Interspecific genomic rearrangements in androgenic plants derived from a *Lolium multiflorum* × *Festuca arundinacea* ($2n = 5x = 35$) hybrid

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Pollen microspores from a hybrid between *Lolium multiflorum* ($2n = 4x = 28$) and *Festuca arundinacea* ($2n = 6x = 42$) were cultured and over 200 androgenic green plants established. In the initial hybrid one chromosome from each of the five homoeologous groups was labelled by a distinct *PGI/2* homoeoallele. Segregation of *PGI/2* alleles in the male gametes and in the androgenic plants indicated chromosome pairing preferences in the hybrid at meiosis. The pentaploid *Festulolium* hybrid genome comprised two homologous sets of *Lolium* (*Lm*) chromosomes and the polyploid set of *F. arundinacea*, namely one genome of *F. pratensis* (*Fp*) and two of *F. glaucescens* (*Fg* and *Fg*₁). There was a high degree of preferential chromosome pairing between the two homologous sets of *L. multiflorum* chromosomes, and each androgenic plant contained a *Lolium PGI/2* allele. Results supported earlier work indicating that gamete viability depended on the presence of a complete *Lolium* genome. Genomic *in situ* hybridization (GISH) was carried out on mitotic chromosome preparations of two androgenic plants using the total genomic DNA of *L. multiflorum* as a probe. Recombinants were observed between chromosomes of all three genomes in the pentaploid hybrid, demonstrating the efficacy of the hybrid as a starting point in introgression breeding programmes.

Keywords: androgenesis, genomic *in situ* hybridization (GISH), *Lolium multiflorum* × *Festuca arundinacea* hybrid, meiosis, phosphoglucosomerase (*PGI/2*).

Introduction

The genera *Lolium* and *Festuca* include most of the major temperate grasses, and together include a range of complementary, agronomically desirable traits (Thomas & Humphreys, 1991). Our ultimate aim is to create homozygous lines from *Festulolium* hybrids which combine the high nutritive value of *Lolium* with the stress tolerance of *Festuca*.

Androgenesis, or anther culture, allows us to produce monoploid plants from which we can eventually develop homozygous plants by chromosome

doubling (Mayer *et al.*, 1995). It can reveal novel characters governed by gene combinations, including recessive alleles, that are otherwise only rarely or never observed. This enables us to identify and select for such hidden traits. We describe here how androgenesis of a very amenable × *Festulolium* pentaploid hybrid genotype which gave rise to a population of aneupolyhaploid plants enabled us to reveal much of the potential genotypic and phenotypic variation that can be generated through meiosis.

The *Festulolium* (5x) hybrid was constructed with one chromosome in each of the five homoeologous groups labelled by a distinct phosphoglucosomerase (*PGI/2*) homoeoallele. We describe the segregation

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of *PGI/2* homoeoalleles in the androgenic population and their use as indicators of chromosome behaviour at meiosis. In addition two androgenic plants, which combine particularly high drought and freezing tolerance with desirable agronomic characters, are characterized cytologically using genomic *in situ* hybridization (GISH).

Materials and methods

The pentaploid hybrid between *L. multiflorum* (*Lm*) ($2n = 4x = 28$) × *F. arundinacea* (*Fa*) ($2n = 6x = 42$) used for anther culture was produced as described by Humphreys (1989), and has a genomic constitution of *LmLmFpFgFg₁* (*Fp* = *F. pratensis*, *FgFg₁* = *F. glaucescens*). The two homologous chromosomes of *Lm* are labelled at the *PGI/2* locus by two alleles *a* and *b*, whereas the three homoeologous chromosomes of *Fa* are labelled by *PGI/2* homoeoalleles *a*⁺, *c* and *e*. The *c* allele labelled a chromosome in the *F. pratensis* (*Fp*) genome, and the *a*⁺ and *e* *PGI/2* alleles labelled chromosomes in each of the two *F. glaucescens* (*Fg* and *Fg₁*) genomes of *F. arundinacea* (Humphreys, 1995). Because the hybrid is very responsive to cell culture (Humphreys & Dalton, 1992) and produces many inflorescences which are male fertile, it is ideally suited for anther culture. The hybrid was used previously in a backcrossing programme with *L. multiflorum* (2x) (Humphreys & Ghesquière, 1994).

