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Introgression of chromosomes of *Festuca arundinacea* var. *glaucescens* into *Lolium multiflorum* revealed by genomic *in situ* hybridisation (GISH)

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Abstract An F_1 hybrid (n=4x=28) between the tetraploid species *Festuca arundinacea* var. glaucescens (GGG′G′) and a synthetic tetraploid *Lolium multiflorum* (*LmLmLmLm*) was backcrossed to diploid *L. multiflorum* to produce triploid (2n=3x=21) BC_1 hybrids (*LmLmG*). At metaphase I of meiosis the triploids had a preponderance of ring bivalents and univalents with some linear and frying-pan trivalents. Genomic in situ hybridisation (GISH) differentiated the *Festuca* chromosomes from *Lolium* and revealed that the bivalents were exclusively between *Lolium* homologues, while the univalents were *Festuca*. Despite the limited amount of homoeologous chiasmata pairing in the triploids, some recombinant chromosomes were recovered in the second backcross when the hybrids were further crossed to diploid *L. multiflorum*. The progeny from the second backcross was predominantly diploid. Genotypes with recombinant chromosomes and chromosome additions involving an extra *Festuca* chromosome were identified using GISH. Changes in plant phenotype were related to the presence of *Festuca* chromatin.

Keywords *Lolium* · *Festuca* · Genomic *in situ* hybridisation · Introgression · Recombination

Introduction

Species within the *Lolium*/*Festuca* complex are adapted to a wide range of ecological conditions and possess complementary characters that are regarded as useful agronomic attributes. The genus *Festuca* consists of geographically diverse species, mainly distributed throughout Europe but also found in the Americas (Mattheij 1982). A polyploid series ranging from the diploid number of chromosomes (2n=2x=14) to decaploid (2n=10x=70) has been documented within the genus (Borrill et al. 1976). The *Lolium* species on the other hand are diploid (2n=2x=14) and are not as widely distributed as *Festuca*. However, the two genera are considered to have diverged from a common ancestor and have a basic chromosome number of x=7. Conventional cytogenetic studies (Jauhar 1975) and more recent studies on ribosomal DNA (rDNA) sites (Thomas et al. 1997a) indicate that while there are close evolutionary links between *Lolium* and *Festuca* species there are clear karyotypic differences. Chandrasekharan and Thomas (1971) deduced from studies of chromosome pairing that the progenitors of the allohexaploid species *Festuca arundinacea* var. genuina (2n=6x=42) include the tetraploid species *F. arundinacea* var. glaucescens (*Fg*) (2n=4x=28) and the diploid species *F. pratensis* (*Fp*) (2n=2x=14). This was confirmed by genomic in situ hybridisation (GISH) studies (Humphreys et al. 1995) and by molecular markers (Sleper and Nelson 1990).

Lolium and *Festuca* species hybridise easily and the high frequency of interspecific chromosome pairing and recombination which occurs in hybrid combinations between the two genera has placed greater emphasis on the introgression of *Festuca* genes into *Lolium*. Combined with this knowledge of chromosome affinities the opportunities for gene transfer between *Lolium* and *Festuca* species have been greatly enhanced by the development of an introgression breeding programme (Morgan et al. 1988; Humphreys 1989). The strategy is to transfer *Festuca* genes characterised for important agronomic traits into a single *Lolium* genotype using a backcross programme. The backcross strategy using the hybrid as the male parent produces gametes with the haploid *Lolium* complement and the least number of *Festuca* chromosomes. Such haplo-gametophytes would develop into viable pollen grains and have a selective advantage over

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gametes with unbalanced chromosome numbers. Consequently, the majority of plants (80%) recovered in a backcross programme are diploid (Morgan et al. 1988). In this way the reconstitution of the recipient *Lolium* genotype and the controlled introgression of *Festuca* genes into *Lolium* can be achieved rapidly. The transfer of *Festuca* genes for drought tolerance and delayed senescence into *Lolium* demonstrates the usefulness of this technique within the complex (Humphreys and Thomas 1993; Thomas et al. 1994). Therefore, by adopting an effective system of gene introgression the potential use of genetic variation that exists within the *Lolium*/*Festuca* complex is greatly increased. The objective is to combine the high forage quality of ryegrasses with the persistence and stress-tolerance of the fescues.

