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Chromosome substitutions and recombination in the amphiploid *Lolium perenne* × *Festuca pratensis* cv Prior (2n=4*x*=28)

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Abstract The synthetic amphiploid cv Prior was created in the early 1970s at the Welsh Plant Breeding Station by crossing colchicine-induced autotetraploids of *Lolium perenne* (2n = 14) and *Festuca pratensis* (2n = 14). Meiosis in the early generations was characterized as stable, with frequent bivalent formation. In situ hybridization of a *L. perenne* total genomic DNA probe to mitotic chromosome spreads of 12 plants, from two extant populations of Prior, demonstrates extensive recombination between the two genomes. Recombination events occur along the whole length of chromosome arms but with a higher frequency in the medial portion. The species origins of chromosomes were assigned by the presence or absence of a fluorescent probe at the centromere. There has been a substitution of *Festuca*-origin chromosomes by those of *Lolium*-origin, resulting in a mean of 17.9 (15–21) *Lolium* and 9.7 (7–13) *Festuca* chromosomes per genotype. Mean chromatin length per genotype comprised 62.1% *Lolium* and 37.9% *Festuca*. On average 9.3 *Lolium* (51.1% of those present) and 3.5 *Festuca* (37.8%) chromosomes had no recombined segments. For chromosomes which did show recombination, fewer alien segments were observed in *Lolium* than in *Festuca* chromosomes. *Festuca* chromosomes in genotypes selected for drought resistance had undergone more recombination than in genotypes from an unselected population, though this difference was not statistically significant for the small sample examined.

Key words *Festuca* · *Lolium* · Recombination · GISH · Chromosome

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Introduction

The commercially valuable grasses *Lolium* (ryegrass) and *Festuca* (fescue) have complementary agronomic traits. Considerable effort has been directed at combining the high forage quality of ryegrasses with the persistence and stress-tolerance of fescues (see review by Thomas and Humphreys 1991). The cultivar Prior is one of several intergeneric amphiploids created at the Welsh Plant Breeding Station in the early seventies, and is the product of crosses made between colchicine-induced autotetraploids of the parental species *Lolium perenne* and *Festuca pratensis*. Seedling dry-weight, tillering, and subsequent field performance and fertility, as judged by anther dehiscence, were used to select plants for polycrossing and seed multiplication (Lewis et al. 1972). The cultivars were not developed commercially in the UK but Prior has received favourable reports in more recent years in terms of yield and ground cover at sites with high summer temperatures and/or severe winters (Thomas & Humphreys 1991).

With the exception of *L. multiflorum* × *L. perenne*, interspecific and intergeneric amphiploids of the *Festulolium* complex have generally been considered genetically unstable. Characteristically they show irregular meiosis and polysomic inheritance, leading to loss of the favourable gene combinations of the parental species (Thomas and Humphreys 1991). Some eastern European *L. multiflorum* × *F. pratensis* amphiploids have, however, proved sufficiently stable for cultivar development (Zwierzykowski et al. 1998).

A high frequency of bivalent formation (0.55 IV, 0.21 III, 11.95 II, 1.33 I) and regular meiosis was initially reported for Prior (Thomas and Thomas 1973). Subsequently, chromosome pairing was reported as fairly stable over four generations, though between- and within-plant variation was high (Osborne et al. 1976). Mean bivalent frequency fell from 10.41 to 9.59, mean quadrivalent frequency varied between 1.07 and 1.45, and mean trivalent frequency increased from 0.58 to 0.93. There were 3–14 bivalents and 0–5 quadrivalents per cell. The level of aneuploidy varied but 93.9% of plants were $2n = 28 \pm 1$.