The androgenic plants were produced following the procedures described by Ward (1993). Great care was taken to isolate and record the origin of different embryoids as they were formed so as to eliminate any possible risk that two or more embryoids might derive from the same microspore and have the same genotype. The *PGI/2* phenotype of all androgenic plants was determined as described by Humphreys (1995).

Two androgenic plants (genotypes 193 and 219) with proven high drought and freezing resistance (Zare, 1996) were selected for detailed cytological examination using genomic *in situ* hybridization (Thomas *et al.*, 1994). Briefly, total genomic DNA of *Lm* labelled with rhodamine-4-dUTP was used as a probe on mitotic chromosome preparations of the two plants. As blocking DNA, ×40 probe concentration of *Fa* DNA was used with the *Lm* probe. Following GISH, chromosome preparations were counterstained with 4',6-diamidino-2-phenylindole (DAPI). Chromosomes containing alien recombinant segments were identified as *Lm*, *Fp*, or *Fg/Fg₁* by the presence of the appropriate GISH labelling on both sides of the centromere.

Results

Phenotypic traits

A total of 221 green androgenic plants, derived from different microspores and thus having different genotypes, were successfully established in soil. The plants had very diverse expression of leaf size, tiller number, growth habit, plant height, root: shoot ratio, growth rate, tillering capacity, and tolerance to cold and drought (Zare, 1996). In particular, 6 per cent of the androgenic plants tested under simulated conditions were found to be more drought or cold tolerant than *F. arundinacea*, the more resistant parent (Humphreys *et al.*, 1996).

Transmission of isozyme homoeoalleles

The frequencies of the five *PGI/2* homoeoalleles in the androgenic population differed significantly (Table 1). This was caused primarily by the high frequency of the *Lolium a* allele (27 per cent) which was recovered at greater frequency than any other *PGI/2* allele (16–19 per cent). The three *Festuca PGI/2* alleles were recovered at similar frequency.

Almost all plants (98 per cent) carried at least one *Lolium* allele, and 10 per cent carried both (Table 2). The recovery of the *Festuca* alleles (*a*⁺, *c* and *e*) was less consistent: 30 per cent carried no *Fg/Fg₁* (*a*⁺ or *e*) allele, and 14 per cent carried both *Fg* and *Fg₁* alleles. The *Fp c* allele was recovered in 44 per cent of the androgenic plants (Table 2).

Further information on transmission of the five alleles is provided (Table 1) by comparing their frequency in the androgenic population with that in a BC₁ population (produced by pollination diploid *L. multiflorum* with the pentaploid × *Festulolium* hybrid, as reported by Humphreys & Ghesquière, 1994). There was no significant difference between the two populations in the frequency of *PGI/2* homoeoalleles. Therefore, there was little or no selection in the BC₁ against gametes with any *Lm*, *Fp*, *Fg* or *Fg₁* *PGI/2* alleles, and hence it is likely that chromosomes from the homoeologous set were transmitted in accordance with their frequency in the male gamete.

Genome structure and recombination revealed by GISH

When the two androgenic plants which combined drought and freezing tolerance were studied using GISH (Fig. 1), the *Lm* DNA probe labelled with rhodamine-4dUTP hybridized preferentially with *Lolium* chromosomes (coloured pink) and with

Table 1 Frequency of *PGI/2* alleles in 221 androgenic plants derived from a pentaploid ($2n = 5x = 35$) hybrid between *Lolium multiflorum* and *Festuca arundinacea* (*PGI/2* allelic constitution = a^+abce), and the transmission of alleles into 117 BC_1 plants. The BC_1 population was produced from the cross between *L. multiflorum* (2x, maternal parent) and the pentaploid hybrid (Humphreys & Ghesquière, 1994). Origin of *PGI/2* alleles: *Fg*, *Festuca glaucescens*; *Lm*, *Lolium multiflorum*; *Fp*, *Festuca pratensis*. Also, summary of χ^2 -values of comparisons between frequencies