Thomas et al. (1994) demonstrated that in *L. multiflorum* (*Lm*)×*F. pratensis* (*Fp*) hybrids and backcross derivatives, genomes of the two species can be discriminated by the use of GISH. The technique has allowed the physical mapping of genes of agronomic importance to specific chromosome segments. GISH has also proved useful in detecting alien chromosome introgressions in a wide range of plants, for example, in wheat (Schwarzacher et al. 1992). The use of GISH to label whole chromosomes or an entire genome in plant hybrids relies on the use of total genomic DNA as a probe rather than cloned sequences and is applied directly onto chromosome preparations (Anamthawat-Jónsson et al. 1995). The technique is species-specific and depends on DNA sequences that have diverged within whole plant genomes. Fluorescent in situ hybridisation of repetitive DNA sequences [e.g. ribosomal RNA genes (rDNA)] is able to determine differences in the positions of gene clusters between related species (Jaing and Gill 1994). In our study we have applied a wheat ribosomal probe (pTa71) to chromosome spreads of the BC_1 triploid (3x) hybrid between *L. multiflorum* and *F. glaucescens* and BC_2 hybrid derivatives as a chromosomal landmark to identify particular *Festuca* chromosomes.

The application of the introgression breeding strategy and the use of the GISH technique are extremely valuable tools for constructing physical and genetic maps (Thomas et al. 1994; King et al. 1998). The approach can elucidate the control of agronomically important characters and characterise novel germplasm. This report describes the results of using these techniques to discriminate between the genomes of the tetraploid species *F. arundinacea* var. *glaucescens* Boiss. (*Fg*) and *L. multiflorum* var. *westerwoldicum* (*Lm*) in hybrids and backcross derivatives.

Introgression from this tetraploid *Festuca* species is more complex than the earlier introgressions for diploid *F. pratensis*. Nevertheless, it is worthwhile as *F. glaucescens* has a number of valuable traits not found in the diploid *Festuca*.

Materials and methods

Plant material

The plant material used in the crossing programme includes *Fg* (2n=4x=28) accession number (Bn354), the synthetic tetraploid *Lm* (2n=4x=28) (Bb 2075) and diploid *Lm* (2n=2x=14) (Bb 2074). The material, which was grown from seed in a glasshouse, was obtained from the Genetic Resources Unit, I.G.E.R., Aberystwyth. The crossing scheme used to introgress *Festuca* chromosome segments from *Fg* into *Lm* is shown in Fig. 1. An F_1 hybrid *LmLmFgFg′* (4x) was produced by hand-emasculating inflorescences of the female *Lm* (4x) parent followed by pollination with *Fg* (4x) as the male parent in plastic bags. The F_1 hybrid (P172/97), which was fertile, was recovered using embryo-rescue techniques 15–18 days after pollination. The first backcross (BC_1) generation of plants were triploid (2n=3x=21), generated by crossing the F_1 hybrid (4x) as the male parent with diploid *Lm*. A fertile triploid hybrid (3x) *LmLmFg* (P175/99) was identified and used as the male parent in crosses with diploid genotypes of *Lm* to produce a second backcross (BC_2) generation of plants (P188/150). The individual plant numbers of the BC_2 generation have been given the prefix G (genotype).