Recent attempts at crop improvement in the forage grasses have concentrated more on the introgression of genes for desirable *Festuca* traits into *Lolium* recipients (e.g. Humphreys and Thomas 1993), and genomic in situ hybridization (GISH) has been used to discriminate chromosomes and chromosome segments of the parental species in *Festulolium* hybrids and introgression lines (Thomas et al. 1994; Humphreys and Pasakinskiene 1996). Successful introgression breeding programmes depend on high levels of homoeologous chromosome pairing and recombination. Unstable amphiploids therefore represent a potentially useful source of recombined genes in backcrossing programmes. Prior is presently being used to introgress *Festuca* genes into *L. perenne* as part of a larger programme to physically map genes for cold tolerance. GISH was carried out to establish the level of recombination between *Lolium* and *Festuca* chromosomes in the cultivar. The Prior genotypes studied here have undergone eight meiotic cycles between 1972 and 1984. A high degree of recombination was expected, but the high frequency of bivalent formation previously reported, as well as the continued fertility, suggested that Prior would have 14 chromosomes of each parental species.

Materials and methods

Six plants (A1–A6) were chosen randomly from among survivors of a 3-month droughting trial carried out at the Institute of Grassland and Environmental Research (IGER). A further six plants (B1–B6) had been used in an isozyme experiment at IGER. They were mature plants grown from seeds of an advanced generation (F_8) and represent a random sample from the Prior seed bank.

Fresh root tips were grown hydroponically from tillers at 23°C; stored in iced-water for 18 h; fixed overnight in a 3:1 solution of ethanol:acetic acid; washed in a 10 mM citric acid-sodium citrate buffer; digested with enzyme solution, 0.15% pectolyase Y-23 (Kikkoman) and 0.15% cellulase R-10 (Onozuka); re-washed in buffer, and squashed in 45% acetic acid.

The *L. perenne* (*Lp*) probe was directly labelled with rhodamine-4-dUTP by nick translation as described by Anamthawat-Jonsson and Heslop-Harrison (1994). *F. pratensis* (*Fp*) blocking-DNA (0.5 μg/μl) was prepared by pressure cooking for 5 min. The protocol for in situ hybridization was that of Leitch et al. (1991). Forty microliters of hybridization mixture containing 100 ng of probe (2–5 μl), and 4 μg of blocking DNA (about 8 μl) were applied to each cell preparation.

The number of *Lolium* and *Festuca* chromosomes, as defined by the presence or absence of fluorescent label at the centromere, was determined in several cells per genotype. Because there was a high degree of uniformity between cells in terms of the number and types of recombined segments observed, a single representative cell was selected for each genotype on the basis of the optimal spread of chromosomes and the clarity of probe labelling. The number and type (terminal vs interstitial) of recombined segments on each chromosome was recorded. A terminal segment includes the telomere and would result from a single recombination event. An interstitial segment does not include the telomere and results from two recombination events within the chromosome arm. An unrecombined *Lolium* or *Festuca* chromosome is one carrying no discernible alien segments. A minimum number of recombination events (MRE) per recombined chromosome was calculated for the *Lolium* and *Festuca* chromosome complements in each genotype, scoring 1 for a terminal and 2 for an interstitial segment. The number of unrecombined chromosomes (URC) in the *Lolium* and *Festuca* chromosome complement of each genotype was expressed as a percentage of the number of chromosomes of that species present and *t*-tests were used to compare the MRE/chromosome and the %URC between *Lolium* and *Festuca* chromosomes (paired) and between populations (unpaired).

The distance from the centromere to the point of recombination on the chromosome arm and the total arm length were measured on projections of the photographic slides for 248 recombination events (122 *Festuca*, 126 *Lolium)*. A frequency distribution of recombination events along the chromosome arms was plotted expressing each distance as a percentage of the total chromosome-arm length. The total lengths of *Lolium* and *Festuca* chromatin were also measured for each genotype from the projections.

Results

Nine genotypes had 28 chromosomes, and three had 27. All 12 genotypes had an excess of *Lolium* chromosomes. There was an average of 17.9 (15–21) *Lolium* chromosomes and 9.7 (7–13) *Festuca* chromosomes per genotype, with no statistically significant difference in this respect between the two populations (Table 1). The total mean chromatin length per genotype was 62.1% (53.7–71.2%) *Lolium* and 37.9% (28.8–46.3%) *Festuca* with no significant difference between the two populations.