<i>PGI/2</i> allele	Origin	Allelic frequency				Comparison	χ^2	<i>P</i>
		Androgenic plants		BC ₁ plants				
		No.	Per cent	No.	Per cent			
<i>a</i> ⁺	Fg	102	19.5	27	12.4	Androgenic vs. BC ₁	8.43	NS
<i>a</i>	Lm	139	26.6	52	23.9			
<i>b</i>	Lm	100	19.1	53	24.3	Androgenic		
<i>c</i>	Fp	97	18.6	50	22.9	All alleles	15.81	<0.01
<i>e</i>	Fg	85	16.2	36	16.5	<i>a, b</i>	6.36	0.05
Total		523		218		<i>a</i> ⁺ , <i>c</i> , <i>e</i>	1.61	NS

introgressed *Lolium* chromosome segments on *Festuca* chromosomes. The *Lm* DNA probe partially hybridized to certain *Festuca* chromosomes (coloured mauve) but failed to hybridize with other *Festuca* chromosomes (coloured blue). The *Lm* genome is known to be structurally closer to *Fp* than to *Fg/Fg₁* (Humphreys & Ghesquière, 1994) and the *Festuca* chromosomes which partially hybridized with the *Lm* probe were therefore ascribed to the *Fp* genome. In consequence, the chromosomes which showed no evidence of hybridization with the *Lm* probe belong to the *Fg* or *Fg₁* genomes. Chromosomes with partial hybridization to the *Lm* probe and carrying segments with no hybridization to the *Lm* probe are likely to be *Fp* chromosomes carrying *Fg/Fg₁* introgressions (for example Fig. 1b(4)).

Genotype 193 had 21 chromosomes of which eight belonged to the *Lm* genome, seven to *Fp* and six to *Fg/Fg₁* (Fig. 1a). Genotype 219 also had 21 chromosomes: seven *Lm*, six *Fp* and eight *Fg/Fg₁* (Fig. 1b). Recombinants involving all three genomes were observed (an example of each combination is illustrated in Fig. 1) and this was confirmed with GISH using total genomic DNA probes of *Fp* and *Fg/Fg₁* in addition to *Lm* (not shown). One of the recombinants in 219 was derived from *Fp*, and replaced most of the long arm of a *Lm* chromosome with a large satellite identified as chromosome 2 (Fig. 1b(3)).

Discussion

Despite high levels of homoeologous chromosome pairing in pentaploid *Festulolium* hybrids (Humphreys, 1989), certain chromosome pairing preferences might be anticipated. For example, the

two homologous *Lm* genomes would be expected to pair preferentially, and earlier meiotic chromosome studies support this conclusion (for example Morgan *et al.*, 1988; Humphreys, 1989).

The analysis of segregation of *PGI/2* alleles in the androgenic population described here provides clear indications as to whether these predictions for chromosome behaviour at meiosis are accurate. The presence of a *Lm PGI/2* allele in 98 per cent of androgenic plants is strong evidence for preferential chromosome pairing and near-regular disjunction between the two *Lm* genomes of the pentaploid hybrid. This adds to evidence from backcross breeding programmes (Morgan *et al.*, 1988; Humphreys & Ghesquière, 1994) that each gamete in the pentaploid hybrid contains a complete *Lm* genome. The low frequency (2 per cent) of plants with no *Lm PGI/2* alleles shows that a complete *Lm* genome is nearly always required to provide gamete viability. On the other hand, the existence of a large proportion of androgenic plants with either no *Fp* (56 per cent) or no *Fg* or *Fg₁* (30 per cent) *PGI/2*-labelled chromosomes indicates that a complete *Festuca* genome is not essential for gamete viability.

A lower than expected recovery of a *PGI/2* allele in the BC_1 would have been evidence of gametophytic selection through pollen competition or zygotic abortion, which can occur in backcross breeding programmes and preclude recovery of certain gene combinations (Humphreys & Thorogood, 1993). However, 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) $\times$ *Festulolium* (5x) hybrids (see Humphreys & Ghesquière, 1994).