GISH techniques

Total genomic DNA was extracted from young actively growing leaves of *Fg* and *Lm* as described by Dellaporta et al. (1983). The methods used for root-tip preparations, DNA labelling, and *in situ* hybridisation were as described by Thomas et al. (1994). Anthers were fixed and squashed as described by King et al. (1998). DNA from *Fg*, which was used as a probe, was labelled with FluoroLink Cy3-dCTP (Amersham) after being sheared to 200–1000 bp by suspending a microfuge tube containing the DNA solution in an ultrasonic bath for 6 min. The probe pTa71 (Gerlach and Bedbrook 1979), which contains the 18S-5.8S-26S rRNA gene cluster, was labelled with fluorescein-12-dCTP. The genomic DNA of *Lm* was autoclaved for 2 min to produce fragments of approximately 200 bp in length and then used as blocker. All preparations were counterstained with DAPI and mounted in Vectashield antifade.

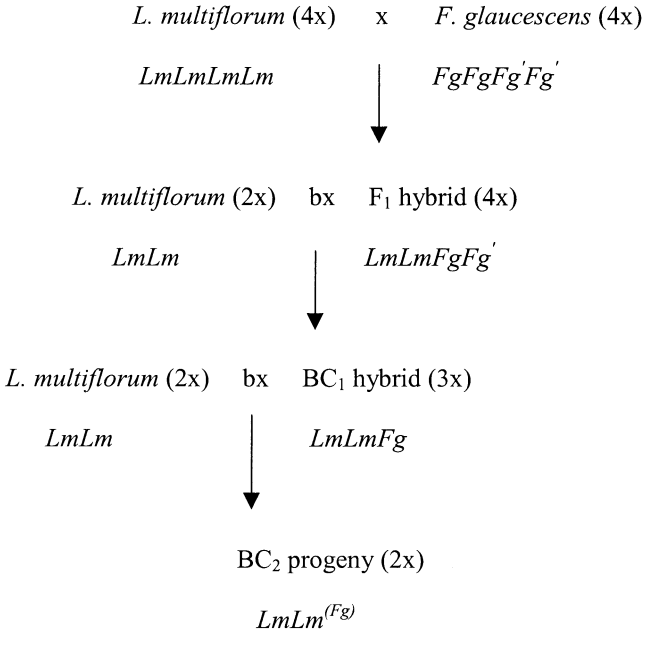

Fig. 1 Crossing scheme for introgression of *Fg* chromosomes into *Lm*

Slide preparations from root-tip meristems and anthers from the BC_1 3x hybrid and BC_2 derivative plants were used as substrate for GISH.

Images of the introgressed fescue segments were examined under a Leitz DM microscope and captured digitally in monochrome with a CoolView CCD camera using Apple Macintosh computer software by Improvision. When Adobe Photoshop was used the monochrome Cy3 images were given a false red colour, the fluorescein images were coloured green and the DAPI images were coloured blue. The images were then combined in Photoshop.

Mitotic and meiotic preparations

Mitotic preparations to determine the chromosome numbers of the BC_2 population were made according to the protocol used by Morgan (1976). Inflorescences of the BC_1 (3x) hybrid used in the study of chromosome pairing relationships were fixed in Carnoy's 6:3:1 (ethanol:chloroform:acetic acid), and anthers were squashed in 1·5% aceto-carmine. Meiosis was studied at metaphase I in 100 pollen mother cells. For GISH studies, inflorescences were fixed in ethanol-acetic acid (3:1) and anthers squashed in 45% acetic acid.

Results

Chromosome pairing studies in the BC_1 3x hybrid (2n=3x=21)

Chromosome pairing associations were studied in the BC_1 triploid *LmLmFg* hybrid (P175/99) at metaphase I of meiosis. The triploid with 21 chromosomes has two homologous chromosome sets of *Lm* and seven *Fg* chromosomes drawn from the two haploid chromosome sets of that species. The mean and range of chromosome associations observed in the triploid is shown in Table 1. Trivalents were observed in 92% of the cells, with a mean of 2.15 trivalents per cell. Two-thirds of the trivalents were linear (chain or V-shape), and the remainder of the frying-pan type. Ring bivalents (mean 4.14) were predominant and exceeded the number of rod bivalents. The total number of trivalents and bivalents observed in each cell never exceeded seven, indicating that there is no pairing between the seven chromosomes of *Fg*. This was confirmed by GISH where all bivalents were *Lm-Lm* and all univalents were *Fg* (Fig. 2a).

