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**Review article**

# Applications of recent advances at the Institute of Grassland and Environmental Research in cytogenetics of the *Lolium/Festuca* complex

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**Abstract.** Recent advances at Institute of Grassland and Environmental Research (Aberystwyth, U.K.) in cytogenetics of the *Lolium/Festuca* complex places us in the advantageous position of being able to map genes of agronomic importance onto chromosome arms using fluorescence in situ hybridization (FISH). The ability to physically map genes leads to the capability for "dissecting" quantitative traits into their different components and will lead to better understanding of the complex physiological processes involved and the identification of their genetic control. By tagging genes of interest, using molecular and morphological markers, it will be possible to select and combine suites of desirable genes in a single genotype and thus produce novel cultivars by conventional breeding procedures. Programmes for introgression depend on the relationships between species and on levels of chromosome pairing. Phylogenetic relationships within the *Lolium/Festuca* complex are being determined using both genomic in situ hybridization (GISH) and FISH. With recent advances in genetic manipulation within the *Lolium/Festuca* complex, opportunities now arise for gene transfer from *Lolium* and *Festuca* species into other important agricultural crops.

**Key words:** androgenesis, drought resistance, fluorescence in situ hybridization (FISH), gene isolation, introgression mapping, *Lolium/Festuca* complex, plant breeding.

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## Introduction

Recent advances in cytogenetics of the *Lolium/Festuca* complex provide new opportunities for understanding and manipulating physiological mechanisms involved in complex quantitative traits. These advances rely on two exceptional properties of the *Lolium/Festuca* complex. On the one hand, the chromosomes of some *Festuca* species, e.g., *F. pratensis*, have sufficient homology with the chromosomes of certain *Lolium* species, e.g., *L. perenne* to permit the chromosomes of the two genera to pair freely and recombine in hybrids. Yet on the other hand, the chromosomes of *Lolium* and *Festuca* retain sufficient structural heterogeneity to enable chromosomes of one species to be discriminated from another, using genomic in situ hybridization – GISH (THOMAS et al. 1994).

Through introgression of *Festuca* genes into *Lolium* or *Lolium* genes into *Festuca*, it is now possible to "dissect" complex traits into their different components and thereby clarify their function. Genes introgressed from *Festuca* into *Lolium* or from *Lolium* into *Festuca* can be detected by GISH and assigned to chromosome arms. In this way introgression maps can be created and germplasm identified for use by physiologists, geneticists, breeders, and molecular biologists.

As one example, we describe a multidisciplinary approach at IGER aimed at establishing the genetic and physiological basis of drought resistance and its transfer from *F. arundinacea* into *L. multiflorum*.

## Dissection of physiological traits conferring drought resistance

Drought is a complex phenomenon, involving not only reduced availability of soil moisture, but also high evaporation load, supra-optimal temperatures, potentially damaging insolation, reduced availability of mineral nutrients, and increased soil hardness. Plants have evolved a number of traits for dealing with each of these stresses. Each trait must involve at least one metabolic pathway, and each pathway a number of enzymes. It is therefore inevitable that the genetic control and heritability of drought resistance is particularly complex. A great disadvantage is that it is difficult to know what traits are most important in particular species and environments, and therefore to develop ideotypes. One approach is to compare expression of putative traits in species known to have contrasting levels of drought resistance. The problem with this is that many of the species' differences will be unrelated to drought resistance.

Another approach is to produce experimental breeding lines that are homozygous except for the genes responsible for a particular trait. The disadvantages are that the process is very time-consuming, that responses may be unique to the common genetic background, and that it is difficult to apply to outbreeders such as forage grasses.

The *Lolium* × *Festuca* hybrids provide us with a convenient (and possibly unique) tool for identifying traits contributing to drought resistance and quantifying their importance in the field. For example, by screening drought susceptible lines of *L. multiflorum* containing introgressed segments of drought resistant *F. arundinacea*, we have isolated élite genotypes that are as drought resistant as *F. arundinacea* (HUMPHREYS, THOMAS 1993). Those so far analysed by GISH have been shown to include a single *F. arundinacea* segment on the same chromosome arm of *Lolium* having a common origin in the *F. pratensis* genome of the hexaploid species (HUMPHREYS, PASAKINSKIENE 1996). Physiological studies are now underway to detect festucoid traits, i.e., those occurring in the élite plants but not in the recurrent (*L. multiflorum*) parent. Preliminary analyses (HUMPHREYS et al. 1997) show that the élite plants have inherited the high adaxial leaf-water conductance of *F. arundinacea*, and the low abaxial conductance of *L. multiflorum*. Whether this contributes to water-use efficiency has yet to be determined.

