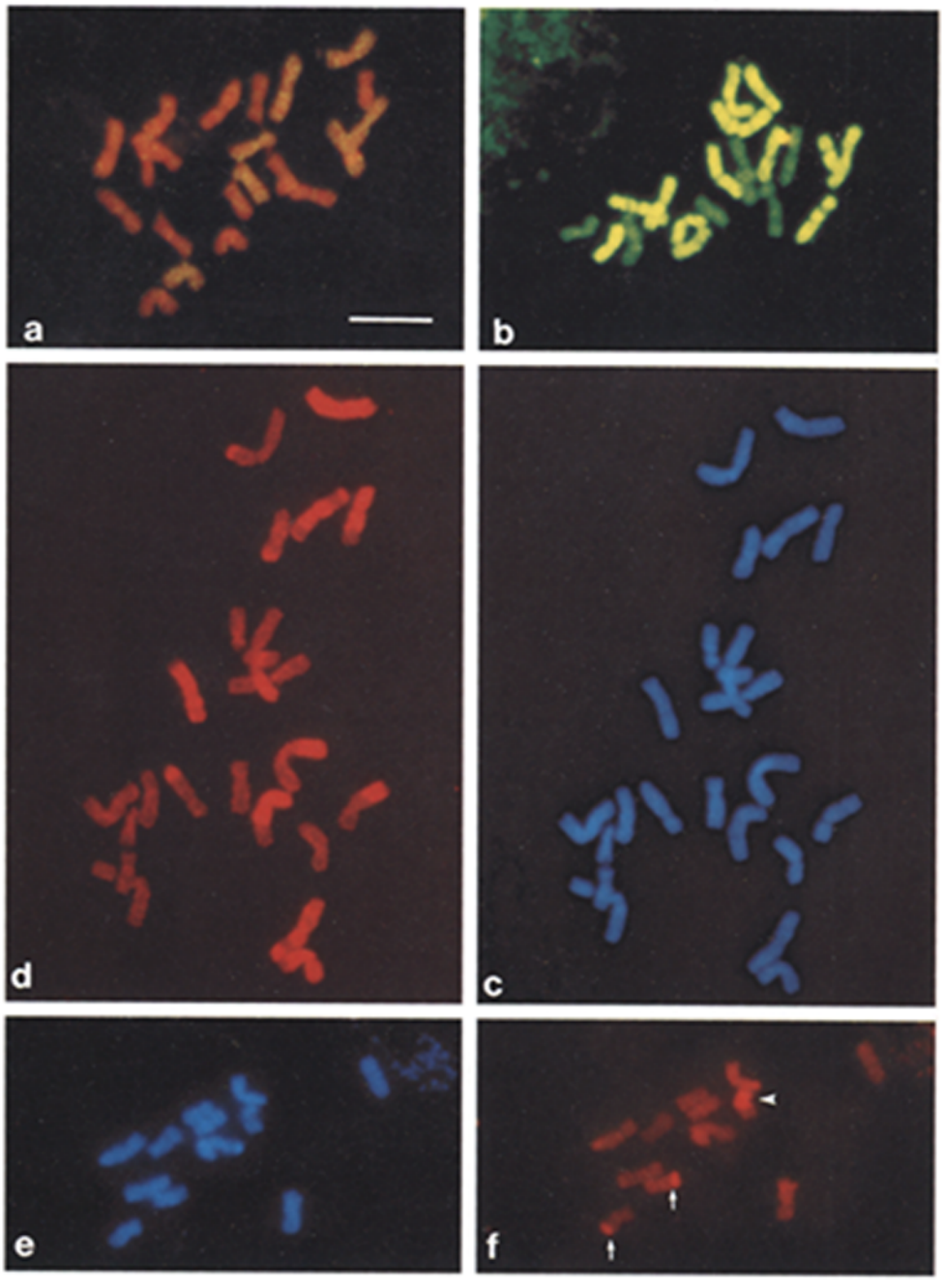

Fig. 1a–f Fluorescence in situ hybridization of root-tip chromosome preparations using total genomic *Festuca* DNA as probe labelled with **a** biotin and **b** digoxigenin each detected with fluorescein, and **d, f** labelled with rhodamine. **a** *LmLmFp*, the 7 *Festuca* chromosomes show hybridization (*yellow*), the *Lolium* chromosomes are counterstained *red* with propidium iodide. **b** *LmFpFp*, the 14 *Festuca* chromosomes are hybridized (*bright yellow*); the *Lolium* chromosomes are *dull*. **c** *Lm* (*LmFp Elmet*) stained with DAPI. **d** The same cell as **c** showing sites of hybridization to *Festuca* chromosomes and chromosome segments (*bright red*); the *Lolium* chromosomes and chromosome segments remain *dull*. **e** An incomplete cell of the *L. multiflorum* introgression line *sid.-sid* stained with DAPI. **f** The same cell as **e** segments of *Festuca* chromosomes show hybridization in the distal parts of the long arms of one pair of chromosomes (*arrows*) that carry the *sid* gene and in the centromeric region of another chromosome (*arrowhead*). Bar: 10 μm

that would have resulted from multiple exchanges, probably spread over a number of generations. The presence of these exchanges clearly demonstrates the level of interspecific recombination that has occurred between the chromosomes of *L. multiflorum* and *F. pratensis* in the generations required to produce a seed stock of the amphiploid derived cultivar 'Elmet'. The detection of narrow segments of *Festuca* DNA in the *Lolium* chromosomes and *Lolium* DNA in the *Festuca* chromosomes shows that the technique is capable of identifying small introgressed segments.