Chromosome numbers in BC_2 progeny and morphology

Chromosome numbers for all 78 progeny plants from the BC_2 population (P188/150) were determined mitotically. Table 2 shows the variation and frequency of chromosome numbers ranging from 2n=2x=14 to 2n=3x=21. The percentage for each class is also given. About 50% of the progeny plants were diploids with 14 chromosomes, slightly less than expected from the direction of cross. Approximately 20% of the progeny had 20 or 21 chromosomes which seems a relatively high proportion when this data is compared with published results of similar backcross programmes (Morgan et al. 1988; Humphreys 1989) and may indicate a selective advantage of diploid pollen. The remainder of the progeny ranged in chromosome number from 15 to 19. Two genotypes were identified as having cells that had twice the number of chromosomes of the normal complement, i.e. 14 and 28 in the one genotype, and 15 and 30 in the other. This observation must be the result of spontaneous chromosome doubling in the zygotic tissue (Newton and Pellew 1929).

A range of morphological forms that fall outside the variability of the recipient *Lolium* parent were identified by recording morphological measurements on both the parents and the BC_2 plants. Four BC_2 genotypes with interesting morphological features were considered for further studies using GISH to establish the presence or absence of *Festuca* introgressions in a *Lolium* background. Two were diploids (2n=2x=14), G24 and G25, and two were addition lines (2n=2x=15), G39 and G60. Each plant could be classified as broad-leaved (range: 0.6–1.0 cm wide) or narrow-leafed (range: 0.3–0.5 cm) and low or high tillering (range: 1–13 and 14–37, respectively). A further two diploid genotypes (G46 and G66) were included in the study as representing plants that were morphologically like *Lm*.

Genomic in situ hybridisation in BC_1 (3x) hybrid and in BC_2 progeny

GISH was carried out on mitotic and meiotic cells of the BC_1 3x hybrid (P175/99) and on mitotic preparations of

Table 1 Mean and range of meiotic configurations in 100 cells in the BC_1 *LmLmFg* triploid hybrid

Parental no.	2n	I	II	Rods / Rings	III	Linear / Frying-pan	Xta
P175/99	21	4.83	4.86	0.72 / 4.14	2.15	1.43 / 0.72	15.21
(range)		(1–9)	(1–7)	(0–3) / (1–7)	(0–6)	(0–3) / (0–3)	(11–20)

Table 2 The frequency of chromosome numbers in BC_2 progeny derived from the cross *Lm*×*LmLmFg*

Chromosome no.	14	15	16	17	18	19	20	21
Frequency	37	13	6	1	2	3	9	7
Percentage (%)	47.43	16.66	7.69	1.28	2.56	3.85	10.26	8.97