Extensive recombination between chromosomes of the two parental species was revealed by GISH (Fig. 1). Recombination events are distributed along the length of the chromosome arms of both species (Fig. 2), with exchanges occurring most frequently in the medial portion of the arms. The distribution is skewed somewhat towards the distal end in *Lolium* chromosomes. Identical patterns of recombination occur in some chromosomes both within the same genotype and in different genotypes (Fig. 1). Further chromosomes with reciprocal patterns of recombination to these can be identified in other genotypes (Fig. 1).

An average of 9.3 (4–13) *Lolium* and 3.5 (2–7) *Festuca* chromosomes showed no recombination. This is 51.1% of *Lolium*-origin chromosomes and 37.8% of *Festuca* origin chromosomes, a statistically significant difference ($P \leqslant 0.05$). The minimum number of recombination events per recombined chromosome was

Table 1 Number of chromosomes of each species origin, recombined segments and the minimum number of recombination events (MRE) in genotypes of cv Prior. Recombined segments are either terminal (T), interstitial (I), or absent (0) in a particular chromosome. Numbers in brackets indicate the number of chromosomes present in the genotype with that combination of recombined segments

Genotype	2n	*L. perenne* origin			*F. pratensis* origin		
		No. chroms.	Recombined segments	MRE	No. chroms.	Recombined segments	MRE
A1	28	15	0(7) T(7) TI(1)	10	13	0(2) T(3) TT(3) TI(3) TII(1) I(1)	25
A2	28	16	0(4) T(3) TI(1) TT(7) II(1)	24	12	0(4) T(2) TT(1) TI(1) TTI(2) I(2)	19
A3	28	18	0(9) T(7) I(2)	11	10	0(2) T(2) TT(1) TI(2) I(1) II(1)	16
A4	28	21	0(10) T(7) TI(1) TT(1) I(2)	16	7	0(3) T(1) TT(1) TI(1) TII(1)	11
A5	27	19	0(13) T(5) I(1)	7	8	0(3) T(4) TT(1)	6
A6	28	20	0(10) T(8) TT(2)	12	8	0(4) T(2) TI(1) I(1)	7
Mean A		18.2			9.7		
B1	28	17	0(13) T(3) TT(1)	5	11	0(3) T(4) TT(4) I(1)	11
B2	27	16	0(6) T(7) I(3)	13	11	0(2) T(5) I(2) TT(1) II(1)	15
B3	28	19	0(8) T(7) TT(2) I(2)	15	9	0(5) T(2) I(2)	6
B4	28	18	0(13) T(4) I(1)	6	10	0(7) T(3)	3
B5	28	20	0(12) T(4) I(2) TI(2)	14	8	0(4) T(2) TT(1) TI(1)	7
B6	27	16	0(6) T(7) I(2) TT(1)	13	11	0(3) T(3) TT(1) TI(2) TTI(1) I(1)	17
Mean B		17.7			9.7		
Mean A+B		17.9			9.7		

significantly higher ($P \leqslant 0.05$) for *Festuca* (1.85) than for *Lolium* (1.37).

Festuca chromosomes of drought-stressed genotypes (A1–A6) exhibit more recombination events per recombined chromosome (2.06) than *Festuca* chromosomes of genotypes in the unselected population (1.65); however, the difference is not statistically significant. For *Lolium* chromosomes there were 1.38 recombination events per recombined chromosome in the stressed population and 1.36 in the other.

There were no inconsistencies between different cells in the number of chromosomes of each species origin, or the number of unrecombined chromosomes, scored from either the same or different preparations of the same genotype. There were a few discrepancies between cells, arising from a difficulty in discriminating between some terminal and subterminal segments as a result of the varying orientation of chromosomes. In genotype B1 the total number of *Festuca* chromosomes was 11 as ascertained from several cells; however, the representative cell used to score recombination had only nine *Festuca* chromosomes present.

Discussion

Genomic in situ hybridization has demonstrated that there are more than the expected 14 *Lolium* chromosomes in all 12 Prior genotypes examined, and an excess of *Lolium* over *Festuca* chromatin. In the single genotype of the *L. multiflorum* × *F. pratensis* (4*x*) amphiploid cv Elmet, studied by Thomas et al. (1994), chromosome substitution was not observed despite extensive recombination between the two genomes. In reciprocal *L. multiflorum* × *F. pratensis* (4*x*) hybrid combinations Zwierzykowski et al. (1998) showed extensive recombination in F_8 populations. The proportion of total genome length occupied by *Lolium* was between 49.2% and 66.7% and therefore less than that reported here in F_8 Prior genotypes, but the species origin of chromosomes was not assigned.