As traits are identified, their contribution to drought resistance can be proven by crossing to a drought susceptible tester genotype, and subsequently correlating presence or absence of *Festuca* genes with drought resistance in the progeny. We expect that different drought resistant plants will express different traits determined by genes located on different chromosomes, and that some introgessed chromosome segments will include genes coding for more than one trait. By "dissecting" the different traits involved in drought resistance, it will be possible to select for genes of major importance and exclude genes of lesser significance. In this way, genes for drought resistance will be transferred from *Festuca* into *Lolium* with little disruption to the *Lolium* genome.

## Gene isolation

The presence of an alien chromosome segment, identified by GISH and shown to confer an adaptive advantage such as drought resistance enables us to investigate the molecular and biochemical basis of adaptation to drought stress.

Selfing plants with alien introgressions will produce lines either homozygous, heterozygous, or homozygous for no alien *Festuca* genes. These lines will provide excellent starting material for studying gene expression. Genomic subtraction techniques (e.g., Representational Difference Analysis, RDA (LISITSYN 1995)) can be used to selectively amplify and clone sequences unique to introgressed chromosome segments. A companion technique, cDNA-RDA (HUBANK, SCHATZ 1994), or alternatively Differential Display (LIANG, PARDEE 1992) can be used to detect differentially regulated mRNAs. A combination of these techniques can be used to identify and isolate clones uniquely expressed from the introgressed segment. Clones isolated by these techniques can be rapidly characterised.

The ability to routinely transform species of the *Lolium/Festuca* complex (DALTON et al. 1995) allows the testing of cloned sequences for adaptive activity. Clones can be expressed in "sense" in *L. multiflorum* and their contribution to adaptation assessed in genotypes lacking the introgressed segment. The same clones can also be introduced into *F. arundinacea*, or *L. multiflorum* lines homozygous for the alien *Festuca* segment, in "antisense" or "short sense" to suppress their normal expression. This will yield strong evidence of function – the ability to confer activity in a negative background, and inhibition of activity in a positive background.

## Cytogenetic studies to evaluate efficiency of gene transfer in *Lolium* × *Festuca* hybrids

Successful breeding programmes aimed at introgression of alien genes from one species into another depend on adequate levels of interspecific chromosome pairing.

The pentaploid hybrid between autotetraploid *L. multiflorum* (2n=4x=28) and *F. arundinacea* (2n=6x=42) has high levels of recombination and moreover is both female and male fertile. It has been used as a male parent in backcross breeding programmes with diploid *L. multiflorum* (HUMPHREYS 1989, HUMPHREYS, GHESQUIERE 1994).

Using GISH, we have determined that *F. arundinacea* was derived from two species, *F. pratensis* (2x) and *F. glaucescens* (4x) (HUMPHREYS et al. 1995). As an alternative breeding strategy for introgression of *Festuca* genes from *F. arundinacea* into *Lolium*, genes may be introduced directly from either of the two progenitors of the hexaploid species. Introgression from *F. pratensis* into *Lolium* (2x) has been effective using as a starting point a triploid hybrid

between autotetraploid *L. multiflorum* (4x) and *F. pratensis* (2x) (THOMAS et al. 1994). However, segregation of introgressed *F. pratensis* genes does not always conform with Mendelian expectations, particularly through the male gamete (HUMPHREYS, THOROGOOD 1993). Introgression from *F. glaucescens* (4x) into *L. multiflorum* (2x) has been achieved by forming an initial tetraploid hybrid between *L. multiflorum* (4x) and *F. glaucescens* (4x) and backcrossing twice onto *L. multiflorum* (2x) (GHESQUIERE et al. 1991).

HUMPHREYS and GHESQUIERE (1994) measured levels of recombination between *L. multiflorum* and each genome which constitutes hexaploid *F. arundinacea*. They created a pentaploid hybrid between *L. multiflorum* and *F. arundinacea* (2n=5x=35) with a single chromosome in each of the three genomes of *F. arundinacea* labelled with different homoeoalleles of the phosphoglucoisomerase (PGI/2) locus. The pentaploid hybrid was backcrossed onto diploid *Lolium* and recombinants recovered in the $BC_2$ involving *Lolium* and each of the three *Festuca* genomes establishing the efficacy of such a backcrossing programme for introgression of *Festuca* genes into *Lolium*.