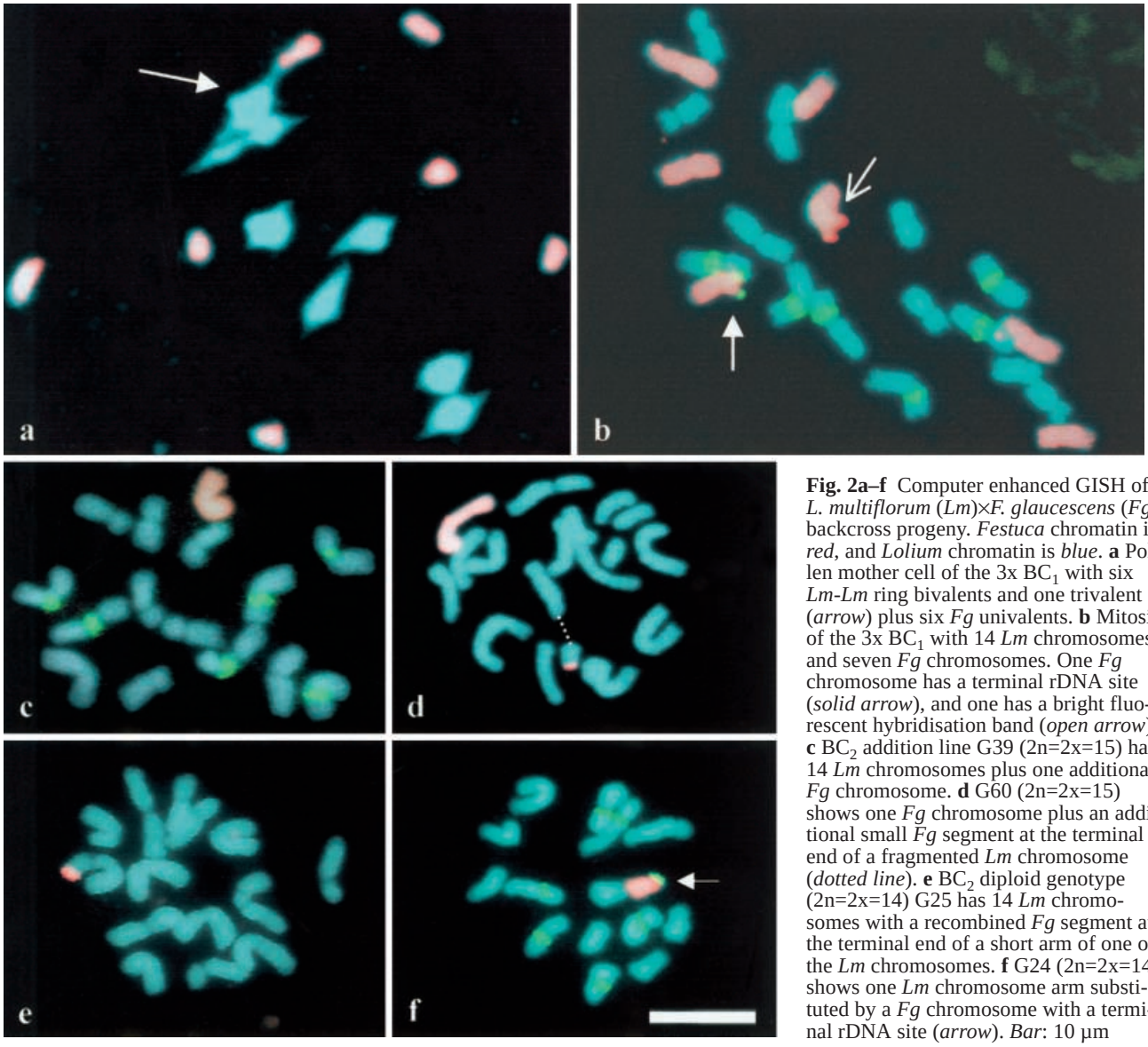

Fig. 2a–f Computer enhanced GISH of *L. multiflorum* (*Lm*)×*F. glaucescens* (*Fg*) backcross progeny. *Festuca* chromatin is *red*, and *Lolium* chromatin is *blue*. **a** Pollen mother cell of the 3x BC_1 with six *Lm-Lm* ring bivalents and one trivalent (*arrow*) plus six *Fg* univalents. **b** Mitosis of the 3x BC_1 with 14 *Lm* chromosomes and seven *Fg* chromosomes. One *Fg* chromosome has a terminal rDNA site (*solid arrow*), and one has a bright fluorescent hybridisation band (*open arrow*). **c** BC_2 addition line G39 (2n=2x=15) has 14 *Lm* chromosomes plus one additional *Fg* chromosome. **d** G60 (2n=2x=15) shows one *Fg* chromosome plus an additional small *Fg* segment at the terminal end of a fragmented *Lm* chromosome (*dotted line*). **e** BC_2 diploid genotype (2n=2x=14) G25 has 14 *Lm* chromosomes with a recombined *Fg* segment at the terminal end of a short arm of one of the *Lm* chromosomes. **f** G24 (2n=2x=14) shows one *Lm* chromosome arm substituted by a *Fg* chromosome with a terminal rDNA site (*arrow*). *Bar*: 10 µm