The excess of *Lolium* chromosomes could have arisen from inadvertent exposure of the hybrid to autotetraploid *L. perenne* pollen during cultivar development or subsequent seed multiplication. This would have introduced a number of autoallotetraploids (*LpLpLpFp)* into the population. Such plants largely form bivalents at meiosis and have high pollen fertility (Jauhar 1993). Crosses between them and the amphiploid (*LpLpFpFp*) could then have given rise to the variable genotypes observed here. Commercial cultivar development is, however, carried out under carefully controlled pollination conditions and care is taken during seed multiplication to avoid contamination by pollen from neighbouring fields.

An alternative hypothesis, consistent with the extensive intergeneric recombination observed in Prior, is that *L. perenne* chromosomes have replaced *F. pratensis* chromosomes over repeated cycles of meiosis with selection in favour of additional *Lolium* chromosomes

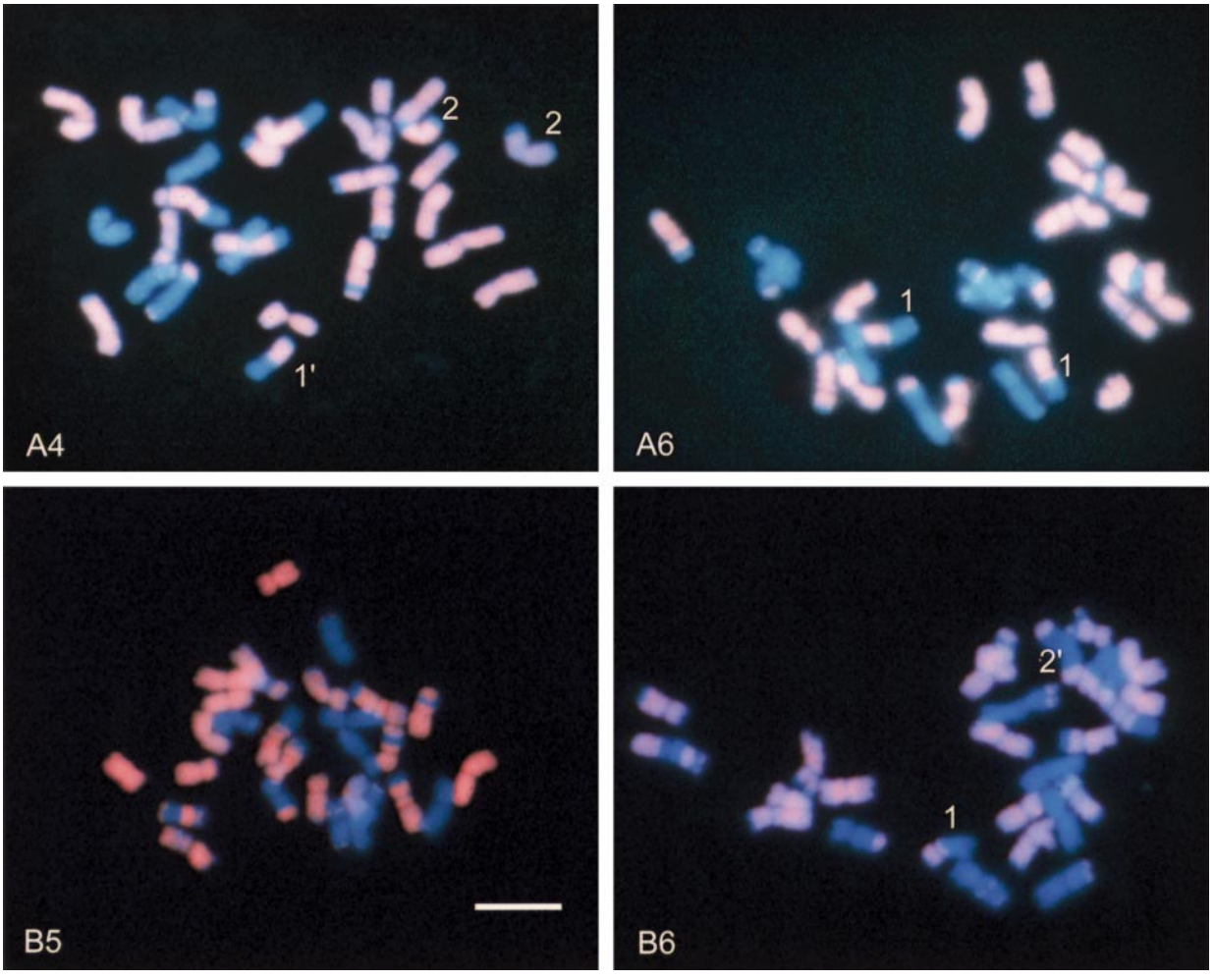

Fig. 1 Genomic *in situ* hybridization of cv Prior genotypes *A4*, *A6*, *B5*, *B6*, using a total genomic probe of *L. perenne*. The probe hybridizes to chromosomes and chromosome segments of *L. perenne* (*pink*) but not *F. pratensis* (*blue*). More than 14 *Lolium* chromosomes, as defined by presence or absence of hybridization at the centromere, are present in all four genotypes. Identical recombination patterns occur in chromosome pairs marked *1 and 2*. Reciprocal recombination events to these occur in chromosomes marked *1′ and 2′*. (Bar = 10 μm)