Interspecific recombination between *Lolium* and either *F. pratensis* or *F. glaucescens* chromosomes carrying the PGI/2 locus is higher in *Lolium* × *F. pratensis* and in *Lolium* × *F. glaucescens* hybrids than in hexaploid *F. arundinacea* when the two fescue species are present in combination (HUMPHREYS 1995, GHESQUIERE pers. comm.). Once the locations of the principal genes involved in stress tolerance in *F. arundinacea* have been determined, it may prove more effective to transfer genes into *Lolium* directly from either *F. pratensis* or *F. glaucescens* rather than from the hexaploid fescue species.

## Exploitation of cytogenetic advances for grass breeding

Grass breeders have exploited the potential of allopolyploidy in the *Lolium/Festuca* complex by creating novel hybrids with extended adaptive ranges for new agronomic niches.

Tetraploid hybrids between *F. pratensis* and *L. multiflorum* and *L. perenne* have extended the ability of grasses with good early growth and high nutritive value to cope with extremes of temperature and moisture (THOMAS, HUMPHREYS 1991). Good agronomic potential has also been demonstrated in octoploid hybrids between *L. multiflorum* and *F. gigantea* which have potential to improve summer growth (HUMPHREYS et al. 1989). Some allopolyploid combinations have proved difficult to maintain with a lack of stability and in-

creasing infertility over generations of seed production. FISH can be used to indicate the extent of recombination between genomes of the different parent species in allopolyploids which can lead to genetic imbalance and progressive deterioration in fertility.

However, use can be made of the unstable properties of amphiploid *Festulolium* cultivars which have undergone considerable chromosome recombination through years of seed multiplication (e.g., *L. multiflorum* × *F. pratensis* cv. Elmet). Such hybrids provide ideal starting points in backcross breeding programmes aimed at introgression of *Festuca* genes into *Lolium* (THOMAS et al. 1994) which rely on high levels of recombination.

Recent interspecific grass breeding has concentrated on introgression and transfer of specific adaptive traits between species rather than combining complete genomes. Breeding programmes based on introgression have been successful in transferring good summer growth from *F. pratensis* into *L. perenne* (HUMPHREYS 1993) and drought tolerance from *F. arundinacea* into *L. multiflorum* (HUMPHREYS, THOMAS 1993). Dry matter yields, under rain-out shelters at Aberystwyth and under natural conditions in Lusignan, France, of Italian ryegrass selections containing genes from tall fescue were increased by 33% and 17%, respectively, compared to control Italian ryegrass cultivars (THOMAS et al. 1995). Improved winter hardiness through the introgression of *F. pratensis* genes into ryegrasses (HUMPHREYS, HONNE 1995) increased tiller survival by 32% compared to control cultivars over two winters in Norway. Work is also in progress to improve nitrogen use efficiency in ryegrass using fescue genes and to improve the nutritive value of tall fescue through gene transfer from ryegrasses.

Gene transfer between species is aided considerably by the construction of genetic maps, the identification of quantitative trait loci (QTL's) and use of FISH. In *L. perenne*, associations with isozyme loci have been identified for a number of traits such as water soluble carbohydrate content (HUMPHREYS 1992), and yield and flowering time (HAYWARD, MCADAM 1988). RFLP's and PCR techniques greatly increase the range of genetic markers available to allow genetic maps to have more detailed and extended coverage of grass genomes (HAYWARD et al. 1994). Nine QTLs concerned with different aspects of the flowering process in ryegrass have been identified of which three control inflorescence emergence (HUMPHREYS et al. 1995). Careful choice of QTLs should minimise undesirable correlated selection responses in marker assisted selection. For example in Linkage Group (LG)1 and LG7 of perennial ryegrass, QTL's for heading date are closely associated with head number, whereas there are separate QTLs for heading date on LG2 and for head number on LG4.