The identification of specific introgressed segments of chromosomes

The recessive allele at the *sid* locus (Thomas 1987) first described by Lewis (1972), which controls delayed senescence, was transferred from a genotype of *F. pratensis* into *L. multiflorum*. As the *sid* gene is recessive, there must be 2 *Lolium* chromosomes with segments of *Festuca* present at the same location in the homozygous progeny *sid.sid*. As can be seen in Fig. 1f, in the genotype studied there are

3 chromosomes with brightly fluorescent segments: 2 of these are clearly a pair of chromosomes, each with a distal segment of *Festuca* chromatin in the long arm, while the third labelled chromosome segment includes the centromere and is present once only. The *sid* gene, therefore, must be in the distal introgressed segments. The location of this segment has been confirmed in another diploid genotype of *L. multiflorum* and by the presence of 4 such chromosomes in a tetraploid nulliplex genotype.

Discussion

GISH has been clearly demonstrated to be a suitable technique for differentiating chromosomes of species within the *Lolium/Festuca* complex. Although studies of genome homology based on chromosome pairing have shown that the genomes of the two diploid species *L. multiflorum* and *F. pratensis* are closely related (Jauhar 1975), the chromosomes of the two species could be readily differentiated through GISH. Use was made of unlabelled DNA of *L. multiflorum* at a ratio of 40:1 of the labelled *F. pratensis* genome probe to block any cross hybridization, thereby enhancing the discriminating power of the technique. The results from the reciprocal triploid hybrids with their different doses of parental genomes illustrate the repeatability and specificity of the technique within this complex of forage grasses. The ability to differentiate genomes through the use of in situ hybridization could prove invaluable in assessing phylogenetic relationships within the polyploid series in the genus *Festuca*.

Greater control over the introgression of genes from the diploid and polyploid species of *Festuca* into *Lolium* through the development of crossing procedures increases the potential use of variation within the *Lolium/Festuca* complex, e.g. the introduction of increased drought tolerance (Humphreys and Thomas 1993). Most of the characters that would be desirable to transfer from a breeding point of view are polygenically controlled. Variation within *Lolium* can be wide, and the response to selection can result in a significant shift in the expression of these characters as demonstrated by Cooper (1969) for date of ear emergence. Therefore, in BC_1 populations derived from species hybrids it is not possible to be categorical that a shift in the expression of polygenic characters is due entirely to the introduction of segments of the alien genome. The discriminatory power of the in situ hybridization technique is such that it will pin-point any segment of alien chromosome introduced and confirm that the introgression of alien genes has taken place. Correlation between the expression of characters of the donor species and the presence of specific alien chromosome segments would locate the genes controlling those characters on chromosomes of the recipient species.

The results reported here for the diploid *L. multiflorum* introgression line involving the *sid* gene illustrate the effectiveness of in situ hybridization in locating a chromosome segment carrying an introgressed gene. The segment of *F. pratensis* chromosome present with the terminal segments in the genotype studied (Fig. 1f) has not been eliminated during backcrossing even though no conscious selection for its retention was imposed. This is not surprising since the genotype was derived from a BC_1 F_2 population. The ability to identify extraneous segments of the *Festuca* genome could be used to effect the full recovery of the *Lolium* genome through marker assisted selection. Backcross progeny could be screened for the presence of the critical alien segment and the absence of any other segments, thus accelerating the reconstitution of the genome of the recipient species.

The detection of introgressed chromosome segments was most obvious in the hybrid *Lm(LmFp Elmet)*. The amphiploid used has been taken through a number of generations of seed multiplication with the potential to recombine at each generation. Since the triploid hybrids *LmFpFp* and *LmLmFp* derived from the crosses *L. multiflorum* (2x) $\times$ *F. pratensis* (4x) and *L. multiflorum* (4x) $\times$ *F. pratensis* (2x), respectively, always showed distinct chromosomes of both species, the heterogeneity observed in the karyotype of the triploid hybrid derived from the amphiploid is unique to that hybrid. The diversity in karyotype structure is due entirely to recombination and indicates the shift in genome organization in the amphiploid that occurred during the generations of seed multiplication. Lewis (1980) reported that chromosome pairing is not confined to homologous pairs in the amphiploid and that there is an appreciable amount of pairing between homoeologues, that would account for the exchanges observed in the triploid hybrid. A comparison of the BC_1 progeny from crosses using the *LmLmFp* and *Lm(LmFp Elmet)* triploids was undertaken by Ward (1993) who found that the BC_1 derived from the *Lm(LmFp Elmet)* triploid was more variable than that derived from *LmLmFp*. There was evidence from the phenotype of the progeny that the introgression of *Festuca* characters was more prevalent in the *Lm(LmFp Elmet)*-derived population than in the *LmLmFp* BC_1. The greater variability of the BC_1 population derived from the *Lm(LmFp Elmet)* triploid than from the *LmLmFp* BC_1 probably reflects the chromosome exchanges accumulated in the amphiploid.

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