at some stage of the life cycle. Recombination between parental genomes in Prior shows that inheritance varies between disomic and tetrasomic. The 14 chromosome pairs of the initial genome, in effect, behave like seven quartets, each comprising two *Lolium* homologues and two *Festuca* homologues. Homologous pairing within a quartet gives rise to euploid gametes which are all hybrid (*FpLp*). Homoeologous pairing gives rise to hybrid (*FpLp*) or monospecific (*LpLp* and *FpFp*) gametes depending on disjunction. Genotypes in the next generation can, therefore, have any combination of *Festuca* and *Lolium* chromosomes. The actual population frequency of each genotype will depend on

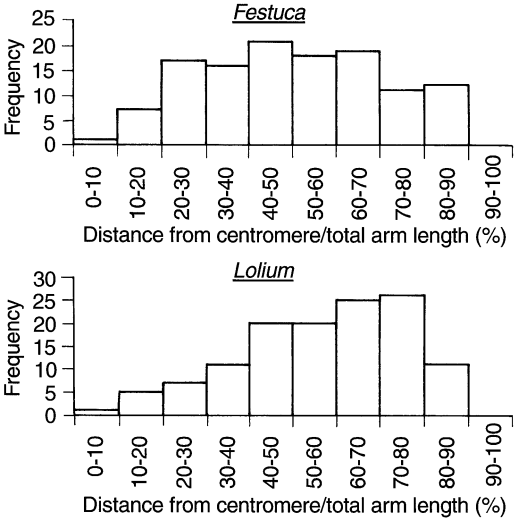

Fig. 2 Distribution frequency of recombination events along the chromosome arm in *Festuca* and *Lolium* chromosomes of cv Prior

both selection and the probability of homoeologous pairing within each chromosome quartet. Theoretically the frequency of balanced genotypes ($14Lp:14Fp$) is increased by more preferential pairing between homologous chromosomes but is decreased by selection against either genome.

Previous work suggests that preferential pairing is only partial. In all possible autoallotriploid hybrids of *L. perenne*, *L. multiflorum*, and *F. pratensis* (*LpLpLm*, *LmLmLp*, *FpFpLm*, *FpFpLp*, *LmLmFp*, *LpLpFp*) a lower trivalent and a higher bivalent frequency occured in those having two *Festuca* genomes than in those with two *Lolium* genomes of the same species (Jauhar 1975), indicating a degree of preferential pairing between chromosomes of the same species. More recently GISH on autoallotriploids of *L. temulentum* and *L. multiflorum* (*LtLtLm* and *LmLmLt*) indicated only limited preferential pairing between chromosomes of the same species, and in this case more preferential pairing occurred between the *L. multiflorum* genomes (Thomas 1994).