FISH can help to locate QTL's on chromosomes and allow direct visual monitoring of introgression between species. For example, in an introgressed line of *L. multiflorum*, derived from a cross between *L. multiflorum* and *F. pratensis*, chromosome segments carrying the *F. pratensis* derived *sid* (senescence induced degradation) allele could be identified (THOMAS et al. 1994). It is of interest that the *Festuca* segment in *Lolium* which carries genes for drought resistance (HUMPHREYS, PASAKINSKIENE 1996) is in a similar position to a QTL which has been shown to affect aftermath heading (HUMPHREYS et al. 1995) and that reduced aftermath heading is one of the features of the drought resistant lines of *L. multiflorum* (THOMAS et al. 1995).

## Introgression mapping

The first stage of introgression mapping currently underway at IGER, involves the production and isolation of 14 *L. perenne* (Lp)/*F. pratensis* (Fp) chromosome arm substitution lines. Each line will have the Lp chromosome complement except for a single chromosome arm being replaced by the homoeologous Fp arm. For example, chromosome arm substitution line 1 will have a long arm of Lp chromosome 1 replaced by a long arm of Fp chromosome 1; while line 14 will have a short arm of Lp chromosome 7 replaced by a short arm of Fp chromosome 7. In this way each of the 14 chromosome arms of Lp are replaced by a homoeologous arm from Fp to give the 14 substitution lines. The lines will be identified by their molecular karyotypes following in situ hybridization (ISH) with cloned repetitive sequences that give chromosomal landmarks, and by genetic markers.

A recombinant series will then be derived from each one of the 14 chromosome arm substitution lines. This is being achieved by crossing each substitution line with normal *Lolium*. In each case, the Fp chromosome arm recombines with the homoeologous Lp chromosome arm in the hybrid. Thus in the next generation, individuals carrying a spectrum of different sized Fp chromosome segments are produced, all derived from that one Fp arm.

These recombinant series will be used to screen and to physically map any major gene, QTL or other genetic marker in the Fp genome that is expressed in the Lp phenotype, and thereby produce a physical map of the Lp and Fp genomes and also facilitate the introgession of characters from Fp into Lp. The first stage of screening will use the chromosome arm substitution lines to locate a particular character to a particular chromosome arm, and then a recombinant series will be produced for that arm. Stage two will involve

screening the recombinant series to identify plants with the smallest Fp segment but which still expresses the character, and this plant will then be backcrossed to Lp. Progeny which carry the Fp segment, and the gene of interest, will be selected by using genetic markers and GISH. Introducing small segments in this way will diminish the chances of carry-over of deleterious Fp genes.

## Tissue and anther culture

Other strategies have been attempted to increase levels of interspecific recombination including the use of tissue culture to induce somatic chromosome rearrangements. The *L. multiflorum* × *F. arundinacea* pentaploid hybrid used in the backcrossing programme described above was very responsive to cell suspension culture (HUMPHREYS, DALTON 1992). Culture conditions induced chromosome aberrations including chromosome breakage and association and led to significantly more *Lolium* × *Festuca* chromosome configurations at meiosis in regenerant plants. A cell culture phase may be used prior to a backcrossing programme to increase recovery of interspecific recombinants. Unfortunately until recently, culture induced chromosome aberrations have led to infertility in *Festulolium* (5x) hybrids and have prevented the use of culture regenerants in backcross breeding programmes.

However, PASAKINSKIENE et al. (1997) report finding fertile novel diploids arising through chromosome segregation and somatic recombination in amphiploids of *L. multiflorum* × *F. arundinacea*.

We have recently attempted to exploit androgenesis as a means for improved selection of genes for stress tolerance in *Festulolium* hybrids (HUMPHREYS et al. 1996). Development from microspores leads to monoploid plants and enables subsequent production of homozygous plants by chromosome doubling.

Microspores as products of meiosis represent a vast array of genetic variation. The pentaploid *L. multiflorum* × *F. arundinacea* hybrid described earlier was very responsive to anther culture, and androgenic plants were found with coacclimation to both drought and freezing stress in excess of the stress tolerant *Festuca* parent (HUMPHREYS et al. 1996).