the six BC_2 genotypes identified above. Using total genomic *Fg* DNA labelled with Cy3 as a probe we were able to easily distinguish the *Fg* chromatin from chromatin of *Lm* (Fig. 2). A pollen mother cell of the 3x hybrid in Fig. 2a shows one trivalent+six bivalents +six univalents, with the *Fg* chromosome occupying the ”handle” position in the frying-pan trivalent.

In Fig. 2b the mitotic preparation from the same plant shows clear differentiation with seven red chromosomes of *Fg* and 14 blue chromosomes of *Lm*. Two of the *Fg* chromosomes are differentiated from the remainder. One of the *Fg* chromosomes has a rDNA site at the end of the short arm (small arrow) and another *Fg* chromosome (large arrow) has a bright band at one end where the Cy3-dCTP signal is very bright. G39 has one *Fg* chromosome in addition to the *Lolium* complement. The plant had broad leaves (0.7 cm) and few tillers (9). Genotype G60 has a small *Fg* segment at the end of one of the *Lolium* chromosomes in addition to a whole *Festuca* chromosome. The leaves of this plant were narrow (0.3 cm), and the tiller number was low (13). There was no rDNA site on the *Fg* chromosomes present within these two genotypes (Fig. 2c, d).

The two diploid genotypes G25 and G24 are morphologically similar in that they have broad leaves (0.7 cm) and high tiller numbers-29 and 33, respectively. G25 is a recombinant with only a small *Fg* segment on the distal end of a *Lm* chromosome (Fig. 2e). This plant was also rhizomatous in its behaviour, an important agronomic trait which is characteristic of *Fg*. G24 also has a recombinant chromosome where the short arm of a *Lm* chromosome is substituted by the short arm of a *Fg* chromosome. The pTa71 probe revealed a distal rDNA site on the *Fg* arm (arrow Fig. 2f). This plant had an unusual prostrate habit and is atypical of the morphology observed in both parents. The two diploid plants, G46 and

G66, that looked like *Lm* showed no evidence of introgression of *Festuca* segments.

Discussion

By combining the complimentary characteristics of the fescues and ryegrasses through introgression, novel germplasm can be developed to enlarge the gene pool available for selection by breeders. Fertile interspecific *Festulolium* hybrids are ideal starting points for use in a backcrossing programme. The products of interspecific recombination from *Fg* were recovered in a modified diploid *Lm* genome in the BC_2. Studies on chromosome pairing in the triploid BC_1 combination revealed that chromosomes of *Lm* and *Fg* do not pair as frequently as in some other hybrids between species of the two genera (Jauhar 1993). However, chromosome pairing between *Fg* and *Lm* is sufficient to allow recombination to take place with the possibility of transferring *Fg* genes into *Lm*.

In the tetraploid F_1 hybrid between *Lm* and *Fg* each *Lm* chromosome has a homologous partner, and these chromosomes pair as bivalents. The two haploid sets of chromosomes from *Fg* are also sufficiently homologous to regularly form seven bivalents (Ghesquiere et al. 1991). The gametes from the F_1, therefore, comprise the haplo-set of *Lm* and seven *Fg* chromosomes representing a basic set drawn from the two *Fg* genomes G and G′. In the triploid hybrid each of the 21 chromosomes can be placed into one of seven homologous/homoeologous groups, with each group containing two homologous *Lm* chromosomes and one homoeologous *Fg* chromosome. The combined number of bivalents and trivalents analysed in each cell never exceeded seven, with the remaining chromosomes being unpaired. This shows that none of the seven chromosomes of *Fg* pair with each other but do occasionally pair with *Lm* chromosomes. The high frequency of bivalents observed shows that pairing is preferential between homologous chromosomes of *Lm* and that any trivalent configurations would include a single *Festuca* chromosome.