In Prior, selection against gametes with additional *Festuca* chromosomes in a subset, or all, of the quartets would lead to the *Lolium*-biased genotypes reported here. Selection at the male gametophyte level has been shown in maize (Ottaviano et al. 1982). Disturbed Mendelian segregation of *F. pratensis* alleles in BC_1 and BC_2 plants derived from reciprocal backcrosses to *L. multiflorum* reveals reduced transmission through the male gamete (Humphreys and Thorogood 1993). Jauhar (1993) has noted that fertility is generally higher in material with more *Lolium* than *Festuca* genomes. The necessity for gamete viability of a complete *L. multiflorum* genome in pentaploid hybrids with *F. arundinacea* has been demonstrated by Humphreys et al. (1998) and noted repeatedly in backcrossing programmes carried out at IGER.

Selection of vigorous seedlings or *Lolium*-biased genotypes by breeders during the development of Prior, or competition between seedlings in the sward during early field trials, could also explain skewed genotypes in the population. *Lolium* species generally show better establishment, growth, persistence, and seedling vigour than *Festuca* species. However, Prior is more stress-tolerant than *L. perenne* suggesting the simultaneous selection of *Festuca* characters. Selection for the retention of *Festuca* genes which confer drought tolerance may explain the greater number of recombination events per recombined *Festuca* chromosome observed in population A. Although not a statistically significant result, it was obtained with only a small sample. The cold and drought tolerance noted in Prior populations in some climates (Thomas and Humphreys 1991) may result from such a selection process in which the effects of *Festuca*-derived genes for stress tolerance are enhanced by improved establishment and vigour derived from the *Lolium* parent.

Recombination events have clearly occurred in the history of Prior as demonstrated by the presence of numerous introgressed chromosome segments of both parental species. The mean number of *Festuca* chromosomes showing no recombination is 3.5 (2–7) per genotype. Recombination events are distributed along the length of the chromosome arms and are most frequent in the medial portion of the arms. Pairs of chromosomes within the same and different genotypes showing identical patterns of recombination, and also pairs from different genotypes showing reciprocal recombination events (Fig. 1), suggest that there may be "hot spots" for recombination between the two genomes. The skew towards the distal end evident in the distribution of recombination events in chromosomes of *Lolium* origin (Fig. 2) may also indicate a difference between the two species in the location of such hot spots.

An accumulation of recombinant chromosomes over successive generations would, in effect, increase the level of homology between *Festuca* and *Lolium* chromosomes belonging to the same quartet. Even if there was an initial degree of preferential pairing between chromosomes of the same species origin, an increase in homoeologous pairing would be expected with a consequent acceleration in interspecific recombination events per generation. Following substitution, forced homoeologous pairing of single *Festuca* chromosomes remaining within quartets would be promoted as parts of trivalents, quadrivalents, or even bivalents with *Lolium* chromosomes. As previously mentioned, frequent bivalent pairing occurs in the autoallotetraploid (Jauhar 1993). An elevated level of homoeologous pairing would increase the number of unbalanced gametes produced and so amplify any selective pressure present. Chromosome substitution would then accelerate in later generations. Whether substitution of remaining *Festuca* chromosomes will occur in future sexual generations of Prior depends upon whether selective pressures continue, or were confined to the initial period of cultivar development.

While contamination by autotetraploid pollen cannot be ruled out, progressive chromosome substitution in Prior is consistent with a model of chromosome behaviour in which pairing between homologues in the hybrid is only partially preferential. If there was a contamination event it could not have been detected by conventional cytogenetic analysis and the value of GISH in breeding applications is demonstrated. Whatever chromosome pairing configurations occur during meiosis in extant Prior, the analysis will be flawed unless the species origin of chromosomes can be distinguished. The high level of recombination between the genomes means that any assumptions regarding homology and homoeology made on the basis of pairing configurations are unsafe.

Declaration These experiments were carried out in accordance with current British Law.

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