Therefore, it is likely that there is no serious barrier to any gene combination in × *Festulolium* hybrids.

The GISH chromosome study of the two androgenic plants demonstrates that chromosomes from *Lm*, *Fp* and *Fg/Fg₁* genomes are present and can be distinguished by their degree of hybridization with the *Lm*-DNA probe (Fig. 1). Recent studies (not shown) using Southern hybridization to determine genome relationships between *Lm* and the different

Table 2 The possible and observed *PGI/2* phenotypes in 221 androgenic plants derived from a (5x) hybrid between *Lolium multiflorum* and *Festuca arundinacea*, with *PGI/2* homoeoallelic constitution = *a⁺abce* (for origin of alleles see Table 1)

No. of alleles	Possible phenotype	Observed frequency of plants	
5	<i>a⁺abce</i>	0	0%
4	<i>a⁺abc</i>	0	
	<i>a⁺abe</i>	0	
	<i>a⁺ace</i>	0	
	<i>a⁺bce</i>	0	
	<i>abce</i>	0	
	Subtotal	0	0%
3	<i>a⁺ab</i>	18	
	<i>a⁺ac</i>	19	
	<i>a⁺ae</i>	13	
	<i>a⁺bc</i>	6	
	<i>a⁺be</i>	16	
	<i>a⁺ce</i>	1	
	<i>abc</i>	3	
	<i>abe</i>	0	
	<i>ace</i>	3	
	<i>bce</i>	3	
	Subtotal	82	37.1%
2	<i>a⁺a</i>	12	
	<i>a⁺b</i>	15	
	<i>a⁺c</i>	0	
	<i>a⁺e</i>	2	
	<i>ab</i>	1	
	<i>ac</i>	43	
	<i>ae</i>	27	
	<i>bc</i>	18	
	<i>be</i>	19	
	<i>ce</i>	1	
	Subtotal	138	62.4%
1	<i>a⁺</i>	0	
	<i>a</i>	0	
	<i>b</i>	1	
	<i>c</i>	0	
	<i>e</i>	0	
	Subtotal	1	0.5%
0	—	0	0%

genomes of *Fa*, support the conclusions made here for close homology between *Lm* and *Fp* and lower homology with *Fg/Fg₁*. This ability to distinguish all three genomes using a single probe facilitates a rapid cytological analysis of hybrids involving these species.

We observed (Fig. 1) a number of reciprocal recombinants involving the *Lm*, *Fp* and *Fg/Fg₁* genomes. In contrast, Humphreys & Ghesquière (1994) were unable to observe any *Lm*–*Fg* or *Lm*–*Fg₁* recombinants among the BC₁ in their breeding programme. We therefore conclude that gametes carrying *Lm*–*Fg/Fg₁* recombinants must face severe selection pressure or, following fertilization, produce nonviable zygotes. Indeed, certain gene combinations resulting from *Lm*–*Fg/Fg₁* chromosome recombination which can be recovered from anther culture may never be transmitted by conventional breeding methods.

In genotype 219, a large *Fp* chromosome recombinant occupies nearly the entire length of the long arm of chromosome two of *Lm* (Fig. 1b(3)). Because this chromosome arm of *Fp* is known to carry genes for drought resistance (Humphreys & Pašakin-

Fig. 1 Genomic *in situ* hybridization using *Lolium multiflorum* total genomic DNA as probe labelled with rhodamine 4dUTP on mitotic preparations of androgenic genotypes (a) 193, (b) 219, both $2n = 21$. Chromosomes which hybridize with the *Lm* probe are *Lm* (coloured pink); chromosomes which partially hybridize with the *Lm* probe are *Fp* (coloured mauve); and chromosomes with no hybridization to the *Lm* probe are *Fg* (coloured blue). Examples of interspecific recombinants are arrowed: (1) *Fg* or *Fg₁* chromosome with *Lm* recombinant, (2) *Lm* chromosome with *Fg* or *Fg₁* recombinant, (3) *Lm* chromosome 2 with *Fp* recombinant and (4) *Fp* chromosome with *Fg* or *Fg₁* recombinant. *Lm*, *Lolium multiflorum*; *Fp*, *Festuca pratensis*; *Fg/Fg₁*, *F. glaucescens*. Bar = 10 μ m.

skienė, 1996), it is likely that these *Fp* genes have also contributed to the genotype's extreme drought resistance.