From a cytogenetic aspect, androgenic plants represent the result of chromosome pairing, recombination and disjunction. They therefore provide an insight into chromosome behaviour at meiosis. HUMPHREYS et al. (1996) described segregation of *Lolium* and *Festuca* PGI/2 homoeoalleles within a population of androgenic plants derived from the pentaploid *Festulolium* hybrid. The segregation of PGI/2 homoeoalleles is considered a good indicator

of chromosome behaviour at meiosis in the pentaploid *Festulolium* hybrid, of at least one homoeologous group. They compared segregation of PGI/2 alleles within the androgenic population and their recovery in BC1 progeny from the backcross breeding programme described by HUMPHREYS and GHESQUIERE (1994). The presence of a *L. multiflorum* PGI/2 allele in nearly every androgenic plant provides strong evidence for preferential chromosome pairing and regular disjunction between the two *L. multiflorum* genomes of the pentaploid hybrid. This adds to evidence from backcross breeding programmes (MORGAN et al. 1988, HUMPHREYS, GHESQUIERE 1994) that each gamete in the pentaploid hybrid contains a complete *L. multiflorum* genome. Furthermore, the absence of plants with no *L. multiflorum* PGI/2 alleles is evidence that a complete *L. multiflorum* genome is required to provide gamete viability.

No difference was observed between the segregation and recovery of the five PGI/2 alleles in androgenic plants with their transmission into the $BC_1$ from *Lm* (2x) × *Festulolium* (5x) hybrids. A lower than expected recovery of a PGI/2 allele in the $BC_1$ would be evidence of gametophytic selection through pollen competition or zygotic abortion which can occur in *Lolium/Festuca* backcross breeding programmes and preclude recovery of certain gene combinations (HUMPHREYS, THOROGOOD 1993).

In order to utilise the variation obtained by anther culture, it will be necessary to restore fertility to selected androgenic plants and to incorporate them in breeding programmes. By chromosome doubling, preferential chromosome pairing between homologous chromosome partners will be encouraged. The selection for the pairing control gene(s) known to be located in one of the *F. arundinacea* genomes will enhance levels of preferential bivalent pairing.

## Phylogenetic studies using FISH labelled specific gene sequences

ISH of repetitive DNA sequences such as ribosomal RNA genes (rDNA) have proved valuable in comparisons of chromosomes of related species. The two wheat ribosomal DNA probes pTa71 and pTa794 are clones of the 18S-5.8S-26S and 5S rRNA genes, respectively (GERLACH, BEDBROOK 1979, GERLACH, DYER 1980). These have been hybridized to the chromosomes of inbreeding and outbreeding *Lolium* species to detect the number and positions of rDNA sites.

After ISH, pTa71 hybridization sites were seen on two pairs of chromosomes in the inbreeding taxa. In the outbreeding species *L. multiflorum* there were three pairs of hybridization sites corresponding with the secondary constrictions

on chromosomes 1, 2 and 3 (THOMAS 1981). However, in the other outbreeders *L. perenne* and *L. rigidum*, there were additional hybridization sites on up to nine chromosomes in all. pTa794 hybridized to two sites in each taxon but the position was different in the inbreeders to that found in the outbreeders. Using this approach, THOMAS et al. (1996) have clarified previously unknown phylogenetic relationships in this genus.

GISH has categorically demonstrated that *F. arundinacea* has evolved from an amphiploid involving early forms of *F. pratensis* and *F. glaucescens* (HUMPHREYS et al. 1995). But what, if any, structural changes have the chromosomes of *F. arundinacea*, *F. pratensis* and *F. glaucescens* undergone subsequently? To answer this question and increase our understanding of chromosome evolution in *Festuca* we have applied the two rDNA probes to chromosome spreads of *F. arundinacea*, and its progenitors *F. pratensis* and *F. glaucescens*.

The number and positions of the hybridization sites in *F. pratensis* and *F. glaucescens* corresponds with those in *F. arundinacea* but there has been a loss of two pTa71 sites from a "*F. glaucescens*" chromosome in *F. arundinacea* and there has been a repositioning of one pTa794 site.

Despite the unstable nature of rDNA sites in some genera, they can nevertheless provide valuable chromosomal landmarks. Physical mapping of rDNA loci can add to our understanding of chromosome evolution and provides a valuable tool for the study of phylogeny.

Other sources of repetitive DNA may also be of value to the cytogeneticist. We have collected a library of suitable probes isolated from a range of plant species (e.g., *Lolium*, *Festuca*, *Triticum*, *Aegilops* and *Secale*). Such probes, used singularly or in combination will give distinctive banding patterns to the chromosomes producing a "molecular karyotype" (for review see JIANG, GILL 1994). Once these karyotypes have been produced, they can be used to identify individual chromosomes in aneuploid stocks and determine genomic relationships and the extent to which the karyotypes may have diverged.

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