In the *Lolium* species chiasmata are preferentially distributed so that in bivalents with two chiasmata, the chiasmata are between different chromosome arms ensuring ring bivalents. This mechanism is manifested in the autotriploids by a predominance of ring bivalents and of linear and frying-pan trivalents with a paucity of rod bivalents and tri-radial trivalents (Thomas et al. 1997a, b). The same is seen in the present autoallotriploids; indeed, no tri-radial trivalents were found.

With two chiasmata per chromosome configuration of three chromosomes either a ring bivalent plus an univalent will be formed, or a linear trivalent, depending on whether there has been a pairing partner switch (PPS) between the two chiasma sites. In the autoallotriploids analysed here, there were more than twice as many bivalents as trivalents, with a preponderance of rings. The trivalents were either linear or frying-pan. In the bivalents there were either no PPSs between the two chiasma sites or an even number of PPSs at pachytene that maintained homologous pairing between *Lolium* chromosomes. Two-thirds of the trivalents were linear, requiring one PPS between two chiasmata and resulting in *Lm*/*Fg* recombinant chromosomes. A third chiasma is needed to form a frying pan trivalent, and the paucity of these trivalents is probably a reflection of the low chiasma frequency in the triploids. Despite these limitations on homoeologous chiasmata pairing, significant numbers of recombinant chromosomes were recovered in the BC_2 genotypes.

In the BC_2 plants, GISH was useful in identifying whole *Fg* chromosomes and *Lm* chromosomes carrying *Fg* segments. Results indicate that these *Fg* chromosome segments carry genes affecting specific traits. For example, a *Fg* segment was present at the proximal end of one *Lm* chromosome in the diploid genotype G25 (Fig. 2e). This plant was identified morphologically as having a high tiller number and a rhizomatous character, usually found in the *Festuca* species. Genotype G24 (Fig. 2f) had a different short arm substitution with a *Fg* rDNA site. It was also high tillering and the growth habit was more prostrate. Both of the short arm substitutions affected tillering capacity, but only one affected the agronomically important rhizomatous character. It has also been shown that different size segments from different *Fg* chromosomes can be inserted, demonstrating that recombination between *Lm* and *Fg* is not restricted to particular chromosome regions.

We intend to screen more characters from a larger backcross population, thus setting up a series of chromosome-arm substitution lines to determine the genetic control of a range of agronomically important traits. Chromosome arms, which carry genes expressing those characters of interest, would be screened and identified, thereby enabling introgression maps to be created. When a recombinant series is analysed, genes can be genetically and physically mapped and assigned to a specific location on a chromosome arm. King et al. (1998, 1999) have demonstrated the importance of developing such a recombinant series in *Lolium*/*Festuca* hybrids. The BC_2 addition lines G39 and G60 exhibit totally different morphological characters that could be linked to chromosomal differences; e.g. G39 has broader leaves, and G60 has much narrower leaves. However, further backcrossing of these addition lines will be carried out to produce chromosome-arm recombinant series for the mapping of introgressed genes.

Genetic variation within *Lolium* can be wide, and the response to selection can result in a significant shift in the expression of morphological characters (Cooper 1969). In our BC_2 population it is not possible to state categorically that a shift in the expression of the characters which may be polygenically controlled is due entirely to the introduction of whole chromosomes or segments of the alien genome. Further investigation will be necessary to confirm the relationships between the *Fg* chromosomes that we have observed in our study and the

effects on the phenotype. This approach of controlled introgression of genes from one species into another can be manipulated in a 'designer breeding' programme for the development of novel germplasm with new combinations of genes.

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