In order to utilize the variation obtained by anther culture, it will be necessary to restore fertility to selected androgenic plants and to incorporate them in breeding programmes. Improved fertility and genome stability should result from colchicine treatment, which will induce chromosome doubling and thereby encourage preferential pairing and regular disjunction between homologous chromosomes.

References

- HUMPHREYS, M. W. 1989. The controlled introgression of *Festuca arundinacea* genes into *Lolium multiflorum*. *Euphytica*, **42**, 105–116.
- HUMPHREYS, M. W. 1995. *Gene Introgression Following Intergeneric Hybridization Within the Lolium/Festuca Complex*. Ph.D. Thesis, University of Wales, Aberystwyth.
- HUMPHREYS, M. W. AND DALTON, S. J. 1992. Stability at the phosphoglucosyltransferase (*PGT*) locus in *Lolium multiflorum* ($2n = 4x = 28$) $\times$ *Festuca arundinacea* ($2n = 6x = 42$) plants regenerated from cell suspension. *Genome*, **35**, 461–467.
- HUMPHREYS, M. W. AND GHESQUIÈRE, M. 1994. Assessing success in gene transfer between *Lolium multiflorum* and *Festuca arundinacea*. *Euphytica*, **77**, 283–289.
- HUMPHREYS, M. W. AND PAŠAKINSKIENĖ, I. 1996. Chromosome painting to locate genes for drought resistance transferred from *Festuca arundinacea* into *Lolium multiflorum*. *Heredity*, **77**, 530–534.
- HUMPHREYS, M. W. AND THOROGOOD, D. 1993. Disturbed Mendelian segregations at isozyme marker loci in early backcrosses of *Lolium multiflorum* $\times$ *Festuca pratensis* hybrids to *L. multiflorum*. *Euphytica*, **66**, 11–18.
- HUMPHREYS, M. W., ZARE, A. G., PAŠKINSKIENĖ, I. AND KERLAN, M.-C. 1996. The potential of anther culture derived plants from a *L. multiflorum* $\times$ *F. arundinacea* ($2n = 5x = 35$) hybrid as a novel forage grass crop. *Proceedings of the Eucarpia Fodder Crops and Amenity Grasses Section*, Plant Breeding and Acclimatization Institute, Radzikow, Poland, Oct. 7–10, 1996, pp. 155–156.
- MAYER, M., GLAND, G., CECCARELLI, S. AND GEIGER, H. H. 1995. Comparison of doubled haploid lines and F_2 bulks for the improvement of barley in the dry areas of North Syria. *Pl. Breed.*, **114**, 45–49.
- MORGAN, W. G., THOMAS, H. AND LEWIS, E. J. 1988. Cytogenetic studies of hybrids between *Festuca gigantea* Vill. and *Lolium multiflorum* Lam. *Pl. Breed.*, **101**, 335–343.
- THOMAS, H. AND HUMPHREYS, M. O. 1991. Progress and potential of interspecific hybrids of *Lolium* and *Festuca*. *J. Agric. Sci. Camb.*, **117**, 1–8.
- THOMAS, H. M., MORGAN, W. G., MEREDITH, M. R., HUMPHREYS, M. W., THOMAS, H. AND LEGGETT, J. M. 1994. Identification of parental and recombined chromosomes in hybrid derivatives of *Lolium multiflorum* $\times$ *Festuca pratensis* by genomic *in situ* hybridization. *Theor. Appl. Genet.*, **88**, 909–913.
- WARD, S. P. 1993. *The Use of Interspecific Hybrids in Forage Grass Breeding*. M.Sc. Thesis, University of Wales, Aberystwyth.
- ZARE, A. G. 1996. *Anther Culture in Intergeneric Hybrids of Forage Grasses*. Ph.D. Thesis, University of Liverpool, Liverpool.